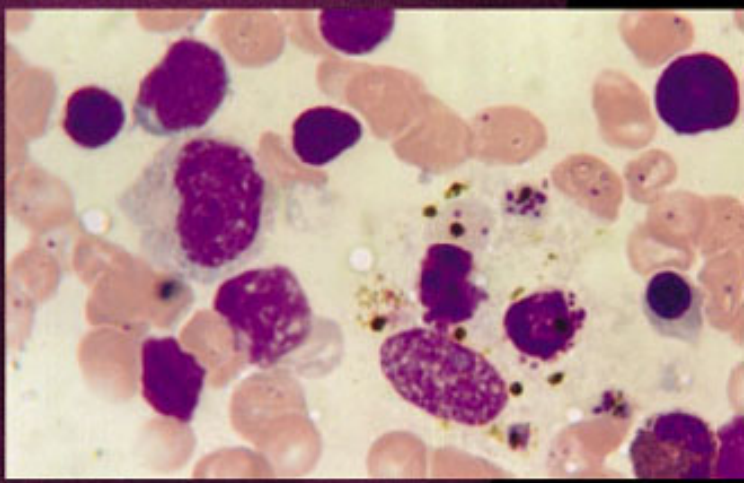
TROPICAL MEDICINE
Science and Practice

Malaria

A Hematological Perspective

Edited by
Saad H Abdalla & Geoffrey Pasvol



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Geoffrey Pasvol and Stephen L Hoffman

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Malaria

A Hematological Perspective

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Volume 4

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MALARIA: A HEMATOLOGICAL PERSPECTIVE

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About the Series Editors

Geoffrey Pasvol is Professor of Infection and Tropical Medicine in the Faculty of Medicine, Imperial College London. He graduated from the University of Cape Town and went to Oxford in 1974 as a Rhodes Scholar to study the relationship between malaria and the hemoglobinopathies at the Nuffield Department of Clinical Medicine. From 1974 to 1979 he worked at the Medical Research Council Laboratories at Fajara in the Gambia on the cellular interactions between the malaria parasite and the red cell, and from 1989 to 1991 on clinical aspects of malaria at the KEMRI Wellcome Unit at Kilifi on the Kenyan coast. He was a Medical Research Council visiting fellow at Harvard Medical School in 1984. He has a special interest in the cellular and molecular interactions in the pathogenesis of malaria and has more recently been involved in the immunopathology of tuberculosis. Professor Pasvol is currently Director of the Wellcome Trust Centre for Clinical Tropical Medicine at Imperial College.

Stephen L Hoffman is the founder and Chief Executive and Scientific Officer of Sanaria Inc, a vaccine development company. After studies at the University of Pennsylvania, Cornell University Medical College, and the London School of Hygiene and Tropical Medicine, and residency training at the University of California, San Diego (UCSD), he cofounded the Tropical Medicine and Traveler's Clinic at UCSD in 1979. In 1980 he joined the US Navy and moved to Indonesia, where he was Director of the Department of Clinical Investigation and Epidemiology at the Naval Medical Research Unit-2 from 1980 to 1984, and conducted studies on typhoid fever, tropical splenomegaly syndrome, severe malaria, cholera, and lymphatic filariasis. In 1985 he joined the malaria vaccine development team at the Naval Medical Research Institute (NMRI) and the Walter Reed Army Institute of Research, and he became the Director of the NMRI program in 1987. Until his retirement in 2001 he worked to improve malaria diagnosis treatment and prevention by studying the epidemiology,

immunology, and genomics of malaria throughout the world and spearheaded efforts to develop malaria vaccines and better methods of chemoprophylaxis. From 2000 to 2002 he led the effort to use genomic and proteomic information to develop immunotherapeutic and other biologic products, and coordinated the *Anopheles gambiae* genome sequencing effort as Senior Vice President of Biologics at Celera Genomics in Rockville, MD. Dr Hoffman is an adjunct professor at the Uniformed Services University of the Health Sciences in Bethesda, Maryland, a visiting professor at the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, and a member of numerous national and international advisory and steering committees, as well as the former president of the American Society of Tropical Medicine and Hygiene.

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This book is dedicated to the memory of my mother, who over the years urged me to complete it. I also wish to extend my thanks to the coauthors and to the numerous colleagues, family members and friends who encouraged me.

— Saad H Abdalla

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Introduction

Malaria is one of the most important transmissible diseases in humans. There is an intimate relationship between the malaria parasites and the blood. The parasites are members of the *Haemosporidiae*, which are obligatory intracellular parasites of red cells. The parasites have adapted in ancient times to live inside the red cells and are widespread in the animal kingdom, with parasites specific to orders as varied as amphibians, birds and mammals. The obvious advantages to parasites of intraerythrocytic parasitism are that it offers them protection from the host's defence mechanisms and transport through the bloodstream to where they can be taken up in the peripheral circulation by blood-sucking female anopheline mosquitoes.

Because of this association between the parasites and red cells, there are numerous consequences to the host's blood extending far beyond the direct effect of parasitized RBCs. Anemia ensues from malaria and has a complicated pathogenesis and major consequences. The incidence and seasonal variation of the anemia, which follow that of malaria, mean that anemia is synonymous with malaria in certain tropical areas.

The severe selective pressure exerted by malaria has had profound effects on human history, especially in the important Mediterranean area in classical times. For example, it is thought that malaria may have been one of the factors responsible for the fall of the Roman Empire.

The selective pressure has been such that malaria is thought to have led to changes in the human genome. The majority of these genetic changes have, not surprisingly, occurred in the very object of parasitization: the red cell, involving red cell antigens, the cytoskeleton, enzymes and hemoglobin. Interestingly, other genetic changes have recently been described which do not relate directly to red cells but have indirect effects on the host that may lead to protection or increased susceptibility to anemia, such as the HLA antigen polymorphisms and the TNF-α promoter polymorphism.

The above are the two major areas of overlap between hematologists and plasmodia, but there are numerous other areas of interest (apart from the diagnostic aspect), such as the role of malaria in the pathogenesis of Burkitt's lymphoma, the immunological imbalance of hyperreactive malarial splenomegaly and its hematological consequences, the role of cytokines in host defences and disease pathology, and the interaction between malaria and iron deficiency or treatment with iron, to name a few.

The aim of this book is to review all the hematological changes and interactions in malaria and, in so doing, to emphasize the importance of malaria as a primarily hematological disease and to increase the awareness and interest amongst hematologists, malariologists and tropical physicians.

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Chapter 1

Laboratory Procedures for Diagnosis of Malaria

DC Warhurst and JE Williams

Introduction

Malaria is caused by blood parasites of the genus *Plasmodium*, carried by *Anopheles* mosquitoes. Four species are regularly recognized in man: *P. falciparum* (responsible for most deaths and referred to as the malignant tertian malaria parasite); *P. vivax* and *P. ovale*, the benign tertian malaria parasites; and *P. malariae*, the quartan malaria parasite. The closest evolutionary relative of *P. falciparum* is *P. reichenowi*, a chimpanzee parasite. This is consistent with the hypothesis that *P. falciparum* has been associated with hominids since their divergence from their closest hominoid relatives. The three other human *Plasmodium* species are extremely close genetically to species found in nonhuman primates; *P. brasilianum* and *P. simium* are found in New World monkeys and, while *P. brasilianum* is equivalent to human *P. malariae* (also found as *P. rodhaini* in great apes in Africa), *P. simium* is very close to *P. vivax* of man (1).

The usual symptoms of malaria are fever, chills and headache, but these are relatively nonspecific. Medical history and details of recent travel of the patient may lead to suspicion of malaria infection. The positive predictive value of clinical diagnosis will of course increase where malaria attacks are more common. Since malaria is a medical emergency, prompt and accurate laboratory diagnosis is the key to effective disease management.

Crucial diagnostic questions which need to be answered are:

(1) Is it malaria?
(2) What species is present?
(3) How dense is the parasitemia (what is the parasite count)?
(4) What stages are present?

There can be no doubt that the first question is the most important, and on this can depend the survival of the patient. A naïve host is at serious risk from any intensity of falciparum malaria parasitemia and urgent action is vital. For patients in malaria-endemic areas, the level of parasitemia and the age of the patient both determine the significance of the detection of malaria parasites, and a case definition for adults may require fever together with a parasite density above a cutoff specific to that location (2). It is quite common for the cause of fever in a semi-immune person to be other than malaria.

Microscopy

Microscopy is widely used for detection, identification and quantification of malaria parasites (3), and remains the gold standard.

Samples and Methods

Blood should, if possible, be taken during or after pyrexia, and before the administration of antimalarial drugs. The sample should ideally be taken direct from the patient's finger or ear and the smears prepared at the bedside or in the clinic (4). If it is necessary to use anticoagulants, then the films should be made as soon as possible, certainly less than three hours after the blood has been drawn. EDTA is superior to other anticoagulants for this purpose.

For routine diagnosis of malaria, two thin and two thick blood films should be prepared. One of the thin films, fixed in methanol and stained using Giemsa's stain, should be adequate for detection of normal parasitemias and for determination of species. Malaria is a notifiable disease in the UK, and the PHLS Malaria Laboratory would appreciate having duplicate thick and thin films for confirmation, with case details on the relevant questionnaire, from every case in the UK.

An unfixed thick film should also be stained using Field's or Giemsa's stain. If problems with diagnosis arise, the two spare films are available for further study. To exclude malaria when the clinical evidence is strong and yet parasites are not found in the films taken initially, the minimal requirement is to examine further films for two subsequent days and preferably during a febrile paroxysm.

Provided that immersion oil is cleared from stained films after examination, using inhibisol or xylol, they can be stored for many years in dry and dark conditions with little or no deterioration.

Stains and Reagents

Commercial stains, when available, should be tested before routine use, to determine optimum dilution and confirm satisfactory staining characteristics. As an alternative to commercial stains, which regularly become unavailable, the staining solutions may be made up as follows:

Table 1. Field's stain. The solutions should be filtered after being allowed to stand overnight.

A		B	
Methylene blue (medicinal)	0.8 g	Eosin	1.0 g
Azure I	0.5 g	Na_2HPO_4	5.0 g
Na_2HPO_4	5.0 g	KH_2PO_4	6.25 g
KH_2PO	6.25 g	Distilled water	500 ml
Distilled water	500 ml		

Table 2. Giemsa's stain. Absolute methanol (Analar) is used for fixation of thin films.

A stable solution can be prepared from azure II eosin and azure II in glycerol and methanol. Filter after standing in the dark in a sealed bottle for one week. Where necessary, stains are diluted using buffered distilled water.

Azure II eosin	3.0 g
Azure II	0.8 g
Glycerol	250 ml
Methanol	250 ml
Phosphate buffered distilled water, pH 7.2:	
KH_2PO_4	0.7 g
Na_2HPO_4	1.0 g
Distilled water	1.0 l

Thin Blood Film

Preparation of the smear, and staining are similar to those used for normal hematology, except that ordinary Giemsa's stain is used, and dilution is made in buffered distilled water (pH 7.2) instead of the usual slightly acidic buffer (pH 6.8) used by hematologists. The buffered water at pH 7.2 must be used. It is only at this alkaline pH that proper differentiation of parasite nuclear and cytoplasmic material takes place, as well as the staining of cytoplasmic and membrane changes in infected RBCs (e.g. Maurer's clefts in falciparum and Schüffner's and James's dots in vivax and ovale, respectively). Both sensitivity and specificity are defective in the use of acidic staining. Ensure that the film has a good "tail" and does not reach the edges of the slide laterally.

For optimal results, a staining tray, constructed so that the slides may be stained face downward in a small volume of solution, helps to prevent precipitation of the stain on the blood film. The stain is diluted in a 20 ml disposable syringe using a 5 cm × 19 g needle (point removed). The syringe method for dilution of Giemsa is strongly recommended, as it prevents the diluted stain from being exposed to air and producing a precipitate. Staining face downward also reduces precipitation, and any that does develop falls away from the smear. Cleanly stained smears are very important when one is searching for small intracellular parasites. More commonly, the slides are stained face upward.

Method (Giemsa's stain)

(1) Allow the film to dry in air and fix with methanol for 1/2 to 1 min.
(2) Tip off excess methanol and place face down on a staining tray.
(3) Using the 20 ml syringe and blunt needle, dilute the stock Giemsa 1 : 10 with buffered distilled water. Mix well and expel air.
(4) Infiltrate the stain, using the syringe and needle, under the slide, taking care not to trap large air bubbles. Stain for 40–45 min.
(5) At the end of the staining time, rinse the slides briefly with tap water and allow them to drain dry in a vertical position.

A ×50 or ×63 oil immersion objective is invaluable for preliminary examination. Where parasites are scanty, they may be more easily found along the

edges and tail of the thin film. Possible parasites should be examined in more detail using the ×100 oil immersion lens.

Thick Blood Film

Thick blood films allow a rapid examination of a relatively large volume of blood, enabling the detection of scanty parasitemias. In unit time, a well-prepared thick blood film gives a ten fold increase in sensitivity over thin films (5), although 40 times the volume of blood is examined. Two staining techniques are usually employed: Field's technique, which demands expertise for the best results, and the slower Giemsa's method, which gives a more predictable but less attractive result.

A dried thick film, several RBCs in depth, is made, and the hemoglobin washed out either before or during the staining process. The malaria parasites in the film are stained with little interference from the large numbers of RBCs present, and can be seen against a clear background where red cell membrane changes seen in vivax and ovale are detectable.

Samples

Ideally, thick smears of venous or capillary blood should be taken without anticoagulants, since they adhere better to the slides and leave a clearer background after lysis. This is often difficult to achieve in routine laboratory work, but care should be exercised that films from anticoagulated specimens are prepared as soon as possible and certainly less than three hours after collection.

Preparation of Thick Blood Film

(1) A drop of blood, 3–5 mm in diameter (3–5 µl), is put into the center of a 76 × 26 mm slide and spread, with the corner of another slide or a swab stick, to cover an oval area of approximately 10–15 mm diameter.
(2) The final density of the smear should allow newsprint to be just visible through it.
(3) Thoroughly dry the smear, horizontally, in an incubator at 37°C for not less than 15 min, ideally for one hour in a dry atmosphere. Films must be absolutely dry before lysis, or else the blood smear is likely to detach from the slide.

Staining Method (Giemsa's Method)

(1) Do *not* fix the dry film, but place it in a Coplin jar containing buffered water (pH 7.2) and allow it to lyse until no hemoglobin can be seen falling away from the smear (usually 3–5 min).
(2) Remove from the water, place face down on the staining dish, and stain with Giemsa's stain diluted 1 : 10 with water, as for the thin film method.
(3) Stain for 30 min; then rinse briefly with tap water and drain dry. Examine the film as described for a thin blood film.

Staining Method (Field's Stain)

(1) Do *not* fix the dry film.
(2) Dip the film for 3 s in Field's stain A (Coplin jar).
(3) Wash gently in tap water and drain off excess water onto absorbent paper.
(4) Dip the film for 3 s into Field's stain B (Coplin jar).
(5) Wash gently in tap water, and allow to drain dry in a vertical position.
(6) When dry, examine as for a thin blood film, paying particular attention to the lower half of the film, where the background is often better. Although part of every thick film is usually unreadable, even the worst film may have a usable area.

Examination of the thick film has for many years been the most rapid way to detect a malaria infection. A total of 200 oil immersion fields should be scanned, which usually takes an expert approximately 6 min.

Blood Films from Suspected Hemorrhagic Fevers or HIV

The recommended technique is as follows:

Thick Films

(1) Fix the dried smear directly in 10% buffered formalin for 10 min.
(2) Wash three times (total 3 min) in buffered water and stain with Giemsa as usual.

Thin Films

(1) Fix in methanol for 5 min.

Discard all washings and methanol into chloros.

Comment

Nuclei of malaria parasites stain darker by this technique, and the characteristic RBC stippling is not always satisfactorily revealed.

Interpretation of Stained Thin Films (see Figs. 1–15 and Table)

The presence of intraerythrocytic bodies, generally consisting of a blue staining cytoplasmic area closely associated with a small reddish-staining nuclear area, and, in the larger, more mature parasites, the presence within the organism of yellow–brown to black malaria pigment, are diagnostic of malaria infection. As the malaria parasite grows within the erythrocyte, and finally divides to give a maximum of 24 infective merozoites, the host cell may show enlargement (*P. vivax* and *P. ovale*), remain the same size, or shrink (*P. falciparum* and *P. malariae*). The erythrocyte membrane may develop surface markings which stain pink with Giemsa at pH 7.2 (*P. vivax* and *P. ovale*). All stages of the parasite

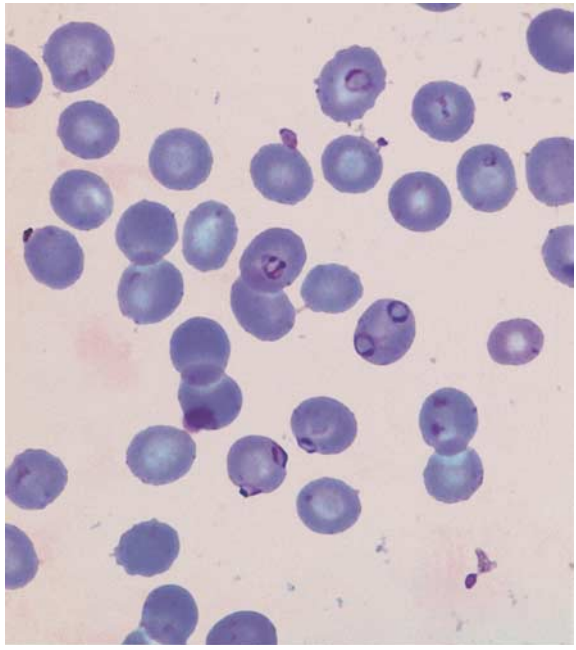
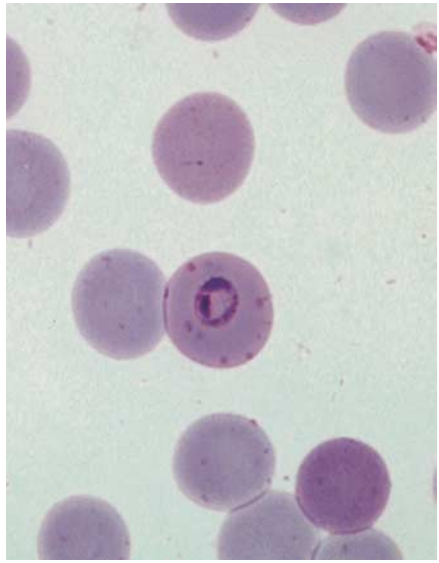


Figure 1



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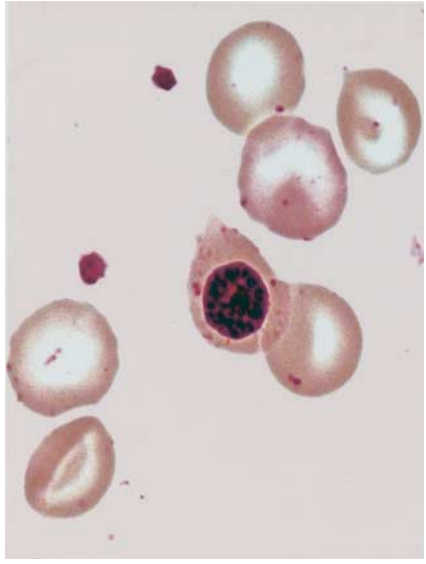

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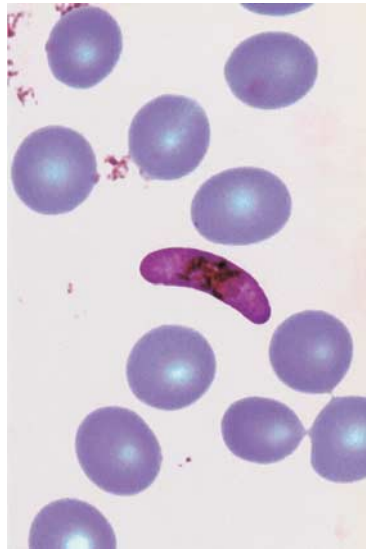

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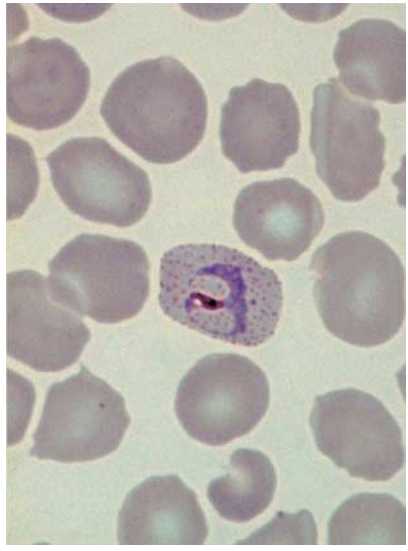

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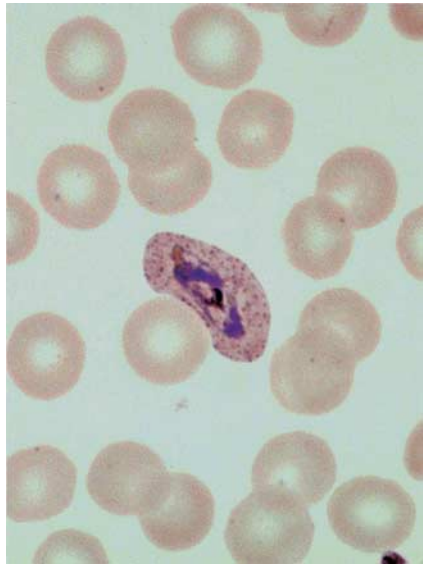

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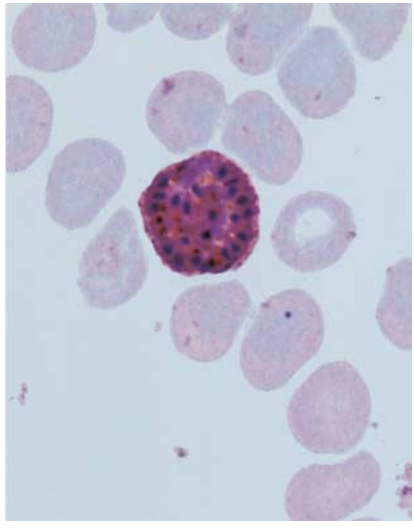

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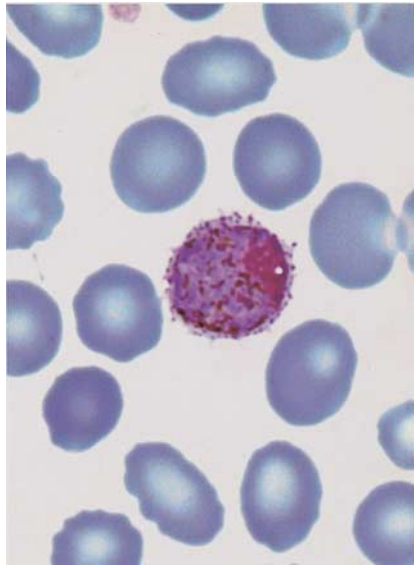

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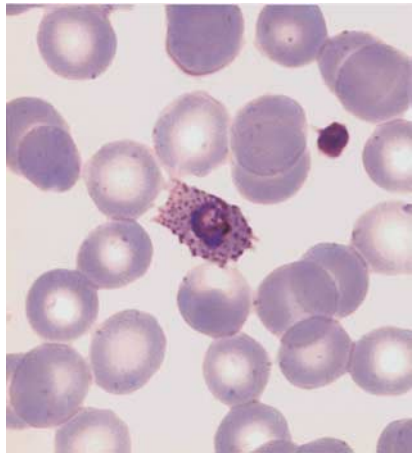

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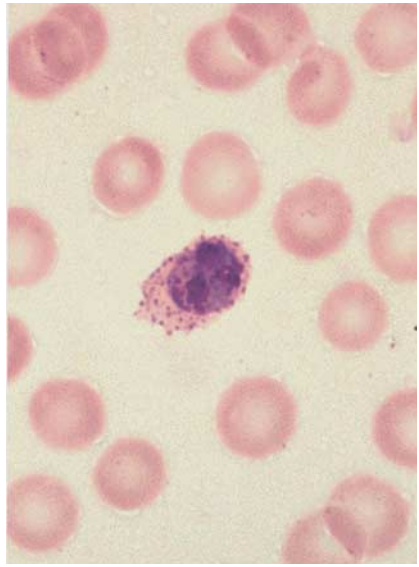

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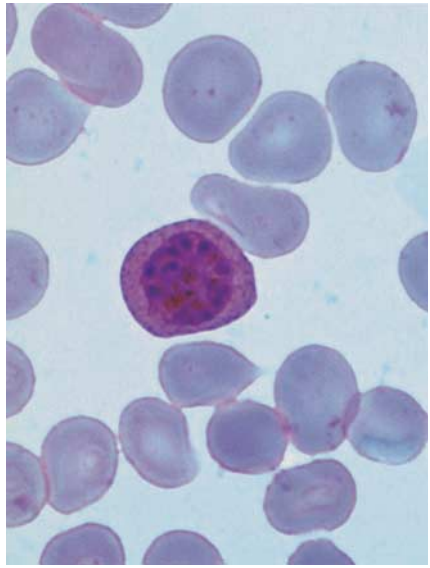

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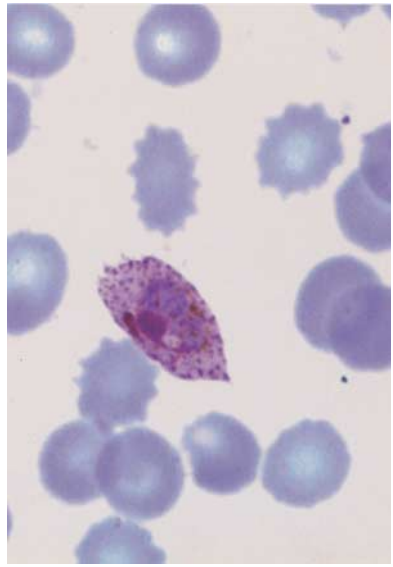

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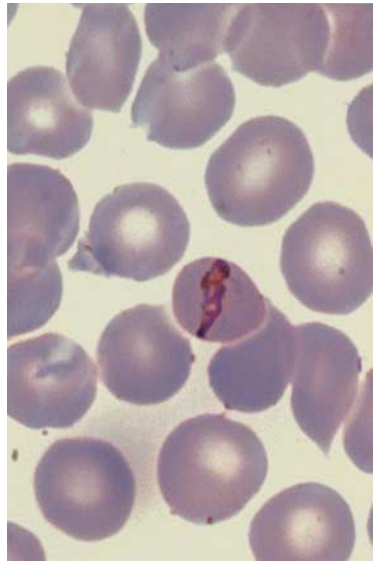

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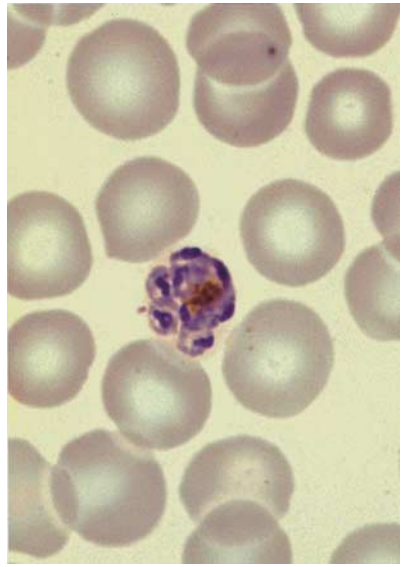

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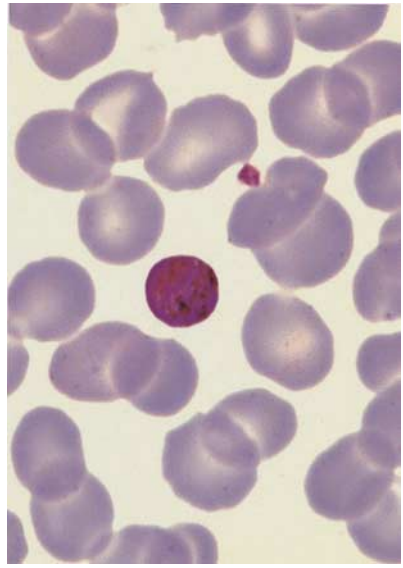

Figure 15

may be seen in the peripheral blood in the case of *P. vivax*, *ovale* and *malariae*, but generally only the ring parasites and (in older infections) the banana-like gametocytes) are found in *P. falciparum*. In infections of *P. falciparum*, a few irregularly spaced intraerythrocytic inclusions of different sizes appear, particularly noticeable in erythrocytes with more mature forms. These are termed Maurer's clefts, and should be distinguished carefully from the finer, much more numerous Schüffner's dots found on the RBC membrane in *P. vivax* and *ovale*. The dots in *P. ovale* are sometimes referred to as James's dots.

Blood which has been in anticoagulant for too long may manifest several changes (6, 3):

(1) The further development of the sexual stages may occur (even within 20 min under the right conditions) and the male microgametes released into the plasma may be mistaken for other organisms, such as *Borrelia*. They may be distinguished from *Borrelia* by the central location of the red-staining nucleus, and the absence of marked repetitive sinuous curves. Under suitable conditions of temperature and several hours' delay, fusion of male and female gametes of *P. vivax* developing *in vitro* may take place with the production of the wormlike ookinetes.
(2) If parasitized blood is left at warm laboratory temperature, reinvasion of released merozoites into red cells may take place, leading, for example, to the occurrence of appreciable numbers of "accolé" (2, 7) forms. This may lead to mistaken diagnosis of *P. falciparum* in a *P. vivax* case, since they are normally more prevalent in *P. falciparum* infections.
(3) Heavier parasitemias left for several hours may lead to a buildup of acid in the blood sample, and the serious deterioration of the already delicate parasitized erythrocytes. Those parasitized with early *P. vivax* forms may shrink or become crenated. Later stages of the parasite may become compact, and the membrane of the infected erythrocyte may become very delicate so that it stretches when the film is prepared.

Importance of Parasitemia Estimation in *P. falciparum* Malaria

An estimate of the percentage of erythrocytes infected is of immediate value to the clinician, especially in the case of *P. falciparum* infections. For example, if parasitemia exceeds 10%, exchange transfusion may be indicated in

Differential diagnosis of *Plasmodium* species of man in Giemsa-stained thin films of peripheral blood				
Species	*falciparum*	*vivax*	*ovale*	*malariae*
Ring forms	0.15–0.5 diameter of RBC which is unaltered in size: cytoplasm very fine in young rings; thick, irregular in old rings. Marginal (accole) forms, forms with two chromatin dots and multiple infections common. (Fig. 1)	0.3–0.5 diameter of RBC which is unaltered in size: cytoplasm, circle, thin.	0.3–0.5 diameter of RBC which is unaltered in size: cytoplasm circle, thicker.	0.3–0.5 diameter of RBC which is unaltered in size: cytoplasm circle, thicker.
Growing forms	RBC unaltered in size, sometimes spotted, pale, parasite compact; pigment dense brown or black mass. (Fig. 2)	RBC enlarged, stippled, parasite amoeboid, vacuolated, pigment fine and scattered, golden brown. (Figs. 5 and 6)	RBC unaltered in size or slightly enlarged; stippled, may be oval and fimbriated. Parasite compact, rounded, pigment fine brown grains. (Figs. 9 and 10)	RBC unaltered or shrunk, parasite compact, rounded or band-shaped, dark brown or black pigment, often concentrates in a line along one edge of band. (Fig. 13)
Mature schizonts	Not usually seen in peripheral blood. RBC unaltered in size, sometimes spotted, pale, parasites about 0.6 of RBC, nuclei or merozoites 8–24. Pigment clumped, black. (Fig. 3)	RBC much enlarged, stippled, parasite large filling enlarged RBC. Nuclei or merozoites 12–24, pigment, a golden-brown central loose mass. (Fig. 7)	RBC frequently oval, fimbriated, stippled. Parasite as for *P. malariae* but does not fill the RBC. Pigment brown central clump. (Fig. 11)	Parasite fills unaltered RBC completely. Nuclei or merozoites 6–12, usually 8, sometimes forming a rosette. Pigment brown or yellowish, central clump. (Fig. 14)
Gametocytes	RBC distorted, parasite crescentic. (Fig. 4)	RBC enlarged, stippled, parasite large, rounded, filling enlarged RBC. (Fig. 8)	RBC slightly enlarged, stippled, parasite round. (Fig. 12)	RBC unaltered, parasite small, round, filling RBC. (Fig. 15)
Stippling (later stages)	Maurer's clefts. (Fig. 2)	Schuffner's dots. (Figs. 5 and 6)	James's dots.	None (fine dots when overstained).

P. falciparum (see Chapters 2 and 12). In addition, the presence of late dividing forms of *P. falciparum* in the peripheral blood is associated with severe disease and indicates a poorer prognosis (7).

Follow-up parasitemia estimation will also give an indication as to response to treatment, and the parasitemia should continue to be estimated until parasites are cleared. On occasion, parasitemia may rise in the first few hours after initiation of treatment, before starting to fall. This does not denote failure of treatment, as some antimalarials are effective only in the trophozoite and early phases of parasite development, when hemoglobin digestion is at its peak.

On the thick film, the white cell count can be taken as an index of the volume of blood which has been examined. A figure of 8000 white cells per µl is commonly taken as a multiplier for field studies. If parasites are counted until 200 white cells have been seen, and the observed parasite number multiplied by 40, this will give an estimate of parasites per µl. Parasitized cell percentage is measured on the thin film. The number of infected cells per 100 red blood cells is counted. Infected cells are those which contain any number of parasites. Sufficient red blood cells are counted in an evenly spread area of the blood film, taking at least 10 fields into account. Use of a 1/9 eyepiece graticule is helpful. Fifty parasitized cells should be counted, or 1800 red blood cells overall.

The detection of gametocytes alone in the blood of a symptomatic untreated patient will give rise to the justified suspicion that an inapparent parasitemia of trophozoites is present and is an indication for treatment. Gametocytes of *P. falciparum* are not counted as part of a parasitemia estimation, and are not considered to be of any clinical significance in post-treatment films.

The re-examination of negative blood films, preferably by another experienced worker, increases the detection sensitivity (8), and in cases where the presence of infection is in doubt, it is recommended that there should be two independent examinations, each on 100 oil immersion thick film fields, to enhance the chance of detection.

Serology

Serology is not used routinely for diagnostic purposes as, although highly sensitive, it may reflect past infection. Blood films are quicker and detect active infection. However, serology (9) is valuable in the following cases:

(1) Transfusion blood screening, and the investigation of transfusion-acquired infections. Approximately 40 000 whole blood donations per year are

discarded in the UK because of suspected malaria exposure during overseas travel. A donor who has visited an endemic area more than six months previously and has not developed antibody is unlikely to be carrying a malaria infection. In the possible case of an antibody-negative donor becoming infective from newly developing dormant hypnozoites in the liver, the organism will be *P. vivax* or *ovale*, not the potentially fatal *P. falciparum*, and the infection in the recipient will be capable of radical cure with chloroquine. Serology is apparently the only satisfactory technique currently available for adequately screening transfusion blood, since an antigen or nucleic acid method would have to detect one malaria parasite in a unit of blood (see also Chapter 12).

(2) Retrospective diagnosis of a febrile illness in someone who has not been previously exposed to malaria.
(3) Investigation of "cryptic" malaria.
(4) In endemic areas, serology is invaluable for epidemiological purposes. The seroconversion rate in children between one and nine years of age is a good indication of intensity of transmission.
(5) Malaria antibody titres are useful in confirming the diagnosis of hyperreactive malarial splenomegaly (HMS) (see Chapter 10).

Source of Antigen

For serological studies, blood stage antigen is used, prepared from primate blood infections or from *P. falciparum* cultures in the laboratory. Falciparum antigen tends to cross-react with antibodies against the other species, although generally at a lower titre, so that it can be used as a general screen. The schizont stage of the blood cycle (obtained from cultures in the case of *P. falciparum*) is used for preparation of antigen, as this gives a more sensitive test. The blood or culture should be washed well with PBS before use to remove serum or plasma proteins.

For indirect fluorescent antibody test (IFAT) antigen, thick films are made on slides, dried, wrapped in tissue and stored frozen under desiccation. This antigen is very stable and will last years provided that thawing is carried out in a desiccator.

For ELISA antigen the infected erythrocytes are lysed with saponin in the presence of protease inhibitors, erythrocyte material is removed by differential centrifugation, and nonidet P40 extracts are prepared and stored frozen.

Antigen prepared for ELISA is relatively unstable and needs to be used within six months, otherwise false negativity is observed. There are problems with quality control when the ELISA plates are produced commercially.

Fluorescent Microscopy

Techniques in fluorescent microscopy can be classified into:

(1) Fluorescent staining on thick or thin films or fresh drops;
(2) Fluorescent staining on centrifugally concentrated blood.

Fluorochrome Dyes

Fluorochrome dyes such as acridine orange (AO) (10) or benzothiocarboxypurine (BCP) are used as stains which bind to nucleic acids (nuclear DNA and cytoplasmic RNA) of the parasite in the blood film. The staining can be carried out in the diluted blood drop before drying (i.e. vital stain) or on the prepared unstained film.

AO and other fluorescent stains, applied to dried films, will stain white cells in addition to malaria parasites, so the operator needs to distinguish parasites from white cell and other cellular debris. BCP applied to and examined in a fresh suspension of blood cells will stain the parasites only.

Although these techniques are reported to be as sensitive and specific for detection of malaria infection as the standard thick and thin film (especially if the parasitemia is more than 100/µl), and are quicker and easier to perform, they have some disadvantages:

(1) Species differentiation is more difficult;
(2) They require specialist equipment and supplies;
(3) The staining is unstable over time and subsequent quality control is impossible;
(4) False positives may be reported owing to the presence of Howell–Jolly bodies.

The sensitivity of these film AO techniques may fall to about 40% on malaria parasitemias below 100/µl. Specificity (for *Plasmodium* in general) is high, though training in cytology, particularly with respect to recognizing WBC debris and Howell–Jolly bodies, is important.

BCP sensitivity and specificity (11) for malaria parasite detection are reported to be in the region of 90%, otherwise the reservations above apply.

The Quantitative Buffy Coat (QBC™)

This test is the only centrifugal fluorescent staining test commercially available. Blood samples in acridine-orange-coated heparinized tubes are centrifuged and the area just below the buffy coat is examined *in situ* under a fluorescent microscope to detect the parasitized cells.

In studies in UK laboratories (12) and under field conditions (13), the sensitivity of the QBC test has been found to be about the same as for Field's stained thick film. It is still necessary to use films for determination of species, and it is recognized that the sensitivity of this test for detection of species other than *P. falciparum* is diminished. Howell–Jolly bodies (nucleic acid remnants in RBCs in megaloblastic anemia or other dyshemopoietic conditions) may give false positive results, as they do in other fluorescent-staining methods (14). If late parasite stages or gametocytes are present, they tend to localize among the highly fluorescing leucocytes of the buffy coat and are easily missed. The QBC technique gives only a rough indication of the intensity of the infection, so for proper diagnostic work thick and thin films ideally are run in parallel. The tubes do not remain readable at their most sensitive for much more than a matter of hours, so for any permanent record and for quality control, slides are necessary. Centrifuging capillary tubes of blood poses a safety hazard. However, it is easier to train workers to use the QBC than to interpret thick films. The technique is quicker than preparation, drying, staining and examination of thick films. At some locations in the UK the technique has been introduced for use on-call, because of its greater simplicity. The cost per capillary tube is in the region of £2.50. This and the cost of the additional apparatus needed probably rule out use in small rural clinics in the developing world.

Limitations of the QBC are the same as those outlined for the slide fluorescent methods (see above). In addition, accurate estimation of the parasitemia is not possible. The localization of other species and late stages of *P. falciparum* among fluorescing buffy coat cells may lead to false negatives.

Antigen Detection Methods

A monoclonal antibody of high affinity against *P. falciparum* histidine-rich protein 2 (HRPII: $AHH[AHHAAD]_2$), found in the membrane of infected erythrocytes and in the plasma of infected persons, has been used in two types

of tests specific for *P. falciparum* infection, one of which, a dipstick method (one example is ParaSight-F^{TM}) (15), is commercially available, costing £4 per test. The technique depends on the capture of the HRPII by a line of monoclonal antibody attached to a dipstick composed of nitrocellulose and fiber. A control dashed line of HRPII is also present on the dipstick to monitor the detection reagent. The bound HRPII is detected by a dye-labeled anti-HRPII polyclonal antibody. A modification using the same principle in a simpler test format has also been introduced as the ICT^{TM} test (16). In field studies the dipstick had >90% sensitivity when tested on blood containing >60 parasites/μl; positivity may sometimes persist for several days after treatment (17), and has rarely been reported in unexposed persons. More importantly, false negative results have been reported in a small number of cases even when parasitemia above 1000/μl was present. It may be that high antibody level in plasma in some samples from endemic areas is rendering the antigen inaccessible, but recent studies indicate that in some areas ∼2% of isolates may lack the HRPII gene. Therefore sensitivity may not be able to exceed 98%. For clinical purposes it cannot be assumed that a negative ParaSight-F or ICT test rules out malaria.

Nevertheless these techniques are invaluable reserve methods for the routine hematology laboratory and can be particularly useful for the detection of *P. falciparum* in mixed infections. The need for microscopic techniques is still emphasized for best laboratory practice. A negative HRPII test does not rule out a *P. falciparum* infection.

As experience in the manufacture of these immunochromatographic and dipstick tests has increased, attempts have been made to include the detection of other species, particularly *P. vivax*, in kits. A modification of the ICT is available which, as well as being anti-HRP2, contains unspecified antibodies against a component of *P. vivax*. The antivivax antibody is not as sensitive as the antifalciparum one, and this is exacerbated by the lower parasitemias seen in such infections. For routine diagnosis this system can be misleading.

A recent review (13) of alternative diagnostic techniques discusses methods based on detection of the parasite-specific lactic dehydrogenase (LDH) enzyme which are becoming available. The latest modification of these methods (OptiMAL^{TM}) detects the enzyme using specific antibody, and can detect *P. falciparum* and *P. vivax*. Sensitivity and specificity for *P. falciparum* appear the same as for the HRP2 techniques. The rheumatoid factor, which can give false positives, especially in the Parasight-F method, is a lesser problem in OptiMAL

(18). In the OptiMal dipstick system the manufacturer states that *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* LDH are detected by two antibodies—a falciparum-specific antibody and a pan-specific antibody. It claims that a positive reaction with the latter and not the former indicates *P. vivax* (or *ovale* or *malariae*). This claim seems likely to be invalid, since both antibodies react well with *P. falciparum*, and according to a recent study (19) the pan-specific antibody detected low *P. falciparum* parasitemia when the falciparum-specific antibody did not react. These authors report seven instances of a single band, indicating *P.vivax* and not *P. falciparum*, in confirmed *P. falciparum* infection. It appears that reaction with the pan-specific band indicates any or all of the four species, while reaction with both bands indicates a *falciparum* infection with or without any or all of the three other species.

Although improvements are in progress, species diagnosis except for *P. falciparum* is still not adequately addressed by any of the antigen tests to date.

Advantages

The advantages of antigen tests include:

(1) Minimal staff training is required.
(2) No laboratory or special equipment is required.
(3) Only a drop of blood is needed.
(4) A result is obtained quickly (10–15 min).
(5) Detection limits are usually comparable with, and occasionally superior to, those of average thick film microscopy. This means that they are a valuable backup in laboratories relying mainly on microscopy.
(6) The result can usually be reviewed at a later date if the dipsticks are carefully stored.
(7) Presence of *P. falciparum* gametocytes (of no clinical interest in treated patients) is claimed to produce negative results.
(8) Parasite lactic dehydrogenase (LDH), produced by all species of *Plasmodium*, detected by the OptiMal test, apparently ceases to be detectable by its enzymatic activity on the death of the parasite. It has been reported that this is also true (20) for the OptiMal test, relying on antigen detection. This means that the test may be used as an indicator of cure, compared to tests based on HRPII, which may remain positive after parasites have cleared from the blood.

Disadvantages

(1) Kits detecting the HRP2 antigen (produced by *P. falciparum* only) are more or less susceptible to false positives with rheumatoid factor (RF). In the Parasight-F test, up to 60% of uninfected RF sera reacted (18). Use of a first antibody which is an IgM (as in the ICT test) reduces the problem.
(2) Antigenemia with HRP2 may persist for 7–14 days after clearance, rendering use as a test of cure unreliable.
(3) Storage of reagents in the ICT system needs to be at 2–8°C.
(4) HRP2 tests may occasionally (up to 2%) be negative in the presence of higher (500–1000/μl) *P. falciparum* parasitemias (~0.02%). There are indications that the HRP2 gene may be lacking or abnormal in these strains.
(5) The OptiMal dipstick system does not behave as the manufacturer claims with respect to diagnosis of species other than *P. falciparum*, and following the manufacturer's instructions closely could lead to missing a parasitemia with this species.
(6) These tests are not capable of an accurate estimate of parasitemia.

Comment

Antigen test methods can be used for the diagnosis of malaria in areas where microscopy is not available, and for urgent malaria diagnosis at night and at weekends, when routine laboratories are closed, and on-call microscopists may be inexperienced in interpretation of malaria slides. However, confirmation, where possible, by standard slide techniques is advisable.

In developing country situations, particularly in epidemic malaria areas, where microscopy is not possible owing to lack of trained staff and equipment, these tests give a significant improvement in diagnosis and are therefore being encouraged by the WHO (21), and in some cases they are available at a reduced price from the manufacturers.

Nucleic Acid Methods

Species Determination

The use of relatively insensitive, though specific, nucleic acid probe methods for diagnosis has apparently fallen out of favor as polymerase chain reaction

(PCR) techniques have proliferated. Although quantitative PCR methods have been described, no extensive malaria studies have been carried out. The most widely used method for epidemiology and clinical research work has been the nested PCR for specific sequences of the small subunit ribosomal DNA devised by Snounou (22). Studies on preparation of samples for diagnostic PCR indicate that use of dried blood spots on glass fiber membrane is very simple (23) and the sensitivity is excellent, though use of filter paper (24) evidently reduces sensitivity. PCR has been regularly used successfully as a gold standard method superior in sensitivity to all other tests, though there is evidence for some intraspecific variation in the blood stage small subunit ribosomal gene in *P. ovale* in S.E. Asia (25).

Drug Resistance Detection

Resistance to mefloquine and chloroquine. Resistance to mefloquine is associated with amplification of the *Pfmdr1* gene (chromosome 5) specifying the multidrug resistance protein PGH-1, in laboratory and field observations. Appreciable resistance is also seen where an unamplified wild-type *Pfmdr1* is present (mammalian stages of *Plasmodium* are haploid). In such cases, the presence of one or more specific mutations in *Pfmdr1* restores mefloquine sensitivity (26), whilst enhancing chloroquine resistance (27) in strains already carrying mutations on chromosome 7 (*crt* gene) (28, 30). Resistance to endoperoxide artemisinin derivatives is mediated by the same changes in the PGH-1 protein as for mefloquine, although the endoperoxides are so active that resistance is not yet a clinical problem.

Pfdhps *and* Pfdhfr *and sulphonamide/antifol combinations*. Resistance to sulphonamides is associated with mutations in the *dhps* gene (chromosome 8) and to antifols such as pyrimethamine and cycloguanil, the active metabolite of proguanil, with mutations in the *dhfr* gene (chromosome 4). Mutations in both genes are responsible for resistance to sulfadoxine/pyrimethamine, the second line treatment drug combination widely used in chloroquine-resistance in Africa.

PCR-RFLP protocols are available for analysis of blood spot samples to detect these mutations (29).

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Chapter 2

Definition, Epidemiology, Diagnosis and Management of the Anemia of Malaria

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Introduction

Malaria is a major cause of morbidity worldwide, with an estimated 250 million cases a year and between 1 and 2 million deaths (1). Conventionally, the major cause of death in malaria has been attributed to the syndrome of cerebral malaria (CM) and its acute accompaniments, e.g. acute respiratory distress and organ failure. Many of the earlier publications on malarial anemia have emphasized pathogenesis but it is only in the last decade that the impact of anemia on morbidity and mortality has been highlighted. Until then little emphasis was placed on the clinical aspects of malarial anemia, an inevitable consequence of malaria of any severity including asymptomatic infected individuals, those with acute disease, and also cases of chronic persisting infections. Such anemia may result in major morbidity. Severe malarial anemia with hypoxia, acidosis and cardiac failure may indeed lead to considerable—hitherto neglected and often unrecognized—mortality. Chronic anemia due to malaria may additionally lead to profound consequences on pregnancy, on birthweight, the growth and development of children, and on the economic productivity of adults in endemic areas. In this chapter the epidemiology, clinical aspects, diagnosis and management of malarial anemia will be discussed.

Definition

A robust definition of anemia due to malaria is elusive. In the majority of malarial infections, the hemoglobin will fall to some degree. Clearly, any hemoglobin that falls below the normal range (conventionally accepted as two

standard deviations below the mean) for age, sex and state of pregnancy in a given population is regarded as abnormal. However, in many populations where malaria is endemic, an accurate reference range is not known. Throughout much of the tropical world the hemoglobin concentration in both sexes and at all ages is certainly lower by about 2 g/dl than that found in temperate non-malarious areas (2). Anemia appears to be an inevitable consequence of malaria and the degree of anemia related to cumulated parasite densities (3). Anemia may occur in the absence of symptoms: Ghanaian schoolchildren with asymptomatic but patent parasitemia had significantly lower hemoglobins than those who remained parasite-free (12.3 vs 11.4 g/dl) (4). Thus, malaria needs to be considered in any anemic patient living in a malaria-endemic area.

One arbitrary definition of malarial anemia in such a setting would be a hemoglobin less than 8 g/dl, which is equivalent to a hematocrit of less than 24% in a parasitemic individual (5). Attribution of low hemoglobin solely to malaria is, however, even more difficult because of the multiple other coexisting causes of anemia that occur in these areas. The presence of peripheral parasitemia by itself cannot implicate malaria as the sole contributory factor. Therefore some definitions have incorporated a minimal parasite density, such as 10 000/µl, to define malarial anemia (6). However, such definitions have mainly been devised for purposes of standardization in comparative studies rather than for purposes of clinical practice.

A definition of severe malarial anemia (SMA) is less problematical. The World Health Organization has defined SMA as a hemoglobin less than 5 g/dL or a hematocrit less than 15% seen in the context of malaria but without specifying parasitemia (7). Microcytosis does not exclude the diagnosis of SMA. Thus any hemoglobin level must be considered in context, taking into account the geography and circumstances in which it is detected. Attribution of anemia to malaria can often only be confirmed by a positive response to specific antimalarial treatment which is not included in any of the case definitions, nor is it of practical use when faced with an acutely anemic patient.

Determinants of the Epidemiology of Malarial Anemia

The prevalence and/or the severity of the anemia in a malaria-endemic area are determined by a number of interacting factors. These include, amongst others:

(1) The species of infecting parasite,
(2) The intensity of transmission (endemicity) and the age and pregnancy state of the host,
(3) Associated host genetic factors, and
(4) Causes of anemia other than malaria.

Malaria is the most common cause of severe anemia in endemic areas. Malaria control measures resulting in a rise in hemoglobin levels confirm this association (8), as well as the observation of more severe anemia at the end of a high malarial transmission season (9).

Parasite Species

Severe anemia is most commonly seen after falciparum infections, followed by that due to *P. vivax*. In pregnancy *P. vivax* was shown to be associated with anemia (hematocrit <30% at any stage) but not as severe as that of *P. falciparum* (10). Severe anemia is less common following attacks caused by malaria due to *P. ovale* and *P. malariae*.

Malarial Endemicity, Age of the Host, and Pregnancy

The endemicity of malaria is important in determining the relative incidence and patterns of complications associated with malaria as well as the age at which these manifestations occur. Malarial endemicity has been defined in terms of whether transmission is continuous and intense (holoendemic), intense but seasonal (hyperendemic), intermittent (hypoendemic) or occasional (epidemic). Endemicity can also be defined according to the spleen rates in children and adults, with high spleen rates being observed in areas of highest transmission. In addition to the above-defined patterns of endemicity, malaria may be either stable (i.e. high transmission without fluctuation over the years) or unstable, where transmission may vary from year to year. Finally, malaria may be observed in the previously unexposed nonimmune child or adult, as seen in travelers or migrants.

In areas of stable malaria, infection can occur during the first few months of life (11), but the major burden of disease is borne by children aged between six months and five years, depending on the intensity of transmission. The majority of these children will not die from malaria, but may recover either

spontaneously or with treatment. A relatively small but significant percentage will develop life-threatening disease and the risk of death. With repeated infections these children eventually develop a degree of immunity which leads to only relatively mild attacks during adolescence and adult life. On the other hand, in areas with unstable malaria, immunity takes longer to develop; acute and severe disease is largely observed in older children and even adults. Finally, in those individuals without previous exposure to malaria, such as travelers, expatriates and migrants, severe acute disease may be seen in any age group.

The relative incidence of malarial anemia in relation to other severe manifestations of malaria appears to vary according to these transmission patterns. The more intense the transmission, the earlier and more frequent SMA occurs. In areas of intense transmission, malaria may not be that infrequent in infants—24% of 446 infants were found to be infected in the first six months of life (11). However, under the most intense transmission conditions, the risks of SMA (and CM) might decrease, presumably because of the earlier acquisition of immunity in those exposed (12).

The intensity of transmission also appears to determine the ratio of SMA to CM seen: the more intense the transmission, the greater the number of SMA cases. In four large studies of hospital admissions with severe malaria, the ratio of SMA to CM varied between 2 : 1 and 1 : 3 (13–16) (Table 1). High ratios (2 : 1) of SMA to CM were found in the rural areas with continual transmission but lower ratios were observed in areas of more intermittent seasonal malaria, such as in an urban setting (16). There may well be other simple explanations for the differences in the ratio of SMA to CM, such as patient referral patterns to hospital, criteria for admission, etc. The reasons for the inverse relationship between SMA and CM are unclear.

Although these age-related differences in morbidity patterns can be explained in terms of the intensity of transmission, age as an independent factor may also play a part in the pattern of the disease. SMA is most often observed in very young children between six months and two years of age living in hyperendemic areas. In a case control study of children with severe malaria in The Gambia, the median age was 1.8 years for those with SMA and 3.6 years for those with CM (13). These differences are not due to lack of earlier exposure to malaria, as shown by the lack of difference of various antimalarial antibody titres in those with severe malaria and those with mild malaria (17), indicating that other factors related to age are involved.

Table 1. The relative incidence of severe malaria anemia (SMA) and cerebral malaria (CM) in different study populations of varying malaria transmission intensity.

	SMA (%)	CM (%)	Ratio	Reference
Gambia, West Africa	34	66	0.5 : 1	13
Kilifi, coastal Kenya	25	12	2 : 1	14
Rural Burkina Faso	47.4	28.9	1.6 : 1	19
Urban Burkina Faso	14.8	53.6	0.3 : 1	19
Rural Zambia	9.5	4.6	2 : 1	16

The complex relationship between the transmission pattern on the one hand, and the age and patterns of morbidity and mortality from malaria on the other, is clearly demonstrated in a study carried out in district hospitals in two contrasting transmission settings in Africa (14). One was in Kilifi, Kenya, with seasonal transmission, and the other in Ifakara, Tanzania, with sustained transmission. There were similar minimum annual rates of severe malaria in children below five years—4.6% in Kilifi and 5.1% in Ifakara. However, there were differences in the age and clinical patterns of disease, with twice as many patients under the age of one year with severe disease (mainly SMA) in Ifakara with a high transmission area compared to Kilifi. There was a fourfold higher rate of CM and a threefold lower rate of SMA in Kilifi compared with Ifakara (18). In another study from Burkina Faso, the mean age of children who died of complications of malaria was significantly higher (4.8 vs 2.2 years) in an urban area where the transmission was low, compared to a rural area with a 20-fold higher transmission rate (19).

Amongst pregnant women, malaria is the main cause of anemia, especially in primigravidae but also in multigravidae (20–22).

Associated Host Genetic Factors

Many red cell polymorphisms, such as the hemoglobin variants S, C and E, thalassemias, glucose-6-phosphate (G6PD) dehydrogenase deficiency and Melanesian ovalocytosis, are common in areas that are or have been endemic for malaria (see Chapter 9). Much epidemiological and cellular evidence has accrued over the years to support the hypothesis that these variants provide

relative protection against malaria. Supportive data has largely focused on parasite rates, densities and clinical disease initially, but more recently (and importantly) on severe disease and mortality. Only a few of these studies have examined the relationship of these variants to the development of malarial anemia. In a large case-controlled study in The Gambia, the frequency of the hemoglobin AS phenotype was ~1% in children with severe malaria (CM and SMA) and 13% in controls with mild diseases other than malaria, indicating a more than 90% protection from severe malaria (13). Data for the gene frequency in patients with SMA alone was not given. In Gambian children with malarial anemia (Hb < 8 g/dl) the prevalence of the sickle cell trait was 2%, significantly lower than the 13% in control children without malaria (Abdalla, unpublished observations).

The case-controlled study in The Gambia by Hill and colleagues also showed that a common West African HLA Class I antigen (B53) was associated with protection (of the order of 40%) against both SMA and CM (13). A novel Class II haplotype (DRB1*1302-DQB1*0501) was found to provide approximately 55% protection against SMA, but not against CM. Both of these Class I and II genotypes were found more frequently in Gambians and in sub-Saharan Africa compared to other racial groups in nonmalarious areas, which would further argue that natural selection by malaria has been responsible. However, the molecular mechanisms by which these polymorphisms provide protection against anemia remain to be clarified. Perhaps protection against the intensity of infection is sufficient. One would presume that since red cells express minimal HLA class I molecules on their surface, protection by the Class I genotype is probably against liver stage parasites, whereas for Class II it would reside with the ability to present blood stage antigen(s) to helper T cells.

Other Nonmalarial Causes of Anemia

In all cases of malarial anemia, especially where hemoglobin levels are reduced at the onset of the illness and where hemoglobin levels fail to recover after specific antimalarial treatment, causes other than malaria should be considered. These would include common bacterial infections such as bacteremia, pneumonia and urinary tract infection, as well as hookworm infestation and HIV infection. Underlying hemoglobinopathies, glucose-6-phosphate dehydrogenase (G6PD) deficiency and hematinic deficiencies also need consideration.

In Kilifi in coastal Kenya, 101 children with severe anemia (Hb 5 g/dl or less) were studied. Malarial anemia was defined in patients with hemoglobin <5 g/dl, with no evidence of hematinic deficiencies or hemoglobinopathy, and a response to antimalarials alone, with a rise in hemoglobin of at least 1 g/dl per week. Although falciparum malaria was the primary cause of severe anemia in 46% of cases, iron deficiency appeared to be responsible in 17%, sickle cell anemia in 10% and undetermined causes in 21% (which might well have been related to malaria) (23). A notable aspect of this study was the difficulty in defining the contribution of malaria to severe anemia where many children had asymptomatic parasitemia. The median age in the malarial anemia group was about two years, but over four years in the group with severe anemia due to other causes. Whereas SMA occurs particularly in very young children living in holoendemic areas, in children over the age of three, hookworm infestation, malnutrition and the anemia of chronic disease were found to be other important contributing factors (24).

Morbidity and Mortality of Severe Malarial Anemia

Morbidity caused by malarial anemia is difficult to quantify but is probably considerable. In The Gambia many children aged 1–4 years were anemic, which was primarily associated with malaria parasitemia, but varied from place to place in villages only a few miles apart, from season to season and from year to year (2, 25). The association of asexual parasitemia, a fall in hemoglobin coincident with the rainy season, and the lower hemoglobin levels observed in children with splenic enlargement implicate malaria as an important cause of anemia in this part of the world.

Although many studies have been able to quantitate overall malarial mortality in children (25, 26), the contribution of SMA to this mortality is more difficult. The mortality of SMA has been estimated to be between 6% (27) and 16% (28), and even as high as 30% (29). More than 50% of SMA deaths occurred within 24 h of admission to hospital (30). SMA is often multifactorial and may be associated with other serious complications (e.g. CM, RD and renal failure) that contribute to severity and hence mortality. In addition, in many anemic patients, peripheral parasitemia may be scanty and, in some cases, absent. In a number of children in Africa who died shortly after admission to hospital and in whom a clinical diagnosis of "pneumonia" had been made because of

apparent RD, hemoglobin estimation and blood film examination indicated that malarial anemia, leading to respiratory distress or cardiac failure, was a far more likely cause of death (31). These observations require confirmation, but highlight the difficulty of making an accurate clinical diagnosis of malarial anemia as a cause of severe disease. However, it would appear that the mortality from SMA is higher than the equivalent degree of anemia due to other causes (32). In a study of severe anemia (Hb $< 5\,g/dl$) in Kenya, the mortality rate in SMA was 8.6% compared to 3.6% in anemia with other causes (23).

Although the mortality rate of SMA may be lower than that of CM and other severe malarial complications, its frequency in areas of high malarial transmission makes it an important overall cause of mortality in malaria. The overall mortality of SMA in certain settings may even equal, if not exceed, that of CM. In children with malaria admitted to two hospitals in Malawi—one in the capital, Blantyre, where malaria transmission is seasonal, and the other in an area of more sustained transmission (Mangochi District Hospital)—malaria-attributed death was similar (about 20 vs 18%, respectively). However, malaria-associated mortality due to anemia at the two sites was contrastingly and significantly different (32 vs 54%), with malaria-associated anemia significantly higher in the rural area of more sustained transmission (33). In a rural hospital in southern Zambia with a stable pattern of malaria transmission, the incidence of SMA was about 10% and CM 5% (i.e. 2 : 1) of pediatric admissions, with 1.5% of patients suffering from both. The case fatality rates were 0.189 in CM compared to about half (0.088) in SMA, but because of the relative incidence the two causes contributed equally to overall mortality (16). These studies establish SMA as an important cause of malarial mortality.

It is important to recognize the relative importance of malarial anemia in morbidity and mortality when it occurs together with the other manifestations of severe malaria. In another, more detailed hospital-based study in Kenya, 28% of 1844 pediatric admissions with severe manifestations of malaria had SMA. The mortality ascribed to SMA alone was found to be 1.3%, whereas the mortality rate was 6% in severely anemic children with CM, 16% in those with RD and 34.7% in those with all three complications (28). It is thus important to recognize these different clinical entities and target treatment appropriately.

The Natural History of Malarial Anemia

The progression of anemia in the context of malaria is variable, but two major clinical patterns emerge, which may well be considered to be the extremes of a continuous spectrum.

(1) In the first, patients suffering a severe acute malarial attack and seen early after the onset of the clinical symptoms may not be initially anemic. However, anemia may develop rapidly during the course of infection, its complications and aftermath, especially if there is initial hyperparasitemia (34). Three phases in the fall of the hematocrit during treatment of acute malaria in the setting of severe acute disease are identifiable. The first results from rehydration, the second correlates with the fall in parasitemia and the third relates to the loss of uninfected red cells (35). The fall in hematocrit may occur for four or five days after initiation of treatment (5), and this fall may well continue after the peripheral blood parasitemia has cleared (36). In this last-mentioned study a brisk reticulocyte response was only observed well after the parasites were cleared: if parasite clearance was delayed, the reticulocyte count did not rise despite a sustained fall in hematocrit. Such a delay in reticulocyte response associated with persistent parasitemia would suggest that the parasites themselves are in some way responsible for the inhibition of erythropoiesis or for the suppression of new red cell release into the peripheral circulation.
(2) The second pattern of development of malarial anemia is observed especially in children living in endemic areas who are found to be anemic when first seen (5). The clinical history is usually one of intermittent low grade fever or general symptoms of ill health during the preceding weeks rather than days. On examination, splenomegaly of varying degrees is usually present and the peripheral blood film shows scanty asexual parasitemia. In many cases gametocytemia and malarial pigment are present in phagocytic cells seen on peripheral blood smear. Attributing malaria as the sole cause of anemia in this setting is often difficult, and may be due to the cumulative effect of a number of factors.

Between these extremes a third intermediate group may be seen, in which an already anemic patient with chronic low grade malaria infection suffers acute exacerbation. The clinical presentation of malarial anemia will therefore be very much dictated by these clinical settings.

Clinical Features of the Anemia of Malaria

In acute malaria the clinical features of anemia are often obscured by other, more dramatic manifestations of severe malaria, such as alteration in consciousness level, hypoglycemia, renal failure, etc. The usual symptoms of anemia, namely tiredness, listlessness and breathlessness, are hardly applicable in these severely ill patients. Congestive cardiac failure as a result of anemia is particularly difficult to recognize in young children with severe disease. Some features, such as a tachycardia, a gallop rhythm, a systolic murmur, basal pulmonary crepitations or evidence of right heart failure with a raised jugulovenous pulse, hepatomegaly and ankle edema with ascites, may be present, but could be ascribed to other complications such as a respiratory infection or fluid overload. It is also important to distinguish between cardiac compromise and respiratory distress (RD), another very important and recently identified manifestation of severe disease which may be often associated with lactic acidosis (31). In such respiratory distress there may be flaring of the alar nasae, intercostal or subcostal recession, deep labored (Kussmaul's) breathing and forced expiration (grunting).

On the other hand, children with chronic malaria and severe anemia in areas of high endemicity may be surprisingly asymptomatic. They may be listless or lethargic but these children seldom appear extremely unwell and a low hemoglobin level may be found coincidentally (37). In some cases both malaria and anemia are asymptomatic, as shown in a study of Ghanaian school children aged 5–15 years, where those with asymptomatic malaria had significantly lower hemoglobin levels (11.4 vs 12.3 g/dl) (4). Although these children may present to clinics, population surveys demonstrate that many children with malaria and varying degrees of anemia are entirely asymptomatic and may be found in the community (3).

Simple clinical correlates of anemia have been used to attempt to diagnose malarial anemia in at-risk populations where resources are limited. The most commonly used are skin (palmar and nailbed) and mucous membrane (conjunctival) pallor, and organomegaly (spleen and liver). In an evaluation of clinical methods to identify moderate and severe anemia, palmar and nailbed pallor had a sensitivity of 95% and conjunctival pallor 84% in detecting a hemoglobin of less than 8 g/dl (38). Identification of a systolic murmur, altered sensorium, splenomegaly or malarial parasitemia was independently predictive of moderate or severe anemia. In a study in Zambia, pallor (assessed by examining

the mucous membranes, palms and nailbed to verify anemia) correlated significantly with measured hemoglobin levels. Retrospective analysis showed that there was a significantly higher mortality in those with CM and pallor (17.6%) as compared to CM without pallor (7.2%). The authors argued that pallor could be used to highlight those with a poorer prognosis and target these patients for earlier and more intensive treatment, where the capability to measure hemoglobin was not available (39). In a recent study on the western Thai border, where the transmission of malaria is lower, the risk factors for malarial (falciparum) anemia (hematocrit less than 30%) were: (1) age less than 5 years, (2) a palpable liver, (3) a palpable spleen, (4) recrudescent infection, (5) being female, (6) a prolonged (> 2 days) history of illness before admission, and (7) pure *P. falciparum*, rather than mixed infection (40). Of interest was, the observation that *P. vivax* malaria appeared to attenuate the anemia due to *P. falciparum*, presumably by modifying the severity of disease.

The above studies also highlight the almost universal presence of hepatosplenomegaly in areas of high malarial endemicity. In a study comparing children protected with antimalarials from birth versus unprotected controls, only about 13% and 6% of protected patients had mild splenomegaly and hepatomegaly respectively, compared to 100% with hepatosplenomegaly, sometimes of considerable size in the unprotected group who also had significantly lower hemoglobin levels (34). In a more recent study, comparison was made between liver and spleen sizes in children with malarial anemia and other causes of anemia. The results showed that the presence or absence of organomegaly does not appear to confirm or exclude malaria as the main cause of anemia with confidence (23). Thus, although certain clinical symptoms and signs of malarial anemia might be helpful in raising the suspicion of anemia, confirmation still requires the accurate measurement of the hemoglobin concentration.

Diagnostic Aspects of the Anemic Patient with Malaria

The diagnosis of malaria is fully discussed in Chapter 1, being mainly based on the thick and thin blood films, amongst other, more recently developed methods. The basic diagnostic tests required in assessment of anemia of malaria should be the examination of a thick film to diagnose malaria, quantitation of parasitemia (by either thick or thin film), and hemoglobin estimation. Other

relatively simple tests that may be helpful include a packed cell volume (PCV), which can be performed using a microhematocrit centrifuge, and a reticulocyte count. The hemoglobin measurement (in g/dl) divided by the hematocrit gives the mean cell hemoglobin (MCH—average 30 pg/red cell), which if low, may indicate whether there is a coexistent microcytic anemia.

A reticulocyte count is especially useful in severe anemia during followup after antimalarial treatment. A brisk reticulocytosis will predict a rapid recovery from anemia. A blood film is useful to look for changes in the red cells such as microcytosis and hypochromia which may be associated with microcytic anemias, or macrocytosis and hypersegmented neutrophils seen in megaloblastic anemias. Such changes might suggest other causes of anemia. Other biochemical and hematological tests, such as liver and renal function, tests for hemolysis and hematinic deficiency, may indicate disease severity or other causes of anemia and may be helpful but are not always available.

Blood Film Appearances

The nonparasitized red cells are usually normocytic and normochromic in patients with falciparum malaria. Spherocytes and red cell fragmentation were not found on blood film examination in two detailed studies and are not common findings (5, 36), despite the report that deparasitization (pitting) of cells may occur *in vivo* (41) (see also Chapter 3). Depending on the parasitemia and duration of infection, there may be excessive pigment in both neutrophils and macrophages, which may indicate a poorer prognosis (42). A raised neutrophil count appears to be an indicator of poor prognosis (43) and may, but not always, indicate coexisting bacterial infection (44). Occasionally there may be phagocytosis of both parasitized and nonparasitized cells by monocytes (see Chapter 5). In the nonimmune patient, a mild to moderate degree of thrombocytopenia was found in 85% of acute malaria due to *P. falciparum* and in 72% of those due to *P. vivax* (45), but may be less frequent in immune and semiimmune populations. However, despite these low platelet counts there are rarely any hemorrhagic manifestations associated with this thrombocytopenia (see Chapter 8).

In "chronic" malaria the red cells often show anisocytosis and poikilocytosis, sometimes with macrocytosis and polychromasia indicating a reticulocytosis (5). In vivax malaria the blood film appearances are usually similar to those in acute falciparum malaria but the anemia is seldom as severe (10).

Reticulocyte Counts

The reticulocyte count is a useful reflection of erythroid activity in response to anemia. The absolute reticulocyte count (percentage reticulocytes multiplied by the red cell count) is a better reflection than the percentage reticulocytocyte count as to whether the erythroid response is appropriate to the degree of anemia. A normal figure would be about 100×10^9/L. In acute malaria the reticulocyte count is usually not raised, and can be low, reflecting bone marrow suppression. The lack of reticulocytosis often persists for a few days after parasites have been cleared, whereupon a brisk response ensues (36). In patients with chronic malaria the percentage reticulocyte count can be normal or raised (5). However, the absolute reticulocyte count is often low or normal and therefore inappropriate to the degree of anemia. Such relative reticulocytopenia has consistently been observed since the 1940s (5, 36, 46–48). In the presence of dyserythropoiesis and ineffective erythropoiesis, there is intramedullary cell death. The peripheral reticulocyte count may therefore be much lower than expected and does not reflect the extent of erythroid hyperplasia.

Reticulocytosis following treatment of malaria is usually a sign of effective parasite clearance—a brisk reticulocytosis is rare in the presence of parasites. The reticulocyte count in acute malaria took five days after treatment to start rising, whilst that in chronic malaria usually starts to rise after three days of initiating effective antimalarial treatment, reaching a peak after about seven days, and absolute reticulocyte counts in excess of 1000×10^9/l (normal up to 100×10^9/l) have occasionally been observed (5). Such a reticulocyte response is rapidly followed by a rise in hemoglobin. Dyserythropoiesis and erythrophagocytosis can persist much longer into convalescence after parasites have been cleared (36). Anemia (<13 g/dl in males and <12 g/dl in females) persisted in 50% of Thai patients with malaria due to *P. falciparum* for up to 28 days (49). This persistent anemia appeared to be due to suppressed erythropoeisis and was related to the degree of hepatic dysfunction. In vivax malaria, the reticulocyte count was within normal levels and not appropriately raised on admission, despite moderate anemia and presumably because of the presence of parasites (50).

The reticulocyte count and the clinical state of the patient thus determine how quickly a patient with malaria is expected to recover. Additional factors such as septicemia, drugs and hematinic deficiency may delay recovery by suppressing erythropoiesis. Prolonged suppression of the reticulocyte response may assist in the decision as to whether transfusion should be given or withheld in severely anemic patients (see below).

Serum Bilirubin

The bilirubin level, especially unconjugated (indirect) bilirubin in serum or plasma, is a good indication of the degree of hemolysis in malaria patients with otherwise normal liver function tests. Bilirubin levels may also be mildly elevated in patients with ineffective erythropoiesis because of increased intramedullary destruction of erythroblasts, as is seen in patients with severe megaloblastic anemias. Mild jaundice and hyperbilirubinemia without bilirubinuria (acholuric jaundice) are commonly observed in malaria and are indicative of hemolysis. Occasionally, deeper jaundice with higher levels of bilirubin and bilirubin excretion in urine and liver enzyme abnormalities are suggestive of hepatocellular damage. This is seen in more severe cases of malaria with liver involvement and especially in blackwater fever (51).

The mean bilirubin levels were found to be about four times the upper limit of normal in CM and twice the upper normal limit in uncomplicated disease in nonimmune, predominantly adult patients in Thailand (36). In Gambian children the mean bilirubin levels were 2.2, 2.7 and 1.3 times the upper limit of the normal levels in children with acute, acute on chronic and chronic malaria respectively (5).

Haptoglobins

Haptoglobin is an acute phase protein made up of 2α and 2β subunits produced by hepatocytes but also by adipose tissue and the lung. One of the main functions of haptoglobin is to bind to free hemoglobin which is cleared from the plasma with a half-life of 10–30 min. When large amounts of the haptoglobin/hemoglobin complex are formed, the haptoglobin falls. Therefore the measurement of haptoglobin is of some use in diagnosing the presence of both intravascular and extravascular hemolysis in malaria.

The haptoglobin/hemoglobin complex is taken up by macrophages so that the heme iron is recycled. There are three major haptoglobin genotypes: Hp 1-1, Hp 2-1 and Hp 2.2. The polymorphism is caused by changes in the β subunit. It appears that the different haptoglobin phenotypes lead to different expressions of haptoglobin. The reference range for serum haptoglobin was found to be significantly lower for those individuals with types 2-1 and 2-2 than for those with type 1-1. Moreover, the frequency of individuals with the Hp 0-0 phenotype decreased by 32%, and similar rises in the proportion of individuals with types

2-2 and 2-1 occurred when sera were retyped using high performance permeation chromatography gel instead of the less sensitive technique of starch gel electrophoresis.

A high proportion (20–40%) of individuals living in malarious areas were found to have ahaptoglobinemia, which was originally thought to be a genetic variant (52). However, later studies suggested that malaria may be the main cause of ahaptoglobinemia (53), which was later confirmed by the reversal of ahaptoglobinemia by effective malaria prophylaxis within 2–8 weeks (54), and by the correlation between malaria endemicity and the frequency of ahaptoglobinemia in the Congo (55). Another study found that subjects with ahaptoglobinemia had no major abnormalities in the haptoglobin genes, but that ahaptoglobinemia was associated with high antimalarial antibody titres (56).

The return of serum haptoglobin to normal and the decrease in numbers of subjects with ahaptoglobinemia have been used as measures of the effectiveness of malaria transmission control by insecticide-treated bed nets (57). Together with CRP, haptoglobin was found to be more reliable than fever or parasitemia as an indicator of clinical malaria in another community-based study (58).

It is assumed that ahaptoglobinemia in areas of high malaria transmission results from low grade continuous intravascular hemolysis caused by asymptomatic parasitemia (54). This explanation may be an oversimplification, as ahaptoglobinemia occurs in areas of high malaria endemicity in adults as well as some children who have no or very few circulating parasites. Ahaptoglobinemia does not always correlate with malariometric measures such as parasitemia or splenomegaly (53, 59). Given that haptoglobin is an acute phase protein, its synthesis should be increased in acute malaria; however, it often takes prolonged periods of time (2–8 weeks) for levels to return to normal after successful treatment of malaria (5, 36). Although the epidemiological evidence strongly favors malaria as the cause of ahaptoglobinemia, the explanation that this is caused by low grade intravascular hemolysis remains to be confirmed.

Serum Ferritin

High serum ferritin concentrations have been found in Asian (36), African (23) and Melanesian (60) patients with malaria. The mechanism leading to these high levels is likely to be complex. Ferritin is an acute phase protein. Thus

the acute phase response in malarial infection, hepatic damage and possible defective ferritin clearance may all contribute. This means that serum ferritin concentration, as a measure of iron stores, must be interpreted carefully in malarious regions, particularly in the context of acute disease (see Chapter 4) but also in aymptomatic malarial parasitemia (61).

Folate

Serum/plasma folate is labile, and a more accurate method of assessing storage folate is to measure red cell folate. Red cell folate is in the form of polyglutamates and is acquired or synthesized during erythropoiesis so that mature erythrocytes do not acquire further folate. Folate deficiency appears to be much less of a problem in Africa than other hematinic deficiencies. Low serum folate was found in only 6% of adolescent Nigerian girls (62). Moreover, in two large-scale studies, B12 deficiency was much more common a cause of megaloblastic anemia than folate deficiency in Zimbabwe (63) and in Kenya (64). In Zairean women, folate deficiency was found to be associated with only 5% of anemias at delivery (65). However, folate deficiency may be a more serious problem in the tropics during pregnancy (66) and in sickle cell disease.

Although 20% of anemias in a study in Benin were associated with folate deficiency, there was no correlation between the deficiency and malaria infection (67). Folate deficiency was rare in Gambian children with malarial anemia (68). It does not therefore appear to be a major contributory factor in the anemia of malaria (see also Chapter 4).

Bone Marrow Appearances

The bone marrow in acute falciparum malaria may be hypocellular, normocellular or hypercellular in nonimmune adults and children with malaria (36, 69, 70). The major changes appear to be a reduction of erythropoiesis which may be absolute or relative, depending on the overall cellularity, sometimes with an increased proportion of lymphocytes, especially in children living in an endemic area (5, 70).

In chronic malaria the bone marrow is often extremely hypercellular, with erythroid hyperplasia demonstrating dyserythropoiesis with functional ineffective erythropoiesis, as indicated by an inadequate reticulocyte response (5)

and perturbation of erythroblast cell cycle kinetics (71). Detailed descriptions of the bone marrow appearances in malaria can be found in Chapter 7.

Management of the Anemia of Malaria

The following proposals for the clinical management of malarial anemia must be taken as guidelines and adapted to local experience, conditions and resources. Management of malarial anemia centers on three main modalities: adequate treatment of the malaria itself, the judicious use of blood transfusion and the use of hematinics. The prevention of anemia due to malaria is a far broader issue and involves consideration of targeted chemoprophylaxis, insecticide impregnated bed nets, nutrition, etc.

Effective treatment of malaria with chemotherapy eliminates parasites and facilitates an appropriate bone marrow response in the majority of cases. However, a major current problem is the increased drug resistance of *P. falciparum* to antimalarials, including chloroquine, Fansidar™ and other antifolate-containing antimalarials. A further problem is that the anemia may be sustained. There may be continued bone marrow dysfunction for a period of 2–4 weeks after clearance of parasitemia in acute malaria (36). In acute disease, if other complications of malaria such as bacterial infection, renal failure, cerebral malaria and acute respiratory distress coexist, anemia may be prolonged and carry a poorer prognosis (28). In "chronic" malaria, recovery is usually rapid after appropriate antimalarial chemotherapy (5).

Antimalarials

It is of great importance that effective antimalarials should be given to anemic patients with malaria, and where possible these should be followed-up in order to ensure that malaria recrudescence or relapse does not occur. Inadequate treatment with an effective antimalarial or treatment with an antimalarial to which resistance has developed will lead to failure of parasite clearance and persistence or deterioration in anemia. Recurrence of parasitemia was found to be a major factor in occurrence of severe anemia in children regardless of whether they were transfused or not (29, 40).

In some parts of Africa chloroquine continues to be used to treat falciparum malaria primarily because of its relative low cost and availability despite the

presence of high levels of resistance. Studies have shown that whilst chloroquine may improve some of the symptoms of malaria, it can result in poor and delayed hematological recovery (72).

Treatment of Coexisting Bacterial Sepsis

Supportive treatment of severe anemia of acute malaria includes rehydration, especially in the presence of severe acidosis (31), other general measures, and the treatment of complications such as coexistent infections (44). Bacteremia and other bacterial infections contribute to the anemia of malaria through many different mechanisms, and the choice of antibiotics in these cases is not irrelevant. Two groups of antibiotics are particularly likely to cause problems: sulfonamide-containing antibiotics such as cotrimoxazole (trimethoprim and sulfamethoxazole—Septrin®), especially when the antifolate-containing drug Fansidar™ is used to treat the malaria, and chloramphenicol. Sulfonamide-containing antibiotics may lead to a severe anemia due to megaloblastic arrest in patients who have borderline folate levels (73). In developed countries the use of cotrimoxazole is restricted to certain indications, such as pneumocystis pneumonia, but these antibiotics are still freely available and are often used in areas where anemia and malaria are common. Chloramphenicol is still widely used in Third World countries because it is effective and cheap. It produces a predictable and dose-dependent suppression of erythropoiesis due to mitochondrial damage, which is readily reversible on stopping the drug but which may delay hematological recovery in patients with severe anemia. This action is distinct from the far rarer, idiosyncratic complication of irreversible aplastic anemia, which occurs in 1 out of 40 000. Therefore, wherever possible, these antibiotics should be avoided in the treatment of patients with severe anemia.

Blood Transfusion

Blood transfusion can be life-saving for severely anemic patients but runs the major risk of fluid overload and transmission of blood-borne infections. In areas where malaria is common, facilities for blood transfusion are often inadequate because of a shortage of donors, reliance on family-directed donations, and inadequate facilities for testing for blood-borne infections (hepatitis and HIV). Therefore transfusion to treat anemia of any kind, especially in children, must

Table 2. Suggested guidelines for the use of blood transfusion in malarial anemia.

Hemoglobin (g/dl)	Comments
>9	Transfusion not indicated.
7–9	Not indicated unless in a patient with an active acute coronary syndrome (93).
5–7	Transfuse if nonimmune and there is a low risk of contaminated blood (74). In semi-immune and immune patients, not indicated.
4–5	Transfuse if respiratory distress present (76, 79). Also if impending heart failure clinically suspected, in presence of other complications such as culture-positive septicemia, cerebral malaria, renal failure, hyperparasitemia, etc.
<4	Transfuse all patients unless stable, good reticulocyte response, little guarantee of uncontaminated blood.

be used selectively. The aim is to reduce morbidity and mortality, and therefore transfusion must be targeted at those patients who are at risk of dying without the risk of blood contamination (Table 2). Respiratory distress (RD) appears to be one of the main risk factors of dying of SMA (30). A recent decision tree has provided a framework for the decision analysis of whether to transfuse or not in an African setting (74). Three crucial factors emerge: the risk of mortality without transfusion, the risk of contamination with blood-borne viruses, and the effectiveness of blood transfusion in, reducing mortality. Severe anemia due to malaria may still be managed without transfusion, especially when hyperparasitemia is the main indication. Moreover, in one study, iron treatment provided better, hematological recovery at 28 days than for those treated with blood transfusion (75). However, these authors emphasized that those with severe anemia and signs of RD needed to be identified quickly and transfused as soon as possible.

Recent studies have focused on attempting to define the group of children who would benefit most from transfusions, especially in the context of the anemia of malaria. The overall mortality in Kenyan children with severe anemia (Hb < 5 g/dl) (not entirely due to malaria but with malaria as a major factor), compared to those with Hb > 5 g/dl, was 18% vs 8% (76). The Gambia, mortality due to SMA was 10.8% (77) and 6% in another study in Kenya (23). Analysis

of data from the first Kenyan study showed an apparent reduction of mortality overall from 31 to 10% in those with severe anemia who were transfused as compared to those not transfused (76). The probability of mortality was reduced significantly in those with hemoglobin < 3.9 g/dl and respiratory distress, from 49% in those not transfused to 13% in those who were transfused, provided they were transfused on the day of admission or within the following day, but not subsequently. The authors recommended that the number of transfused children be reduced by targeting those children with hemoglobin < 3.9 g/dl and those with RD irrespective of hemoglobin levels (76). This study provides a benchmark for the debate with regard to transfusion in children with severe anemia in the tropics. It is, however, probably insufficient to use any set value for hemoglobin as an absolute indication for transfusion, but only as a guideline (Table 2). Thus patients with more longstanding anemia, without signs of cardiac or respiratory compromise and evidence of a regenerative bone marrow judged by reticulocytosis or polychromasia on a peripheral blood film, may not necessarily require transfusion (77). A recent review from the Cochrane Library still maintains that there is insufficient published data to be sure whether routinely giving blood to clinically stable children with severe anemia in malaria reduces death or results in increased hematocrit at one-month followup (78).

Balanced against the benefits of transfusion are the risks. The major risks are those of blood-borne infections, such as hepatitis B, hepatitis C, HIV and HTLV 1. Thus the provision of safe blood becomes a serious issue that is often a major obstacle in areas where the prevalence of these infections is high.

On occasion transfusions may be thought hazardous in severely anemic children showing signs of cardiac compromise, and either withheld or given with diuretics. In these cases, it is important to consider whether the signs are those of respiratory distress (RD) rather than cardiac failure. Clinically the distinction is difficult. Three separate clinical entities are indications for transfusion:

(1) *Acidosis*. A recent careful study showed that those children with severe acidosis, oxygen debt, RD and prostration (unable to sit unaided) were also hypovolemic. Those with hemoglobins over 5 g/dl should be given crystalloid, but not transfused unless the hemoglobin falls. In that case they should be given whole blood (10 ml/kg over 1 h). In these patients, rapid rehydration or transfusion, without the use of diuretics, may lead to general clinical improvement (31, 79). However, these studies were carried out in a

setting where central venous pressure, lactate and arterial oxygen tension could be measured. In the setting of rural hospitals in the tropics without these facilities, the clinician has to decide through clinical observation alone whether the possible benefits of rapid transfusion are worth the risk of cardiovascular embarrassment.

(2) *Cardiac failure*. Anemia leading to incipient or established cardiac failure requires the slow transfusion of packed cells given with diuretics.

(3) *Severe anemia (< 4 g/dl)*. Any child with a hemoglobin of less than 4 g/dl should probably be transfused on the basis of inadequate oxygen delivery to the tissues.

Clinical decisions should not be based on absolute hemoglobin values alone. Factors such as the presence of other clinical complications (the most important of these is RD with signs of acidosis), those that prolong anemia (e.g. bacterial infections, hyperparasitemia), the bone marrow response to anemia (reticulocytosis, avoidance of bone-marrow-depressing drugs) and the prompt availability of a safe blood supply, must be taken into account. (See also Chapter 12.)

Exchange Transfusion (see also Chapter 12)

Malaria is one of the few human pathogens that invade red blood cells. Assuming a hematocrit of 45%, the red cell volume is 2.5 litres of the 5.5-litre blood volume in an adult male. Therefore, in an individual with a 30% parasitemia due to *P. falciparum*, around 800 ml of packed cells contain parasites. There is no other protozoan that produces this order of parasite load in humans. Viable malaria parasites are largely confined to the vascular compartment and their red cell localization allows accessible removal of parasites in peripheral blood by exchange transfusion (ET). In addition to parasitized red cells, infected blood contains secreted parasite products such as putative "toxins", high levels of proinflammatory mediators such as TNF, immune complexes and breakdown products of the human host cell such as hemoglobin, which circulate in severe disease and are amenable to removal. Furthermore, reduction in red cell deformability of noninfected cells might also contribute to pathology (80). Therefore the removal of whole blood would include parasitized red cells, uninfected cells and plasma followed by replacement with fresh uninfected, more deformable, fresh red cells might be advantageous in severe malaria.

These theoretical arguments in favor of ET in the treatment of severe malaria might at first appear compelling, but the following questions must be raised in relation to ET:

(1) Is the removal of circulating parasites by ET any more advantageous than parasite destruction by antimalarials? Parasitemia is certainly more rapidly reduced using ET, often without the secondary rise that sometimes occurs after starting antimalarials. In many studies using ET, parasitemia has been reduced from extremely high levels of sometimes over 70% to below 5% in 2–4 h, whereas the mean parasite clearance time in severe malaria using conventional antimalarials may be anything up to 100 h. Using automated ET, parasites can be removed even more rapidly. However, the pathological effects of malaria are due to sequestration and schizogony of sequestered parasites leading to cytokine release and not to the circulating ring forms. Thus reduction of ring stage parasites may be reassuring but purely cosmetic.
(2) Is the removal of red cells alone (erythrocytopheresis) better than removal of whole blood? Removal of whole blood leads to reduction in parasites as well as to plasma-containing parasite products such as circulating antigens, released cytokines as well as coagulation factors, white cells and platelets. Red cell exchange, on the other hand, leads to removal of red cells only and return of other components. Although removal of parasites and their products may be advisable, there is insufficient evidence as to the effects of removal of cytokines and white blood cells on the course of recovery. Removal of platelets and plasma will certainly lead to the need to replace these in some cases with donor platelets and fresh frozen plasma (FFP). The benefits of whole blood exchange in the presence of DIC are debatable.
(3) Is automated ET preferable to manual ET? Automated ET is less likely to lead to extreme volume changes than manual ET. It is programmable and can lead to more efficient reduction in parasitemia in a shorter period of time. Automated ET also introduces the choice between whole blood and red cell (erythrocytopheresis) exchange, which is not possible with manual ET, where only packed cells are usually provided. Therefore, where available, automated ET may well be preferable to manual ET.

The debate regarding the role of ET in the treatment of severe malaria remains ongoing and controversial, and will remain unresolved since it is

unlikely that a carefully conducted clinical trial comparing treatment with and without ET will ever be performed. Anecdotal reports of extremely high parasitemias and complications of severe malaria in individuals who have ultimately survived are often cited in favor of ET. Failures of ET, however, are unlikely to be published as case reports; equally, cases with high parasitemias who have not had ET and survived are seldom reported. One retrospective comparative study of ET failed to show significant benefit but the size of the study was small, those who received ET were more severely ill, there was the lack of a transfusion protocol and there existed the possibility of observer bias (81).

Therefore the use of ET, as with so many other issues in clinical practice, remains one of considered judgement (Table 3). A meta-analysis of ET found that it did not appear to improve the survival rate, but those given ET were significantly more ill and thus not strictly comparable with the control group (82). A reasonable approach as to the use of ET in nonimmune patients would be to exchange all individuals with parasitemias greater than 30% (83). This threshold can be reduced to 10% in the presence of any severe complication, such as coma, renal failure, deep jaundice, pulmonary edema, acidemia or severe anemia. Pregnancy and old age (>65 years) are further reasons for a reduction in the threshold for ET. Such an approach seems appropriate where facilities are adequate for ET. It must be appreciated, however, that in many situations where it might be most needed, for example in the tropics, the use of ET is not practicable because of lack of facilities, unavailability of safe blood and prohibitive costs.

Hematinics

There is no evidence that repeated attacks of malaria lead to iron deficiency (Chapter 4). However, iron deficiency is the commonest cause of anemia worldwide and is particularly found in areas where malaria is also endemic. Patients given iron made a significantly better recovery from SMA measured at 28 days post-treatment than those given blood transfusion, although the difference was small (hematocrit 33.0% vs 31.3%) (75). In the decision analysis of whether to administer iron supplementation, there is also the controversy as to whether iron deficiency protects against malaria, and therefore that iron repletion may lead to recrudescence of malaria. However, in field studies iron supplementation has not led to an increase in the prevalence of malaria (84), but did result in significantly better hematological recovery.

Table 3. Exchange transfusions in falciparum malaria.

Indications (83)	Hyperparasitemia in nonimmunes of greater than 30%. Between 10 and 30% if: Complications present, especially CM, RD or SMA. Reduce threshold in: • Patients over 65 years old or serious underlying condition, especially ischemic heart disease • Pregnancy.
What are the endpoints for ET?	To reduce parasitemia to at least half or less than 5%.
Where should ET be carried out?	In a unit with ITU and adequate monitoring facilities.
Manual versus automated exchange	Manual exchange: • Relatively easy to apply • Only whole blood exchange possible • Labour-intensive Automated exchange: • Limited availability • Special expertise needed • Whole blood or erythrocytapheresis possible • Minimal volume changes • Rapid exchange possible

As far as folate is concerned, there is no evidence that the hemolysis associated with malaria leads to folate deficiency or that folate supplementation leads to improved hematological recovery (68, 84). Theoretically, folate may reduce the effectiveness of antifolate antimalarials (see Chapter 4).

Antihelminthics

Helminthic infestations such as those by hookworm and whipworm (*Trichuris*) are common in many malaria-endemic areas and can lead to anemia in their own right due to gastrointestinal blood loss. Thus, treatment of patients with malarial anemia using antihelminthics may be appropriate. However, setting aside these two helminth infections, patients with other worm infestations and patients with falciparum malaria in Thailand were found to have lower

hemoglobins than those without (85). The mechanism by which these worm infestations reduced hemoglobin concentrations was thought to be due to modulation of the immune response.

Followup

The monitoring of patients after malarial anemia is important. Unfortunately, the majority of patients with SMA are not followed up until full recovery because of limited resources. The purposes of monitoring are to ensure that parasitemia has cleared, that there is an adequate reticulocyte response with a rising hemoglobin level, and that there are no other factors that may delay hematological recovery.

Prevention

A Malarial Vaccine

A major advance in the prevention of malarial anemia would be the development of an effective malarial vaccine. This lies beyond the scope of this chapter. Some caution is necessary, as in a primate vaccine model, vaccination was found to increase anemia.

Insecticide-Impregnated Bednets

Hemoglobin levels have been found to be a useful parameter in judging the efficacy of insecticide-impregnated bednets. In some studies, notably in areas of intense transmission, a significant increase in hemoglobin was observed in individuals using insecticide-impregnated bednets (86–88). This was especially pronounced in children in Tanzania (89). However, an increase in hemoglobin less was not noted in others where the transmission was not that intense (90).

Antimalarial Chemoprophylaxis

The most appropriate setting for antimalarial chemoprophylaxis is in pregnant primigravidae which results in higher hemoglobin levels, less placental malaria and higher birthweight. Malarial chemoprophylaxis is also appropriate for individuals in an endemic area with sickle cell disease (HbSS).

The administration of antimalarials to otherwise normal children living in an endemic area is a changing scenario but is not currently used. Initially, continuous prophylaxis was favored, but then with increasing drug resistance, it fell from favor. Chemoprophylaxis has recently been used, either continuously or intermittently, in children and pregnant mothers. In infants given weekly Deltaprim® (pyrimethamine plus dapsone) continuously, there was a 57% reduction in severe anemia (hematocrit <25%) during the period of administration, but this was followed by an increased risk of anemia and malarial episodes, presumably because of the impaired development of acquired immunity (91). Given intermittently at 2, 3 and 9 months of age in a randomized controlled trial alongside EPI vaccination, antimalarials (sulfadoxine/pyrimethamine) reduced clinical malaria by 50% and SMA by 50% (8). Intermittent sulfadoxine/pyrimethamine (up to three doses) resulted in 85% protective efficacy against peripheral parasitemia and 39% protection against severe anemia (hemoglobin <8 g/dl) in primigravid women in Kenya (92).

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Chapter 3

Pathogenesis of the Anemia of Malaria

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> It is well known that the anaemic conditions which follow the summer–autumn fevers are more slowly and with greater difficulty overcome than those which succeed other malarial infections. Whether this difficulty in recovering depends alone on the fact that the states of anaemia induced are more serious than the others, and the lesions of the haemopoietic organs, which occur during the acute infection, deeper; or whether on the other hand it must be attributed to the persistance of a condition of post infective poisoning, we cannot now decide.
>
> —Marchiafava and Bignami, 1894 (1)

Introduction

The pathogenesis of the anemia of malaria is complex, and this has been recognized from the earliest days of malariology, as indicated in the above quotation. The anemia of malaria can certainly not be explained by the hemolysis of parasitized red cells alone: it is frequently disproportional to the degree of parasitemia, is often worst at a time when the parasitemia is waning, and may persist or deteriorate during periods of low-grade parasitemia. The balance between red cell production and destruction normally determines hemoglobin levels, and in malaria, anemia may arise from the combination of increased red cell loss through hemolysis and underproduction of red cells. Such a combination will lead to accelerated development of anemia. In patients with malaria the marrow response to the anemia is often suboptimal, which might also contribute to both the degree of and the delayed recovery from anemia (2, 3). A familiar example occurs in parvovirus infections in patients with hemolytic anemias (e.g. sickle cell disease), where acute suppression of erythropoiesis

combined with brisk hemolysis can result in potentially fatal anemia. The anemia of malaria serves as another good example.

Anemia in Different Clinical Settings of Malaria

One of the most confusing aspects of understanding the anemia of malaria is the inaccurate extrapolation of pathogenic mechanisms identified in a specific clinical situation to others. It is therefore important to define these settings, as often each one is different. Thus rapidly progressive anemia may occur in acute malaria, a febrile illness of sudden onset in a previously healthy individual with minimal or no previous exposure, and hence no immunity to malaria. The parasitemia is usually but not always high. This picture is seen in patients of all age groups from nonmalarious areas, traveling or immigrating to a malarious one, and in young children living in a malaria-endemic area. Within this definition of acute malaria there may also be a number of pathogenic factors operating. Thus acute malaria with consequent severe anemia may be otherwise uncomplicated, or may be complicated with cerebral malaria (CM) or other organ dysfunctions such as acute renal failure.

At the other extreme, a more insidious type of anemia is seen in patients in endemic areas (often older children) with repeated previous exposure to malaria and a certain degree of antidisease immunity. These usually present toward the end of the malaria transmission season with secondary features such as malaise, lassitude, general ill-health and other symptoms. The parasitemia is usually low; sometimes there may be very few asexual parasites with many gametocytes seen. Splenomegaly is common and children may be afebrile, and the clinical effects of anemia dominate the picture at presentation. These two widely differing clinical presentations have been called "acute" and "chronic" malarial anemia (2). These are clearly two ends of a continuous spectrum and some cases present with features of both, an acute infection superimposed on a chronic background.

The severity, incidence and predominant age of onset of severe malarial anemia (SMA) are dependent on the intensity of malaria transmission. Thus, in areas of high and stable malaria transmission, early and frequent exposure leads to a relatively early onset of the syndrome between the ages of one and three years and quicker development of immunity. In areas of less stable, seasonal or epidemic malaria, immunity takes longer to develop and older children are more likely to be anemic.

Mechanisms of Anemia in Malaria (Table 1)

As mentioned, the anemia of malaria is due to a combination of premature red cell destruction and inadequate red cell production. The two mechanisms often overlap and each may be the result of different pathways. Red cell destruction, for example, involves both infected and uninfected cells whose lifespan may be shortened by nonimmune and immune mechanisms. Inadequate production may be due to the suppression of erythropoiesis, or dysfunctional erythropoiesis (dyserythropoiesis), where the bone marrow erythroid cells are increased but mature red cell output is inadequate. In order to explain the different factors that operate in the anemia of malaria, a model is proposed consisting of three phases which may overlap: (1) an initial phase where the drop in parasitemia after treatment is usually commensurate with the drop

Table 1. Mechanisms of anemia in malaria.

Increased red cell destruction		
Parasitized cells	Nonparasitized cells	
	Nonimmune	Immune
Hemoglobin digestion by parasite Destruction of PRBCs during schizogony Membrane damage Reduced deformability Immune recognition	Reticuloendothelial hyperplasia Reduced deformabillity Pitting of parasitized cells	Complement-mediated Immunoglobulin-mediated
Decreased red cell production		
Acute malaria	Chronic malaria	
Suppression of erythropoiesis by proinflammatory cytokine oversecretion Block in iron incorporation Dyserythropoiesis as a minor component	Dyserythropoiesis as a major component	

in hemoglobin; (2) a second phase of continuing hemolysis, probably mostly related to removal of NPRBC; (3) a prolonged course of anemia with delayed reticulocyte response due to bone marrow suppression or dysfunction. The three elements of this postulate will be examined next.

First Phase: Immediate Red Cell Destruction

Destruction of Parasitized Cells by Maturation of Parasites

The loss of circulating hemoglobin occurs even before the parasitized cells are destroyed, as up to 75% of the hemoglobin may be digested by the parasite (4). Most parasitized red cells are then destroyed, either by rupture at schizogony or by premature phagocytosis by monocytes and macrophages.

Our knowledge of the destruction of red cells purely caused by parasite action in man is limited to a few observations on induced malaria used in the treatment of neurosyphilis (5–7), or data on patients with naturally acquired malaria who have been treated with drugs later found to be ineffective for treatment of malaria (8). Mathematical models based on the analysis of data from these patients have been used to calculate the kinetics of red cell loss and production in malaria, but their conclusions have not been consensual. The first conclusion from these studies is that in the early acute phase the observed multiplication rate of *P. falciparum* is likely to be about 10-fold (8), exceptionally between 10- and 20-fold (5), and this is different from the theoretical multiplicative potential of 16–32-fold (the number of merozoites per mature schizont). This high rate of multiplication is likely to lead to extremely high parasitemias in the naïve host if untreated, but is attenuated after several cycles and in the semi-immune host. The second main conclusion is that using peripheral parasitemia leads to underestimation of the parasite burden and consequently of RBC destruction, because of sequestration, especially in highly synchronous infections (6, 8). Fluctuations in peripheral parasitemia occurring at about 26 h into the cycle were mainly due to sequestration of the maturing forms of the parasite. This was followed by an increase in parasitemia after 48 h, when merozoites released from mature schizonts invaded red cells to give rise to a new brood of ring forms. The loss of RBCs was proportional to the sequestered schizonts, and rising parasitemia subsequently resulted in proportional increasing hemolysis. There was no evidence of removal of ring-infected forms of the parasite from the circulation and also little evidence of

active removal of other, more mature forms of the parasite in these patients. Initially parasitized red cells were lost at the time of schizont rupture.

Two other studies based on reanalysis of data on patients with induced malaria have reached different conclusions. The analysis by Gravenor and colleagues concluded that sequestration leads to underestimation of the true parasite burden, and if corrections are made for this, the anemia in acute malaria would be accounted for by the true parasite burden causing red cell loss together with ineffective erythropoiesis (6). On the other hand, the analysis by Jakeman concluded that the anemia in induced acute malaria could be accounted for by the destruction of NPRBCs, which was estimated to be 8.5 times those of PRBCs (7). The falling hemoglobin in these patients coincided with rising parasitemia, and improvement after reduction of parasitemia. The authors also observed that in the early stages of infection higher peaks of parasitemia are seen and these become attenuated later in the infection. They suggested that with growing immunity, the successful invasion rate is reduced to one per ruptured schizont (7). These authors discounted dyserythropoisis as a possible factor in the anemia, suggesting that in their model there is a postulated increase in erythropoiesis of between 30 and 37%. The suggested mechanism of operation of dyserythropoiesis is, however, through ineffectiveness of the response to anemia, whereby new cell production would be expected to be much higher than these stated figures in order to compensate for the anemia (3, 9).

These models have limitations, as by their very nature the infections have to be controlled. In the clinical setting, in patients with acute malaria, red cell destruction is determined by the immune status of the subject. Previous immunity will lead to quicker clearance of parasitemia, but there is also evidence that in hyperparasitemia there may be nonspecific RES activation without previous immunity (to be discussed later). Other factors, such as circulatory changes, especially dehydration and rehydration, may also complicate the measurement of hemoglobin status (10).

Action of Antimalarials

Antimalarials act at different stages of the intraerythrocytic development of the malarial parasite. Quinine and mefloquine do not appear to inhibit the development of more mature ring forms and their subsequent sequestration, whereas chloroquine and, to a more marked degree, artemesinin

derivatives, act on ring forms and lead to accelerated clearance and inhibition of sequestration (8, 11). This means that the dynamics of parasitized red cell loss in acute malaria following different treatments may not be comparable. Moreover, because these compounds are inactive against sequestered, more mature forms (11), there may be an underestimate of the parasite burden when peripheral parasitemia is cleared but more mature forms are sequestered.

Several studies have shown that in acute malaria there is often a correlation between admission parasitemia and the initial drop in hemoglobin after treatment. This was shown in children with acute noncerebral malaria treated with chloroquine (2) and also in CM treated with quinine in both adults (12) and children (13).

Whether parasites destroyed by antimalarials are removed from RBCs or whether the whole RBCs are removed is not known. A less-than-expected drop in hemoglobin has been observed in some patients with hyperparasitemia treated with artemesinin. In these patients there was also a sharp increase in the number of red cells lacking parasites but with surface RESA antigens, and the authors suggested that the parasites killed by the antimalarial are removed by pitting but without destruction of the deparasitized RBCs (see below) (14).

Most of these dynamic changes can be seen in the acute phase of malaria, but in other cases where there is previous host immunity or the onset is insidious, there is no correlation between the degree of anemia and peripheral parasitemia.

Removal by the RE System

Pitting is a process of removing particulate inclusions within red cells, such as Howell–Jolly bodies, Heinz bodies or intracellular malaria parasites, from red cells circulating through the spleen. Although pitting may not result in immediate red cell destruction, it may lead to loss of red cell membrane, with shortening of their lifespan. Evidence from work in simian malaria suggested that deparasitization of ring-infected red cells by "pitting" occurs *in vivo* (15, 16). It is also postulated that this process occurs in naturally acquired falciparum infections. Invading merozoites deposit ring-infected erythrocyte surface antigen (RESA) on the membrane of the invaded cell, thus leaving a marker of invasion which can be detected by immunofluorescence (16). Red cells of Thai patients with acute complicated malaria and those with uncomplicated malaria who nevertheless needed hospitalization showed the presence of red

cells with abundant surface RESA but no intracellular parasites at presentation and before treatment. Since this phenomenon was not observed *in vitro*, the authors concluded that the ring forms had been removed presumably by the spleen without damage to the red cell by pitting. However, these "pitted" cells may themselves have a reduced life expectancy and contribute to the fall in hemoglobin (14). Another study suggested that deparasitization may account for the discrepancy between the expected fall in hemoglobin as a result of removal of parasitized cells and the higher observed values for hemoglobin in some patients with hyperparasitemia after effective antimalarial treatment. RESA-positive unparasitized RBCs, found at presentation, increased rapidly in number within 24 h of treatment with artesunate, which acts on the ring stage of the parasite, but more slowly on treatment with quinine, which acts at later stages, suggesting that deparasitization may save the cells from at least immediate destruction (17).

Most available evidence implicates the spleen and bone marrow as favored sites for PRBC destruction in A/j mice infected with *P. berghei* (18) as well as in humans dying of severe falciparum malaria (19, 20 and N. Francis, personal communication). This generally applies to more mature forms of the parasites.

In acute but uncomplicated malaria in nonimmune subjects, the spleen does not appear to play an active role in removal of parasitized RBCs until it starts to enlarge and becomes palpable (8). However, in more severe and complicated infections, circulating ring forms may be removed by the spleen, as was shown by a postmortem study of a patient dying of severe malaria where there was a predominance of ring-infected forms in macrophages in the spleen (20).

A number of changes in the PRBCs may lead to their enhanced removal by the spleen. These include decreased deformability (21), increased uptake of immunoglobulins from nonimmune (22, 23) or immune sera (24–26) or through changes in red cell membrane lipid composition (27).

The deformability of PRBCs as measured by the time and pressure required to pass through a 3 mm pipette was reduced in ring-infected and to a greater extent in more mature parasites (21). Ring forms required 50% more time or increase in pressure and mature form 4–6-fold increase in these parameters to pass through these micropipettes. The changes were similar in parasites taken directly from patients and those from cultured parasites. The authors concluded that the increased deformability of ring PRBCs was perhaps sufficient to negotiate the microvasculature of the spleen and bone marrow but

that of more mature forms would lead to their inability to pass through these, leading to their ultimate destruction in the RES (21).

Increased uptake of immunoglobulins by parasitized cells from nonimmune (22, 23) and immune sera (24–26) has been demonstrated by a variety of methods. The uptake of immunoglobulins was more marked on more mature parasite forms and led to selective phagocytosis, agglutination and rosetting of these cells around human monocytes (26). These findings suggest that immunoglobulin binding of mature RBCs infected with more mature parasites may act in specific as well as nonspecific manners, according to the different stages of host immunity.

The role of adhesion molecules such as ICAM-1 and CD36 in PRBC rosetting and adhesion to deep vasculature is now well established. Interactions between phagocytic cells and PRBCs that are independent of immunoglobulins and mediated by adhesion have also been described (28–30). A detailed study concluded that CD36-mediated interaction between monocytes and PRBCs leads to their phagocytosis and without induction of TNF release, suggesting that this mechanism of PRBC phagocytosis plays a protective role which is less likely to be associated with serious side effects (30).

Changes in red cell membrane of PRBCs have also been implicated in contributing to their premature removal. Normally there is asymmetry in the phospholipid content of the red cell lipid bilayer, with phosphatidylserine (PS) and phosphatidylcholine (PC) being expressed in the inner leaflet only, and phosphatidylethanolamine (PE) in the outer. PS, which is a procoagulant and also is recognized by macrophages, was found to be expressed on the surface of PRBCs in monkeys infected with *P. knowlesi*, and this was associated with infection with malaria (27). Later studies revealed that these changes were not found in 30% of acutely infected monkeys, although they were consistently found in monkeys with chronic infections and also in splenectomized uninfected monkeys. The authors suggested that the spleen plays an important role in maintaining the exclusive inner distribution of PS in the normal erythrocyte membrane and that the changes seen in malaria-infected monkeys reflect a dysfunction in that organ associated with malaria (31). Another study, utilizing the effect of phospholipase A2, which causes nonlytic hydrolysis of PE but not PS, failed to confirm the loss of asymmetry in *P. knowlesei*–infected PRB (32). It is therefore not clear whether the loss of lipid bilayer asymmetry plays a part in

contributing to recognition of PRBCs by macrophages and their destruction in human malaria.

Second Phase: Destruction of Nonparasitized Red Cells (NPRBCs)

The concept that NPRBCs are also removed prematurely from the circulation in malaria is not new (33, 34). Hemolysis of NPRBCs has been suggested as a major factor accounting for the degree of anemia in excess of that expected from the parasitemia. A number of observations support this hypothesis:

(1) The hemoglobin may continue to fall after clearance of parasitemia (33–35).
(2) Based on studies of induced malaria in neurosyphilis, Jakeman *et al*. (7) deduced that most of the anemia developing in the first 14 days of infection could be explained on the basis of destruction of NPRBCs. The fall in hemoglobin in these nonimmune patients exceeds that projected on calculation of parasitemia despite considering sequestration, and it was estimated that 8.5 times as many nonparasitized RBCs as parasitized RBCs were destroyed (7).
(3) Histology of bone marrow and the spleen in animal malaria (18, 33) and bone marrows of infected patients often shows phagocytosis of PRBCs as well as NPRBCs (2, 36).

The two main types of mechanisms of RBC destruction are immune- and nonimmune-mediated. They depend on the acuteness of infection and also prior exposure to malaria.

Nonimmune Mechanisms of Red Cell Loss

A variety of nonimmune-mediated pathways have been proposed to explain increased clearance of autologous or allogeneic transfused red cells in patients with malaria. They include increased reticuloendothelial clearance (discussed further in Chapters 5 and 10) and damage to nonparasitized cells mediated in a variety of ways. Some of the postulated mechanisms by which NPRBCs may be recognized and removed from the circulating red cell mass are shown in Table 1.

Nonspecific Increased Reticuloendothelial and Splenic Function (Hypersplenism)

The clearance of NPRBCs from treated *P. berghei*–infected mice transfused into uninfected mice was more rapid than the clearance of autologous red cells, suggesting an intrinsic red cell defect (37). Red cell survival studies in man also suggested accelerated removal of uninfected transfused RBCs (38), and this has been confirmed in cross-transfusion experiments in patients recovering from *P. falciparum* infection (39). In these studies the reduction in survival of chromium-labeled autologous cells was modest in patients, most of whom had uncomplicated malaria (mean survival 56.8 vs 89.6 d in normals) (39). By contrast, when labeled compatible cells taken from an uninfected donor were transfused into patients convalescing from malaria, the survival of these cells was even shorter than that of autologous cells (39). There was also accelerated removal of erythrocytes coated with anti-D (40). In patients with malaria in Thailand, there was also marked and significantly increased clearance of heat-damaged chromium-labeled erythrocytes in anemic patients with splenomegaly as compared to those without splenomegaly, and the values for damaged RBC clearance returned to normal six weeks after treatment of malaria (41). Taken together, these studies suggest a defect extrinsic to red cells, such as increased reticuloendothelial system (RES) activity and hypersplenism. However, the cross-transfusion studies do not exclude the possibility of opsonization or changes in surface charge of these NPRBCs, which could occur shortly after transfusion. Certainly, the increased rate of vascular clearance of injected particles such as colloidal carbon in animals with malaria (42), and of ^{125}I-labeled microaggregated serum albumin in man (43), would suggest RES hyperactivity.

The important factors that lead to enhanced clearance of red cells by the spleen include macrophage proliferation and activation in the RES leading to splenic enlargement. These changes may be governed by a complex array of cytokine and cellular responses (Fig. 1), and these are fully discussed in Chapters 5 and 6. Splenic enlargement may take several cycles of parasite development in the first attack. Thereafter splenic enlargement may become chronic and persist throughout the transmission season even though patients may not have symptomatic malaria (see Chapter 10).

The spleen may play a role in modulating the severity of anemia. Splenectomy in mice infected with rodent malaria delays but does not prevent severe

anemia, although parasitemia is often exacerbated (44). Also, erythrophagocytosis of PRBCs and NPRBCs in the spleen as well as other reticuloendothelial tissues is a prominent feature of both human and animal malarias (33). An increase in phagocytosis of PRBCs and NPRBCs occurs in the spleens of rats infected with *P. berghei* and attains a peak between 10 and 15 days after the onset of parasitemia and corresponds in time with the fall in hemoglobin (33). A similar observation was made in hamsters infected with the same parasite (45, 46).

These observations point to the enhanced clearance of NPRBCs by the RES. Whether this is partly mechanical (filtration by a large spleen) or much more subtly mediated by specific red-cell–macrophage interactions is unclear. Several changes in NPRBCs which may contribute to their accelerated removal have been described, including decreased deformability, changes in lipid composition of the RBC membrane and DIC.

Decreased Deformability of Red Cells

Decreased deformability of PRBCs under fluid shear stress during maturation of *P. falciparum* in erythrocytes has been demonstrated using a rheoscope (47). Reduced deformability of erythrocytes irrespective of the level of parasitemia has also been shown to occur in patients with uncomplicated falciparum malaria (48, 49). Decreased deformability of red cells may lead to enhanced clearance by the spleen, perhaps because of delayed transit through the sinusoids. In Thai adult patients with severe falciparum malaria, the degree of reduced red cell deformability as demonstrated by laser defraction under low shear stress correlated highly with the severity of disease and with prognosis (50, 51). Reduced deformability at high shear level similar to those in the spleen correlated with the degree of anemia and persisted for a two-week period and before a complete return of hemoglobin to normal levels (52). However, in a smaller group of Dutch students returning to Holland with less severe malaria, less deformable red cells were also found which persisted for up to four weeks, until the hemoglobin had returned to normal (52). The authors concluded that reduced deformability may be an important factor in the increased removal of NPRBCs by the spleen, thus contributing to anemia. The causes of this decreased deformability are not known but could include increased peroxidation of lipid by monocyte products (53) or the binding of a heat-stable exoantigen to the red cell membrane which reduces their deformability (54), as normal donor red cells incubated with plasma from patients with

severe malaria or supernatants from continuous culture of *P. falciparum* showed reduced deformability (52).

Disseminated Intravascular Coagulation (DIC)

DIC is associated with a microangiopathic hemolytic anemia and red cell fragmentation. It is another mechanism proposed for destruction of nonparasitized red cells. Although DIC is rare, it has been described in a minority of cases of malaria and is not implicated in the anemia seen in most cases of falciparum malaria (see Chapter 8).

Changes in the Red Cell Membrane

Lipid peroxidation is another mechanism by which uninfected red cells may be damaged. Increased lipid peroxidation was demonstrated in infected as well as noninfected RBC cultures of *P. falciparum*, coincubated with activated monocytes from normal donors. The cells were also found to be more susceptible to peroxide lysis (53). Increased lipid peroxidation of red cell membrane in acute malaria was also demonstrated in a study of patients with acute malaria (55). There was a significant increase in erythrocyte thiobarbituric acid-reactive substance (ETBAR), indicative of increased oxidant stress and lipid peroxidation in erythrocytes of patients with malaria when compared to controls. There was also a decrease of some of the intracellular and extracellular antioxidants, including tocopherol, reduced glutathione and ascorbate, in patients when compared to controls. A correlation between hemolytic indices and ETBAR levels was found, suggesting that increased oxidant stress may play a part in the destruction of red cells and hence the degree of anemia.

Immune Mechanisms of Red Cell Loss

Perhaps the most controversial area in the pathogenesis of malarial anemia has been the existence of immune-mediated mechanisms of hemolysis and, if true, its relative role in the pathogenesis of anemia. There is now convincing evidence of an increase in RBC-associated immunoproteins (immunoglobulins and/or complement) on red cells in both animal and human malarias. In mice infected with *P. berghei*, IgG that reacted preferentially but not exclusively with parasitized red cells and led to phagocytosis of these cells *in vitro* was detected

(56, 57). In patients in areas of high malaria endemicity, there is a correlation between the presence of red-cell-associated immunoproteins and current or recent malaria. This has been demonstrated by either the direct antiglobulin test (DAT) (2, 24, 58–61), IgG quantitation using radioiodine-labeled antihuman antibodies (58, 62, 63), or fluorocytometry (64). In a community study in the Gambia, DAT positivity in children up to 13 years of age was found to be 12.8% in all subjects studied and 95.7% in the subgroup with malaria parasitemia (58). In another study in Kenya, a mixed hospital and community study, the DAT positivity was 17.3% overall and 70% in children with malaria (60). In three studies in children with malaria in the Gambia, the coincidence of parasitemia and DAT was 39% (24), 60% (60) and 70% (59). In Thailand, the incidence of a positive DAT in uncomplicated malaria was considerably higher (16.4%) than that found in normal Europeans (0.007–0.01%) and in healthy Thai controls (4.5%) (61).

Some studies have concluded that immune mechanisms are unlikely to contribute to the anemia of malaria because of a low rate of DAT positivity in their patients (61, 65, 66) or because of the lack of correlation between levels of circulating immune complexes and anemia (65). Others have come to a different conclusion. Facer and colleagues found a strong correlation between red cell sensitization and anemia (59). Anemia was more common in those children with IgG1 on their red cells than those with IgG2 or 4 (67). More recently, Waitumbi and colleagues demonstrated a significant association between severe anemia and red cell changes including IgG deposition and alterations in complement regulatory proteins on RBCs of Kenyan children with malaria (64). The overall conclusion, even from studies that argue a role for immune hemolysis, is that there is a higher rate of positivity in anemic patients (24, 59, 62) but there is no absolute correlation between a positive DAT and anemia, as there are patients who are not anemic but who have a positive DAT, and others who are anemic but with a negative DAT. The inference is that there is no direct evidence that these immunoproteins cause accelerated RBC destruction.

Before the role of immune factors in the pathogenesis of NPRBC destruction is dismissed, it is important to understand the different factors that may contribute to the lack of correlation between anemia and a positive DAT. Immune destruction of red cells (infected and uninfected) can occur because of deposition of IgG or because of complement activation. In turn the IgG deposited may

be directed against RBC membrane components (an autoantibody), or against foreign (in this case parasite) antigen bound to the RBC membrane. On the other hand, complement activation may occur through the classical pathway, or through the non-antibody-dependent pathway by recognition of pathogenic microbial determinants and activation through the alternative pathway. RBC destruction by complement activation may then proceed by activation of the lytic complement cascade, or by deposition of complement components on the red cell surface that may be recognized by complement receptors on phagocytic cells. Therefore data should be analyzed separately for IgG positivity and complement component positivity. Moreover, the endemic pattern of malaria will affect the intensity of transmission and the age of first exposure to malaria, which will in turn heavily influence immune protein deposition on red cells. Age therefore has two effects on the immune status of the patient, as it affects the state of maturity of the immune system and because exposure to malaria increases with age. The parasitemia at presentation will also affect the balance of antigens and antibodies. The following discussion will focus on these variables.

Age, Malaria Endemicity and Parasitemia

Studies that showed high rates of positive DAT in an area of high malaria endemicity in the Gambia, but without discriminating between differences of opsonization with IgG or complement (2, 59, 67), have failed to find a correlation between age or parasitemia and DAT positivity. Analysis of data on the basis of age and correlation with the presence or absence of IgG on red cells in those with positive DAT was attempted in one study only (24). Of 68 Gambian children with malaria, DAT positivity due to IgG with or without complement components was confined to the age group between 16 and 60 months, which was significantly older than those with a negative DAT whereas sensitization with complement alone was also common and did not correlate with age (Table 2). The study from Ibadan, a large city with a less stable pattern of malaria transmission than the Gambia, showed a significantly positive correlation between RBC membrane-associated IgG and malaria, but not between the IgG amount on RBCs and parasitemia and age (62).

We therefore feel that the question of the importance of IgG binding to RBCs in malaria could only be resolved by carefully planned studies comparing IgG DAT positivity rates in areas with different patterns of malaria transmission, stratified according to age and parasitemia.

Table 2. Correlations between means of age, parasitemia, Hb and reticulocyte counts in Gambian children with malaria and various immunoproteins on their red cells (*adapted from Abdalla and Weatherall, 1982*).

DAT	IgG ± C3	C3 or C4	Negative	All
Age (months)	33.9 $P < 0.05$	20.4	21.33	25.2
Parasitemia ($\times 10^{12}$/l00)	0.331 $P < 0.05$	0.117	0.130	0.191
Hb (g/dl)	7.13	5.51	5.92	6.14
Ret ($\times 10^{12}$/l)	0.202	0.242	0.192	0.209

Quantitation and Subgroup Specificity of Immunoglobulin Sensitization

The presence of immunoglobulins on red cells does not always lead to accelerated removal of these cells, as this depends on the amount and IgG subclass of the antibody (68, 69); for example, IgG1 and IgG3 are more efficient in causing hemolysis at a lower level of sensitization than IgG2 and 4 (68). It has also been estimated that the minimal number of molecules of IgG per red cell needed to cause removal of sensitized cells is 1000 molecules for IgG1, whereas fewer molecules are needed for IgG3. IgG2 and 4 antibodies do not lead to hemolysis (69). Thus increases in IgG coating on all cell surfaces can produce a positive DAT without compromising red cell survival (70).

The three published studies on the quantitation of total IgG on the red cells of patients with malaria all agree that there is an increase in IgG on red cells of patients with malaria above those in uninfected controls (61–63). The study in Ibadan found a significant difference in the mean number of IgG molecules on red cells in patients with malaria and normal controls (629 vs 395 per red cell, $P < 0.05$), and a significant negative correlation between the number of IgG molecules and anemia (62), but again the correlation in individual patients was not absolute.

An increase in RBC-associated Ig was also found in patients with malaria as compared to normal controls, but without correlation with levels of IgG

and severity of anemia or with reticulocytosis, suggesting that in a majority of patients, increased IgG on RBCs was not of a sufficient amount to induce phagocytosis (63).

The mean RBC-associated IgG in Thai adult and children with acute malaria was not found to be significantly raised, but these patients were all considerably older than those in the the African studies, as even the children were between 8 and 16 years. Although some of these patients were resident in malaria-endemic areas, the age at presentation is suggestive of little previous immunity to malaria (61).

It can be concluded that the increased number of IgG molecules on red cells is a malaria-associated phenomenon which may not necessarily lead to anemia. Factors other than the amount of IgG on cells that can cause this lack of correlation include the IgG subclass and the state of activation of the RES. Subclass analysis of IgG was carried out in only one study, and it showed correlation between the presence of IgG1 on red cells and anemia. Interestingly also, patients with the IgG1 subtype on their cells were more likely to be of the age group of 8 months to 4 years than older children (67).

Although the amount of IgG on the red cells of patients with malaria and a positive DAT may not, under normal circumstances, be sufficient to lead to removal by nonactivated macrophages from uninfected individuals, it may be sufficient for such recognition and removal by an enlarged spleen and with activated macrophages. This may be another factor to account for the lack of absolute correlation between IgG coating and anemia. Thus immunological factors, whilst playing a part in the anemia of malaria in some patients (59, 63, 64), do not account for the severe anemia in patients with malaria in all cases.

Role of Complement Activation

It remains possible that complement activation could cause hemolysis and yet be undetected by crude antibodies, particularly if a sensitized subpopulation of cells were removed from the circulation before the assay was performed. Using well-characterized reagents, Merry and Phillips (personal communication) were unable to detect red cell membrane-bound complement components in both severe malaria and blackwater fever in patients from Thailand. However, Facer found severe anemia and erythrophagocytosis by PBMC in a patients with C3b coating of red cells, suggesting that complement activation may play a part in the anemia at least in some patients (71).

Antigen Specificity of IgG on Red Cells of Patients with a Positive DAT and Malaria

Where this has been studied, the antigens recognized by antibodies on red cells of patients with falciparum malaria are specific to parasite rather than RBC antigens (24, 26, 67). This was shown by correlation between high titre antischizont antibodies and DAT positivity in Gambian children with positive DAT, and by specific reactions between IgG eluates from their RBCs with schizonts of *P. falciparum* in an indirect fluorescent antibody test, but not with *P. falciparum* gametocytes or *P. malariae* schizonts, and with no evidence of specificity against blood group antigens (67).

The presence of antibodies with specific reactivity against *P. falciparum* schizonts was also shown in another study (24). Using normal adult PBMC and an *in vitro* phagocytic assay, the presence of opsonizing antibodies to schizont-infected red cells was demonstrated in sera from healthy DAT-negative Gambian adults and Gambian children with acute malaria who were IgG DAT-positive, but not in sera of DAT-negative children with acute malaria. There was no significant increase in opsonizing activity when the sera of DAT-negative children were tested during convalescence. There was also increased opsonizing activity in eluates prepared from red cells of children with a positive DAT (24). In this study schizont specificity of these antibodies was shown by the specific phagocytosis of schizonts from short term cultures of autologous parasites incubated with autologous serum, but not when these cultures were incubated with nonimmune AB sera and the absence of phagocytosis of nonparasitized cells (Fig. 13, Chapter 5).

Using ^{125}I-labeled anti-IgG, another study showed an increased IgG on autologous schizont-infected RBCs in short term cultures in sera from patients with acute malaria and a positive IgG but not from those with a negative DAT (26). Using a laboratory strain of parasite, similar results were obtained with sera from patients with a positive IgG and also from immune adult Gambian sera. Increased phagocytosis was observed in both an autologous and a laboratory strain of parasites preincubated with sera from two children with a positive DAT but not from those with a negative DAT (26).

The above studies strongly suggest that the positive DAT due to IgG in children with acute malaria is caused by a reaction between antimalarial IgG and malaria antigens on the surface of red cells. As the DAT positivity persists for a week or two after clearance of parasitemia, it is unlikely that the

reaction is solely due to the presence of parasitized RBCs recognized by antibodies (24). These antigens must therefore either arise by export from inside the cells and persist on apparently uninfected red cells, as shown for the RESA antigen on deparasitized cells (16), or they may be deposited from circulating soluble malaria antigens which are also known to be present and persist in the circulation for a period after parasitemia is cleared (72). Adherence of parasite exoantigens to NPRBCs has been shown directly or indirectly in several studies. The incubation of NPRBCs with culture supernatants of malaria parasites led to decrease in the deformability of these cells when they were passed through a narrow pipette, suggesting that binding of these exo-antigens led to this decreased deformability (54). Alternatively, the parasite antigen, EBA 175, which was shown to bind to glycophorin A and is recognized by antibodies, may also be a candidate for this reaction (73). It is postulated therefore that the positive DAT in acute malaria is found only in children with a combination of a high parasite burden resulting in RBC coating with parasite antigen, but also with developing antiparasite antibodies that bind to these antigens. Children with a positive DAT caused by IgG would then be in the process of developing an immunological response to *P. falciparum*. It can therefore be hypothesized that the coexistence of high antigenemia and antischizont antibodies is needed to produce a positive DAT. This is most likely to occur in heavily parasitized children in hyperendemic areas who are developing immunity to malaria. Nonimmune adults with high parasitemias will lack these antibodies, and immune subjects with low grade parasitemia will lack the circulating antigen, explaining why they are DAT-negative (see Fig. 1).

Conclusions Regarding the Significance of the Positive DAT in Malaria

In many areas where malaria is highly endemic, a positive DAT is common, as are other tests for autoantibodies, such as antinuclear and anti-single-stranded DNA (74), but this should not be construed as evidence for autoimmune disease. An antibody could also be directed against a parasite antigen specifically adsorbed onto the surface of the infected cells, as is the case with the 175 kD erythrocyte binding antigen (EBA) (75), as has been suggested previously (76). Alternatively, malaria antigen–antibody complexes containing complement may bind to the surface of uninfected cells via the C3b receptor (59, 67). Administration of prednisolone at a dose of 40 mg daily in patients with *P. falciparum* malaria did not lead to a significant prolongation of the

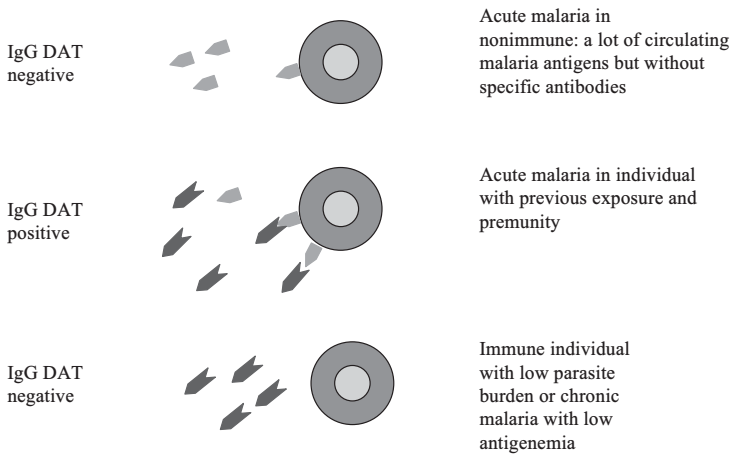

Figure 1 Proposed mechanisms for positive IgG DAT in malaria. A prerequisite is the presence of a large amount of antigen (◀) on the red cells (◎) either deposited from the circulation or exported from infected cells, and specific antibody (◀) formed by previous repeated exposure to malaria.

radioisotopically measured red cell lifespan of these patients compared to controls not given prednisolone (77), but it is well known from clinical practice that corticosteroids may take a few days to cause reduction of red cell lysis in patients with autoimmune hemolytic anemia.

The following conclusions can be made from the summary of the data presented from the studies of DAT in patients with malaria (see also Fig. 1):

(1) *DAT positivity is relatively common in malaria-endemic areas*.
(2) *The mechanisms of complement or IgG association with red cells signify different processes*. DAT positivity due to IgG is likely to be more specific, in that the antibodies appear to be directed against parasite determinants, whereas complement may be activated in a nonspecific manner as well as through formation of immune complexes.
(3) *The rate of DAT positivity may correlate with previous exposure to malaria and to transmission patterns and frequency*. Studies that have yielded the highest positive DAT rates or the equivalent by other techniques have been those in children from West Africa (mainly the Gambia) (24, 58, 59, 62) and Kenya (60, 64). By contrast, studies of nonimmune Thai adults and older children not living in a malaria-endemic area yielded low rates of positivity (61).

(4) *The methods of detection of immunoproteins are different*. Higher positive DAT rates were obtained in studies using microtitre plates, which have a greater sensitivity and are easier to interpret. Many of the negative reports do not specify the techniques used.
(5) The lack of correlation between IgG positive DAT and anemia may be due to insufficient coating with IgG or coating with inactive complement components (C3d) which are insufficient to contribute to anemia. In some cases, however, the amount of IgG may be sufficiently high and is of the appropriate IgG subtype to lead to hemolysis. Similarly, the presence of active C3b on the cells of some patients may lead to hemolysis.
(6) Based on the findings discussed, it is postulated that the DAT IgG positivity is due to a reaction between specific antiparasite antibodies and parasite antigens on red cells, and that therefore the correct combination of high parasitemia and some immunity is needed to lead to a positive IgG DAT.

Specific Syndromes Associated with Severe Acute Anemia in Malaria

Blackwater Fever

"*As regards the pathogenesis of hemoglobinurea (of malaria) everything points out to the conclusion that through a predisposition of the individual varying in degree, a premature necrosis of the red blood corpuscles takes place together with loss of haemoglobin after the invasion of the parasites and that this is the case even in the corpuscles, which have not been invaded by the amoebae, perhaps through the agency of poisonous substances*." (78)

Blackwater fever (BWF) is a term used to indicate the passage of black urine in a patient with malaria. It is associated with acute intravascular hemolysis, hemoglobinuria, jaundice and severe anemia, and sometimes with renal failure and a high mortality rate. BWF was often seen in the past in semi-immune expatriates and associated with the erratic and repeated use of quinine (35). The incidence of BWF declined rapidly after the introduction of antimalarials other than quinine. Foy and Kondi reported a reduction in the incidence of BWF as a proportion of total malaria cases in a hospital in Salonika, from 2.3–9.4% between 1933 and 1941, to 0.4–1.4% between 1942 and 1946. They attributed

this reduction to the increasing use of atebrine instead of quinine (79). This decline continued with the introduction of chloroquine in 1950 (80).

JWW Stephens published a monograph on BWF in 1937, including a large body of documentation implicating quinine and malaria in the etiology of BWF (81). However, the actual pathogenesis of BWF remains elusive and this may well be due to the reduced opportunity to study this syndrome, which became rare after quinine ceased to be the main prophylaxis and, later, treatment for malaria. However, in recent years the incidence of BWF has appeared to be rising with increased use of quinine because of chloroquine resistance of most isolates of *P. falciparum.*

Other antimalarials have also been associated with BWF. They include atebrine (quinacrine) (82) and, more recently, halofantrine (83–86) and mefloquine (86, 87). Hemoglobinurea (but without further details) has also been reported in patients treated with artemesinin and derivatives, in combination with mefloquine in treatment of uncomplicated malaria in one report (88) and in 7 out of 284 adult Vietnamese patients treated for severe malaria (89). Further evidence for implicating Artemesinin and derivatives in classical BWF is therefore awaited.

It has been suggested, with justification, that the term "blackwater fever" should be restricted to cases of severe intravascular hemolysis in semi-immune expatriates living for a prolonged period in a malaria-endemic area, and with intermittent use of antimalarials. They often present with low-grade malaria parasitemia but with overwhelming intravascular hemolysis (86). Intravascular hemolysis is known to occur in cases of overwhelming malaria (90) and also in association with G6PD deficiency (89, 91) and the mechanism of hemolysis in these cases is better understood. Thus, in the study of 50 cases of hemoglobinurea from Vietnam, 54% of the cases were found to have G6PD deficiency, 32% had malaria alone without prior use of antimalarials and 56% had been treated with quinine. There was a considerable overlap between these three factors, suggesting that in areas where there is a high rate of G6PD deficiency, it may be difficult to distinguish between intravascular hemolysis due to G6PD deficiency and BWF (89).

Another study of 38 patients with hemoglobinurea from Zaire found that 4 patients had severe G6PD deficiency, and in 28 patients the clinical picture was that of classical BWF. Quinine was used as a curative treatment in a significantly higher proportion of patients with BWF than in patients with uncomplicated

malaria. Quinine was used in subtherapeutic doses before presentation and 50% of patients were nonimmune. The authors concluded that BWF may be considered a major complication of malaria with an unusually synchronous lysis of infected red cells that may be unrelated to the use of quinine (91).

The way in which quinine can lead to BWF is not fully understood. Normal blood transfused into patients with BWF appeared to be rapidly destroyed, but blood from patients with BWF appeared to have normal survival when transfused into normal individuals, suggesting an extrinsic mechanism of cell lysis such an antibody-mediated phenomenon (92, 93). A suggestion that malaria parasites altered the antigenic makeup of cells, which were then recognized by hemolysins, was made (94) but not substantiated. A therapeutic trial of prednisolone in five patients with BWF with a favorable response to this treatment is also in favor of a possible immune origin of BWF (95).

Although the mechanisms by which quinine and other related drugs may cause hemolysis in BWF are still not clear, several can be postulated. The formation of drug–antibody immune complexes and complement activation rather than IgG binding has been proposed as a mechanism to explain the small amount of drug needed on re-exposure to drug and the absence of direct Coombs positivity in these patients tested before the availability of reliable wide spectrum antiglobulin reagents (96). Idiosyncratic hypersensitivity to the drug was demonstrated in a patient with no previous history of malaria or quinine ingestion who developed BWF after the third dose of quinine for treatment of malaria. *In vitro* incubation of the patient's RBCs with quinine showed abrupt hemolysis in 36 h, whereas hemolysis took over one week to occur in normal control red cells (97). However, this is unlikely to explain the majority of cases of BWF, and the most feasible explanation is through an immune mechanism, as will be discussed below.

The evidence for the presence of antiquinine antibodies attached to red cells is scanty (80), and a recent work where antidrug antibodies were screened for showed antihalofantrine antibodies in only 1 of 21 cases of BWF (86), but details of the techniques used are lacking. Quinine is well known to produce immune thrombocytopenia with demonstration of antibodies that attach to platelets only in the presence of the drug (98, 99). This was demonstrated by *in vitro* agglutination of platelets in the presence of drugs and serum from patients as well as by induction of thrombocytopenia by passive transfer in subjects pretreated with quinine (98). Quinine is also known to be associated with multiple

antibody production to quinine in combination with other target antigens, including those on red cells, platelets, leukocytes and vascular endothelium. It has also been strongly associated with a positive lupus anticoagulant in elderly patients (100–102). More recently, the intermittent use of quinine for nocturnal cramp has been implicated in causing the hemolytic uremic syndrome with thrombocytopenia, red cell fragmentation and microangiopathic hemolytic anemia (103–105). Thus failure to detect quinine antibodies in patients with BWF may be due to a simple methodological error, as these antibodies would be only detected when quinine is added to red cells *in vitro* and may not be detected in other ways. This interesting hypothesis can be easily tested.

The management of BWF is mainly supportive. A blood transfusion should be given if the anemia is severe and renal failure may require dialysis. Although there are no consensual recommendations in the literature, it appears logical not to continue with the drug thought to be implicated, or with similar drugs that may cross-react. Artemesinin or derivatives may be a safer choice, although there is no data to support this approach (86). Similarly, Prednisolone has been reported to be effective in reducing the duration of hemolysis in doses of 40–60 mg/day, but all evidence also appears anecdotal (106).

Hemophagocytosis-like Syndrome in Patients with Malaria

The hemophagocytic syndromes (HSs) are conditions associated with increased macrophage activation and hemophagocytosis. The underlying pathology is often related to a deregulation of T-lymphocytes and excessive production of cytokines. The full-blown syndrome is associated with fever, hepatosplenomegaly, cytopenias, coagulopathy and a number of biochemical abnormalities, and may be associated with certain viral or other infections. Diagnosis is confirmed by examination of the bone marrow, which shows an increased number of macrophages with phagocytoses of hemopoietic cells (107).

A modest degree of hemophagocytosis is often seen in the marrow of patients with malaria (see Chapter 5). There are however, a few case reports in the literature of an extreme form of hemophagocytosis resembling virally induced hemophagocytosis or malignant histiocytosis, and these include cases with falciparum (108–111) as well as vivax (112) malaria. In these cases there was a variable degree of pancytopenia, increased ferritin and triglyceride levels and phagocytosis of cells other than erythrocytes. As these cases are rare, it

may be that they are caused by an extreme reaction to falciparum mitogens, as is seen in HSs with a genetic predisposition. The other possibility is that these patients may have had a viral infection coexistent with a herpes virus (such as EBV), although this was excluded in two of the studies and resolution was obtained after treatment of the malaria.

Hyperreactive Malaria Splenomegaly

This is fully discussed in Chapter 10.

Third Phase: Decreased Red Cell Production

The following is a summary of factors that may contribute to the severity and duration of anemia through reduced erythropoiesis in patients with malaria (this topic will be fully discussed in Chapter 7, dealing with the bone marrow). Inadequate erythropoiesis can result from reduced production of erythroblasts (erythroid hypoplasia) or from normal or increased production of erythroblasts but with intramedullary cell death (ineffective erythropoiesis). The latter is often associated with dyserythropoiesis, which is a morphological description of abnormal red cell maturation. Dyserythropoietic feature may coexist with erythroid hypoplasia in acute malaria, but the predominant picture is that of reduced erythropoiesis. It is therefore important to distinguish between the picture seen in chronic malaria where there is erythroid hyperplasia, dyserythropoiesis and ineffective erythropoiesis, and that in acute malaria where dyserythropoietic changes may occur but in the context of erythroid suppression (2).

In Acute Malaria

Although red cell destruction plays a major role in the anemia of acute malaria, reduced production of erythrocytes by the bone marrow is an important factor in maintaining anemia, which usually takes 3–4 weeks to resolve. Several processes have been implicated in the reduced erythropoiesis, including suppression of erythropoiesis, dyserythropoiesis and disturbances in iron metabolism (3). In the acute phase of the disease in nonimmune individuals, there is suppression of hemopoiesis. Ferrokinetic studies in malaria have

shown decreased iron incorporation into erythroid precursors during acute infection (113). During the asexual parasitemia the serum iron was low and the total iron binding capacity was normal, and these changes were found together with normal or increased stainable storage iron in bone marrow macrophages but not in erythroblasts, indicating a block of erythroblast iron incorporation (2, 12). This picture is similar to that seen in the anemia of inflammation or of chronic disease. Suppression of erythropoiesis in acute malaria has also been observed (2, 36, 113) and may be a result of TNF-α overproduction (114) (see Chapter 6). Finally, dyserythropoietic features, although more florid in chronic malaria, are also seen in acute disease (12, 36, 115) but are thought to be a less important feature than in chronic malaria, where dyserythropoiesis and ineffective erythropoiesis are more important (3, 9, 116).

In Chronic Malaria

By contrast, in the more chronic phase of the disease seen in some children in endemic areas, the bone marrow shows erythroid hyperplasia but with evidence of morphological dyserythropoiesis and resultant functional ineffective erythropoiesis (2, 3, 9, 116). Detailed morphological findings are demonstrated in Chapter 7. Factors responsible for dyserythropoiesis remain to be elucidated but do not appear to be directly related to the presence of parasites in the bone marrow, or to hematenic deficiency (Chapter 4) (although the latter may be of importance in the management of severely anemic patients in areas where these deficiencies coexist with malaria).

Dyserythropoiesis in chronic malaria is thought to contribute to both the slow recovery observed after a single attack of malaria and the persistent anemia in individuals subject to prolonged low grade parasitemia (2, 3, 9). However, dyserythropoiesis is not specific to malaria and varying degrees of dyserythropoiesis are seen in other acute illnesses, in the congenital dyserythropoietic anemias and in the acquired myelodysplastic syndromes. A specific type of dyserythropoiesis with the presence of megaloblasts in the marrow is seen in B12 and folate deficiency. Dyserythropoiesis may also occur in the marrow of patients with vivax malaria (117, 118).

Studies of marrow function strongly suggested that dyserythropoiesis reflects ineffective red cell production (115, 116). Gambian children whose marrows showed dyserythropoietic changes had an increased proportion of red cell precursors in G2, with an arrest during the progress of cell division through

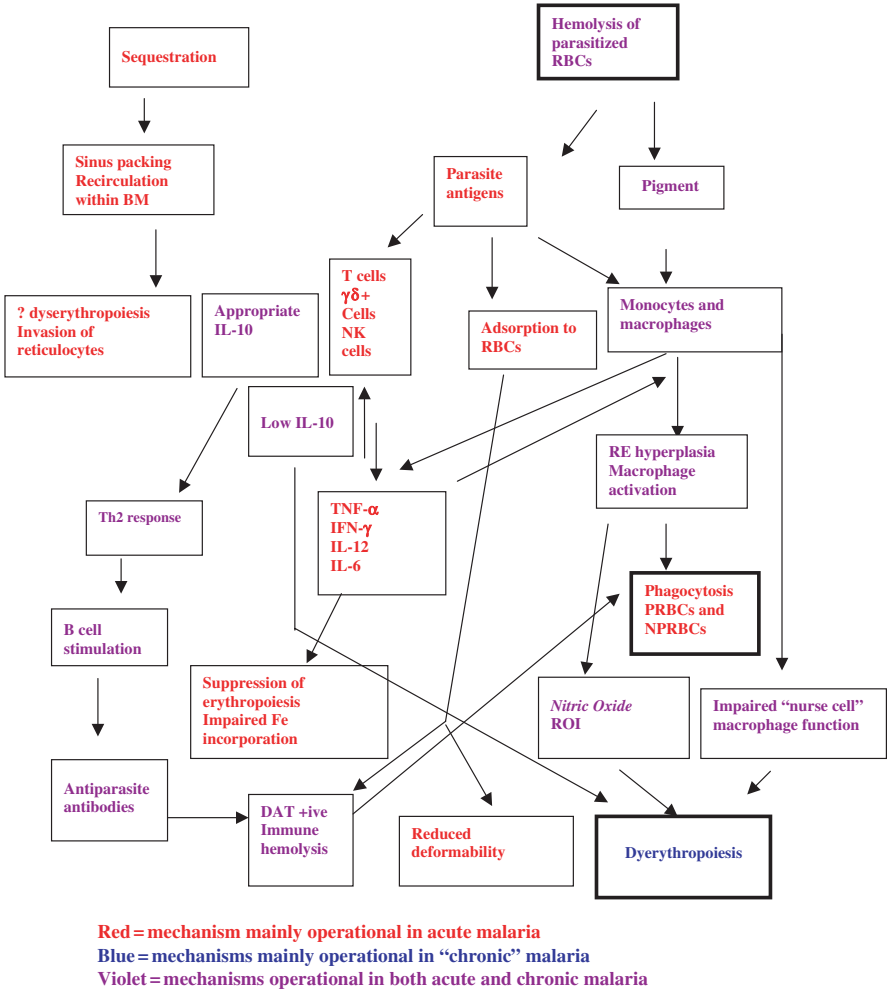

Figure 2 Mechanisms of anemia in malaria.

the S phase (116). These marrow abnormalities contrast with Fleming's description of florid megaloblastic erythropoiesis in African women who have been severely anemic with malaria, pregnancy and folate deficiency (119).

Studies in the Gambia did not find evidence of hematinic deficiency in patients with malaria and dyserythropoiesis (2, 120), despite the presence of

giant metamyelocytes, and in a few cases, megaloblastic changes (9). There was also no evidence of cellular B12 or folate deficiencies in these patients as shown by the normal deoxyuridine suppression test; abnormal results with this test are specific to megaloblastosis caused by reduced production of thymidylate from uridylate seen in B12 or folate deficiency (121).

It is difficult to assess the contribution made by dyserythropoiesis to the anemia of malaria. Dyserythropoiesis has been linked to persistent anemia in Gambian children and it has been speculated that repeated attacks of malaria caused chronic marrow damage (2). The mechanism by which the marrow is damaged is unknown. During the anemic phase of malaria in mice, plasma levels of erythropoietin were raised equally in both fatal and nonfatal infections. Similarly, erythropoietin levels in malaria were found to be raised, although in some cases not appropriately so compared to the degree of anemia (see Chapter 7).

Conclusions

A model for the pathogenesis of anemia of malaria is proposed, in which there are three phases: an initial phase caused by destruction of parasitized cells by parasites or action of antimalarial drugs, a second phase of destruction of NPRBCs by the RES via immune or nonimmune recognition, and a third phase of bone marrow dysfunction. In acute malaria the three phases may follow each other and may overlap, whereas in chronic malaria the anemia is mainly the result of a prolonged third phase of ineffective erythropoiesis.

In acute falciparum malaria the most important mechanisms leading to anemia appear to be the destruction of parasitized and also nonparasitized red cells. Uninfected red cell destruction may play a variable role and the difficulties in quantitation of the extent of damage to uninfected cells is in part caused by the inability to quantitate the total parasite burden accurately because of sequestration. The mechanisms of red cell destruction include reticuloendothelial hyperplasia, reduced deformability of noninfected red cells, membrane changes and immune mechanisms. Some or all of these changes can result from release of parasite antigens that may adhere to noninfected red cells, causing these alterations or leading to immune recognition. In addition, suppression of erythropoiesis may play a part in increasing the severity and lengthening the duration of the anemia.

In chronic malaria there is minimal evidence that hemolysis plays a major role in the anemia, which nevertheless appears to continue in the presence of low grade parasitemia and to improve rapidly with effective antimalarial treatment. Bone marrow dysfunction in the form of dyserythropoiesis appears to play a major role in this type of anemia.

The complex interactions between malaria parasites and host responses and their relationship to anemia are depicted in Fig. 2.

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Chapter 4

Iron and Folate in Malaria

Saad H Abdalla

Introduction

Malaria is endemic in parts of the world where many other conditions causing anemia, such as nutritional deficiencies, inherited red cell variants and infectious diseases, coexist. These conditions complicate clinical presentations, diagnostic tests and management of patients with malaria. The most important of these are nutritional factors.

Interactions between the nutritional state and malaria are complex. Deficiencies of nutritional factors may be deleterious to the host and could equally be so to the parasite, for example by reducing its multiplication rate. Most notable of these nutritional factors are iron and folate.

Iron and Malaria

In some tropical areas, iron deficiency and malaria are singly or in combination the commonest causes of anemia, and they may interact in many ways. This section will deal with three main topics:

(1) The iron metabolism of malaria parasites, which is important for understanding the biology of the parasite and the mode of action of many antimalarial drugs and in the development of new ones.
(2) The assessment of the iron status during malarial infection or in communities where many healthy individuals may have subclinical parasitemia which alters conventional iron-related parameters and often lead to difficulties in defining the iron status.

(3) The effects of iron deficiency and its treatment on the clinical course of malaria.

Iron Metabolism of Malaria Parasites

Plasmodia are obligate intraerythrocytic parasites and therefore have to obtain their nutrients either from the breakdown and incorporation of the host's intracellular components or by transport from the host's plasma. An estimated 25–75% of the hemoglobin, a major source of protein, is digested in the acid parasitophorous food vacuole during the growth phase of the parasite (1).

Like all organisms, plasmodia require a source of iron for essential enzymatic, respiratory and redox reactions. Possible sources of iron for plasmodia include heme iron from the breakdown of hemoglobin, plasma transferrin-bound iron, intracellular ferritin, a labile intraerythrocytic pool of iron, or from nonheme intracellular transit or storage iron (2).

To obtain iron from plasma transferrin, plasmodia would need to induce transferrin binding to the red cells, as transferrin receptors are lost in mature red cells. Various experiments have shown that parasitized red blood cells (PRBCs) will take up radiolabeled transferrin-bound iron but have failed to demonstrate any specificity of this process. The lack of specificity has been shown by the poor correlation between the uptake of radiolabeled iron and parasitemia in culture (3), the large number of parasite antigens that bind to transferrin (4), the increased binding of other proteins such as lactoferrin and albumin to PRBCs and the lack of inhibition of iron uptake by PRBCs in the presence of excess nonradiolabeled transferrin (5).

A transferrin-independent mechanism for obtaining iron from serum has also been proposed, as there was uptake of radiolabeled ferrous (Fe) citrate or Fe nitriloacetic acid into infected and noninfected red cells, despite depletion using immunoadsorption by a factor of 50–100 of transferrin from the culture medium. The radioactive label was found in nonparasitized as well as in PRBCs, and radioactive iron was concentrated in the parasite-rich Percol™ fractions of the cultures (6), but another study suggested that the uptake of iron from plasma by PRBCs was an *in vitro* artefact due to the presence of large molecular weight aggregates of iron salts not normally found in serum (7).

Plasmodia incorporate host cytoplasm into the parasitophorous food vacuole where a hemoglobinase breaks down hemoglobin and the heme is sequestered into the insoluble malaria pigment hemozoin, by the action of

a heme polymerase. It has been postulated that plasmodia are unable to utilize heme iron despite the presence of a large amount in hemoglobin. Free ferriprotoporphyrin IX (FP IX) generated during heme breakdown is extremely toxic, and causes red cell lysis (8). In addition, *P. falciparum* lacks the heme oxidase needed for further degradation of heme (9). The inability of plasmodia to utilize iron through this pathway has now been challenged. It has recently been demonstrated that only 50% of FP IX produced from degradation of heme could be accounted for in hemozoin, with no net loss of iron within parasitized red cells. This was initially shown in *P. berghei* (10) and more recently also in *P. falciparum*-infected red cells (11, 12). The authors also suggested that an alternative mechanism of heme degradation by glutathione could occur. The fate of the balance of the FP IX, and consequently also iron, is not known, but it is likely that at least some of this iron is utilized by the parasites (11, 12). Experimental data using iron chelators (see the following subsection), together with data showing growth of malaria parasites *in vitro* despite extracellular iron deprivation, also appear to favor an intracellular source of iron for the parasites.

Iron Chelators and Malaria

A fundamental observation has been that the iron chelator desferrioxamine (DFO) inhibits the growth of malaria parasites *in vitro* (13). Three mechanisms by which iron chelators cause inhibition of parasite growth have been proposed: by depletion of plasma transferrin-bound iron (3, 13), by a toxic effect of the iron chelator itself (7), or by chelation of an intraerthrocytic labile iron pool (14).

It has been demonstrated that parasite growth was independent of the level of serum transferrin-bound iron, and the action of DFO appeared to be independent of its binding to extracellular iron (7). Preincubation of DFO with iron reduces the inhibitory effect of DFO, which suggested that DFO might be directly toxic to parasites, whereas ferrioxamine, the iron-containing compound, is not. Iron chelators appeared only to be effective as antimalarials if they were capable of entering the parasite cytosol and their toxicity to parasites appeared to be stage-specific. Inhibition of parasite growth was found to be reversible if chelators were present only at a late stage of parasite development, but not when present at the ring stage (15, 16).

Recent work using three different iron chelators, DFO, 2′,2′-bipyridyl (BIP) and aminophenol II, with different permeation properties, and calcein as an indicator of iron showed that these chelators appeared to act by permeation into the parasite cytosol, chelating a labile iron pool. This suggests that the action of the chelators was either on a labile intraerythrocytic iron transit pool or on iron released from the digestion of hemoglobin (14).

The effects of iron chelators in human malaria have now been assessed in clinical studies based on several preclinical observations. *In vitro* DFO suppresses the growth of *P. falciparum* at concentrations between 5 and 20 µmol/L (7, 13, 15). A mean plasma concentration of DFO of 20 µmol/L was achieved in seven non-iron-loaded human volunteers at a parenteral dose of 100 mg/kg/day given as a continuous infusion. Experimental work on mice with *P. vinkei* (17) and *P. berghei* (18) and in Aotus monkeys with *P. falciparum* (19) has shown a reduction in parasitemia when DFO was administered in doses of 60–125 mg/kg/day.

In a double-blind placebo-controlled crossover study, either the placebo or DFO was administered to immune adults with asymptomatic malaria in addition to conventional antimalarial chemotherapy with quinine. DFO was administered in doses of 100 mg/kg/day by continuous subcutaneous infusion (20). The mean plasma DFO concentration achieved in 25 subjects was 6.9 ± 0.6 µmol/L at 36 h and 7.72 ± 0.62 µmol/L at 72 h. In the 25 subjects who completed the study, there was a reduction of parasitemia in both treatment groups, but the rate of reduction of parasitemia with DFO was tenfold that of the control both at first treatment and on crossover ($P < 0.006$). Parasitemia recurred in 19 of 24 subjects followed up between 1 and 6 months.

The same group also carried out a randomized double-blind placebo-controlled trial study in children with cerebral malaria (CM). All the children received standard quinine and supportive therapy in addition to the DFO or placebo. The median time to recovery of consciousness was 20.2 vs 43.1 h in the DFO versus placebo group ($P = 0.38$). However, in the subgroup with deep coma (Glasgow coma score 0–2) the median recovery time was significantly shorter in the DFO group (68.2 vs 24.1 h; $P < 0.03$). Although the rate of parasite clearance from the blood was twice that in patients given DFO compared to those given the placebo, this did not result in significantly reduced mortality (17% vs 22%) (21). The authors also found that there was a slower recovery from coma in children with a transferrin saturation index (TSI) of >43% compared

to those with TSI < 43% in those treated with quinine alone, whereas there was no difference in recovery time in those who were also given DFO whatever the level of TSI. This suggests not only that the high TSI in CM may be a significant association with severity of disease and prognosis, but also that reduction of the duration of coma in those given DFO may be by the chelator acting as a scavenger for free iron. The significance of the main finding of this study of reduction in the duration of severe coma was disputed, as the duration of coma was far greater in the control group compared to those of other studies in children with CM in Malawi (22).

DFO given as a continuous infusion as a single agent was also found to be effective in reducing parasitemia in vivax malaria in Thailand (23).

Other than a direct antimalarial action DFO and also possibly the antioxidant effects as discussed above, it has also been suggested that DFO may downregulate the Th2 response in favor of prolongation of a Th1 effect, thereby enhancing accelerated parasite clearance and recovery from coma. A retrospective study of Zambian children with CM showed that there was a significant rise in IL-4 from baseline levels on day 4 after antimalarial treatment in those given a placebo but no such rise in those given DFO, with no significant differences in the levels of neopterin or IL-6 in the two groups (24).

The possible benefits of DFO in CM, however, do not appear to be of sufficient magnitude to warrant its large scale use, mainly because of its cost and route of administration. Other investigators have therefore studied the antimalarial effects of oral active iron chelators. A class of oral chelators, α-ketohydroxypyridines (KHPs), have been developed, and the most potent of these, deferiprone, also known as L1, has been in clinical use for a while (25). These compounds have antimalarial activity against *P. falciparum in vitro* at concentrations of between 10 and 100 µmol/L. The effects were independent of the iron content in the serum and were abrogated by saturating the drugs with iron prior to addition to the medium, indicating an effect on intracellular iron similar to that seen with DFO (26). Subsequent studies using L1 have failed to show an *in vivo* effect in a rodent model of malaria (27) and in humans (28). In the latter study, adult immune Zambians with low grade asymptomatic parasitemia were treated in a double-blind placebo-controlled crossover study with L1 at doses of 75 and 100 mg/kg/day in three divided doses, achieving levels of 108.9 ± 24.9 µmol/L, which are within the levels found to be effective *in vitro* but with no effect on parasitemia (28). Postulated causes of failure of

L1 to reduce parasitemia *in vivo* include the variability of drug concentration over a 24 h period and the reduced lipophilicity of L1 when compared to DFO. The use of iron chelators in treatment of malaria has added to our knowledge of the biology of plasmodia, but there is no convincing evidence as yet that this class of compounds is useful in the clinical management of malaria.

Iron and Antimalarials

The effects of some antimalarials appear to be related to their blockade of parasite-induced iron detoxification leading to damage to the parasite or lysis of infected cells.

Chloroquine has many mechanisms of action as an antimalarial (Table 1). It acts as a weak base and is concentrated in the acidified food vacuole and is thought to act mainly by inhibiting the polymerization of FP to hemozoin (29), although inhibition of glutathione degradation of heme has also been proposed

Table 1. Some proposed mechanisms of action of some antimalarials in relation to parasite iron metabolism. *Adapted from Rosenthal and Meshnick, 1996* (2).

Chloroquine and other aminoquinolones	1. Alkalinization of food vacuole 2. Formation of toxic compounds when bound to heme 3. Inhibition of heme-dependent protein synthesis 4. Prevention of iron release from hemoglobin 5. Inhibition of food vacuole protease activity 6. Blockage of hemozoin formation by inhibition of heme polymerization
Endoperoxides Artemether Artesunate Artemesinin	1. Cleavage of endoperoxide bridge by iron and heme, with formation of free radicals 2. Formation of covalent bonds with parasite proteins and alkylation of receptor proteins
Iron chelators	1. Chelation of iron pools in PRBCs or intraparasitic with iron deprivation to parasites 2. Free radical scavenger with effects on clinical course of disease

as an additional possible mechanism of action (30). Some of the proposed mechanisms of the action of chloroquine are listed in Table 1 and may also apply to other aminoquinolones such as quinine and mefloquine.

Artemesinin and other related compounds that recently have been widely used in the treatment of chloroquine-resistant malaria appear also to be active through an iron-dependent mechanism (2).

Because many antimalarials have an effect on parasite iron metabolism, it is important to consider the possibility of reduced efficacy of these antimalarials when iron supplementation is given at the time of treating malaria (see the subsection "The Effects of Iron Deficiency and Iron Treatment on the Clinical Course of Malaria").

The other important consideration is the possible interaction between conventional antimalarials when used in combination with iron chelators. The combination of chloroquine and DFO, for example, appears to be antagonistic in cultures of newly isolated *P. falciparum* (31). The possible mode of this interaction was shown by studying the effects of DFO alone, and the interactions between DFO and chloroquine on hematin polymerization. Whereas chloroquine causes inhibition of hematin polymerization by parasites, DFO can initiate and enhance polymerization, and the combination of the two drugs appeared to be antagonistic (32).

Iron Parameters in Patients with Malaria

Malaria and iron deficiency are the commonest causes of anemia in the tropics. Because of the complex interactions between conditions causing anemia and iron-related parameters, it is often necessary to use a number of parameters to assess the iron status in these settings. The parameters commonly used to assess the iron status are:

(1) Hematological parameters: the mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), blood film appearances of red cell hypochromia and microcytosis and, less commonly, bone marrow aspiration to assess bone marrow hemosiderin.
(2) Biochemical measurements: serum iron, transferrin and ferritin, and less commonly used parameters such as erythrocytic protoporphyrin (EPP) and the soluble serum transferrin receptor.

All of these parameters, however, have limitations in areas were anemia is common and is of multiple etiology.

Hematological Parameters Used to Assess the Iron Status in Patients with Malaria

In patients with acute malaria or with frequent exposure to malaria, changes in red cell indices such as MCV, MCH and MCHC may be attributable to malaria or to other coexisting factors such as iron deficiency. A study using manual methods of calculating red cell indices showed that there were no significant differences between MCV and MCHC values in children protected from birth with chloroquine as opposed to those not receiving prophylaxis (33). In a later study of Gambian children using electronic red cell sizing equipment, there were no significant differences in the mean levels of MCV and MCHC between a group of children with acute malaria and those with chronic malaria (as defined in Chapter 3) at presentation. However, another group of children with features of both acute and chronic malaria had significantly higher mean MCV and MCHC than those with chronic malaria at presentation, although these were within normal limits. There was also a significant rise in MCV in all children after treatment of malaria and coincident with marked reticulocytosis, suggesting that this was the cause of the increased MCV (34).

Two studies have independently shown that MCV, and MCH, are valid indicators of severe iron deficiency anemia in areas where malaria is endemic. In the first of these studies on Kenyan children with severe anemia (Hb 5 g/dl or less), the contribution of iron deficiency to the anemia was defined by response to therapy with oral iron, measurement of serum ferritin and serum iron, and exclusion of major hemoglobinopathies. The MCH and MCV were found to be significantly lower in those with iron deficiency anemia (IDA) as compared to other cases with severe malarial anemia (SMA), presumed SMA and anemia of undetermined causes. This study therefore shows that these parameters are valid in diagnosis of iron deficiency in this population with hyperendemic malaria (35). The second study, from India (Orissa), categorized 102 children aged 2–12 years with acute malaria into those with severe (Hb < 7 g/dl), moderate (7–10 g/dl) and mild (Hb > 10 g/dl) anemia. It found significantly lower mean MCV levels in those with severe anemia as compared to the other groups, and a high rate of absent iron stores in the bone marrow, suggesting that iron deficiency played an important part in the severity of the anemia (36).

Three patterns of iron distribution in the bone marrow in malaria can be discerned. The first pattern is that seen in patients with secondary anemia, the so-called anemia of chronic disease, thought to be mediated by proinflammatory cytokines such as IL-6 and TNF-α (see Chapter 6). This pattern shows an increase in bone marrow storage iron in macrophages with a reduction in sideroblasts and is seen on presentation in most cases of acute malaria (37, 38), and persisted or was first seen during convalescence in some cases (38). The second pattern, seen in some patients with all forms of malaria, is that of absent iron stores, reflecting coexisting iron deficiency (34). In the third pattern there is variability in the iron stores in macrophages with a normal or increased number of sideroblasts, sometimes with ring sideroblasts, seen mainly in patients with chronic malaria (34, 37, 39) and often associated with dyserythropoiesis. These different patterns suggest a different pathogenesis of bone marrow pathology in different clinical settings of malaria (see Chapter 7).

Biochemical Parameters Used to Assess the Iron Status in Patients with Malaria

The common biochemical parameters used to assess the iron status are the assays of serum ferritin and of serum iron and transferrin. The transferrin binding index (TBI) or transferrin saturation index (TSI) can then be calculated as a percentage of iron bound to transferrin as a proportion of the total iron binding capacity. Other, less-often-used parameters include erythrocyte protoporphyrins (EPPs) and the soluble serum transferrin receptor (sTfR). These assays have limitations in acute malaria but may be more useful in chronic malaria.

As compared to normal, significantly lower mean serum iron levels with normal mean TIBC were found in Gambian children with acute malaria (34, 37) and in Thai patients of mixed ages (38) but without CM. The mean serum iron levels increased slowly after treatment of malaria to reach normal levels by day 4 (38). By contrast, patients with CM have normal or increased serum iron levels at presentation with normal TIBC (21, 38), as do those with secondary malaria (40), chronic malaria (37, 39) or severe but otherwise uncomplicated anemia (35).

Exceptions to the above patterns of serum iron and transferrin levels were found in studies in Orissa, India. In children aged 2–12 years, no significant differences were found between the mean serum iron levels in children with

acute malaria irrespective of severity of malaria (41) or degree of anemia (36), and those of normal controls. The TSI was significantly higher in children with severe malaria and those with severe anemia compared to those with mild malaria and controls due to a lower mean transferrin level in both cases (36, 41). The apparent discrepancy between these studies and others quoted above may be due to inclusion of patients with CM in the category of severe malaria in these studies, whereas other studies separated these cases from others with SMA.

TNF-α and other inflammatory cytokines are now implicated in the changes in iron metabolism with a block in iron erythroblast incorporation and low serum iron as seen in the anemia of acute or chronic disease (Chapter 6). This is in keeping with changes seen in patients with acute uncomplicated malaria, but not in those with CM. The increased serum iron and TSI in patients with CM is, however, not easily explained by invoking the same cytokines in pathogenesis of the disease and further work is needed to explain this discrepancy. In this context, it is of interest to note that in a study of Vietnamese adults with severe malaria, CM without other organ damage was associated with less marked increase in proinflammatory cytokine levels than in patients with other complications such as renal failure with or without CM (42). Therefore, future studies should examine these parameters in otherwise uncomplicated CM.

Serum ferritin levels appear to be directly related to the severity of malaria (41) and its complications, and the highest levels were found in children with CM (21) and Thai adults with CM or severe malaria (38). More modest but significant increases in serum ferritin were seen in Kenyan children with SMA (35) (Table 2).

The mean levels of serum ferritin rose significantly during the first 48 h after treatment of Thai patients with severe malaria with antimalarials and remained raised at 7 days, reaching near normal levels by 30 days and normal within 90 days. Analysis of isoferritins in this study showed that the source of ferritin in patients with extremely high serum levels was mainly derived from the spleen and binding to concavalin was low in two cases, indicating origin from damaged cells (38).

Erythrocyte protoporphyrin (EPP) levels are raised in iron deficiency because of the block in the final stage of production of heme. High EPP levels are said to reflect tissue iron deficiency. However, like many other parameters used to detect iron deficiency, EPP may also be raised in other conditions where heme synthesis is impaired, such as in the anemia of chronic disease (43, 44). Elevated mean EPP was found in Togolese children with malaria who were

Table 2. Serum ferritin values in studies on patients with malaria.

Study	Category of patients		Number of patients	Serum ferritin (mg/l)
Phillips (1986): Thailand, mainly adults (38)	Cerebral malaria		11	374.0 (209.6)[a]
	Uncomplicated malaria		23	177.3 (36.0)
Newton (1997): Kenyan children, Hb 5 g/dl or less (35)	Severe malaria anemia		24	398 (271–653)[b]
	Presumed SMA		22	165 (82–320)[b]
Das (1997): Children 2–12 years, Orissa, India (41)	Controls		50	27.3 (2)[a]
	Asymptomatic malaria		23	39.1 (7.4)[a]
	Mild malaria		50	165.2 (16)[a]
	Severe malaria		50	410.0 (33.3)[a]
Gordeuk (1995): Zambian children with cerebral malaria (21)	Trans sat <43%	Q	25	358 (90–1422)[b]
		Q + DFO	23	301 (98–919)[b]
	Trans sat >43%	Q	14	560 (303–1035)[b]
		Q+ DFO	19	711 (322–1569)[b]

Q = quinine; DFO = desferrioxamine.
(a) Mean (±2SD); (b) mean (range).

aged 0.5–3 years, with parasitemias above 3000/µl. The authors speculated that these results may be influenced by breakdown products of heme caused by malaria parasites, with a similar fluorescence pattern to EPP (45). In Zanzibari schoolchildren, there was no increase in mean levels of EPP in those with asymptomatic parasitemia, but the expected negative correlation between EPP and hemoglobin level suggestive of iron deficiency was attenuated in those with parasitemias above 5000/µl, thereby reducing the usefulness of EPP in the diagnosis of iron deficiency in patients with higher parasitemias (46). Other studies are needed to assess the possible usefulness of measurement of EPP in various clinical settings of malaria.

The serum soluble transferrin receptor (sTfR) is a truncated version of the cellular receptor. Levels of sTfR are increased in patients with iron deficiency, but are also increased in conditions were there is erythroid hyperplasia, such as in patients with hemolytic anemias (47, 48), ineffective erythropoiesis (49), and congenital iron-loading anemias associated with dyserythropoiesis (50).

Table 3. Serum soluble transferrin receptor (STfR) in malaria.

Author	Patient category	Number	STfR mg/l	Comments
Kuvidibila (1995): Zaire, aged 0.5–16 years (51)	Clinical malaria	17	5.04 (2.41)	Differences not significant
	Subclinical malaria	8	6.17 (3.46)	
	Controls	15	5.11 (1.54)	
Kividibila (1999): Zaire, aged 0.5–16 years (52)	Cerebral malaria	15	5.56 (2.65)	4/15 >7.3 mg/l
	Noncerebral malaria	14	5.49 (2.18)	1/14
	Controls	15	4.83 (1.38)	2/15
Williams (1999): Vanuatu, children (54)	Controls	76	2.45 (2.3–2.7)	Significant reduction in acute malaria $P = 0.003$
	Asymptomatic malaria	18	2.5 (2–3.1)	
	Clinical malaria	21	1.7 (1.2–2.3)	
Mockenhaupt (1999): Nigeria, children, aged 0.8–7 years (53)	Malaria-negative	37	2.99 (2.7–3.3)	Significant increase and proportionate to increasing parasitemia
	PCR only	29	3.48 (3.0–4.0)	
	<1 P/F	42	3.48 (3.1–3.8)	
	>1 P/F	50	4.0 (3.7–4.3)	

There is apparently total disagreement as to the effects of malaria on the serum levels of sTfR in published studies (Table 3). No significant differences were found in the means of sTfR levels in Zairian children with symptomatic malaria, compared to those with asymptomatic malaria and uninfected controls (51), nor between those with CM compared to those without CM (52). By contrast, in a study of 168 nonhospitalized Nigerian children in an area of stable malaria, aged 0.8–7 years, those with asymptomatic or mild malaria were found to have significantly higher mean levels of sTfR compared to those without malaria, with a significant correlation between the level of parasitemia and mean sTfR levels (53). Another recent study, from Vanuatu in the South Pacific, found that the mean level of sTfR was significantly lower in children with acute malaria than in those without malaria (54).

How are these results to be reconciled? Possible explanations are that in acute clinical malaria, suppression of erythropoiesis may be associated with

reduction of serum sTfR, whilst in areas of stable malaria, in patients with chronic malaria with dyserythropoietic bone marrow and ineffective erythropoiesis, the serum sTfR is likely to be increased because of erythroid hyperplasia. These changes, which are further complicated by the common coincidence of iron deficiency, make the results of sTfR difficult to interpret in these populations, and also imply that the diagnostic value of sTfR levels as an indicator of iron deficiency is misleading in areas where malaria is common.

It can therefore be seen that in acute malaria it is extremely difficult to assess a patient's iron status from individual iron-related parameters, as is the case with other acute infectious diseases. In a study where the iron status was validated by the presence or absence of bone marrow iron stores, most parameters measured were reasonable in excluding iron deficiency (34). The best parameter in predicting iron deficiency was the combination of a subnormal serum iron and a raised transferrin, with a better predictive value than a TIBC of less than 15%. This is presumably due to the low levels of serum iron in acute malaria resulting in very low levels of TBI despite normal levels of transferrin (Table 4 (34)).

The theoretical possibility that frequent attacks of malaria may lead to iron loss was raised in a study from northern Liberia, where the mean Hb A2 was significantly lower in children living in a malaria-holoendemic area than in those where malaria was hypoendemic (55). Usually Hb A2 is used in the diagnosis of the β thalassemia trait where the levels are raised, but Hb A2

Table 4. The usefulness of various parameters in correctly diagnosing iron deficiency in children with malaria (from 34).

	Predictive value			
	Positive results (%)	Negative result (%)	Sensitivity (%)	Specificity (%)
MCV	23	81	45.3	65.2
MCHC	45.5	94.5	70.7	86.9
Se Fe	42.8	100	100	47.8
TIBC	53.8	88.9	77.8	72.7
Se Fe and TIBC	72.7	90.9	80	87
TSI	56.3	94.1	90	69.7

percentages may also be reduced in chronic iron deficiency (56). Administration of chloroquine over two years as malaria prophylaxis led to an increase of Hb A2 to the normal range seen in the hypoendemic area. The authors concluded that the findings were consistent with the hypothesis that chronic malaria induces iron deficiency (55). Proposed mechanisms of iron loss in malaria include the reduction in bioavailable iron, which is locked up in malaria pigment, and loss of hemoglobin and therefore iron in the urine through repeated intravascular hemolysis. These theoretical possibilities have not been proven, but a small study failed to find any urinary hemosiderin in the urine of Gambian children with acute or chronic malaria (unpublished observation, Abdalla).

Validation of iron deficiency by examination of bone marrow iron stores is the most reliable way to truly define the coincidence of iron deficiency in patients with malaria in areas of high malaria endemicity. Absent bone marrow iron stores were found in 28% of Gambian children with malaria and anemia (39), and in 15% Kenyan children (35). In population studies, the use of bone marrow is not possible, but some studies using a number of parameters in asymptomatic subjects in areas of high malaria endemicity give an estimate of the extent of background iron deficiency in the population. Thus the incidence of iron deficiency was found to be low in the Gidra-speaking population in the lowlands of Papua New Guinea (57) and in Vanuatu (54) because of a high dietary iron intake. These studies therefore do not support the hypothesis that repeated malaria is associated with an increased incidence of iron deficiency, but that the high rate of iron deficiency of between 20 and 30% is related to other factors such as hookworm or dietary deficiency.

The validity of parameters for diagnosis of iron deficiency in epidemiological studies of healthy populations is important for defining strategies for the reduction of iron deficiency anemia in areas where there is a high prevalence of malaria and where a significant proportion of the population may have asymptomatic parasitemia. The incidence of iron deficiency anemia in asymptomatic schoolchildren was determined in a large-scale study of 3605 schoolchildren in Zanzibar where IDA was defined as the presence of anemia, low ferritin and high EPP levels (46). The rate of IDA was 24% in children with no malaria parasitemia and 21–30% in those with asymptomatic parasitemia. There was no correlation between parasite density and ferritin levels below a parasitemia of 1000 parasites per µL blood. There was a slight increase in ferritin at a rate of 1.5 mg/l per 1000 parasites/µL. EPP levels showed an inverse relationship with Hb irrespective of high or low parasitemia, but the negative correlation

between EPP and Hb was attenuated in children with higher parasitemia. The authors' conclusions from their study and review of the literature were that EPP, serum ferritin and Hb were valid indicators of the iron status in those with asymptomatic malaria, especially those with parasitemias of 1000/μL or less, although higher levels of parasitemia might interfere with the ferritin levels (46).

In conclusion, it can be seen that the determination of the iron status in subjects with coexistent iron deficiency and malaria can be difficult. The most reliable indicators are the MCV, bone marrow iron and response to oral iron. Of these, the most feasible in field settings is the response to oral iron. Where facilities are available, other tests that can be used should include the serum ferritin (where a low level would be diagnostic of iron deficiency but may underestimate its true incidence), and the serum iron in conjunction with other parameters (see Table 5).

The Effects of Iron Deficiency and Iron Treatment on the Clinical Course of Malaria

Whether iron deficiency reduces and iron treatment increases susceptibility to malaria have remained controversial (Table 6). The original observation by Masawe in 1974 was that patients with iron deficiency were more likely to suffer from clinical malaria after treatment of iron deficiency than controls with other anemias (58). In a study of Somali nomads attending a refeeding center, the Murrays found a lower incidence of malaria in subjects who were iron-deficient (0/26) than in those who were iron-replete (16/64). The subjects were then treated in a single-blind oral iron versus placebo-controlled trial. There was a significantly higher incidence of fever and attacks of malaria in the iron group (13/71) as compared to the placebo group (1/66) (59). In a study of infants in their first year of life, Oppenheimer found that babies born with low Hb (which correlates with low iron stores) had a lower incidence of admission with malaria and also bacterial infections, and also of parasitemia in field surveys (60). By contrast, a study of Gambian children aged 1–9 years found no correlation between the iron status measured at the beginning of the rainy season and subsequent attacks of malaria (61). Oppenheimer suggested that the differences in these studies might indicate that iron deficiency is more protective in the malaria-naïve infants than in the older children with previous exposure to malaria (62).

Table 5. Changes in the parameters used to assess iron deficiency in malaria and in the anemia of chronic disease.

Parameter	Acute malaria	Chronic malaria	Iron deficiency	Anemia of chronic disease	Comments
Hemoglobin	Mild to severe decrease	Moderate to severe decrease	Mild to severe decrease	Mild to moderate decrease	Nonspecific parameter
MCV	Unaffected	May rise with recovery reticulocytosis	Reduced	Low normal to reduced	Also reduced in thalassemias
MCHC	Unaffected	Unaffected Reduced with reticulocytosis	Low normal or reduced	normal	Best single parameter in predicting iron deficiency in areas where thalassemia is common
Serum iron	Reduced in uncomplicated cases May be normal or increased in CM	Usually normal	Reduced	Reduced	Affected by acute phase response
Transferrin	Reduced or normal	Normal or increased	Increased	Normal or reduced	Reduced in liver disease
Ferritin	Moderately to markedly increased	Some modest increase with low parasitemia	reduced	Normal or increased	Acute phase response
Erythrocyte protoporphyrin	Unknown? Interference by heme breakdown	Unknown	Increased	May be increased	Awaiting further studies
Soluble transferrin receptor	Normal or reduced	Unknown? Theoretically increased	Increased	Normal	Increased in erythroid hyperplasia
Bone marrow sideroblasts	Reduced	Normal, sometimes increased	None	Reduced	
Macrophage iron	Increased	Increased	None	Increased	

Table 6. Studies of effects of iron supplementation on malaria.

Author	No	Age	Design	Results/conclusions
Oppenheimer (1986) (64) Papua New Guinea	486	2 m – 1 y	IM Fe 150 mg stat at 2 months	**(1) Higher prevalence of malaria in Fe group; (2) lower Hb in Fe group; (3) greater reticulocytosis in relation to malaria. IM replacement in infants may be deleterious.**
Bates (1987) (65) Rural Gambian schoolchildren	190	5–14 y	Oral Fe 100–200 mg twice weekly (+vitamins) vs placebo	*No effect on clinical malaria. Higher parasitemias in supplemented group.*
Smith (1989) (66) Gambian children	213	0.5–5 y	Iron-deficient children given iron or placebo during rainy season	***Significant hematological improvement in iron group. Increased risk of clinical malaria in iron-replete group.***
Harvey (1989) (67) Papua New Guinea		Prepubescent schoolchildren	Blind oral iron 200 mg BD vs placebo Hb 8–12 g/dl	***No effects on parasitemia, spleen size or clinical malaria.***
van Hensbroek (1995) (68) Gambian children	600	Mean 51.7 m	CQ (25 mg/kg) vs fansidar (single dose) oral Fe 27.5–41.25 mg TDS vs placebo 1 OD vs folate 5 mg OD	***Best fansidar plus iron. Worst placebo plus chloroquine. Folate may reduce effectiveness of Fansidar.***
Nwanyanwu (1996) (69) Malawian children	222	<5 y mean 24 m	Clinical mild to moderate malaria Fansidar stat PO on enrolment + (1) daily FeSO4 (4.8 mg/kg), (2) Fe once a week, (3) no Fe	*(1) Effective malaria treatment improves Hb of all; (2) daily iron benefit to those Hb <8 but not those above; (3) weekly iron no different from placebo; (4) longer parasite clearance with daily Fe treatment.*
van der Hombergh (1996) Tanzania (70)	100		Anemia Hb5 g/dl or less +malaria, antimalarials plus either iron supplements or placebo	**(1) No benefit from added iron to effects on Hb; (2) no effects on parasitemia; (3) increased morbidity in iron group from other causes.**
Menendez (1997) Tanzanian infants (71)	832	8–24 w	(1) Fe (2 mg/kg OD PO + Deltaprim PW, (2) Fe + placebo, (3) Deltaprim + placebo, (4) placebo only	***Iron supplement effective in anemia reduction without increasing malaria. Malaria prophylaxis reduced malaria during study period but increased incidence after study period.***
Berger (2000) (72) Togolese children	163	6–36 m	Oral iron supplement 2–3 mg/kg for 3 months vs placebo	*No increase in malaria index in iron group. Nonsignificant increase in number with parasite densities >3000 and 10 000/Cmm.*

Key: **Studies showing deleterious effect of iron supplementation.**
Studies showing iron therapy beneficial.
Studies showing some increased parasitemias in those given iron but without obvious deleterious clinical effect.

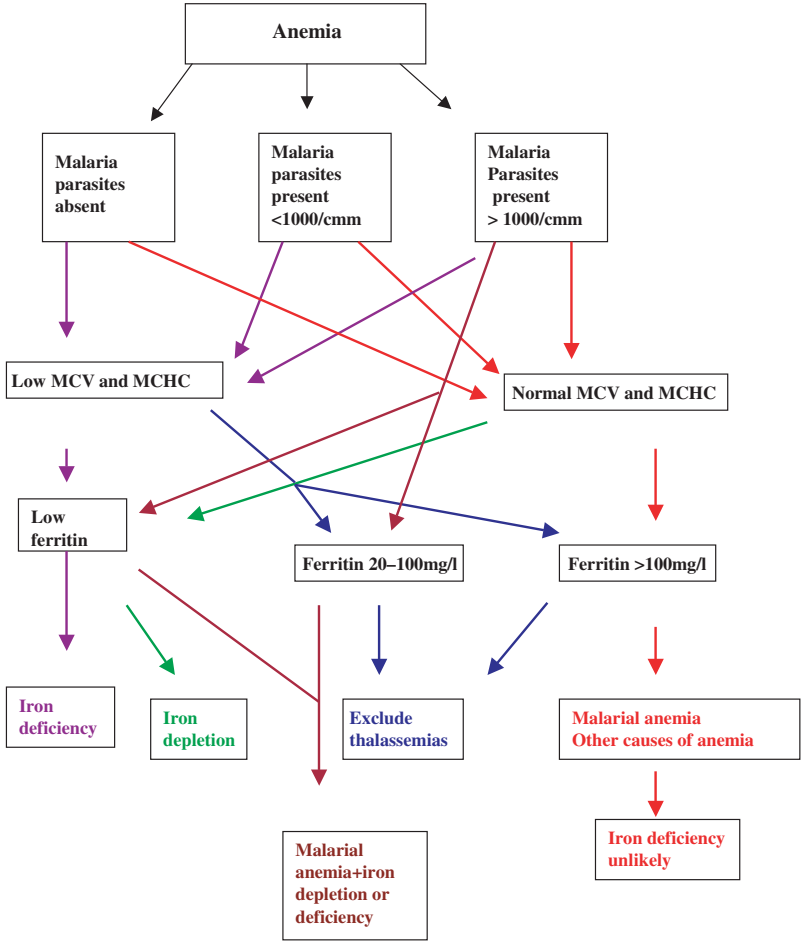

Figure 1 Algorithm for the diagnosis of iron deficiency in areas with high malaria endemicity, by using parasitemia, red cell mean corpuscular volume (MCV), mean corpuscular cell hemoglobin concentration (MCHC) and serum ferritin (SF).

The effects of a single dose of IM iron dextran given to infants aged 2 months in PNG were studied in a large scale long term double-blind placebo-controlled study (63). Although there was no immediate increase in clinical malaria in these infants on the first followup at 7 days, there was a significantly higher rate of malaria slide positivity and spleen rates in the iron dextran group after

6 months (odds ratio dextran vs placebo 1.78 CI 1.02–3.1 slide positive, OR 1.51 CI 0.95–2.33 spleen rate) and 12 months (odds ratio dextran vs placebo 1.95 CI 1.21–3.13 slide positive, OR 1.51 CI 1.02–2.39 spleen rate). The malaria-associated admission rate was also higher in the iron dextran group (25%) than in the placebo group (17%), but without an effect on parasite densities. There was also a higher admission rate with pneumonia in association with malaria and increased mortality in the iron group (63, 64).

The results and conclusions of the various studies as to the effects of iron supplementation on the rate of acquisition of malaria or on the clinical course of acute malaria treated with antimalarials can appear contradictory and confusing. This may be due to the variety of their settings and designs (Table 6). However, taken together the results of these studies can be summarized as follows:

(1) Parenteral iron supplements when given to infants (63, 64) and primigravidae during pregnancy (73) may lead to increased prevalence of malaria, anemia and pneumonia.
(2) Daily oral iron supplementation does not appear to have any effect on malariometric indices in older children with some malaria immunity (67) and may be of benefit and lead to improvement in hemoglobin levels, provided malaria is treated with trimethoprim/sulphonamide combinations or other effective antimalarial (69, 71) but not chloroquine, which was less effective in clearing parasitemia (68). The benefit is more marked in those with Hb < 8 g/dl than in those who are less severely anaemic (69).
(3) Oral iron given to children with iron deficiency during the malaria transmission season leads to significant improvement in hemoglobin but an increased incidence of clinical malaria (66).
(4) There may be slower clearance of parasitemia when iron is used together with otherwise effective treatment of malaria (69), and there may also be a higher rate of parasitemia without a clinical effect in oral iron supplemented children without treatment or prophylaxis (65).

Conclusions

It can be concluded that oral iron supplementation may increase some malariometric measures in individuals in areas where malaria is endemic, and that

this is more likely to be the case in infants and young children with low immunity. However, in patients with severe anemia and especially in those with a low MCV, the effects on improvement in hemoglobin because of the high prevalence of iron deficiency outweigh withholding iron therapy. In these cases, provided malaria is treated with an adequate antimalarial, oral iron supplementation is safe. Parenteral iron should, however, be used with caution and should only be used after clearance of parasitemia and where there is a clear indication for its administration.

Folate and Malaria

There are three areas of interest in relation to folate metabolism and malaria. They are:

(1) Parasite folate metabolism in relation to the action of some antimalarials and the mechanisms of antifolate drug resistance in malaria parasites.
(2) The contribution of folate deficiency to the anemia of malaria and whether it should be prescribed to subjects with malarial anemia in tropical areas.
(3) The risk of precipitating folate deficiency when antifolate antimalarials are used in prophylaxis or an overdose.

Folate Metabolism in Malaria Parasites and Effects of Antimalarials

Folates are a group of pteroylpolyglutamates involved in the key one-carbon transfer reactions that are essential to many synthetic pathways, including DNA synthesis. Preformed or *de novo* synthesized folate undergoes further changes that are important for its functions, such as the formation of polyglutamates essential for cellular retention. Folates are synthesized *de novo* by micro-organisms such as bacteria, malaria parasites and plants but not by mammalian tissue and other higher organisms. Some strains of *P. falciparum* can not only synthesize folate but also salvage and utilize preformed folate (74, 75).

The formation and further modifications of folate in plasmodia are depicted in simplified form in Fig. 2. Antimalarials that act on the folate pathway include the sulphonamides sulphadoxine and dapsone, analogs of para-amino benzoic acid (which causes inhibition of dihydropteroate synthetase (DHPS) and formation of dihydropteroate), and the dihydrofolate reductase (DHFR) inhibitors, pyrimethamine and proguanil. The DHFR inhibitors are weak

inhibitors of mammalian DHFR but act strongly against the plasmodial and microbial enzymes. The actions of the combinations of sulphonamides and pyrimethamine (Fansidar®), or dapsone and pyrimethamine (Maloprim®), are synergistic as they act on different stages of the folate biosynthesis pathway (76).

Over the last 25 years, there has been a major increase in resistance to the antifolates, especially the combination of the two drugs sulphadoxine and pyrimethamine (Fansidar), by *P. falciparum*. Drug resistance in these cases is due to point mutations in the genes of the relevant enzymes. A full discussion on malaria parasite drug resistance is beyond the scope of this review and the reader is referred to a recent reviews for further discussion (77, 78).

A recent study was aimed at explaining the impact of the ability of some parasites to utilize preformed serum folate in relation to the mechanisms of action of combined sulphadoxine/pyrimethamine as an effective antimalarial combination (75). Parasites that utilize preformed folate when grown in a medium containing folate levels equivalent to those found in the serum are resistant to the action of sulphadoxine, and this resistance can be ended by

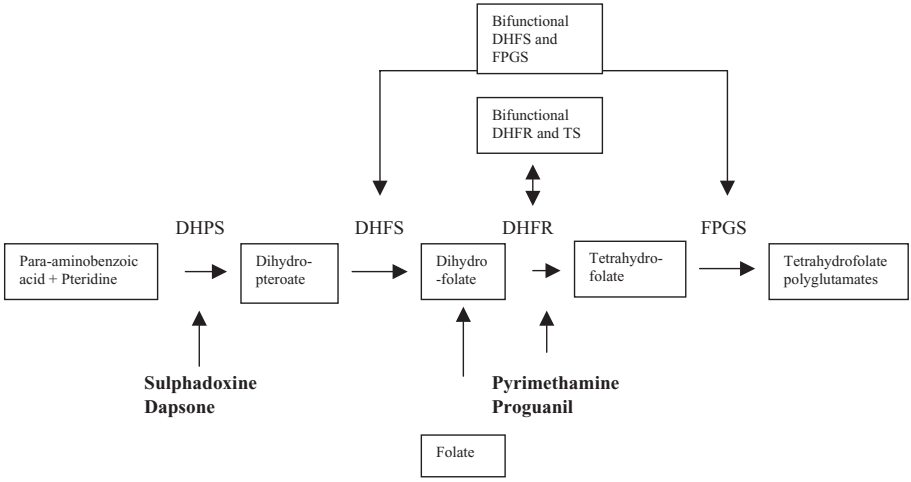

Figure 2 Schematic diagram of the folate biosynthesis and pathways in Plasmodia, also showing the action of antifolate drugs. DHPS = dihydropteroate synthetase; DHFS = dihydrofolate synthetase; DHFR = dihydrofolate reductase; FPGS = folylpolyglutamate synthetase; TS = thymidylate synthetase. *Adapted from Salcedo et al. (2001)* (76).

addition of pyrimethamine at levels insufficient to inhibit parasite DHFR. In addition to a blockade of the action of DHFR, pyrimethamine, in the presence of exogenous folate, appeared to act by blocking folate uptake or utilization.

The inhibition of the action of the combined antimalarials by addition of folate to the culture medium appears puzzling, as the red cell is an extremely folate-rich environment. Most of the folate in red cells is in the form of polyglutamates, which the parasite is apparently unable to utilize because they lack the appropriate pteroylglutamate hydrolase enzyme (79). However, about one third of red cell folate is in the form of monoglutamates (80) and it is therefore surprising that this rich source of folate is not utilized by the parasites.

Folate Deficiency and Folate Supplementation and the Anemia of Malaria

Folate deficiency may limit the erythropoietic response in patients with chronic hemolysis, and folate supplements are used to counteract this, as is the case in patients with sickle cell disease. It has therefore been thought that because of the extensive hemolysis believed to occur in malaria, folate deficiency may play a part in the anemia of malaria, and that folate supplements should be routinely used (81). Fleming suggested that, in pregnant women in West Africa, folate deficiency was an important cause of anemia in association with malaria, and that folate supplementation was necessary for to improving hemoglobin during pregnancy (82).

Earlier studies suggesting a role for folate deficiency in malarial anemia were based on the observations of macrocytosis in the peripheral blood (83), and the presence of hyperplastic erythropoiesis with some megaloblastic features (83, 84). In fact, Knüttgen in 1963 commented on these findings, and whilst recognizing that these changes were not due to B_{12} or folate deficiency, suggested that they might denote a perturbance in the folic acid metabolic pathway (85). These changes were also observed in later studies, and frank megaloblastic changes as well as giant metamyelocytes have been reported in the bone marrows of anaemic children with malaria, coexistent with other dyserythropoietic changes (39), and were more marked in children with chronic, rather than acute, malaria. Despite these findings, several studies have failed to confirm that folate deficiency plays an important role in severe anemia associated with malaria. Serum and red cell folate were measured in 77 Gambian children with severe malarial anemia (mean age 1.98 years; mean Hb 5.36 g/dl). None of the

patients had low serum folate and only two had low red cell folate below the reference ranges. The mean red cell folate was high (622.2 µg/l) at presentation, and in a subgroup of 15 patients the red cell folate showed a significant rise 1–2 weeks after treatment of malaria (mean at presentation 466.2 µg/l; after treatment, 599 µg/l ($p = 0.01$) (34). Other studies have also shown that folate levels are not lowered in acute malaria (35) and that there is a rise of red cell folate after treatment of malaria due to increase in reticulocytes which contain more folate than normocytes (80, 86, 87).

The deoxyuridine suppression test carried out on aspirated bone marrow is a sensitive and specific method which detects the efficiency with which methylation of deoxyuridylate to thymidylate occurs at the cellular level, and this conversion is dependent on the presence of adequate intracellular folate and vitamin B_{12}. This test was found to be normal in most Gambian children with malarial anemia despite the presence of megaloblastic changes associated with dyserythropoiesis, thus confirming conclusively that folate deficiency is not a major feature of the anemia caused by malaria (88).

Folate supplementation was not found to be of benefit in improving hemoglobin levels during pregnancy in a randomized trial in Kampala, Uganda, an area of low malaria endemicity (89). In Nigeria, where malaria is hyperendemic, florid megaloblastic anemia has been documented in pregnant primigravidae in association with malaria. Although the incidence of megaloblastic anemia was reduced in a group of women receiving prophylactic folate throughout pregnancy, there was no significant improvement in hemoglobin levels when compared to controls who did not receive a supplement (82). Moreover, in the same study, red cell folate was found to be higher in patients with malaria than in those without malaria. Therefore the contribution of folate deficiency to the anemia of malaria during pregnancy remains unproven. The question is of scientific rather than practical importance, as in any case it is recommended that folate supplementation should be given to all women during pregnancy, primarily to prevent neural tube defects in the fetus.

A randomized study of chloroquine versus Fansidar, combined with either iron treatment, folate or placebo in Gambian children with acute malaria and anemia (68; see also Table 6), showed that there was no benefit, in terms of hematological recovery, from the addition of folate. The authors also found a significantly higher failure rate of clearance of parasites at days 7 and 28 after treatment with Fansidar and folate (30.1%) than for those given Fansidar and

placebo (15.4%). This suggests that the coadministration of folate together with antifolate antimalarials may reduce the effectiveness of these drugs and may theoretically also lead to eventual increase of resistance to these drugs.

It has also been suggested that folate deficiency may protect against malaria. Ten Rhesus monkeys with induced dietary folate deficiency were resistant to clinical disease after intravenous injection of *P. cynomolgi*, whereas folate-replete monkeys developed the disease and five untreated monkeys died of malaria (90, 91).

It can therefore be concluded that there is no justification for using folate supplements routinely in patients with SMA except in pregnancy. Furthermore, folate supplements should only be given, with caution, to patients with malaria who are receiving drugs containing antifolates, and preferably only after treatment of malaria and clearance of parasitemia.

Folate Deficiency During Malaria Prophylaxis with Antifolate Drugs

The antifolate antimalarials pyrimethamine, proguanil and cycloproguanil are weak inhibitors of human dihydrofolate reductase but much stronger inhibitors of parasite DHFR (92). However, toxicity due to pyrimethamine can occur due to overdosage, reduced clearance such as in renal failure, or prolonged use (93–96). In addition, in patients with borderline folate deficiency, these drugs may precipitate an acute deficiency. *In vitro* experiments using the deoxyuridine suppression test, on human bone marrows, showed that pyrimethamine in concentrations achieved therapeutically, inhibited human DHFR activity and consequent methylation of deoxyuridine, and that this impairment was aggravated in bone marrows of patients with B_{12} or folate deficiency (97). This observation may have important clinical implications for populations with borderline folate levels.

Conclusions

Several important interactions between hematenics in the host and the malaria parasite have been identified in this chapter. Malaria parasites may use nutrients from their intracellular environment or may need to utilize specially

evolved mechanisms to import these hematinics for which receptors are lost in mature erythrocytes. In the case of iron there is increasing evidence that the malaria parasite may be able to use some of the intracellular iron rather than utilize extracellular iron. In the case of folate, existing evidence suggests that the parasites are generally unable to utilize this rich source of folate as they lack the polyglutamate hydrolase, but when challenged with sulphonamides they may be able to utilize external monoglutamates; however, conclusive evidence for this remains forthcoming.

Many antimalarials target metabolic pathways within the parasites either to produce toxicity of breakdown products or to deplete parasites of essential components for growth and in particular by targeting iron and folate metabolic pathways in the parasites. Because of increasing parasite resistance to current drugs, more attention is being paid to drugs that target these pathways, such as iron chelating agents and other antifolate drugs.

In the tropics, facilities for accurate diagnosis of the cause of anemia may be lacking, the opportunities for patient access to medical care may be limited to a single visit, and followup rudimentary. In these situations, the practising physician may be faced with a decision as to whether to cover all possibilities and treat anaemic patients with antimalarials, iron and folate. However, there may be both theoretical and real dangers in giving hematinics together with antimalarials, as these may be antagonistic. The danger of giving oral iron with effective antimalarials appears to be less of a problem. There is overwhelming evidence that iron deficiency of whatever cause is extremely common in areas where malaria is endemic and treatment of malaria facilitates contact of the patient with health services and an opportunity for the treatment of other causes of anemia. This approach should be encouraged in the most severely anaemic patients. However, the use of intramuscular iron in this setting may be associated with increased morbidity and should be reserved for special circumstances. On the other hand, as the incidence and significance of folate deficiency is less well documented in the same populations, and there are potential problems with slow parasite clearance and resistance to antifolate drugs, folate should preferably not be administered at the same time as drugs containing an antifolate component, and if necessary treatment with folate should be deferred until after cessation of therapy. Where other drugs such as aminoquinolones are given, this restriction may be less important.

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Chapter 5

Leukocytes in Malaria

Saad H Abdalla

> It may be well to repeat here that malaria, by itself, does not determine this sort of leucocytosis, which is found to a very pronounced degree in inflammatory diseases for instance.
>
> —Marchiafava and Bignami, 1894 (1)

Introduction

Leukocytes play a vital role in defence against malaria. Initial nonspecific responses include phagocytosis, cytotoxity and cytokine production. With repeated infections, there ensues specific immunity which has a predominant antibody-dependent component. A full discussion on immunity in malaria is beyond the scope of this book and the reader is referred to some recent publications (2, 3).

Leukocyte changes in malaria are variable and depend on many factors, such as the acuteness of infection, the parasitemia, the severity of the disease, the state of host immunity to malaria and concurrent infections. Circulating white cells represent only a small proportion of the total body leukocyte pool, and numerical changes in the peripheral blood in malaria often represent redistribution of these cells between different vascular and tissue compartments.

Numerical Changes in Peripheral Blood Leukocytes

Total Leukocyte Counts

The total white cell count is usually within the normal range, or there may be a slight leukopenia in adults with acute falciparum malaria. In a small proportion of children and adults with severe and complicated malaria, leukocytosis may also occur (4).

Leukopenia appears to be a relatively common finding in in nonimmune adults with malaria. In 404 American servicemen from Vietnam with either falciparum or vivax malaria, 31% had "leukopenia" below 5×10^9/l whilst 63.5% had a normal WBC, and 5.3% had leukocytosis above 10×10^9/l. The leukopenia was unlikely to be related to drug prophylaxis, as similar results were obtained from subjects from Panama with vivax malaria who were not on prophylaxis (5). Similar results were obtained in another study but, interestingly, leukopenia was less common in blacks than in whites (12% vs 22%) and leukocytosis was seen more frequently in blacks than in whites (27% vs 9%) (6). These results are the opposite of those expected, as neutropenia is commonly seen in blacks of African origin (7). These studies overestimate the incidence of leukopenia, as they set the lower limits of leukocyte counts at 5×10^9/l, as opposed to those quoted by Wintrobe, of 4.3×10^9/l (8). The total white cell count was found to be normal in 26/30 adults with acute uncomplicated malaria due to *either P. falciparum* or *P. vivax* three patients had mild leukopenia and one had leukocytosis (9).

Normal or slightly increased white cell counts were found in children living in malaria-endemic areas. In Gambian children the total WBC was found to be moderately raised in acute malaria relative to those of patients with "chronic malaria" (as defined in Chapter 3) (12.7×10^9/l vs 11.2×10^9/l). The leucocyte count returned to normal within three days after antimalarial therapy (10). Similar findings were reported in Indian children, although the counts were lower (8.4 and 6.6 respectively) for acute and chronic malaria (11). A significantly higher WBC was found in adults in Thailand with cerebral malaria when compared to those with uncomplicated malaria (mean values 10.6 and 6.2 respectively (12), and in Melanesian adults with severe and complicated malaria the WCC was raised in 26.5% (mean 9.4, range 2.8–41.2) (13).

By contrast, the WCC was found to be normal in children with chronic malaria (14) and in Thai adults with vivax malaria (15).

Neutrophils

Changes in the total WBC in malaria are generally reflected by changes in the neutrophil counts. Although neutropenia, due to increased neutrophil margination (16), is said to be common in acute malaria, in most cases the neutrophil counts were rarely below 1.5×10^9/l (4). Neutrophilia was not commonly seen in nonimmune adults with malaria, but in some cases, despite the normal neutrophil counts, toxic changes and a left shift were seen in circulating neutrophils (17, 18). In adults with acute uncomplicated vivax or falciparum malaria from Singapore, the neutrophil count was normal in all cases (9).

In acute malaria in children in the Gambia, the neutrophil count was significantly higher at presentation (mean level 6.7×10^9/l) when compared to those with chronic malaria (mean neutrophils counts 3.8×10^9/l). The neutrophil counts decreased by day 3 (mean 3.3×10^9/l). These changes were not observed in children with chronic malaria who presented with anemia (14). Similar results were seen in another study in the Gambia, where relative granulocytosis was found in 23% of children with acute malaria as opposed to 8% with chronic malaria (19), whereas neutrophilia was observed in 5 out of 9 patients with cerebral malaria in adults from Thailand (20). Another study from Thailand showed that the neutrophil count was higher at presentation in patients with severe malaria (altered consciousness, hyperparasitemia or severe anemia) than in those with uncomplicated malaria (5.5×10^9 as opposed to 3.3×10^9), returning to normal values by day 7 after treatment (21). Neutrophilia was also found in patients with severe malaria and cerebral malaria in the absence of bacterial infection and was found to be associated with poor prognosis (22, 23). Leukemoid reactions have also been described in severe malaria due to *P. falciparum*, but this appears to be a rare occurrence (24, 25). Marked neutrophilia is therefore uncommon in uncomplicated malaria and may indicate a more serious prognosis.

It is important to decide whether neutrophilia in a patient with acute malaria is due to concommitant bacterial infection and not only a reaction to malaria, as neutrophilia in acute malaria may indicate either a heavy infection or, in some cases, bacterial infection. In two studies on Thai adults, the incidence of culture-positive septicemias was found to be 9% in those with CM (12) and 3% in those with severe malaria (22). In the Gambia there was an increased incidence of septicemia with enteric organisms in children with acute malaria (26). More recently, a review of data on 783 Kenyan children with severe malaria showed

that the incidence of bacteremia was 7.8% overall and 12% in those below the age of 30 months. The mortality was 33.3% in those with bacteremia, which was threefold that of all cases (27). These observations clearly have important clinical implications and pre-emptive measures, including performance of blood cultures and administration of widespectrum antibiotics (27), especially in at-risk groups of patients whether identified by age or by presence of neutrophilia.

Lymphocytes and Subsets

In the pre-1970s literature the lymphocyte numbers were said to be normal but more recent literature shows that lymphopenia, which is sometimes profound, is a common finding in acute malaria in nonimmune adults (28–31) and is less severe in children in malaria-endemic areas (14, 32, 33). In all cases the lymphopenia appears to resolve within 72 h of initiation of antimalarial treatment (14, 33, 34) and in some cases there is a rebound mild lymphocytosis more frequently seen in children with acute malaria (10, 14, 35).

Differences between the observed peripheral blood lymphocyte responses in the different studies can be attributed to:

(1) The different patterns of malaria, whether chronic, acute uncomplicated or complicated.
(2) The age of the patients. This is of extreme importance because of (a) major changes in the reference ranges for lymphocyte numbers in children between the ages of 0 and 10 years (mean 7.3 at 1 year compared with 3.1 at 10 years) (36), which are also different from that of the adult normal reference ranges, and because (b) there are major differences in the immunological response between malaria-naïve adults and children to their first exposure.

Studies of lymphocyte subsets have found that the major changes in lymphocyte numbers are in the T cell compartment. There is a reduction in T cells with a relative increase in null cells in acute falciparum malaria (32, 33), and these changes are reversed after treatment.

Studies of T cell subsets in peripheral blood have also shown that there is a reduction in both CD4+ and CD8+ T cells in acute falciparum or vivax malaria in one study (28) or in acute falciparum malaria with a reduction in natural killer (NK) cells; and a rebound of CD4+ cells to levels above normal by day 7 post-treatment in another (34). In children with cerebral malaria (CM) there was

a profound reduction in CD4+ cells compared to those with uncomplicated malaria, with a relative increase in the CD8/CD4 ratio (37). Interestingly, the same study found an increase in the percentage of NK cells in peripheral blood of children with uncomplicated malaria compared to those with CM, suggesting that the NK response may correlate with protection against severe disease.

A community-based study of lymphocyte subsets in children in Guinea-Bissau showed an inverse correlation between CD4+ cell percentage and parasitemia, and a significant reduction in CD4+ and CD8+ cells in symptomatic children with high parasitemia when compared to those who were uninfected or had asymptomatic parasitemia (38). A longitudinal study of lymphocyte subsets carried out in Burkina Faso, however, did not show any significant differences in lymphocyte subpopulations according to age and presence or absence of clinical malaria (39).

The transient reduction in the circulating lymphocytes in malaria may theoretically be due to either destruction or redistribution of lymphocytes. In favor of lymphocyte destruction is the finding of lymphotoxic factors in the serum of adult Thai patients with acute malaria (40), active at 15°C but not at 37°C (41), although another study found reactivity at 37°C when using a larger panel of lymphocytes (28). In addition, an increased rate of spontaneous apoptosis of peripheral blood lymphocytes was found to occur in *in vitro* cultured peripheral blood mononuclear cells (PBMCs) from subjects living in a malaria-endemic area in Senegal. The rate of apoptosis was age- and parasite-dependent and increased by *in vitro* exposure of PBMCs to malaria parasite extract (42). There was a reduction in apoptosis when IL-2, IL-4, IL-6 or IL-10 were added to the cultured cells. It has been suggested that CD 95, or Fas, a membrane glycoprotein of the TNF receptor superfamily, which mediates apoptosis by interactions with the Fas ligand, may play a role in apoptosis of lymphocytes in malaria (43, 44). Increased levels of the Fas ligand associated with low lymphocyte and T cell counts were found in sera of nonimmune adults with falciparum malaria (44), and increased expression of CD 95, or Fas, was found on incubation of lymphocytes with malaria antigens (43). However, the authors concluded that CD 95- and Fas-mediated apoptosis was not thought to be the main or only factor leading to apoptosis of lymphocytes as there was no correlation between the two measures (43).

An increased number of preapoptotic cells that were Annexin V+ and 7AAD-negative in peripheral blood of children with falciparum malaria was also found in the early stages of treatment. Stimulation with immobilized CD

3 antibody to activate these cells led to an increase in necrotic cells at a later stage, indicating increased susceptibility of these cells to activation-induced apoptosis (35).

Redistribution or reallocation of lymphocytes in different compartments has also been suggested as an explanation of changes in lymphocyte counts in malaria. Adult patients with acute malaria in the Sudan were found to have increased levels of plasma activation (soluble IL-2 receptor) and inflammation (the adhesion molecules soluble ICAM-1 and ELAM-1) markers, but with a depletion of T cells with a high expression of LFA-1, the T cell receptor for ICAM-1 (45, 46). There was a significant positive correlation between the adhesion molecules and soluble IL-2 receptor levels, but a negative one with the number of activated cells with a high surface expression of LFA-1. The authors suggested that a state of endothelial inflammation associated with adherence of activated T cells occurs and that the peripheral lymphopenia may represent reallocation of these cells to inflamed endothelium in acute falciparum malaria (47). Other studies have also shown that the peripheral blood lymphocytes of children in an endemic area show an increase in activation markers HLA-DR and CD 69, peaking two days after the start of treatment for acute falciparum malaria. These cells, which were CD 8-positive, also produced Ifn-γ *in vitro* (35).

In addition to the above observations, several other lines of evidence suggest that redistribution of lymphocytes is responsible for the changes in lymphocyte count:

(1) Studies of murine malaria have shown that there is an overall increase in splenic lymphocytes despite the different proportional changes (48).
(2) There is a rapid reversal in lymphopenia in adults with acute malaria after effective treatment, suggestive of sequestration followed by release (9, 13, 32, 33).
(3) The proportion of lymphocytes in the bone marrow of children with acute malaria is increased despite peripheral lymphopenia, and this was not seen in children with chronic malaria (10, 49). It is of course likely also that the two phenomena of lymphocyte destruction and redistribution may coexist and contribute to peripheral lymphopenia to different degrees in the different settings in patients with acute malaria.

γδ+ T Lymphocytes

γδ+ T cells normally constitute 4% of circulating T cells but appear to be preferentially localized in the red pulp of the spleen, accounting for 17% of T cells (50). They lack both CD4 and CD8 antigens, and the α and β chains of the T cell receptors, are CD3+ and also express HLA-DR. They are thought to mediate nonspecific cytotoxicity in a non-MHC-related way. γδ+ T cells respond to highly conserved antigens such as heat shock proteins and may act as a first line of defence against a variety of infections (51). They appear to act as a nonspecific first line of defence in malaria and most studies agree that there is an increase in their numbers in acute infections, although the extent of the increase and its duration may be variable in the various clinical settings.

In subjects with minimal prior exposure to malaria, γδ+ T cells appear to be increased in proportion to other lymphocytes at presentation, and this response may be long-lasting. This was shown in patients with acute falciparum malaria in Thailand, at or soon after presentation, and the increase was maintained for at least 28 days (29). In primary malaria in nonimmune overseas visitors returning to France, this increase in numbers of γδ+ T cells was maintained for up to 9 months (52). The percentages of γδ+ cells were transiently increased in patients with vivax malaria and the magnitude of increase correlated positively with previous exposure and severity of disease (54). By contrast, γδ+ cells were found to be reduced in number as part of the general lymphopenia found in the initial stages of infections in subjects with induced malaria, and although there was no increase during the phase of patent parasitemia, these cells exhibited activation markers (55). γδ+ cells were not increased in Ghanaian children in the acute phase of complicated or uncomplicated malaria, but increased 1–2 weeks after treatment (53). γδ+ T cell numbers were not increased in asymptomatic children and adults living in a holoendemic area for malaria, although γδ+ cells isolated from these individuals proliferated *in vitro* on incubation with *P. falciparum* (56).

A high relative number of γδ+ cells was found in the spleens of patients who died of CM (50, 57) and also in experimental simian and murine malaria (57).

γδ+ cells proliferate preferentially when incubated with *P. falciparum* merozoites, even in malaria-naïve individuals (52, 56, 58, 59). The most potent activator of γδ+ cells appeared to be a schizont-associated heat-stable antigen (60). Although some authors found reactivity to malaria antigen to be confined to the V γ 9 subset of these cells (58), another study found extensive junctional

diversity in the δ mRNAs of this expanded population, inferring a polyclonal expansion against multiple distinct antigens (61).

The significance of expansion of $\gamma\delta+$ cells in acute malaria remains controversial. There is evidence that these cells may act to control the initial infection in nonprimed individuals by a direct effect on extracellular merozoites (62). $\gamma\delta+$ cells are strongly stimulated by parasite products following schizogony and produce a number of proinflammatory cytokines, mainly TNF-α and interferon-γ, but also TNF-β, transforming growth factor β (TGF-β) and interleukin-8 (IL-8), with variable expression of IL-2, IL-5 and IL-10 RNAs. These cells stimulated with falciparum antigen or T cell mitogens inhibited the growth of *P. falciparum in vitro*, and this inhibition requires cell-to-cell contact (63, 64). Another study demonstrated that incubation of $\gamma\delta+$ cells from malaria-naïve donors with live SIRBCs led to significant production of IL-2, whereas incubation with PF schizont extract did not (65).

$\gamma\delta+$ cells may therefore represent one of the first lines of defence against malaria infections in the nonimmune host, by direct effects on the parasites or by cytokine release to stimulate other cells to kill the parasites, but in semi-immune populations they do not seem to play a part in controlling parasitemia. $\gamma\delta+$ T cells are not the only cells thought to respond in a nonspecific way to malaria antigens. Several studies have also shown proliferative responses to malaria antigens in the CD4+ compartment of T cells from malaria-naïve individuals (66).

In addition to a protective role, $\gamma\delta+$ may contribute to the pathogenesis of severe malaria. Thus mice which were depleted of $\gamma\delta+$ by monoclonal antibody treatment were found to be protected against experimental CM when infected with the *P. berghei* ANKA strain, which causes CM in untreated mice (67).

Monocytes

An important early observation in the pathology of malaria was the marked reticuloendothelial hyperplasia, specifically of macrophages leading to hepatosplenomegaly (68). Also, monocytosis was a feature of acute malaria in man and in monkeys (4).

Monocytosis was found in 20% of cases of malaria in American servicemen with acute falciparum or vivax malaria (5) and is also common in children with acute falciparum malaria in hyperendemic areas (11, 14), and resolved rapidly within 3–7 days of treatment. In children with acute malaria in India, the

absolute monocyte count exceeded 1.5×10^9/l in 21% of cases (11). Monocytosis was less common in children with chronic malaria (14, 20).

The phagocytic function of monocytes will be discussed in a later section.

Eosinophils

The absolute eosinophil count was increased above the normal range in 7% of American servicemen with acute malaria. Eosinophilia was found in 6% before treatment and in 30% after treatment of malaria (5). In Gambian children with acute uncomplicated malaria there was a reduction or absence of eosinophils in the peripheral blood at presentation, and these returned to at least normal numbers in all cases 3–7 days after treatment of malaria. Marked rebound eosinophilia was seen in two cases that were later found to have strongyloidiasis after treatment of malaria (14). No such changes were seen in patients with chronic malarial anemia.

In a study of adults in Thailand with acute malaria in which atopy and geohelminth infestation were excluded, eosinopenia was found in all and persisted until discharge from hospital (median 5 days), with rebound eosinophilia reaching a peak about one month after the initial diagnosis of malaria (69).

Another study from Thailand found that eosinophil counts were elevated in 11% of patients with acute malaria at presentation and 93% had elevated eosinophil counts by day 7 after treatment. There was then a marked reduction of eosinophil counts by day 14, followed by another increase by day 28 (70). 54 of the 70 (77%) patients were found to have intestinal parasites. There was a significant positive correlation between the eosinophil count on day 7 and hemoglobin concentration on day 28 whether patients had intestinal parasites or not. The authors suggested that an eosinophilic response was associated with hastened recovery from the effects of malaria and that this might be interpreted as signifying the onset of a Th-2 protective response. In a prospective follow-up of children in Ghana, eosinophil counts increased in 7/9 children who subsequently acquired asymptomatic malaria and decreased in 7/9 who had symptomatic malaria, suggesting that the eosinophilic response signified a protection against effects of parasitemia (71). In the same study, hospitalized children with CM, SMA and uncomplicated malaria had low eosinophil counts acutely followed by eosinophilia 30 days after initiation of treatment. Despite the low eosinophil counts, levels of the eosinophilic cationic protein and protein X,

derived from eosinophils, were increased at presentation and were significantly higher in patients with CM than in the other two groups. There was a positive correlation between the serum levels of eosinophil-associated products and those of TNF and the soluble IL-2 receptor, suggesting that eosinophilic degranulation occurs as part of an inflammatory response (71).

The mechanisms by which peripheral blood eosinopenia occurs in acute malaria are not known. The findings of Kurtzhals and colleagues above suggest that increased destruction rather than decreased production of eosinophils is responsible for the eosinopenia in severe malaria. Eosinopenia certainly does not appear to be related to underproduction of eosinophils, as the bone marrow of Gambian children was found to contain a normal or increased number of eosinophils and precursors at presentation (14). Eosinopenia is a feature of the acute inflammatory response and is a useful feature for distinguishing between severe leukemoid reactions and chronic myeloid leukemia (personal observation), and may be related to the different cytokines and chemokines released in the acute phase response. Although it has been suggested that the eosinopenia with increased bone marrow precursors may indicate a certain degree of dyseosinopoiesis (69), this is unlikely as no morphological dysplastic changes were seen in eosinophils and their precursors in the bone marrow of Gambian children with acute falciparum malaria (S. Abdalla, personal observation).

The significance of the eosinophilic changes in acute malaria in the bone marrow and peripheral blood is not known. Experimental *in vitro* work has shown that eosinophil products inhibit the growth of *P. falciparum* (72). Work in murine malaria has shown that eosinophils phagocytose *P. yoelii* infected mouse red cells coated with immune serum (73). Moreover, mice injected with body fluid of *Ascaris suum* to induce eosinophilia were partially protected against the *P. bergheii* challenge (74). Recently a significant reduction in the odds ratio of suffering from severe malaria was found in a retrospective case control study in those coinfected with *Ascaris lumbricoides*, as compared to those who were not, suggesting a protective role of this helminth against human CM (75).

Eosinophil production and migration are controlled by a variety of cytokines, mainly those characteristic of a Th-2 response, in particular Il-5. The persistence of eosinophilia after treatment of malaria may protect against reinfection, as has been suggested, but may also denote a Th-2 response, reflecting the effect of other cytokines leading to the production of protective antibodies. In this respect it is interesting to note that the changes in eosinophil counts in Gambian children appear to occur earlier than in Thai adults. It could be

postulated that this quicker response may reflect premunition due to prior repeated exposure to malaria, which is more likely to be seen in children in hyperendemic areas than in acutely infected adults.

From the practical diagnostic point of view the changes in the eosinophil count may cause some difficulties. Rebound eosinophilia after treatment of malaria may not require further investigation to exclude intestinal infestations unless the eosinophilia persists for more than six weeks after treatment of malaria (69). On the other hand, the absence of eosinophilia at presentation of acute malaria may delay the diagnosis of intestinal infestations (14).

Basophils

There does not appear to be any literature relating to changes in basophils in malaria.

Bone Marrow Changes

This section will be confined to changes in the white cells in malaria, as erythropoiesis is discussed in Chapter 7.

Granulocyte Series

Earlier studies reviewed by Maegraith suggest that there is a reduction of the granulocytic series in marrows of patients with acute malaria, but an increase in lymphocytes and plasma cells. Phagocytic cell hyperplasia with ingestion of parasitized red cells and pigment were also observed (4).

Studies on bone marrows of Gambian children with falciparum malaria showed that there was an overall reduction in the proportion of granulocytic cells in acute and chronic malaria, associated with increased erythropoiesis in chronic malaria and an increased proportion of lymphocytes in acute malaria (10, 14). This reduction appeared to be more severe in the myelocyte and metamyelocyte stages, suggesting a block in or slower maturation. Studies on Thai adults with CM and also those with vivax malaria also showed a slight reduction in the proportion of neutrophil granulocytic series in the bone marrow (14, 19).

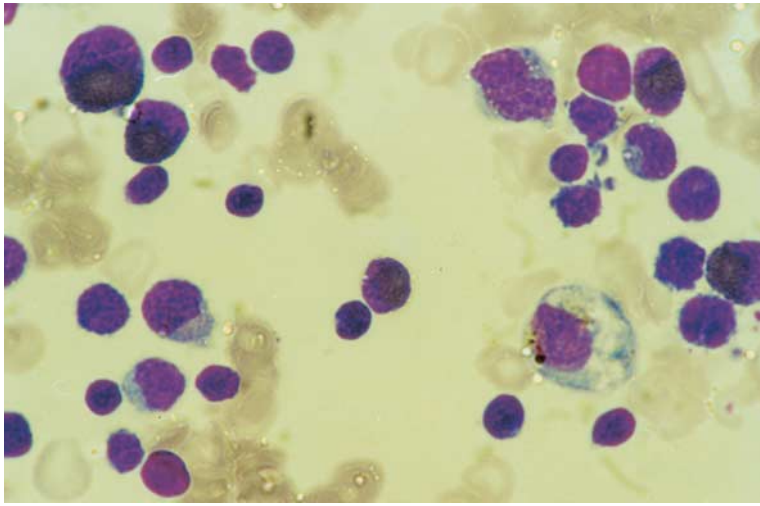

Figure 1 Bone marrow in acute malaria: an increased number of eosinophils and precursors. There is also a monocyte with ingested malaria pigment and vacuolation in cytoplasm.

In contrast to the peripheral blood results, eosinophils and their precursors were found to be increased in numbers in the bone marrows of Gambian children with acute malaria, but not in those with chronic malaria (Fig. 1) (14). Eosinophils and precursors were also often increased in marrows of patients with CM (20) and those with vivax malaria (15).

Monocytes and Macrophages

Studies by Knüttgen (76) in 10 European nonimmune patients and 10 semi-immune Africans with falciparum malaria showed that there was in general no increase in macrophages in the bone marrow except in one fatal nonimmune European.

The proportion of monocytes and of macrophages were found to be increased in bone marrow aspirates from children with acute, subacute and, to a lesser extent, chronic malaria (14). The number of macrophages was also increased in the bone marrow of most patients with CM (15) and vivax malaria (20). Phagocytosis of cells and parasite products will be reviewed in a later section.

Lymphocytes and Plasma Cells

Lymphocytes were noted to be increased in the bone marrow in studies summarized by Maegraith (4) and also in children with acute malaria in the Gambia (14) (Fig. 2). In the latter study plasma cells numbers were not increased. By contrast, the lymphocyte percentage was rarely raised in adult Thais with CM or vivax malaria, although in both cases there was an increase in plasma cells, which was less marked in those with vivax malaria (15, 20).

Significance of the White Cell Changes in the Bone Marrow in Comparison with Those in the Blood

The changes in white cells in the bone marrow and peripheral blood in the various cell types appear to be reciprocal rather than parallel. Thus, in acute malaria there may be (1) peripheral lymphopenia but with no decrease

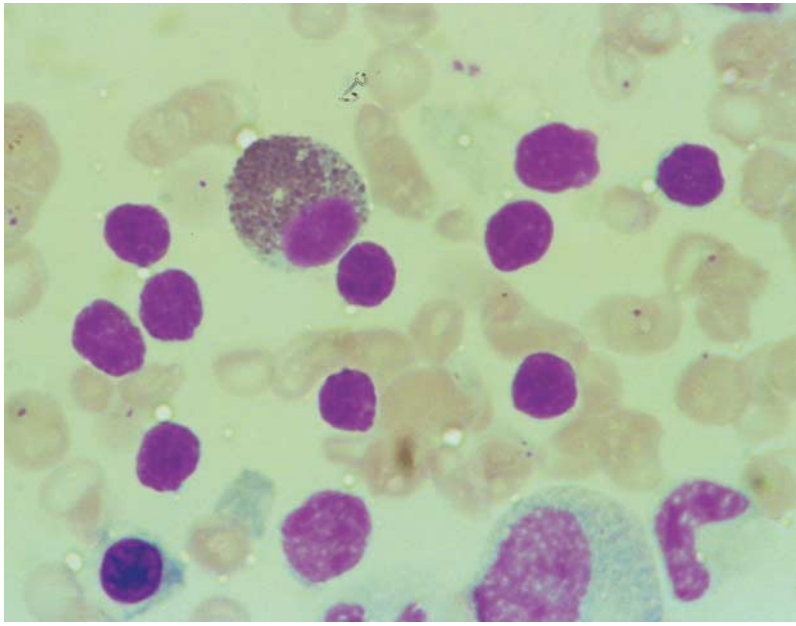

Figure 2 Bone marrow in acute malaria: an increased number of lymphocytes. An eosinophilic myelocyte is also seen.

or increase in marrow lymphocytes, (2) modest monocytosis in the blood with increased macrophage numbers and activity in the bone marrow, and (3) peripheral blood eosinopenia with increased precursors in BM. In addition, most of the changes observed are often reversed within a few days of treatment with antimalarials and in some cases with a rebound eosinophilia which may then persist. These acute changes are highly suggestive of compartmentalization and redistribution of these cells.

These conclusions are supported by studies of murine malaria which show that reticuloendothelial hyperplasia and hepatosplenomegaly result from the recruitment of peripheral blood monocytes from the circulation rather than by local proliferation (77).

Morphological Changes in White Cells in Malaria

A number of morphological changes are often seen in the peripheral blood and bone marrow leucocytes in patients with malaria (Table 1 and Figs. 3–12).

Other than phagocytosis of parasites and pigment, many of the morphological features seen in the white cells in malaria are not specific. These morphological changes may result from either dysmyelopoiesis, activation of cells or damage to cells. Cell activation and phagocytosis will be discussed separately below.

Hypersegmented neutrophils (HSNs) and giant metamyelocytes (GMMCs) (Fig. 3) are considered to be features of megaloblastic anemias. HSNs and GMMCs were found in the peripheral blood and BM of patients with malaria and were not found to be related to hematinic deficiencies as red cell folate and serum B_{12} levels in these patients were normal (10, 14, 78). The deoxyuridine suppression test, which detects tissue deficiency of B_{12} or folate, was normal in the patients with GMMCs, indicating that these changes were not caused by deficiencies in these vitamins (78). GMMCs were more commonly seen in patients with chronic malaria and severe dyserythropoiesis than in those with acute malaria (10). The presence of GMMCs may therefore represent a "dysgranulopoiesis" equivalent to the dyserythropoieis seen in the red cell series (10, 79, 80). Occassional neutrophils with a ring nucleus are also seen and may denote dysgranulopoiesis (Fig. 4).

The presence of reactive lymphocytes and occasional plasma cells and plasmacytoid lymphocytes in the circulation in patients with malaria has been

Table 1. Morphological white cell changes in malaria.

Morphological changes seen	Postulated significance	Other conditions where feature is observed
Vacuolation of neutrophils, monocytes and macrophages (Figs. 1, 6(a) and 6(b))	Effects of activation and phagocytosis	Bacterial infection
Hypersegmented neutrophils	Dysmyelopoiesis	Megaloblastic anemias
Giant metamyelocytes (Fig. 3)	Dysmyelopoiesis	Megaloblastic anemias
Ring neutrophils (Fig. 4).	Dysmyelopoiesis	Myelodysplasia
Reactive or atypical lymphocyte (Fig. 5)	? EBV-infected B cell escape from T cells ? Blastogenic transformation of T cells ? Activated NK cells	Viral infections: EBV, CMV, etc. Toxoplasmosis
Phagocytosis of malaria pigment (Figs. 6(a), 6(b) and 9)	Non-antibody-dependent	Malaria-specific
Phagocytosis of parasitized RBCs (Figs. 8 and 9)	Non-antibody-dependent or antibody-dependent response	Malaria-specific
Phagocytosis of noninfected RBCs (Fig. 10)	Macrophage activation Autoantibodies or antibodies to malaria antigen on RBCs	Autoimmune haemolytic anemias
Phagocytosis of nucleated cells (Figs. 11 and 12)	Macrophage activation	Hemophagocytic syndromes Ineffective hemopoiesis
Smear cells (Fig. 4)	? Spontaneous apoptosis	Whooping cough CLL Other conditions with quick cell turnover

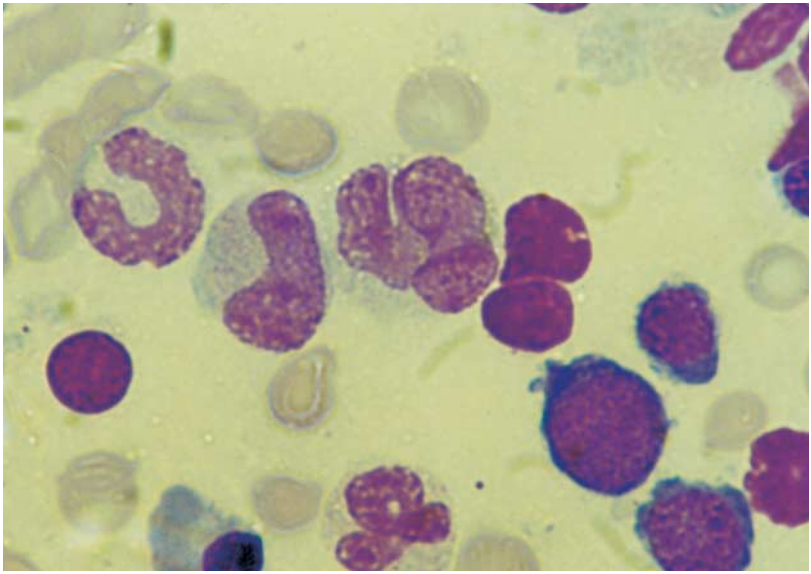

Figure 3 Bone marrow in chronic malaria: a giant metamyelocyte.

noted by several observers (9, 10, 14, 19) (Fig. 8). These cells are more common in acute malaria and increase in the circulation by day 3 or 4 and then decline. An increase in the fraction of large unstained cells (LUCs) in automated cell counters that classify white cells by their peroxidase reaction and size has also been described in acute malaria and may be due to the presence of these cells (81). The nature of these cells has not been studied but they may represent activated T cells responding to malaria antigens or mitogens, or they may result from reactivation of certain latent viruses such as EBV or CMV. In this context it is of interest to note that an increase in EBV-carrying B cells is found in the circulation in acute malaria (82). In another study EBV-infected cells in normal individuals and in patients with acute malaria were found to be large and carrying IgM and IgD (83).

The presence of an increased number of smear cells has been noted in the bone marrow and peripheral blood of patients with acute malaria (10) (Fig. 4). Smear cells, which are common in two conditions characterized by marked lymphocytosis, chronic lymphocytic leukemia and whooping cough, are thought to be fragile cells disrupted during blood film preparation, and

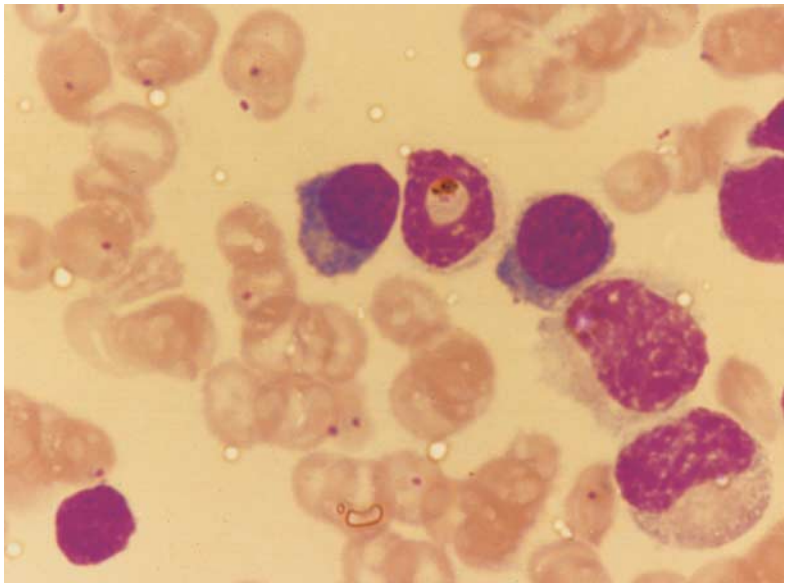

Figure 4 Peripheral blood in acute malaria: a ring neutrophil with central malaria pigment. There is also lymphocytosis and a few smear cells.

the use of albumin-precoated slides can decrease their number. The significance of this finding is not clear. The cells may also be seen in the BM of hypercellular marrows, such as in hemolytic anemias and other conditions. As discussed before, there is increased spontaneous apoptosis when lymphocytes from malaria-infected patients are incubated *in vitro* or when normal lymphocytes are incubated with malaria parasite culture supernatants (42). Whether smear cells represent a population of cells that are more susceptible to apoptosis is an interesting postulate.

Ultrastructural Changes of White Cells

Ultrastructural studies of peripheral blood neutrophils in acute falciparum malaria show changes which reflect some of those seen in peripheral blood under light microscopy, such as vacuoles containing electron-dense material, presumably pigment, and possibly also parasite material (20). Some of these cells show degenerative changes in the cytoplasm and nucleus (84).

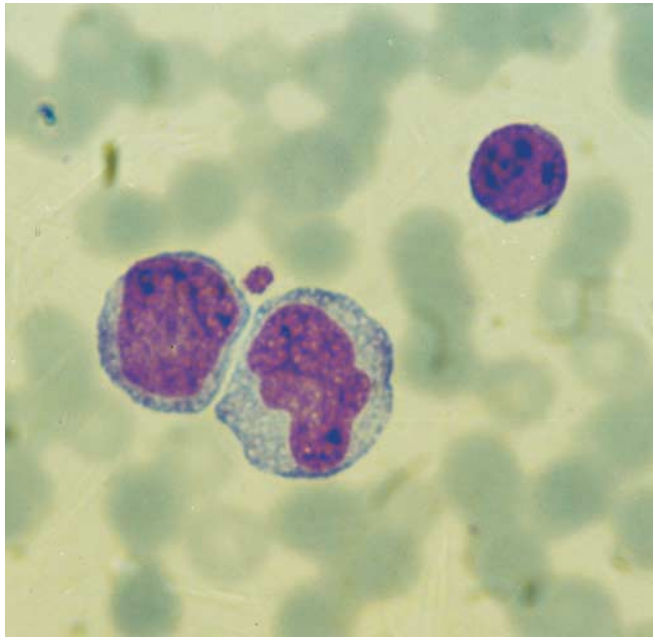

Figure 5 Peripheral blood in acute malaria: reactive lymphocytes.

These changes are also seen in BM neutrophils in CM together with lipid droplets, rounded inclusions and occasional parasite associated with pigment (20). Some peripheral blood monocytes showed dense or, sometimes, heterogeneous clumping of the chromatin, with cytoplasmic vacuolation but intact cytoplasmic organelles (84). Phagocytosis of parasitized and nonparasitized RBCs and of nucleated red cells was seen in macrophages in the bone marrow, and these showed varying degrees of degradation (20). Some of the phagocytosed parasitized red cells showed surface knobs which were connected to the walls of the phagocytic vacuoles by fine strands (85).

In peripheral blood some lymphocytes showed abnormal distribution of heterochromatin and some plasma blasts showed swollen mitochondria and numerous dense granules connected to long parallel cisterna of rough endoplasmic reticulum, not seen in normal cells (84). In some cases of vivax malaria, groups of up to 10 plasma cells were sometimes seen clustered around blood vessels or macrophages (15).

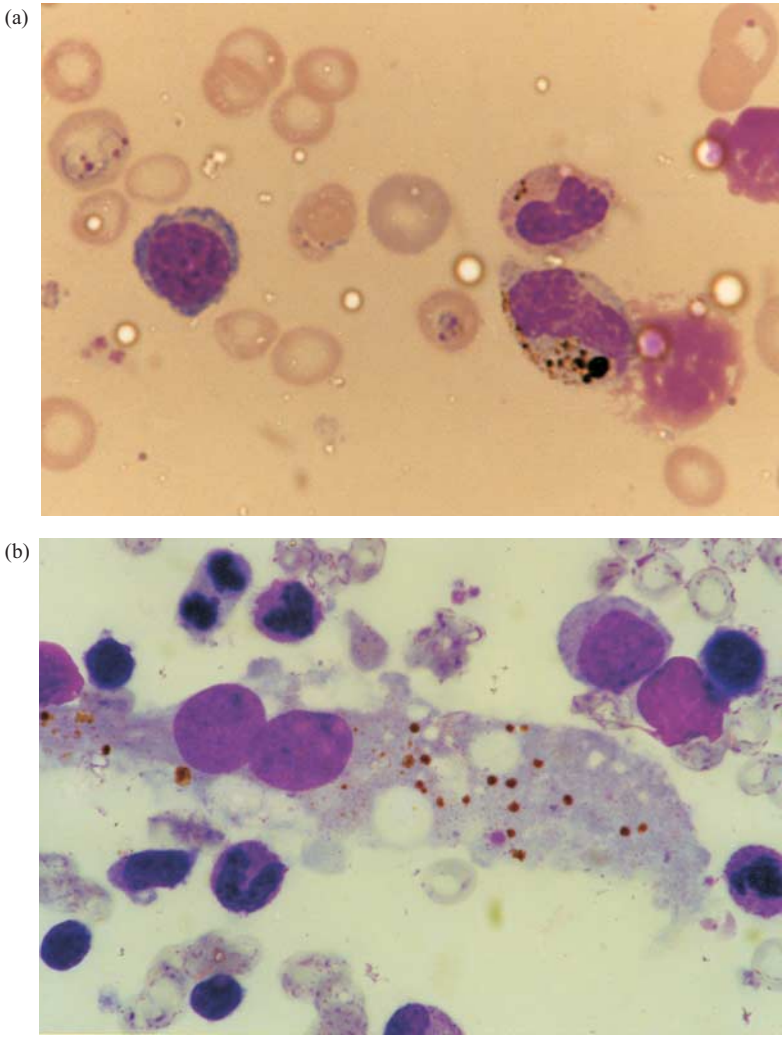

Figure 6 Phagocytosis of malaria pigment in acute malaria by (a) peripheral blood monocytes and (b) bone marrow macrophage, which also shows cytoplasmic vacuolation.

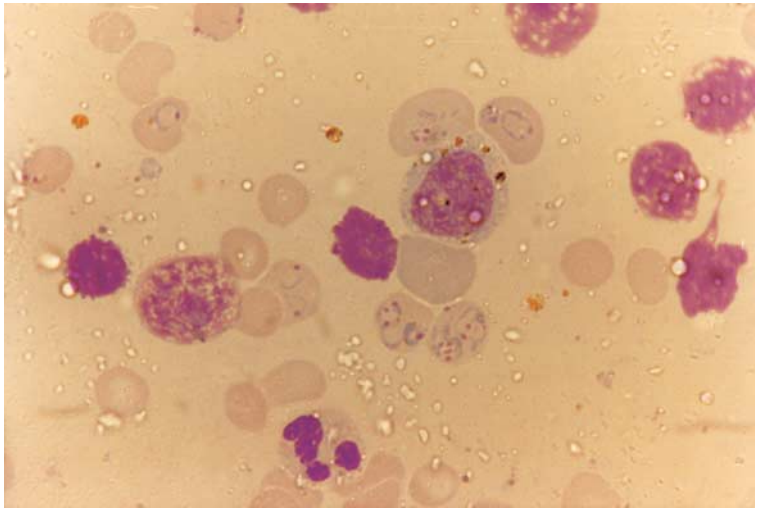

Figure 7 Peripheral blood in acute malaria: a pigment-containing monocyte and many smear cells.

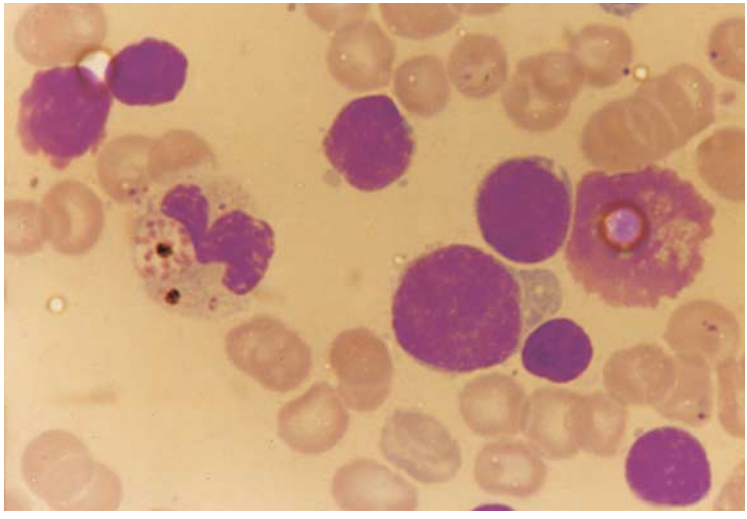

Figure 8 Phagocytosis of schizont-infected RBCs by monocyte in bone marrow in acute malaria.

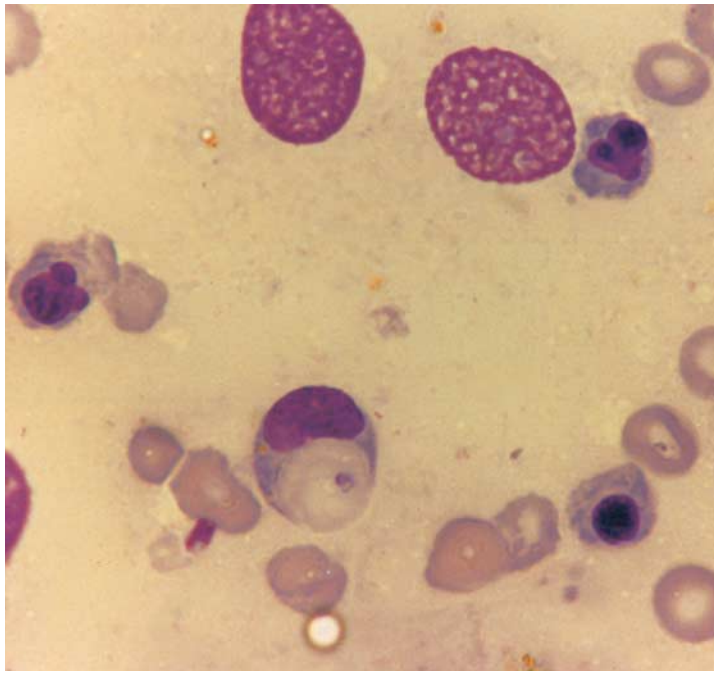

Figure 9 Bone marrow in acute malaria: a monocyte with ingested ring-infected erythrocyte, and dyserythropoiesis.

Knoblike structures were seen on the cytoplasmic surfaces of peripheral blood neutrophils and promyelocytes and some lymphocytes, with ultrastructural similarity to those found on parasitized RBCs (84). These structures may play a similar role to those on parasitized red cells—mediating adhesion of cells to endothelium. Phagocytic cell adhesion to endothelial cells is the hallmark of a murine CM model but not usually a feature of human CM (86). It is of interest, however, that monocytes were found to completely fill and possibly occlude capillary vessels in the lung in a fatal case of acute respiratory distress syndrome associated with malaria (87).

Phagocytosis in Malaria

Phagocytosis by neutrophils, monocytes and tissue macrophages as a major mechanism in the host defence against malaria has been noted from the earliest

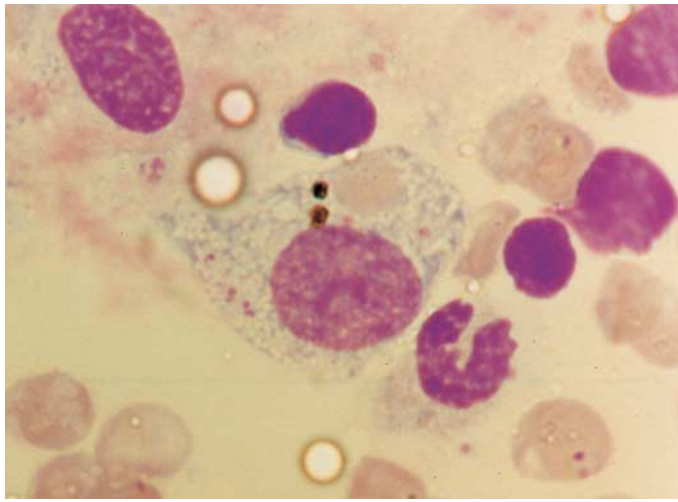

Figure 10 Bone marrow in acute malaria: phagocytosis of noninfected RBCs by macrophage.

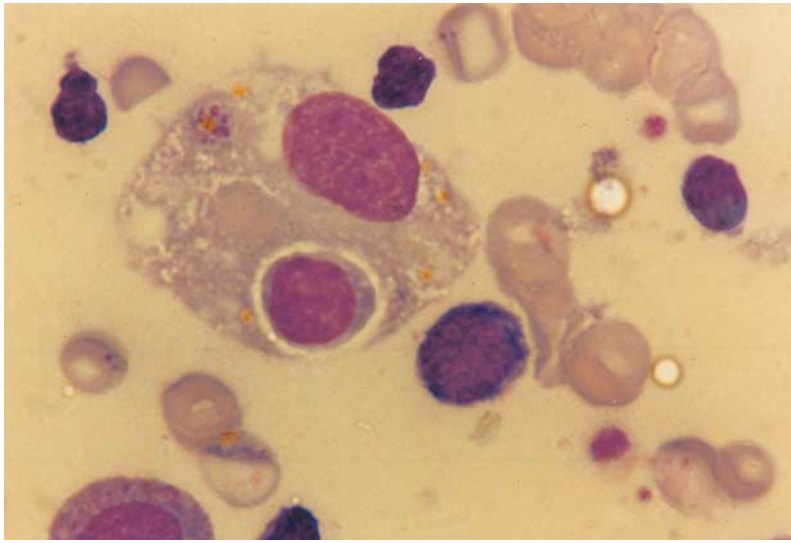

Figure 11 Bone marrow in acute malaria: phagocytosis of nucleated and other cells. Phagocytosis of lymphocyte, noninfected, schizont-infected RBCs and malaria pigment.

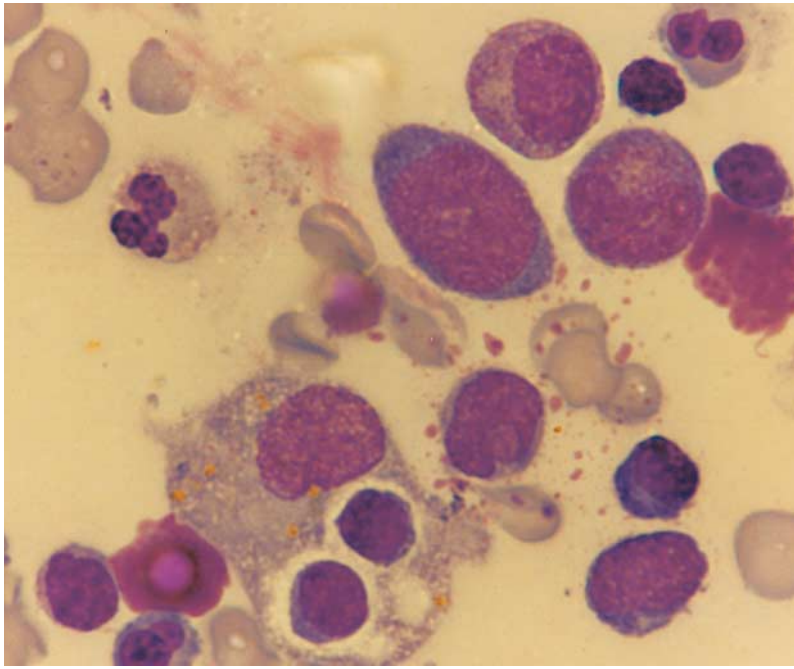

Figure 12 Nucleated cell phagocytosis in acute malaria. Phagocytosis of two nucleated cells. The macrophage also contains malaria pigment.

of studies (1, 68). The particles that are ingested include merozoites, malaria pigment (hemozoin), parasitized red cells, nonparasitized red cells, platelets, and occasionally other nucleated cells.

Morphological Features of Phagocytosis in Malaria

Phagocytosis of falciparum-infected red cells by peripheral blood monocytes is a rarely reported phenomenon and appears to occur only in patients with high parasitemias at presentation (88). A positive correlation between phagocytosis of parasitized and of nonparasitized cells by peripheral blood monocytes was also found in Gambian children with acute malaria and C3b on their red cells, suggesting that complement activation may play a role in this phenomenon (89). A prerequisite for parasitized red cell phagocytosis by

monocytes is the presence of circulating, more mature forms of the parasites, as ring-infected red cells do not appear to be phagocytosed either *in vivo* or *in vitro*; all studies report phagocytosis of the more mature forms (88–90) and therefore PRBC phagocytosis is seen only in patients with circulating schizonts. Phagocytosis of parasitized red cells by peripheral blood polymorphs is rare (90, 91).

Most of the observations on *in vivo* phagocytosis in humans are from bone marrow biopsies. Phagocytosis of parasitized red cells by macrophages in the bone marrow of adult nonimmune patients with malaria correlated with the severity of infection. Marked phagocytosis was seen only in one fatal case, whereas phagocytosis was rarely seen in the lighter infections (79). In a study of Gambian children with malaria, phagocytosis of parasitized and nonparasitized red cells by bone marrow macrophages correlated with high parasitemias but not with the presence or absence of a positive direct antiglobulin test (14) (Figs. 8–10). In CM in adults in Thailand, macrophages in the bone marrow showed marked phagocytic activity, with 1–12 parasitized RBCs in each macrophage and also some nonparasitized red cells and occasional nucleated red cells and plasma cells (20). Phagocytosis of noninfected red cells appeared to predominate in patients with vivax malaria. In 2 out of 9 patients there was phagocytosis of neutrophils, plasma cells and nucleated RBCs (15).

Phagocytosis by granulocytic cells was also reported in the above studies. Whereas phagocytosis involved intact schizont-infected red cells in monocytes and macrophages, only phagocytosis of merozoites was seen in neutrophils (10).

Phagocytosis of pigment is by contrast a frequent occurrence in the peripheral blood and bone marrow monocytes (Figs. 1, 6(a), 7, 8 and 10) and neutrophils (Fig. 4), and also in bone marrow macrophages (Figs. 6(b), 11 and 12). In one study, pigment in macrophages/monocytes was found in over two thirds of cases with high parasitemia and in a half of those with chronic malaria (10). Phagocytosis of noninfected red cells (Figs. 10 and 11) is also observed in some patients with acute falciparum malaria but is less common than infected RBC phagocytosis, and is not seen in the absence of the latter phenomenon. Increased phagocytosis of nonparasitized RBCs appears to be due to activation of macrophages by cytokines released during acute malaria and may play a part in the severity of the anemia following malaria (see Chapters 3 and 6).

Similarly, phagocytosis of nucleated cells, including erythroblasts, neutrophils, lymphocytes and plasma cells, has been observed in both falciparum (10, 14; Fig. 15) and vivax malaria (15). This phenomenon may contribute to

ineffective erythropoiesis by premature destruction of erythroblasts. Rarely, hemophagocytosis may be severe and mimic the viral hemophagocytic syndrome where there is a marked phagocytosis of all cells resulting in pancytopenia (92).

Kinetics and Significance of Malaria Pigment in Phagocytic Cells

Malaria pigment in peripheral blood phagocytes persists after parasite clearance, and the amount of pigment may better correlate with parasite burden than peripheral parasitemia in falciparum malaria because of sequestration of mature parasites.

A study of clearance kinetics of pigment-containing peripheral blood neutrophils and monocytes in 27 adult Vietnamese patients with severe malaria showed that the median clearance time was 216 h for pigment-containing monocytes, compared to 96 h for parasitized red cells and 72 h for pigment-containing neutrophils in venous blood. Using fingerprick samples, the pigment-containing monocyte clearance was even longer, at 12 days. The authors also found that symptoms and clinical and laboratory features persisted beyond parasite clearance but cleared before pigment clearance (93). Another study, from Gabon, divided patients into three groups: children with severe malaria (CSM), children with mild malaria (CMM) and adults with mild malaria (AMM). Whereas there was no significant difference in parasite clearance between the three groups, there was a marked difference in both monocyte and neutrophil pigment clearance between them (monocytes—CSM $= 21 \pm 4.4$ days, CMM $= 15 \pm 7.2$, AMM $= 9 \pm 7$; neutrophils—CSM $= 6.3 \pm 6.2$, CMM $= 2.0 \pm 3.8$, AMM $= 0.2 \pm 0.7$, respectively) (94).

The prognostic value of quantitation of malaria pigment was studied in 300 Vietnamese adults with severe malaria. There was a better correlation between the percentage of pigment-containing neutrophils ($7.7 \pm 5.9\%$) and monocytes ($8.8 \pm 5.9\%$) than with parasitemia. The relative risk of death was 6.2 (95% CI 3.2–11.8) when 5% or more of neutrophils contained pigment, and this showed a better correlation of prognosis than parasitemia of more than 100 000 per microlitre (RR 1.8, CI 1.0–3.3). Because neutrophils are shorter-lived than momnocytes in the circulation, the presence of pigment-containing neutrophils and their number correlated better with the acuteness and severity of malaria than that of pigment-containing monocytes (95).

Two studies from Africa in areas of high malaria endemicity have quantitatively assessed malaria pigment-containing leukocytes in various settings of clinical malaria. In the first study, from Gabon, the median number of monocytes containing pigment was 24% in children with severe malaria, 7% in those with mild malaria and 1% in adults with mild malaria, and the figures for neutrophils were 2.8%, 3.2% and 2.3%, respectively (94). In the second study, from Ibadan in Nigeria, a high proportion of patients with various degrees of severity of malaria, as well as healthy asymptomatic children, had malaria pigment in circulating leukocytes. The percentage of pigment-containing monocytes was as follows: children with CM = 53%, uncomplicated malaria = 17%, asymptomatic malaria = 27% and healthy controls = 28.5%; and for pigment-containing neutrophils, 27%, 9%, 6.5% and 2%, respectively (96).

The above results show high rates of pigment-containing leukocytes in African children, even healthy ones, and much higher levels than in adults from Southeast Asia. The following tentative conclusions can be made:

(1) There is a correlation between the number of pigment-containing monocytes and neutrophils in all the studies and disease severity and presumably also parasite burden.
(2) The number of pigment-containing neutrophils may be a better reflection of the acuteness of the disease.
(3) The presence of pigment in phagocytes and their persistence may be better-correlated with clinical symptoms in areas of hypoendemic or epidemic malaria or in nonimmune individuals than in those of holoendemic malaria where many asymptomatic subjects may continue to have pigment in monocytes.
(4) The much lower number of pigment-containing phagocytes in Vietnamese adults with severe malaria compared to that of children in Nigeria may indicate tolerance to a higher parasite burden in these children and suggests the development of antitoxic immunity prior to the onset of antiparasite immunity.

Phagocytic Cell Function in Malaria

Phagocytic cells include neutrophils and monocytes, as well as tissue macrophages. The function of monocytes/macrophages is central to defence against all infectious organisms, in clearance of necrotic or apoptotic cells, in

tumor cytotoxity and as antigen-processing and -presenting cells. In addition, macrophages play a central role as "nurse" cells in the erythroblastic island in erythropoiesis. The complex functions of these cells involve different processes, such as phagocytosis (antibody-mediated or nonspecific), release of lysozyme and other cytotoxic molecules, release of nitric oxide and reactive oxygen intermediates, and release of cytokines and growth factors. The determinants as to the pathways leading to either of these processes are complex but there is increasing evidence that the major proinflammatory cytokines interferon-γ and TNF-α are important in upregulating the phagocytic function of these cells as well as the production of nitric oxide (NO) and reactive oxygen intermediates (ROI), whereas IL-10 and IL-4 downregulate the effects of the proinflammatory cytokines. There is also evidence that the timing of exposure to these cytokines is important in determining their effects, and that macrophages are resistant to conflicting signals by responding only to the initial stimulating cytokines (97).

In Vivo *Phagocytosis*

Splenic Fc receptor function was determined in twenty patients with acute falciparum malaria in Thailand by measuring the clearance of autologous ^{51}Cr IgG coated red cells (29). There was a significant direct correlation between labelled cell clearance on the one hand and both the absolute parasitemia and hematocrit on the other. Clearance of coated cells was accelerated after the clearance of parasitemia with a return to normal in 6–8 weeks after the acute episode. There was a failure to increase clearance in patients with high parasitemias probably due to reticulo-endothelial blockade by increased antigen load. The conclusions from this study are that increased phagocytosis probably contributes to the control of infection as well the anemia in falciparum infection (29).

In Vitro *Phagocytosis*

In vitro phagocytosis of pigment, merozoites, schizont-infected RBCs and noninfected RBCs have been investigated by several observers in order to elucidate some of the mechanisms of phagocytosis in malaria. Phagocytosis of merozoites by naïve human monocytes can occur in the absence of specific immune sera (98), but can be increased by preincubation of monocytes with immune sera (99). Phagocytosis of parasitized red cells is confined to the more

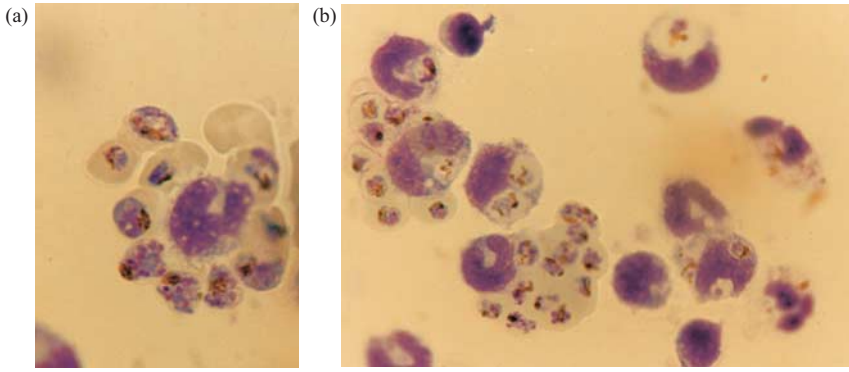

Figure 13 *In vitro* agglutination, phagocytosis, and rosetting of SIRBCs: SIRBCs incubated with either sera from children with positive DAT and acute malaria, or adult Gambian immune sera, or eluates from RBCs from children with positive DAT and malaria showed these phenomena (103).

mature forms, especially schizont-infected cells. Phagocytosis by monocytes and neutrophils of schizonts occurs to a small degree in the presence of nonimmune serum but is markedly enhanced by incubation of schizonts with immune sera (100–103) and sera from children with a positive direct antiglobulin test (DAT) or eluates from their red cells, but not from those with acute malaria and a negative DAT (100) (Figs. 13(a) and 13(b)).

Although the importance of reticuloendothelial hyperplasia and phagocytosis both in protection and in the anemia of malaria has been recognized for a long time, the mechanisms by which early recruitment of macrophages and reticuloendothelial hyperplasia occurs were not known. It is now clear that enhancement of phagocytosis in malaria is caused by release of phagocytosis-stimulating cytokines, mainly by T cells. That this was a T-cell-dependent process was first demonstrated in 1978 by Roberts, who showed that splenomegaly, enhanced phagocytosis and anemia in mice infected with malaria were thymus-dependent processes. Thymus-deficient nude mice showed minimal splenomegaly and carbon particle clearance compared to normal or B-cell-deficient mice infected with *P. yoellii*. These responses were restored by thymus reconstitution (104).

The enhancement of peritoneal macrophages phagocytosis of IgG-coated RBCs by supernatants of splenic T cells primed with either BCG or *P. chabaudii* led to the conclusion that these changes are mediated by release of lymphokines

(105). The first evidence of the role of released parasite products was provided by the demonstration of enhanced phagocytosis of IgG-sensitized RBCs by human peripheral blood monocytes preincubated with boiled supernatants of *P. falciparum* cultures. This process was shown to be dependent on the presence of T cells capable of DNA synthesis, suggesting a mitogenic effect of the supernatants on T-cell-releasing substances that activate macrophages (106). Since that time there have been many publications on the effects of cytokines in macrophage activation in malaria, and this subject will be dealt with in detail in Chapter 6.

Despite the demonstration of monocyte activation and increased phagocytosis *in vitro*, there is some indication that this state of activation is not seen in circulating human monocytes. *Ex vivo* peripheral blood monocytes from patients with malaria in the acute phase showed reduced phagocytosis of anti-D coated red cells compared to normal controls, and this reduced phagocytic function persists for two weeks after treatment of malaria (100). Another study on volunteers with sporozoite-induced malaria in a vaccine trial showed various effects of malaria and their antigens on phagocytic function at different stages of the infection. Monocyte-derived macrophages (MDMs) were normally found to exhibit a variable range of phagocytic capacity. MDMs from the individuals during the parasitemic phase showed a reduction in the population of MDMs with high phagocytic capacity compared to MDMs from normal individuals. This was reversed during convalescence, with an increase in MDMs with high phagocytic capacity (107). Limited exposure of MDMs to schizonts caused an increase in the population of more actively phagocytosing cells from normal controls, but a further reduction in MDMs from patients with malaria. Exposure to larger numbers of shizonts caused reduction or even paralysis of phagocytosis, in normal MDMs. The authors interpreted these findings as indicating that increasing exposure to malaria schizonts can lead to a blockade against both specific and nonspecific phagocytic functions of these cells in acute malaria. Alternatively, the reduced phagocytic capacity of circulating monocytes may indicate the homing of more active cells to reticuloendothelial organs during malaria infection.

Other Changes Related to Phagocytic Cells in Malaria

The chemotactic responses of freshly isolated human peripheral blood monocytes and neutrophils from patients with primary acute malaria due to

P. falciparum, *P. vivax* and *P. ovale* were found to be significantly suppressed in half the patients at presentation but improved to normal levels by day 7 after treatment (108). There was no reduction in phagocytosis or killing of *Candida albicans*. The possible explanations are that reduced chemotaxis of peripheral blood phagocytes in malaria may indicate redistribution of leukocyte population and migration of more active cells to spleen and other tissues, or suppression of chemotaxis by the parasites or their products (108).

The serum levels of polymorphonuclear elastase were found to be raised in patients with severe malaria and the levels correlated with markers of hepatic and renal dysfunction and parasitemia, indicating that neutrophil activation may contribute to the pathogenesis of severe malaria (109). Human neutrophil elastase can degrade parasite circumsporozoite protein of *P. vivax* and at low concentrations can also interfere with or reverse cytoadherence of mature forms of *P. falciparum* (110). Western blot analysis of infected red cells showed degradation by neutrophil elastase of a high molecular weight malarial antigen associated with the membrane, suggesting a possible protective effect of elastase on the human host.

Other evidence suggests that neutrophils may also be involved in protection against malaria. Zymosan-activated neutrophils, on incubation with parasites, were found to inhibit the *in vitro* growth of *P. falciparum*, perhaps related to production of singlet oxygen (111).

The nonphagocytic killing of malaria parasites by human leukocytes has also been demonstrated by a variety of mechanisms. Crisis forms, which are degenerate ring forms, are seen in some patients recovering from malaria and can be induced by incubation of serum from immune individuals with malaria parasites in culture (112). Crisis form induction has been demonstrated *in vitro* by incubation of gamma interferon (IFN-γ)-stimulated human monocytes and MDMs (105). However, IFN-γ and TNF may not be sufficient to induce loss of infectivity during crisis in *P. cynomolgi* in its natural simian host, and other unidentified factors appear to be required (113).

Conclusions

The changes in the white cells in malaria vary in different clinical settings of the disease. In the most severe forms of the disease there is an increase in neutrophils which is sometimes marked, monocyte and macrophage hyperplasia with increased phagocytosis of parasitized and nonparasitized cells, peripheral

Table 2. Summary of molecules and cells involved in the response to malaria.

Triggers	Soluble antigen GPI anchor Pigment Other, undefined malaria antigens Products of red cell destruction	
Primary response	$\gamma\delta+$ T cells Other T cells Macrophages	TNFα IFN-γ IL-12
Effector mechanism	Proinflammatory cytokine release TNFα, IFN-γ, IL-12	
	Protective effects	Harmful effects
	Killing or inhibition of parasites Activation of phagocytosis NO production	Increased adhesion molecules Increased sequestration Hemophagocytosis (NO production)
Secondary response	IL-6 in response to TNFα	Enhanced proinflammatory response
		Anti-inflammatory: feedback inhibition of proinflammatory cytokines Acute phase proteins may help neutralize cytokines Stimulation of T or B cell specific immune responses
	IL-10	Feedback inhibition of proinflammatory cytokines
	IL-4 IL-5	Induction of specific immunoglobulins Modulation of responses toward Th2
Modulators	TGF-β	Early and marked response inhibits IFN-γ and may be harmful Late production may modulate response

eosinopenia, and sometimes severe lymphopenia. In less severe forms the changes may be attenuated, and in mild malaria there may be no major changes in the white cells apart from transient lymphopenia. In chronic malaria some of the changes seen may be similar to those in megaloblastic anemias and may denote dysmyelopoiesis.

The changes in the white cells in malaria can be attributed to changes in cytokines and may be initially produced by nonspecific responses to malaria antigen by $\gamma\delta+$ cells or other T cells, and do not require previous priming. These responses are geared in the main toward activation of the phagocytic cells in order to contain the infection, until the refining of specific immune responses.

The percentage and persistence of malaria pigment-bearing white cells in the circulation may be an important indication of parasite burden and also prognosis in nonimmune individuals, but may be less important in assessing prognosis or even disease acuteness in areas with more sustained infections.

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Chapter 6

Cytokine Changes in Malaria

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Proinflammatory Cytokines and Malaria

Cytokines, released mainly by leukocytes, play both protective and pathological roles in malaria. Cytokine responses may be broadly classified as either proinflammatory (Th-1), with production of cytokines such as IFN-γ, IL-1, IL-6 and TNF-α, or anti-inflammatory (Th-2), with production of cytokines such as IL-10, IL-4 and IL-5. The subject has been extensively reviewed recently and the reader is referred to the reviews for further reading (1, 2).

In malaria there are different pathways by which cytokines are triggered, and an overlap in the actions of the cytokines themselves and the cells that produce them. The variability of the responses according to the age, immune status and genetic makeup of the patient adds further complexity. The following review is biased toward a hematological perspective on the role of cytokines in malaria and is not meant to be comprehensive. The proinflammatory cytokines to be discussed in this section include TNF-α, IL-1, IL-6, IL-2, IL-12, IFN-γ and the chemokine IL-8.

Tumor Necrosis Factor

TNF-α is a cytokine produced by monocytes and macrophages, and also by other cells, including T cells and neutrophils. There is now a large body of evidence that there is increased production of TNF-α in acute malaria whether due to *P. falciparum* or *P. vivax*. Several studies have shown that there is a correlation between serum TNF-α levels and parasitemia and disease severity (3, 4). High serum levels of TNF-α were found in patients with CM (5, 6), and TNF-α levels

are raised in most patients with other severe complications of malaria, including severe organ damage (4, 5), hyerparasitemia (4) and severe anemia (4, 7), although another study failed to find an association with severe anemia (8). Extremely high levels of serum TNF-α, up to ten times those found in patients with uncomplicated malaria, were found to be related to fatal outcome in CM (6). However, comparably high levels of TNF-α have been found in the sera of patients with vivax malaria, in which severe or fatal disease is rare, suggesting that the cytokine is not the only cause and other factors, such as sequestration leading to local effects, may play a part in severe disease seen in falciparum malaria (9). Others have also found that despite high levels of TNF-α associated with complicated malaria, there is no direct correlation between TNF-α levels and sequelae or outcome (4). Significantly higher levels of serum TNF-α and other proinflammatory cytokines were found in patients with severe malaria and organ damage compared to those with otherwise uncomplicated cerebral malaria in Vietnamese adults (10), suggesting that the pathology of CM may be different from that of other organ damage.

Coculture of falciparum-infected red cells and PBMCs from normal donors led to TNF-α secretion by these cells, and there was a sharp rise in TNF-α production shortly after schizont rupture. The authors suggested that malaria fever is mediated, at least in part, through paroxysmal TNF release associated with schizont rupture (11). Further work showed that two exoantigens of *P. falciparum*, Ag1 and Ag7, were responsible for the cytokine-releasing activity from human monocytes (12), but other antigens may also cause release of these cytokines.

TNF-α may be protective to the host or may contribute to the pathology of severe malaria. The cytokine may act in malaria and other conditions either directly on cells or infectious organisms, or by causing secondary release of other cytokines or increased production of nitric oxide. TNF-α-containing sera were found to be cytotoxic to cultured *P. falciparum* (13). Recombinant TNF-α injected into mice with malaria also led to accelerated recovery from the disease (14, 15), but was not found to be inhibitory to the parasites *in vitro* (15), suggesting that effector cells or other additional factors are needed to inhibit the parasite growth.

The harmful effects of TNF-α overproduction in malaria may result from the upregulation of expression of endothelial adhesion molecules such as ICAM-1 or other adhesion molecules, leading to enhanced sequestration of parasitized

red cells (16), to hypoglycemia (6), to direct damage to cells or tissues, and, in murine studies, to anemia through macrophage activation leading to increased phagocytosis, suppression of erythropoiesis and dyserythropoiesis (17).

Theoretically TNF-α overproduction in malaria can contribute to reduced red cell production through two different mechanisms: suppression of eryrthopoiesis and dyserythropoiesis. In man, TNF-α has been shown to suppress erythropoiesis through inhibition of BFUe and CFUe (18–20) and there is a direct correlation between TNF-α levels and anemia in patients with rheumatoid arthritis (21). In a murine model, TNF-α causes suppression of erythropoiesis which could be counteracted by administration of erythropoietin (22). Also, the suppressive effect of macrophages from patients with rheumatoid arthritis on human BFUe and CFUe was shown to be mediated by TNF-α (23). Other studies have also suggested that TNF-α and other proinflammatory cytokines contribute to the anemia of chronic disease by the suppression of erythropoiesis and reduced erythroblast iron incorporation (24). In severe acute malaria, there is often suppression of erythropoiesis and changes in iron distribution between erythroblasts and macrophages similar to those seen in the anemia of chronic disease (25), and TNF-α may therefore play a part in suppressing erythropoiesis in these cases (see Chapter 7).

TNF-α was shown to stimulate erythropoiesis at low levels of infusion in rats, but the erythroid hyperplasia was associated with dysplastic changes and ineffective erythropoiesis (26). Morphological dyserythropoiesis has also been demonstrated in acute malaria in human studies (25, 27–30), but superimposed on a background of suppressed erythropoiesis. This is similar to the experimental situation in murine malaria where dyserythropoiesis was seen in the bone marrow of mice infected with malaria and also injected with TNF-α (17).

The major contribution of dyserythropoiesis in malaria is, however, thought to be in children with chronic low grade parasitemia where there is marked erythroid hyperplasia, ineffective erythropoiesis and reticulocytopenia (25). Although TNF-α levels were not measured in these children, the clinical picture of low grade parasitemia and absence of systemic manifestations such as fever is unlike that of acute malaria where the serum TNF-α levels are raised, suggesting that TNF-α is unlikely to be the cause of dyserythropoiesis in these patients, and that other factors are involved.

As severe malaria is relatively uncommon in relation to the number of infected individuals in endemic areas, genetic polymorphisms may account

for this variation in severity. In the case of the TNF-α gene, a polymophism 308 nucleotide upstream from the transcription initiation site in the TNF promoter has been described due to a point mutation. These alleles are called TNF1 and TNF2 and the less common TNF2 allele is associated with elevated serum TNF-α. In a case-controlled study of Gambian children, homozygotes for the TNF2 allele were found to have a sevenfold increased risk of death or severe neurological sequelae due to CM (31).

Unlike CM, there is a paucity of data, other than in experimental murine malaria, to implicate TNF-α in the pathogenesis of malarial anemia. Serum TNF-α levels were not elevated in Gambian children with severe anemia (8). In the same study SMA was found to be associated with another allele, the TNF-238 A allele, with a 2.5-fold risk of developing SMA ($P < 0.001$), suggesting separate genetic factors close to the TNF gene responsible for SMA and CM (8).

In another study, a reduced IL-10 to TNF-α ratio was found to be a risk factor for both SMA and CM. Whereas carriers of the TNF1 allele had IL-10 to TNF-α ratios of more than 1, carriers of the promoter TNF-238A and TNF-238A/TNF2 polymorphisms consistently had IL-10 to TNF-α ratios of less than 1, and this association was highly significant, suggesting that the polymorphisms are associated with the reduced ability to mount an anti-inflammatory response, which in turn is related to disease severity and complications (32).

The TNF2 allele is strongly associated with the high-TNF-producing autoimmune MHC haplotype HLA-A1, B8, DR3. However, despite this linkage disequilibrium, disease severity in Gambian children was found to be independent of HLA class I and class II variations, suggesting that the regulatory polymorphisms of cytokine genes, rather than the association with HLA types, affect the outcome of severe infection (8). Moreover, the maintenance of the TNF2 allele at a gene frequency of 0.16 in The Gambia implies that the increased risk of CM in homozygotes is counterbalanced by some other biological advantage (31).

To summarize, extremely high levels of TNF-α are found in peripheral blood of patients with fatal CM and complicated malaria with organ damage but not always in patients with otherwise uncomplicated CM or severe anemia. High levels of TNF-α may also be found in vivax malaria and severe falciparum malaria but there is no correlation with disease severity. Although TNF-α, can lead to suppression of erythropoiesis and increased phagocytosis of infected

and uninfected red cells in acute malaria, there is no evidence that it plays a part in SMA seen in chronic malaria.

Interleukin 1 (IL-1)

IL-1 is a cytokine produced mainly by monocytes and macrophages, but can also be produced by T cells and other cells. IL-1 induces IL-2 and IL-2R production by T cells and activation of these cells. In addition, IL-1 has pyrogenic properties and causes upregulation of some intercellular adhesion molecules, and its functions and effect show a certain degree of overlap with those of TNF-α.

Increased levels of IL-1 with a direct correlation with disease severity were found in Gambian children with malaria (6), and IL-1 may synergize with TNF to produce some of the pathological effects in malaria (33). In another study in Gambian children with severe malaria, both admission TNF-α and IL-1 levels correlated with lactic acid levels, and sustained lactic acidosis, measures which have previously been found to be the most significant prognostic factors (34). By contrast, another study, also in Gambian children, showed no positive correlation between disease severity and IL-1 levels but a significant one with increased levels of the IL-1 receptor antagonist (35). Another study, on adults from Brazil, found that IL-1 levels were increased in only 2 of 35 patients with acute malaria. There was also a reduced proliferative response of these patients' T lymphocyte to PHA stimulation, and this increased when IL-1 was added to the culture medium (36). The authors suggested that there is a deficiency of IL-1 production in acute malaria and that this may contribute to the antigen-specific immunosuppression seen in this condition. Il-1 levels were also not raised in subjects with induced experimental malaria, although these subjects were treated as soon as they became parasitemic (37).

Addition of IL-1 to cultured human endothelial cells enhances the adhesiveness of parasitized RBCs to these cells by 50% (38). Patients with severe malaria had increased levels of serum-soluble ICAM-1, and the addition of soluble malaria antigens to human mononuclear cells led to increased expression of IL-1 RNA after four hours of incubation and increase in ICAM-1 production. It was postulated that IL-1 may contribute to the pathology of CM by increasing sequestration through increased expression of adhesion molecules (39).

Interferon Gamma

Interferon gamma (IFN-γ) is an important macrophage activator produced by T cells. IFN-γ production is stimulated by IL-12 and leads to activation of macrophages with enhancement of killing of intracellular organisms, increased phagocytosis (antibody-dependent and -independent) and induction of inducible nitric oxide synthase (iNOS) leading to nitric oxide (NO) production. The action of IFN-γ may be by stimulation of macrophages. IFN-γ (and other cytokines) may also upregulate ICAM-1 (and other adhesion molecules) (40).

Clinical Data

Druilhe and coworkers first demonstrated circulating interferon-like activity in the sera of patients who had malaria with all four species of human malaria parasites, in 1983. 87 of 100 sera contained an inhibitor to the cytopathic effect of the vesicular stomatitis virus, and titres were higher in nonimmune Europeans than in subjects from a hyperendemic area. The inhibition of cytopathic activity was neutralized by antibodies to IFN-γ (41). IFN-γ-activated monocytes from malaria-naïve donors were shown to inhibit parasite growth *in vitro* and to cause induction of crisis forms of the parasites, and these effects were inhibited by antibodies to IFN-γ (42).

High production of IFN-γ appears to correlate with lack of previous exposure in acute falciparum malaria. Thus high serum IFN-γ levels were found in nonimmune German travelers returning with falciparum malaria (3), in Thai adults with acute malaria, at presentation (43) and in adult Indians in the Amazon with malaria, and especially those with high parasitemias (44). In Tanzanian patients with clinical malaria of any severity, measurement of urinary neopterin as a surrogate marker for IFN-γ production showed that there was higher production in children than in adults (45). IFN-γ levels increased abruptly a day before the onset of fever and parasitemia in subjects with induced malaria (37). On the other hand, IFN-γ levels were not raised in patients with uncomplicated imported malaria, irrespective of parasitemia (46). A longitudinal study of cytokine levels in Gabon, where malaria is holoendemic, found positive correlations between IFN-γ levels and parasitemia and presence of clinical malaria, and a negative one with age (47).

The levels of IFN-γ do not always correlate with disease severity or mortality (3, 44, 48). Similarly, no correlation between disease severity and serum levels of IFN-γ was found in Burundi adults with severe malaria with or without CM (49), nor in children with mild malaria or CM in Guadeloupe, where malaria is mesoendemic (50).

Other studies have found a correlation between high serum IFN-γ and morbidity and mortality. In Papua New Guinea, serum levels of IFN-γ and also TNF-α were found to be significantly higher in children who died of CM than in those with mild malaria (51), and in Vietnamese adults high serum IFN-γ and other proinflammatory cytokine levels correlated with severe malaria with organ damage but not with otherwise uncomplicated CM (10). The serum levels of IFN-γ were not raised in patients with *P. vivax* malaria (3) in one study but were found to correlate with the intensity of fever and parasitemia in another (52).

Cytokine responses can be transient and may account for the lack of correlation between cytokine levels and disease severity in the different studies. Neopterin, a pteridine derivative found in body fluids after monocyte activation with IFN-γ, is stable and has been used as a more sensitive index of recent IFN-γ production. Thus serum IFN-γ levels and urine neopterin levels were found to be increased in Thai patients with acute malaria at presentation, and neopterin levels continued to rise during the first five days but started to fall thereafter. Neopterin levels correlated with serum IFN-γ levels and both levels correlated inversely with the morbidity and number of previous attacks of malaria, suggesting a reduced IFN-γ production with increasing immunity (53). Persistence of high serum neopterin levels to seven days and low levels of IL-4 after treatment of malaria in Zambian children were found to be associated with prolonged anemia (54), and a 10-fold rise in serum neopterin was associated with a 50-fold increased risk of severe anemia, suggesting that macrophage activation through high production of IFN-γ may result in anemia (55).

Experimental Data: Factors Causing Release of IFN-γ from Lymphocytes

$\gamma\delta+$ T cells were found to be the main source of IFN-γ when human PBMCs from unprimed donors were incubated with schizont-infected red cell extracts (56) or with a soluble schizont-derived antigen, and this effect was enhanced by the addition of IL-1, IL-10 and IL-12 (57). Another study found that $\alpha\beta$

unprimed T cells produced IFN-γ in response to a membrane-bound antigen, independent of the exoantigen responsible for TNF release (58). Crude malaria antigen led to increased production of IFN-γ from cells of patients with acute malaria but without lymphoproliferation (48). Lysates of *P. falciparum* parasitized human erythrocytes and several synthetic peptide antigens of the parasite were also found to stimulate U937 cells (a human macrophage cell line) to secrete neopterin after incubation for 48 h, and this effect was enhanced by the addition of human IFN-γ (59). Similar results were obtained in monocytes from 2 of 3 normal healthy donors cultured with the antigens for 7 days.

The pattern of IFN-γ production induced by *P. falciparum* crude schizont extract appears to be dependent on previous exposure to malaria. A higher level of IFN-γ production was found in Europeans recently recovering from primary malaria than in immune adult Gabonese living in a malaria-endemic area (60). The number of IFN-γ-producing cells and the amount of IFN-γ produced were, however, increased by CD 8+ cell depletion, indicating the presence of *P. falciparum* antigen-specific suppressor T cells in the immune individuals (47). On the other hand, the response of peripheral blood lymphocytes of Gabonese children to liver stage or merozoite antigens was more marked in those who either had mild malaria or prospectively had less severe or less frequent reinfection than those who presented with severe malaria or also subsequently had more frequent and severe reinfection (61). These observations suggest that an early response to malaria by IFN-γ production contributes to protection rather than pathology. IFN-γ production by placental intervillous mononuclear cells in response to malaria antigen was also found to correlate with gravidity and absence of susceptibility to malaria in pregnant women (62).

Crude schizont extract was found to lead to release of low levels of IFN-γ from lymphocytes of nonimmune and malaria-immune individuals, whereas soluble malaria antigen was found to lead to release of the same cytokine only in a proportion of immune individuals (63). An exoantigen of molecular weight 70 KD caused nonimmune CD4+ and CD8+ cells to produce IFN-γ (64). In another study Riley and colleagues found that PBMC responses to partially purified soluble antigen were depressed in the acute phase of the disease but were regained in about half the children during convalescence and persisted in some for a long period (65). The same workers found that these responses were depressed in pregnant women and the suppression was more marked in primigravidae (66), although other studies failed to show this

(67). The response to crude antigen of cord blood cells was low (67). There was a correlation between poor pregnancy outcomes and maternal anemia on one hand and increased proinflammatory cytokines and low IL-10 production on the other (68). Responses to purified extracts of exoantigens showed that children whose cells responded to one of these antigens (Ag7) with production of IFN-γ subsequently had more symptoms than those who did not (69).

In vitro experiments have shown that inhibition of *P. falciparum* growth in culture occurred only in the presence of both T cells and adherent cells but with neither alone. The effect was partially dependent on release of IFN-γ by CD4+ cells produced in response to an antigen released during schizont rupture, and as shown by IFN-γ neutralization experiments. However, a CD8+ T-cell dependent but IFN-γ-independent mechanism was also postulated (70).

Conclusions

IFN-γ is produced by cells, mainly $\gamma\delta$+ cells, in response to a number of plasmodial antigens and leads to activation of macrophages to increased phagocytosis and cytotoxicity. An early acute response may be beneficial, but sustained release of the cytokine can contribute to anemia. There is evidence that in the process of increasing immunity, antigen-specific suppressor CD8+ T cells may be generated and may attenuate the response to antigen, thereby reducing the possible deleterious effect of sustained IFN-γ release.

Interleukin 6 (IL-6)

IL-6 is a pleiotropic cytokine and is a member of a family of the neuropoietic cytokines, so called because of their effects on nervous and hemopoietic systems (71). Members of this family share the GP 130 receptor for signal transduction as a part of a heterodimeric receptor complex, usually with another specific receptor for each cytokine. In the case of IL-6 this is an IL-6 binding alpha chain, which also occurs in a soluble form. Many cells, including monocytes and endothelial cells, produce IL-6 and TNF-α, and other proinflammatory cytokines trigger its production. IL-6 plays an important role in the immune response, including proliferation of antigen-specific cytolytic T cells and NK cells, and in B cell growth, differentiation and immunoglobulin secretion, the acute phase response and in hematopoiesis (72). IL-6 and related cytokines are released as part of the proinflammatory immune response, but they also

play a pivotal role in limiting this response by causing the release of acute phase proteins which neutralize the action of proinflammatory cytokines, and by downregulating the production of TNF-α, IL-1 and phospholipase A2 (71).

There appears to be agreement in most studies that the IL-6 level is increased in patients with acute malaria, but its role in the pathogenesis of the disease remains controversial. High levels of IL-6 were found in experimental murine CM (ECM), but also in nonlethal malaria. The rise in IL-6 levels was produced in response to TNF-α and IFN-γ, as shown by prevention of this increase by prior treatment with anti-IFN-γ and TNF-α antibodies. Anti-IL-6 antibodies did not prevent ECM but reduced the occurrence of hypergammaglobulinemia usually seen in these mice. The authors concluded that the IL-6 is not directly implicated in the pathology of ECM (73).

IL-6 inhibits the development of hepatic stages of murine malaria in intact mice or isolated hepatocytes (74), through stimulation of its production by TNF-α (75) or IL-1 (76), and may account for the protective role of these cytokines against *P. yoellii* and *P. berghei* (77). There is also evidence that IL-6 may accelerate immunity to *P. chabaudi* through increased production of specific antiplasmodial immunoglobulins (78).

Many reports suggest that IL-6 may play a part in the pathology of severe human malaria as levels of IL-6 correlated with both TNF-α levels in patients with *P. falciparum* malaria and disease severity. Levels of IL-6 were also increased together with TNF-α and IL-1 in parallel with parasitemia in patients with *P. falciparum*, but not in *P. vivax* (3). High levels of IL-6 were found in Senegalese adults with severe malaria, including CM and organ failure (79); in Gambian children with CM (35); in adult Thais with complicated malaria (80); and in Gabonese children with severe malaria (81). It has been proposed that proinflammatory cytokines such as IFN-γ, TNF-α and IL-1 may be responsible for the raised IL-6 levels in these patients (86). It has also been suggested that IL-6 production triggered by TNF-α causes feedback inhibition of TNF-α production by T cells in malaria (83).

The plasma levels of IL-6, the soluble IL-6 receptor (sIL-6r), the soluble GP 130, leukemia inhibitory factor (LIF) and the ciliary neurotrophic factor (CNTF) were measured in 32 cases of imported severe malaria. IL-6 and sIL-6r were significantly higher in patients with CM and acute renal failure than in those with other severe complications. There was a drop in IL-6 levels within 24 h of initiation of therapy and a rise in sIL-6r. There was a significant reduction

in CNTF levels in patients with renal failure and CM, with a return to normal levels after therapy. There were no changes in levels of gp 130 or LIF. The authors concluded that the shedding of sIL-6r may lower the IL-6 levels. The results could indicate that these changes in cytokines may either contribute to or result from CM and acute renal failure in severe malaria (84).

Various cytokine levels were measured in 287 Vietnamese adults with severe malaria. Significantly higher levels of IL-6, IL-10, TNF-α plasma levels and IL-6 : IL-10 ratios were found in patients who died as compared to matched surviving controls. Interestingly, CM without systemic organ involvement was not associated with the highest cytokine levels. In patients who died there was a relative rise in IL-6 levels and a fall in IL-10 levels, leading to higher IL-6 to IL-10 ratios terminally. The authors concluded that the imbalance between the proinflammatory and the anti-inflammatory response in acute malaria may be an important determinant of mortality associated with organ failure but not in otherwise uncomplicated CM (10).

In summary, IL-6 levels are raised in severe malaria and there is general agreement that the levels correlate with disease severity. The disagreement is mainly in the interpretation of this finding. On the one hand, the high IL-6 levels, which generally rise in parallel with those of other proinflammatory cytokines, may contribute to disease severity. On the other hand, IL-6 production, in response to these proinflammatory cytokines, may represent the first step in the downregulation of this initial response, and form the first stage toward the formation of specific antibodies. The failure of this process to progress further, by failure of production of IL-10, may lead to a more serious outcome, but may not necessarily implicate the increased levels of IL-6 as being causative of this outcome.

Chemokines and Receptors

Chemokines are a family of low molecular weight proinflammatory cytokines that are attractants and activators of leukocytes. About 40 chemokines have been characterized and they include interleukin 8 (IL-8), macrophage inflammatory protein 1 (MIP-1), platelet factor 4 (PF4) and regulated upon activation, normal T-cell expressed, presumed secreted (RANTES). These chemokines have specific receptors but, in addition, the Duffy blood group antigen was found to be a promiscuous receptor for many chemokines, and the name "duffy antigen receptor for chemokines" (DARC) has been

adopted for this antigen. Moreover, the Duffy blood group antigen on red cells is also known to be the receptor for merozoites of *P. vivax* and the absence of expression of Duffy antigens in West Africans leads to absolute protection against infection with this parasite.

IL-8 is a chemokine that activates and attracts neutrophils. It is released by several cell types, including monocytes, macrophages and T-lymphocytes, in response to an inflammatory stimulus. High IL-8 levels, similar to those in patients with fatal gram-negative sepsis, were found in six Thai patients with acute malaria (85). IL-8 levels were found to correlate with parasitemia in patients with severe imported falciparum malaria (86) and also with disease severity in Gabonese children (81).

IL-8 is one of the cytokines frequently produced by V γ9+ T cells when they are coincubated *with P. falciparum* schizonts and supernatants of malaria cultures (56), and also from monocytes, granulocytes, T cells and large granular lymphocytes (LGLs) (87). A significantly higher production of IL-8 RNA was also found to be expressed in the hemozoin-laden macrophages from malaria infected, as opposed to noninfected, placentas (88).

The role of IL-8 in the pathogenesis of malaria remains to be elucidated. As marked neutrophil responses amounting to leukemoid reactions are rare in malaria, it could be postulated that the neutrophils and IL-8 do not normally play a major role in the pathology of the more common complications of malaria. However, IL-8 may theoretically play a part in the pathology of acute respiratory distress syndrome (ARDS) in nonimmune patients with malaria, as this cytokine has been implicated in ARDS due to other diseases where there is accumulation of neutrophils and monocytes in the lung. It is also tempting to postulate that IL-8 levels and responses may be different in Duffy-negative individuals. A recent study of knockout mice with a Duffy null genotype showed that severe lung pathology occurred at a lower level of endotoxin administration in these mice than in their wild type litter mates (89), which may indicate that the Duffy antigen in red cells may play a part in dampening down the acute neutrophil-mediated response. It would be of interest to investigate the relevance of this finding in individuals lacking RBC Duffy antigen expression in relation to both susceptibility to such pathology and levels of circulating IL-8.

Interleukin 12 (IL-12)

IL-12 is a proinflammatory cytokine which activates cytotoxic and NK cells. It acts on T cells to produce IFN-γ, and it has been postulated that the *in vivo* effects observed after IL-12 administration are caused by its stimulation of IFN-γ production (90). The other biological effects of recombinant IL-12 independent of induction of IFN-γ include tumor toxicity, anemia but with trilineage bone marrow hyperplasia in squirrel monkeys, lymphadenopathy and splenomegaly (91). A phase 1 trial in humans showed that intravenous IL-12 causes pancytopenia and increased levels of circulating IFN-γ and neopterin (92). IL-12 injected into mice caused a redistribution of hemopoiesis from the bone marrow to the spleen (93). It appears also to have a stimulatory effect on erythropoiesis. IL-12 with or without added erythropoietin had no effect on *in vitro* mouse erythroid colony growth; there was enhancement of colony growth in a dose-dependent fashion only when either IL-4 or stem cell factor (SCF) was added to the cultures (94). The authors suggested that the inhibitory effect of IL-12 therefore appears to be an indirect one, possibly through stimulation of IFN-γ production.

IL-12 injection prior to malaria infection was found to protect mice against challenge with *P. yoellii* sporozoites by enhancing IFN-γ production and in part through an NO-dependent pathway (95). Similar findings were described in mice infected with *P. chabaudii* (96) and monkeys infected with *P. cynomolgi* (97). IL-12 intraperitoneally injected simultaneously with infection with *P. chabaudii* in susceptible A/J mice was found to enhance bone marrow and splenic erythropoiesis measured by BFUe and CFUe assays, and there was a significant increase in hematocrit, reticulocytes, increased precursors in marrow and spleen as well as of circulating BFUe (98). Higher doses of IL-12 caused enhanced splenic erythropoiesis and reduced bone marrow erythropoiesis. Thus IL-12 appears to have a protective action against malaria in susceptible mice that is independent of the enhanced development of protective immunity. The same authors also showed that B6 mice, which appear to be more resistant to the same parasite, have a higher capacity to produce IL-12. Thus IL-12 may have a complex role in protection in murine malaria, acting by enhancing erythropoiesis at low doses and by stimulation of immunity through IFN-γ production at higher doses (99).

Modest increases in serum IL-12 levels were found in adult Thai patients with severe malaria. The levels did not correlate with parasitemia and persisted over a 14-day period (100). In a case control study in Ghanaian children with severe versus mild malaria, serum levels of IL-12 and IL-12 production capacity from mitogen-stimulated whole blood were significantly lower in severe malaria than in mild malaria (61). There was an inverse correlation between serum IL-12 levels on the one hand and parasitemia and pigment-laden monocytes on the other. The authors suggested that heavy parasitemia and macrophage loading with hemozoin may impair the capacity to produce IL-12. Similar findings of significantly reduced levels of IL-12 were made in Gabonese children with severe malaria, compared to levels in mild malaria (101). The significance of these findings will remain to be further elucidated in severe malarial anemia, especially in the context of chronic malaria.

Anti-inflammatory Cytokines

These cytokines are characteristic of the Th-2 response seen in inflammatory response. They include IL-10, IL-4 and IL-5, and TGF-β.

Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine produced by monocytes, T cells and other cells in response to several proinflammatory cytokines, in particular TNF-α production by monocytes/macrophages. IL-10 downregulates the production of these proinflammatory cytokines and may therefore protect against harmful effects caused by their overproduction. IL-10 is also a B cell proliferation and maturation factor. EBV produces an analog to human IL-10 (102) which is biologically active. IL-10 or its viral analog may therefore have a role in some human B cell non-Hodgkin's lymphoma (103) (see Chapter 11).

In Vivo *Levels*

Elevated serum IL-10 levels were found in all patients with imported acute malaria and in adult Thai patients with acute malaria (43, 104). Although serum levels in mild malaria were markedly raised, they were about threefold higher in patients with severe malaria and CM (104) and correlated with disease severity (48). The levels returned to normal after 7 days of treatment of malaria. In

the Thai study, the levels of IFN-γ and IL-10 rose in parallel and returned to normal within 14 days.

A preliminary study in Ghana showed that in children with SMA (Hb $<$ 5 g/dl) circulating IL-10 levels were significantly lower at presentation than those in children with either uncomplicated malaria or CM. In a subsequent study, children were subdivided into groups according to whether they were conscious or not, and also into categories of no anemia, mild anemia and severe anemia. Fully conscious patients with severe anemia had the lowest levels of IL-10 and also the highest ratio of TNF to IL-10 ratios (105). The TNF levels were not significantly different between the three groups of patients. The authors postulated that IL-10 underproduction in response to TNF may play an important role in severe malaria anemia, but not apparently through downregulation of TNF production. Another study found significantly higher ratios of IL-10 to TNF-α in Kenyan children with severe or moderate parasitemias but otherwise uncomplicated malaria compared to those with severe anemia. However, the changes appeared to be mainly due to raised TNF-α levels rather than a reduction in IL-10, as IL-10 in the anemic children was also raised to levels similar to those of children with high parasitemia but no severe manifestations (106).

Similarly, significantly lower levels of IL-10 and higher IL-6 to IL-10 ratios were seen in patients with severe malaria in Vietnam. In patients who died, the levels of IL-10 showed a further drop as compared to those in matched controls, suggesting that a deficiency in IL-10 production may be a factor in mortality (10).

Taken together these results point to an appropriate IL-10 response that is needed to protect against the harmful effects of TNF-α overproduction, by reducing the possibility of severe anemia and by reducing mortality. Further investigations are needed to define the IL-10 responses in relation to the anemia in chronic malaria in different epidemiological settings.

Ex Vivo

PBMCs from acute malaria patients produce IL-10 and IFN-γ without lymphoproliferation in response to incubation with malaria-antigen. Recombinant IL-10 prevented malaria-antigen-induced TNF production and antigen-specific lymphoproliferation in convalescent patients' PBMCs, the later response being incompletely restored by addition of anti-IL-10, suggesting a role of IL-10 in feedback reduction in proinflammatory cytokines but without contributing to

defects in antigen-specific responses (48). In another study, exogenous IL-10 inhibited malaria-antigen-induced proinflammatory cytokine production by reducing mRNA accumulation of these cytokines. The effect was maximal when IL-10 was added within 2 h of antigen stimulation. Proinflammatory cytokine production was increased by the addition of anti-IL-10. Kinetic studies showed that whereas the proinflammatory cytokines were produced within 2–4 h of stimulation, IL-10 was only detectable after 8 h (107).

A case control study in Gabonese children with mild or severe malaria showed increased *in vitro* production of IL-10 by PBMCs taken in the acute phase and incubated with *P. falciparum* liver stage antigen (LSA-1) or asexual stage antigens, and the amount of IL-10 produced correlated with accelerated parasite clearance. The authors suggested that increased IL-10 production can lead to accelerated parasite clearance and more rapid recovery from effects of malaria (108). Further evidence for the potential protective effect of IL-10 production was shown in a study on adult Kenyan volunteers. There was a correlation between the increased production of IL-10 by PBMNCs in response to LSA-1 recombinant antigen and subsequent resistance to reinfection with *P. falciparum*, indicating a protective role of IL-10 production and also of immunity to LSA (109).

Experimental Work

Experimental work on mice infected with *P. chabaudii* showed a sequential Th-1, then Th-2 response during peak parasitemia, followed by subpatency. Purified spleen cells from these mice produced IL-10 *in vitro*. In B-cell-depleted mice, there was no subpatency, no IL-10 production and no downregulation of the Th-1 responses, indicating the roles of B cells and IL-10 in this response (110). IL-10 knockout mice also showed increased pathology and mortality when infected either with *P. chabaudi chabaudi* as compared to wild type animals (111).

Postulated Mechanisms of Protective Effects of IL-10 in Patients with Malaria

In the above studies it is postulated that an appropriate IL-10 response leads to quicker recovery and less complications from acute malaria. Protective effects may result from the downregulation of TNF-α and possibly also other proinflammatory cytokines, resulting in switching from a Th-1 to a Th-2

response with stimulation of antibody production, and also possibly by direct stimulation of erythropoiesis.

Interleukin 4 (IL-4)

IL-4 is produced by activated T cells of the Th-2 subtype and mast cells. It acts on B cells to induce maturation and regulation of antibody production. The levels of IL-4 in the serum of patients with malaria have been measured in several studies and in general they had either not been elevated (105, 106) or serum levels did not correlate with disease severity (50), but in one study IL-4 levels correlated with the duration of fever prior to presentation (112). In another study, serum levels of IL-4 at presentation and follow-up showed a fall with increasing serum neopterin levels in patients who remained anemic, suggesting that the persistence of a Th-1 response in these patients may have contributed to the anemia (54). The same group also found that there was a major reduction in the risk of developing anemia if there is a rise in IL-4 (55).

The possible enhancement of antibody-mediated immunity by IL-4 was illustrated by two studies. Using *P. falciparum* 155/RESA as an immunogen *in vitro*. IL-4 mRNA and intracellular IL-4 were induced in T cells from individuals with elevated concentrations of naturally acquired antibodies to the antigen (113). In individuals with high falciparum-specific IgE, there was a higher ratio of lymphocytes producing IL-4 than those producing IFN-γ after stimulation with a polyclonal T cell mitogen, leucoagglutinin. (114). On the other hand, non-antibody-dependent killing of asexual *P. falciparum* parasites by human monocytes and macrophages was suppressed when these cells were pretreated with recombinant human IL-4 (115), suggesting a mechanism of immune evasion by the parasite.

The importance of IL-4 in the development of specific T cell immunity has recently been highlighted. In BALB-c mice immunized with attenuated sporozoites of *P. yeolii*, antigen-specific CD8+ T cell responses were generated only in the presence of IL-4 synthesized by CD4+ cells (116). In humans IL-4 is produced by memory CD4+ cells in volunteers immunized with attenuated sporozoites of *P. falciparum*, and these responses were elicited by parasitized RBC antigens as well as sporozoite antigens (117). Further work has also suggested that the maintenance of protracted protection against malaria is dependent on responses produced by memory CD4+ cells, to sporozoite, liver stage

and asexual stage antigens, leading to IL-4-dependent stimulation of specific CD8+ cells (118).

Interleukin 5 (IL-5)

IL-5 is an anti-inflammatory cytokine involved in Th-2 responses. It is a colony-stimulating factor, as well as a differentiating and activating factor for eosinophils. In general most studies show no rise in serum IL-5 levels in acute malaria (105, 106). The bone marrow eosinophilia in patients with acute malaria does not therefore appear to be associated with increased circulating levels of IL-5, and no studies have attempted to correlate IL-5 levels with peripheral eosinophilia following treatment of malaria. The possible TH-2 effects of IL-5 in malaria may be seen in the eosinophil changes described in Chapter 5. Further work on the association of IL-5 levels and eosinophil counts during and after treatment of acute malaria is needed to elucidate the significance of these changes.

Transforming Growth Factor Beta (TGF-β)

TGF-β is a multifunctional factor produced by a variety of cells, including platelets, monocytes, macrophages, placental cells and a number of cell lines.

A role of TGF-β in modulating the severity of murine malaria has been suggested. In BALB/c mice, lethal infections with *P. berghei* led to reduced levels of TGF-β, whereas in resolving nonlethal infections *with P. chabaudii chabaudii* or *P. yoelli* there was increased production of TGF-β. Administration of anti-TGF-β antibodies to BALB/c mice led to increased severity of infection with *P. berghei* and exacerbated infection with resolving *P. chabaudii chabaudii* but not *P. yoellii*, and led to increased parasitemia. Conversely, treatment of BALB-c mice with recombinant TGF-β slowed parasite proliferation and led to increased survival. Thus TGF-β appears to produce protective responses in this experimental model (119). Apparently contradictory results were reported from another study. The susceptibility of BALB/c mice to *P. bergheii* was attributed to an early production of TGF-β with inhibition of IFN-γ and NO at the early stages of infection (120). Moreover, administration of recombinant TGF-β led to increased morbidity and mortality in another strain of mice, normally resistant to lethal infection (121), and the lethal effect of *P. bergheii* in BALB/c mice was abolished by administration of anti-TGF-β. A possible explanation for these

apparently contradictory findings is that a large amount of TGF-β given early in the course of infection may inhibit the protective responses, whereas later production of TGF-β may be protective. It has been postulated that TGF-β may act to facilitate switching between Th-1 and Th-2 responses and may therefore have a dual role in malaria and other infections. At an early stage low concentrations of TGF-β may stimulate the production of proinflammatory cytokines, whereas at higher concentrations and later in the course of the infection, it may cause an anti-inflammatory response (120).

Circulating serum TGF-β levels were significantly lower in Thai adults with acute malaria than those in healthy controls and the levels were inversely correlated with the levels of TNF-α, although not with parasitemia, and returned slowly to normal within 21 days (43). The significance of these findings and also whether these low levels are due to decreased production or enhanced clearance, or tissue accumulation of TGF-β in *acute P. falciparum* malaria remain to be elucidated.

Other Cytokines

Granulocyte–macrophage-colony-stimulating factor (GM-CSF) levels were found to be increased in some patients with malaria but the levels did not correlate with disease severity or parasitemia (122). In lethal mouse malaria there was a temporary increase in GM-CSF on day 2 of the infection (123). More recently, the protective effects of GM-CSF in murine malaria were investigated by comparing GM-CSF knockout mice with wild type mice infected with *P. chabaudi.* The knockout mice had higher peak parasitemias, more frequent recrudescences and a high mortality associated with a lesser degree of splenomegaly and leukocytosis and higher TNF-α levels than the wild type infected mice. The authors concluded that GM-CSF plays a role in resistance to *P. chabaudi* by regulating both the cellular and cytokine responses (124).

A significant rise in the granulocyte-colony-stimulating factor (G-CSF) was found in patients with complicated falciparum malaria on admission, with levels falling to normal within seven days. There was a significant correlation with parasitemia but not with neutrophil counts (125). A protective effect of G-CSF in *P. berghei* XAT infection in CBA mice was shown by suppression of parasitemia in mice injected subcutaneously with repeated doses of recombinant human G-CSF, compared to control mice. This suppressive effect was reduced

by treatment with antibodies to either IFN-γ or TNF-α, suggesting that the protective effects may, at least in part, be due to G-CSF-dependent release of these cytokines (126).

Macrophage-colony-stimulating factor (M-CSF) levels were elevated in uncomplicated and severe *P. falciparum* and *P. vivax* malaria. The levels of M-CSF were highest in patients with severe malaria and correlated with platelet counts, suggesting that macrophage activation as a result of high M-CSF contributed to the thrombocytopenia (127).

The macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine secreted by lymphocytes and possibly other cells on administration of low dose corticosteroids. A recent study showed that the ingestion of *P. chabaudii*–infected red cells or malaria pigment by mouse macrophages leads to release of MIF. There was also a correlation between disease severity and circulating MIF levels in *P. chabaudii*–infected Balb/c mice, and a parallel increase of immunoreactive MIF with increasing pigment and parasitized cell load in the liver. Elevated levels were also found in the spleen and bone marrows of these mice and correlated with more advanced disease. The authors suggested that MIF, which is a potent inhibitor of red cell and white cell colonies, is a possible candidate involved in the pathophysiology of malarial anemia (128).

Nitric Oxide and iNOS

Nitric oxide (NO) is a bioactive molecule involved as a neurotransmitter and vasodilator. There are three mammalian isoforms of nitric oxide synthase (NOS): NOS3 or eNOS produced by vascular endothelium; NOS1 or nNOS produced by neurones; and NOS2 or iNOS produced by monocytes, macrophages and other cells. The first two are thought to produce NO constitutively, but iNOS is capable of high capacity sustainable NO production when induced (129). The triggers for NO production by iNOS are cytokines, mainly IFN-γ possibly induced by IL-12 (94, 96), TNF-α, IL-1 and lymphotoxin (130), and microbial products, especially LPS (129). The glycosylphosphotidyl inositol (GPI) anchor from *P. falciparum* was found to induce iNOS from macrophages and vascular endothelium (131), whereas NO production *in vitro* was found to be reduced in mouse cells which had ingested malaria pigment (132). It has been postulated that NO may play a role in CM by feedback suppression of nNOS in neurones and blockage of neurotransmission (133), and in malaria

immunosuppression (134). NO may also play a protective role against blood stage parasites in murine malaria (96) and may also act in killing of gametocytes (135) and in induction of the crisis form seen in certain malaria-immune patients (136). It was shown to suppress the growth of *P. falciparum in vitro* (137, 138).

Two studies in adults with severe malaria, in Thailand (139) and Vietnam (140), have shown that serum and urine levels of NO metabolites or reactive nitrogen intermediates (RNIs) were raised, but with no correlation between their levels and disease severity. The levels returned to those found in normal controls within 48 h (139). There was also no correlation between NO metabolites in CSF (139), or serum RNI (140) and depth and duration of coma. A small but significant increase of RNI levels was found in fatal cases as compared to matched survivors in the Vietnamese study. There was also a correlation between serum creatinine and RNI, and the association between RNI and fatality was abolished when serum creatinine was added as a covariate in a multivariate analysis.

Serum RNI levels were not raised in nonimmune adults with imported uncomplicated malaria (141). Another study, on semi-immune and nonimmune adults with imported malaria, found markedly higher levels of iNOS RNA in whole blood from patients with mild malaria when compared to those with severe malaria. Normal healthy controls showed no iNOS RNA. Further work showed that the cells predominantly expressing iNOS RNA were monocytes, and retrospective analysis showed that patients with mild malaria had significantly higher monocyte counts at presentation than those with severe malaria (142). These results taken together suggest that monocyte-derived iNOS leading to NO production is protective in malaria.

Studies on children appear to indicate that a higher capacity to produce NO is protective against severe malaria. Increased production of NO and iNOS expression correlated negatively with disease severity in Tanzanian children (143). Increased capacity for iNOS and NO production by cultured PBMCs of healthy Gabonese children with known malaria histories showed that those with a history of mild malaria had significantly higher levels of iNOS RNA and NO production from fresh monocytes and also PBMCs after one week of culture, and lower levels of serum TNF-α when compared to the SM group (144). Another study, on children in PNG with malaria of varying severity, found that the highest levels of RNI were found in children with fatal CM and

also in asymptomatic malaria. However, there was a significantly higher ratio of RNI to serum TNF levels in asymptomatic malaria than in fatal CM (51).

The possible role of chronic NO production in the anemia of malaria was also highlighted. Urinary RNI was found to be significantly higher in asymptomatic Tanzanian children with malaria than in controls in a surgical ward who were similarly exposed to but did not have malaria. There was a negative correlation between the RNI levels and Hb in children with malaria, but this was not found to be an independent predictor in a multivariate analysis as both high RNI and parasitemia negatively correlated with hemoglobin levels (145). In a further study the effects of age on children with asymptomatic parasitemia stratified against thick film positivity or thick film negativity, PCR positivity (PCR+) and PCR negativity (PCR−) were studied by measuring urinary and plasma RNI and leukocyte iNOS (145). There was no difference in the levels of these parameters between PCR+ and PCR− slide positive patients. There were two peaks of increased NO production, the first in infancy and the second after five years of age, ages associated in both groups with less severe complications of malaria, suggestive of a protective role of NO against malaria.

Polymorphisms associated with the iNOS2 promoter have been studied to find out whether there is a genetic basis for increased NO production, which may in turn be protective in malaria. The promoter region of the iNOS gene contains a highly repetitive pentanucleotide microsatellite sequence with a variable number of repeats (146). In one study in Gabon, a polymorphic point mutation in the promoter region was significantly associated with mild malaria and protection against reinfection. This polymorphism was not found in German subjects studied as controls (147). In Gambians there was a bimodal distribution of numbers of repeats of the microsatellites with a trough at 11 repeats. The short allele phenotype was significantly commoner in children who died of CM than in those who survived, and those with anemia and malaria, controls with mild malaria or children with mild disease other than malaria (148). Another study, on Tanzanian children, found no significant association with disease severity, and measures of NO production on the one hand and either of these polymorphisms on the other (149).

The above studies suggest that a high capacity to produce NO, in part genetically determined, may be protective against malaria, but may also be associated with severe disease in certain circumstances, especially in those with high TNF-α levels. It also appears that prolonged production of NO may contribute

to the anemia seen in chronic malaria, and it is tempting to speculate that NO may be one of the main direct causes of dyserythopoiesis seen in these patients (see Chapter 7).

Triggers of Cytokine Release in Malaria

Cytokine release occurs as a result of schizont rupture and release of parasite antigens, merozoites, pigment and cellular debris from remains of infected RBCs. Early work has shown a variety of mitogenic and activating effects on human and murine cells by malaria parasites and their products. Three groups of products related to parasites have been implicated in the effects on host cells and somewhat loosely termed "malaria toxin." The active substances produced by malaria parasites that produce effects on human cells include malaria pigment, the GPI protein anchor and a number of exoantigens, some of which have been characterized further.

Soluble malaria antigens were found to be mitogenic to B (150, 151) and T cells (152), but also *P. falciparum* merozoites or soluble antigens were found to lead to direct activation of peripheral blood neutrophils and monocytes from malaria-naïve individuals to produce oxygen radicals (153). In another experiment, incubation of *P. falciparum*–infected red cells with peripheral blood monocytes resulted in stimulation of oxidative bursts seen by immunoelectron microscopy at the junction between monocytes and infected red cells. The authors postulated that the association of an infected RBC ligand with CD 36, a putative sequestration receptor, mediates this reaction, as similar results were obtained by using an anti–CD 36 monoclonal receptor which leads to activation of the appropriate signaling pathway (154).

In addition to direct effects of malaria parasite products on phagocytic cells, a T-cell-mediated increase in the phagocytic capacity of peripheral blood monocytes from nonimmune individuals incubated with malaria culture supernatants was demonstrated (155), and this effect may be due to release of cytokines. Antigens of molecular weights 250, 70 and 18 kilodaltons released during the final stages of the parasite cycle were found to lead to lymphoproliferation and secretion of IFN-γ by CD+4 and CD+8 T lymphocytes from malaria-naïve donors (64). Incubation of peripheral blood mononuclear cells with purified schizont antigen from *P. falciparum* led to a dose-dependent release of IFN-γ, mainly by CD 4+ cells, and IFN-γ production was found

to be significantly higher in nonimmune individuals with a single attack of malaria than in immune adults living in a malaria-endemic area (60).

P. falciparum heat-stable antigen was found to induce TNF-α production by human peripheral blood monocytes as well as from a murine macrophage line and resident peritoneal macrophages. Immunization of mice showed that this effect was inhibited and was independent of LPS-induced TNF-α release by monocytes and macrophages (12).

It has recently been postulated that most of the effects of malaria antigens on white and other cells can be produced by the isolated glycosylphosphatidylinositol (GPI) anchor attached to many plasmodial antigens (156). GPI was found to induce TNF-α and IL-1 production by macrophages through protein C kinase transduction. *In vivo* administration of plasmodial GPI to mice led to cytokine release, fever and hypoglycemia. In further studies, plasmodial GPI induced iNOS expression in macrophages and vascular endothelial cells by a protein C-dependent and through a tyrosine kinase-signaling pathway (157). Similarly, GPI led to upregulation of ICAM-1 and VCAM-1 and E selectin in vascular endothelial cells (VECs) through the same pathway. This resulted in increased leukocyte and parasite cytoadhesion to VECs (158).

Whereas GPI appears to lead to stimulation and upregulation of various cellular functions, another parasite product, hemozoin, has a different effect. In various experimental systems, hemozoin ingestion by macrophages was found to lead to failure to ingest opsonized RBCs, kill bacteria, fungi and tumor cells, and failure to produce oxidative bursts. These effects were produced without loss of viability or ability to adhere. The effects of hemozoin were dependent on the presence of membrane-associated unsaturated lipids in these preparations, and ingestion of hemozoin results in lipid peroxidation and generation of 4-hydroxyl-trans-nonenal (HNE). HNE is an alkylating agent and leads to enzyme inhibition, modulation of gene expression and inhibition of protein kinase C (PKC) and NADPH oxidase (NOX) (159).

The varying effects of GPI and other malaria antigens (on the one hand) and malaria pigment (on the other) on macrophages may help to explain the different effects of malaria at different stages and presentations of the disease. Thus, in acute malaria the immediate effects of antigens and GPI may be to induce the production of proinflammatory cytokines with overproduction of TNF-α, IL-1 and IL-6. On the other hand, the accumulation of ingested pigment may not be accompanied by the release of large amounts of cytokines, but may

Table 1. Some of the effects of hemozoin on monocytes and macrophages, possibly leading to immunosuppression. Increased susceptibility to bacterial sepsis impaired eryrthopoiesis (159).

Mechanisms	Measured effects
Inhibition of PKC through ROS and lipoperoxides and HNE	Impaired phagocytosis of opsonized red cells
Impairment of NADPH oxidase	Intracellular bacterial, fungal or tumor cell killing
Increased release of TNF and IL-1	Failure of production of oxidative bursts No loss in viability Increased adhesion to plastic Impaired expression of class II MHC expression Impaired expression of ICAM-1; CD54, Integrin and CD11c

impair macrophage function, including antigen processing, oxidative bursts, phagocytosis and cytotoxity as well as function as nurse cells to erythroblasts. It is tempting therefore to postulate that dyserythropoiesis may be another factor that results from this impairment of macrophage function.

Liver stage antigen-1 (LSA-1) as well as asexual blood stage antigens have also been shown to stimulate production of IL-10, IFN-γ and TNF-α from acute phase PBMNCs in Gabonese children with mild acute malaria (108). However, significantly faster parasite clearance time was associated with increased production of IL-10 but not IFN-γ or TNF-α from the PBMNCs, in response to LSA-1 but not asexual stage malaria antigens. In adult volunteers in Kenya, responses of PBMNCs to recombinant LSA-1 antigens were commonly found to lead to production of IL-10, IFN-γ and TNF-α by these cells. Only the degree of production of IL-10, however, was predictive of resistance to reinfection after eradication treatment in the subsequent malaria season (109).

Table 2. Summary of molecules and cells involved in the response to malaria.

Triggers	Soluble antigen GPI anchor Pigment Other, undefined malaria antigens Products of red cell destruction	
Primary response	$\gamma\delta+$ T cells Other T cells Macrophages	TNF-α IFN-γ IL-12
Effector mechanism	Proinflammatory cytokine release TNF-α, IFN-γ, IL-12	
	Protective effects	Harmful effects
	Killing or inhibition of parasites Activation of phagocytosis NO production	Increased adhesion molecules Increased sequestration Hemophagocytosis (NO production)
Secondary response	IL-6 in response to TNF-α	Enhanced proinflammatory response
		Anti-inflammatory: feedback inhibition of proinflammatory cytokines Acute phase proteins may help neutralize cytokines Stimulation of T or B cell specific immune responses
	IL-10	Feedback inhibition of proinflammatory cytokines
	IL-4	Induction of specific immunoglobulins
	IL-5	Modulation of responses toward Th2
Modulators	TGF-β	Early and marked response inhibits IFN-γ and may be harmful Late production may modulate response

Table 3. Proinflammatory cytokines in malaria.

Proinflammatory cytokines				
Cytokine	Cells that produce cytokine	Triggers	General effects	Specific effects in malaria
TNF-α	Monocytes and Macrophages	Schizont rupture and release of antigens GPI anchor	Hypoglycemia Suppression of erythropoiesis (dyserythropoiesis) Organ damage	May be protective but may also be harmful when overproduced
IL-1	Monocytes and macrophages	As TNF-α GPI anchor	Actions overlap and synergize with those of TNF-α	
IFN-γ	T cells, mainly $\gamma\delta+$ T cells	Crude schizont lysate and purified and synthetic antigens Soluble exoantigens	Macrophage activation Upregulation of ICAMs	Phagocytosis, protective in early phases; sustained release may contribute to severe anemia
IL-6	Monocytes and macrophages Endothelial cells Other cells	GPI anchor	Proliferation of antigen-specific T cells, B cell growth and differentiation acute phase response hemopoiesis	May be pivotal in downregulating TNF and may be protective in stimulating immune response
IL-12	Macrophages		Leads to production of IFN-γ from T cells Differentiation of Th1 helper cells	May be protective in enhancing early interferon production Stimulates erythropoiesis
IL-8	T cells Mononcytes and macrophages		Chemoattractant and activator of neutrophils	Role not clear ? contributes to ARDS

Table 4. Anti-inflammatory cytokines in malaria.

Anti-inflammatory cytokines				
IL-10	Monocytes T cells	LSA-1 and schizont antigens	Downregulates production of proinflammatory cytokines B cell proliferation and maturation	Underproduction in malaria may lead to prolongation of Th-1 response, delayed recovery and severe anemia
IL-4	Activated CD4+ T cells Mast cells	CSA	Enhancement of antibody-mediated immunity Development of T-cell-specific immune response	Failure or inadequate response may lead to prolonged anemia
IL-5	Th2 CD4+ T cells		Eosinophil differentiation and activation; terminal B cell differentiation to Ig secretion	Insufficient data, but production may be protective
TGF-β	Wide variety		Immune response	Large amount produced early in infection may delay recovery; late production may enhance protection

Conclusions

The review of cytokines highlights the complexity of host responses to malaria parasites. These responses are dependent on state of immunity or premunition, age, genetic background, nutritional status as well as parasite factors, such as the parasitemia, genetic changes in parasites and ability to manipulate the host.

It must be emphasized that most studies on malaria in humans are snapshots of a wide and mobile canvas, on which different players come and go. Taken together the studies demonstrate the enormous diversity and redundancy of some of the host responses to the invader. Thus no oversimplification

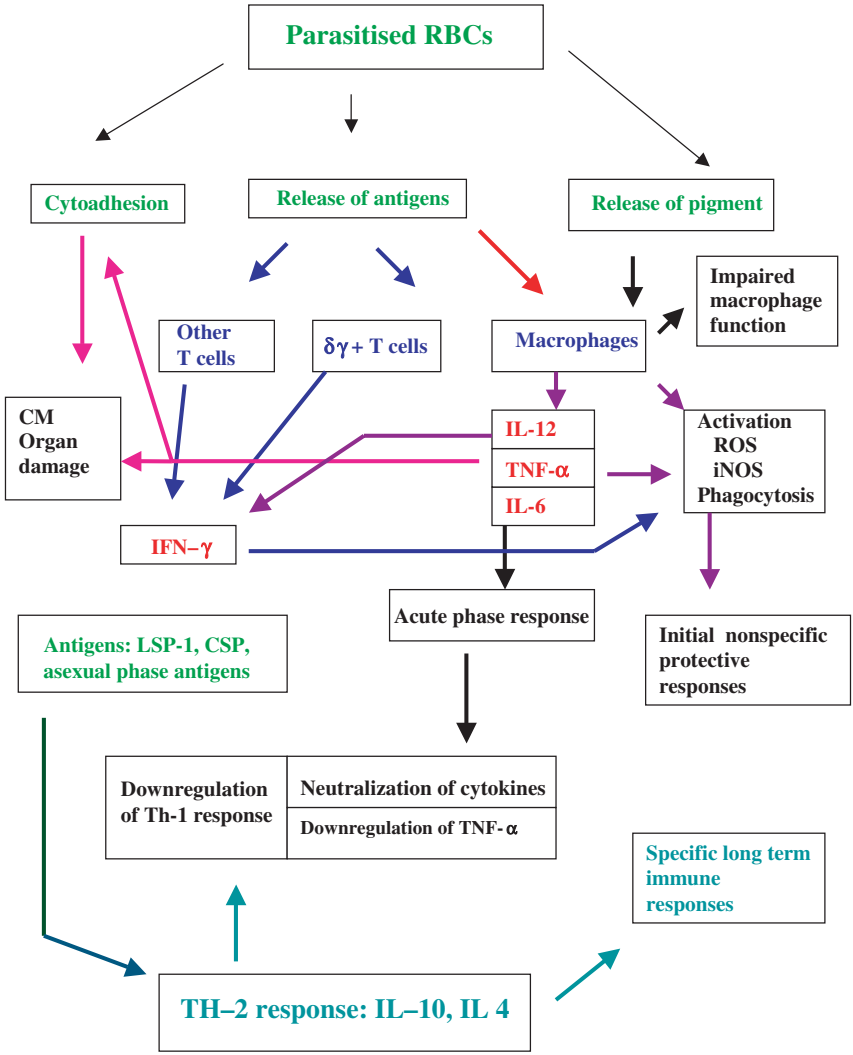

Figure 1 The effects of parasites and their products on cells and release of cytokines in malaria.

can convince one that a particular response, whether through a cytokine, a cell or other molecule, is *protective* or *harmful*. Sometimes excessive production may be protective, and at other times it may cause pathology.

In the complex interactions some important findings emerge: in acute malaria in totally unprimed individuals, the ability to mount a quick nonspecific Th-1 response, the main manifestation being increased phagocytosis, may be life-saving. These responses, mediated probably by release of malaria antigens at schizogony, and affecting unprimed $\gamma\delta+$, or other T cells, are characterized by a massive release of a multiplicity of proinflammatory cytokines such as TNF-α, IFN-γ and IL-6 .The ability to survive this cytokine storm depends on a number of factors relating to the host and parasite but aims at the initial containment of the invader and the institution of specific immune responses. The first step in this process is to downregulate the production of proinflammatory cytokines, and IL-6 and IL-10 play important roles in this switch. During this initial phase anemia occurs as a result of hemolysis of parasitized cells, but also as a result of activation of phagocytosis. Noninfected red cells fall victims to this indiscriminate excess reaction, and the anemia is worsened by bone marrow suppression by these cytokines.

This pattern of acute malaria is attenuated with subsequent repeated attacks but in the interim the host's response is refined with the onset of a phase of "antitoxic" immunity. It is often at this stage that children with repeated malaria appear to suffer from severe anemia in hyperendemic areas, and this anemia is clearly related to bone marrow dysfunction caused by factors often associated with a low parasite load. Candidates for causing this dysfunction, mainly seen as dyserythropoiesis, are low grade continuous production of proinflammatory cytokines (mainly IFN-γ resulting in NO production), and failure in IL-10 production or a switch to a Th-2 response. Some evidence suggests that this switch may correlate with the formation of anti-liver-stage antigen immunity. The mechanisms involved in these processes are clearly different and further work is needed to elucidate this intricate cytokine network in the anemia of chronic malaria.

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Chapter 7

The Bone Marrow in Human Malaria

Saad H Abdalla and Sunitha N Wickramasinghe

> The marrow of the flat bones (ribs) is of a reddish-brown colour, that of the long bones (the femur) is for the most part reddish brown in the two upper and lower thirds, while in the middle third it has a yellowish, jelly-like appearance. Its consistence is usually very soft, indeed it is almost fluid. The endoglobular parasitic forms generally fill up the lumen of the blood vessels of the bone marrow; the adult forms and those sporulating are the most common; in some cases also the crescent-shaped forms preponderate. Besides this we find parasitic forms outside the blood-vessels, where, nevertheless, the most prevalent shapes are (inside), as in the spleen, the large phagocytes, mostly in a state of necrosis. In some cases the vessels of the marrow contain great quantities of the nucleated red blood-corpuscles, in which parasites are never found.
>
> —Marchiafava and Bignami, 1894 (1)

Introduction

This chapter will discuss the distribution of the different stages of maturation of malaria parasites in the bone marrow compared with that in the circulation, the value of bone marrow aspiration in the diagnosis of malaria, and the morphological and functional changes in hemopoiesis with a special emphasis on erythropoiesis.

Parasite Stages in the Bone Marrow

Asexual Stages

P. falciparum is unique amongst human malaria parasites in that the more mature asexual phases are not normally seen in the circulation and sequester

in deep vascular tissues. Sequestration is thought to play a major role in some of the severe manifestations of malaria, such as cerebral malaria and other vital organ involvement (2).

The site, severity and clinical manifestations of sequestration are dependent on parasite-related factors such as the parasite load and the ability to sequester, and host-related factors such as the preferential site of sequestration, which may in turn be due to variability in expression of adhesion molecules and to secretion of cytokines. The effects of sequestration on an organ may also depend on anatomical factors. Thus organs with end-arteriolar blood supply and no collateral circulation, such as the brain and the kidney, may be more susceptible to anoxic effects caused by massive sequestration. On the other hand, the reticuloendothelial organs, such as the liver, the spleen and bone marrow with open sinusoidal vascular systems, may encourage stasis but may not be as susceptible to anoxia. These organs are also rich in macrophages and may therefore be able to clear adherent parasites and prevent massive sequestration.

In benign tertian malaria (due to *P. vivax*) the parasite distribution is the same in the peripheral blood as in other organs (1). In human falciparum malaria there are limited data on organ-specific sequestration in the various clinical presentations of the disease. Postmortem observations have shown that the stage distribution of malignant tertian malaria due to *P. falciparum* was variable in different organs, with three main patterns of distribution (1).

(1) Patients with high parasitemia and accumulation of parasites of all stages in all organs.
(2) Those where the parasites predominate in the brain microvasculature.
(3) Those where there is a greater accumulation of parasites in the spleen and bone marrow and sometimes also in the small intestine.

Patients with the second pattern of parasite distribution often presented with cerebral symptoms, whereas those with the third pattern usually presented with gastrointestinal or other generalized symptoms (the so-called algid malaria) (1). In uncomplicated malaria there is no particular predisposition for more mature stages of the parasite to favor the reticuloendothelial organs (spleen and bone marrow), as shown by splenic aspirates in which where there was a lack of increase in pigmented and sporocytic phases (1). Although it has been stated that the bone marrow and the spleen are favored sites for the

asexual development of *P. falciparum*, Maegraith considered that there was no solid evidence for this belief in his review of the literature on the subject (3).

Studies on nonimmune Europeans (4, 5) and Gambian children (6) with acute falciparum malaria concluded that in patients with low parasitemias, the only asexual forms seen in the bone marrow and peripheral blood were the small ring forms. With increasing parasitemias there was an increase in the number of trophozoites and occasional schizonts in the bone marrow and to a lesser degree in the peripheral blood, suggesting that the bone marrow could not be one of the primary sites of schizogony (4–6).

The bone marrow is organized into loose vascular spaces, the sinusoids, and parenchymal space where hemopoiesis takes place. Normally the parenchyma is extravascular and therefore not directly accessible to circulating parasites. An ultrastructural study of the bone marrow of Thai patients with cerebral malaria showed that there was packing of bone marrow sinusoids with parasitized red blood cells (PRBCs), mainly trophozoites and some schizonts, and sometimes luminal noninfected RBCs. The PRBCs were adherent to the vascular endothelial cells via knobs on the red cell surface as well as by complex interdigitation with the endothelial cells (Fig. 11). Moreover, in some cases PRBCs were also found in the parenchymal space of the bone marrow, suggesting that there has been damage to the integrity of the sinusoids with leakage of PRBCs, or that there is reversed diapedesis of cells from sinusoids to parenchyma, which may in turn lead to parasitization of the parenchymal reticulocytes (7). Such sinus packing was not seen in bone marrow from Gambian children with severe anemia with or without high parasitemias (Wickramasinghe, personal observation). It therefore appears that the bone marrow is not a major site for sequestration and development of the asexual erythrocytic cycle in uncomplicated falciparum malaria and in those without severe manifestations other than anemia. However, sequestration in the bone marrow may occur in individuals with CM and possibly other severe forms of malaria.

Pigment, and less frequently more mature forms of parasites, including schizonts and merozoites, are seen inside bone marrow macrophages of patients with acute and also more chronic malaria (see Chapter 5). The origin of the cells containing these phagocytosed particles is not known. Bone marrow macrophages are known to protrude cytoplasmic processes into the sinusoids and these processes may interact with and phagocytose PRBCs. The phagocytosed particles may therefore either originate locally from parasitized RBCs that are freely circulating in the sinusoids or that have cytoadhered and

then been rapidly removed from sinusoids by bone marrow macrophages, or the macrophages may have engulfed parasite material elsewhere and then migrated to the bone marrow through the bloodstream, although this is difficult to prove. Increased phagocytosis of mature parasite forms has been easier to demonstrate in the spleen in animal malarias. Postmortem studies in fatal cases show active phagocytosis in the splenic cords in both human and simian malarias (3). The finding of circulating schizonts in splenectomized individuals and experimental work on monkeys (8, 9) suggest that removal of adherent schizonts in macrophage-rich tissues is the most likely explanation for the accumulation of pigment in RE tissues. Preferential sequestration in various organs of more mature parasite forms may not therefore be the only explanation for the absence of these stages in bone marrow and splenic aspirates but can theoretically be explained by the rapid removal of parasitized RBCs from the microvasculature in these macrophage-rich tissues. In the case of heavy infections such as those seen in CM, the capacity of the reticuloendothelial system may be exceeded and thus account for the presence of sequestered parasites in larger numbers in these RE organs.

Sexual Stages

Although the bone marrow appears not to favor asexual stage parasite development, it often contains immature gametocytes (Fig. 1) and appears to favor gametocytogenesis (6, 10, 11). The reasons for the marrow being a favored site for gametocytogenesis are not known. Gametocytogenesis can be induced under certain circumstances *in vitro* (6, 12) or develop *in vivo* after drug therapy or during recovery (3).

The mechanism of retention of immature gametocytes in the bone marrow appears to be distinct from that of the retention of asexual parasite stages and remains unclear. Gametocytes develop from trophozoites, which presumably have started to sequester (13). However, both mature gametocytes which circulate freely and immature gametocytes which appear to be retained in vasculature of tissues such as bone marrow and the spleen lack knobs that are normally associated with sequestration in the asexual stages (13). It can be hypothesized that the milieu of the bone marrow sinusoid is conducive to the development of gametocytes. In this context recent observations that induction of erythropoiesis favors the development of male gametocytes (14) are of interest, and it can be postulated that molecules produced by the host in the bone marrow

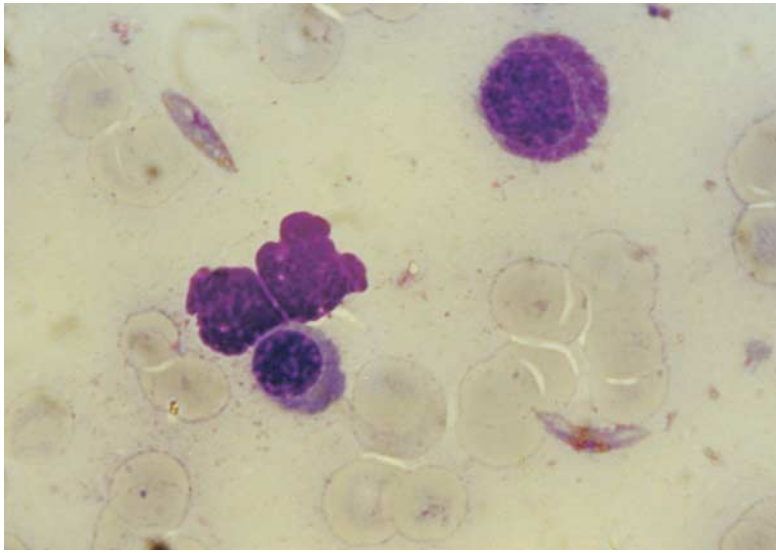

Figure 1 Immature gametocytes in the bone marrow aspirate of a Gambian child with chronic malaria.

during early stages of recovery from malarial anemia may be conducive to gametocytogenesis and may also determine the sex of the gametocytes produced. Another interesting observation is that the intracellular gametocytes do not appear to attract phagocytosis in the same way as the maturing asexual stages do (15). This may be related to the lack of knobs and or other parasite determinants on the surface of infected RBCs. One fact that remains unexplained is that, despite the lack of knobs, the immature gametocytes appear to sequester whereas the mature gametocytes circulate freely. This cannot be simply explained by the lack of deformability of immature gametocytes, and alternative explanations are needed.

The Diagnostic Value of Bone Marrow Aspiration in Malaria

There is scanty literature on the diagnostic value of bone marrow aspirates in malaria. This is presumably because bone marrow aspiration is a specialized procedure to carry out in areas of malaria endemicity and because the diagnosis

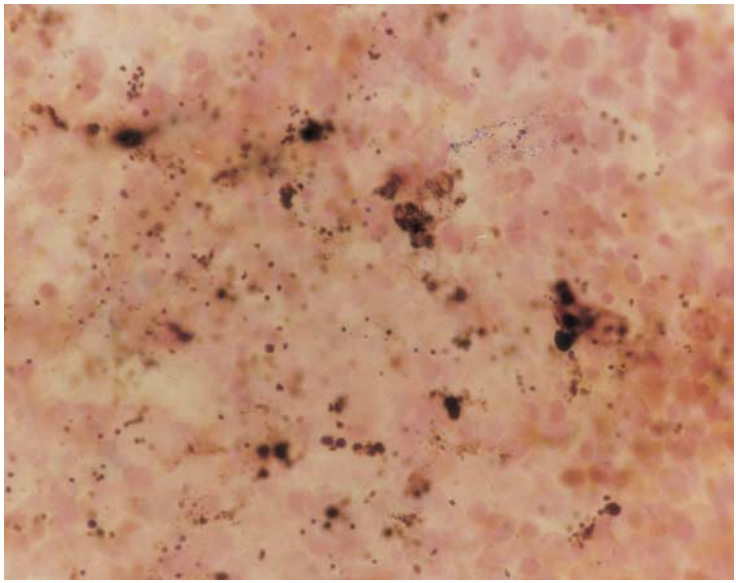

Figure 2 Low power view of bone marrow fragment in acute malaria showing malaria pigment.

of malaria is often made on peripheral blood smears. As discussed above, the parasite stages and numbers seen in the bone marrow reflect those in the peripheral blood apart from the presence of immature gametocytes. Repeated blood sampling during the febrile stages of disease is preferable to performing a bone marrow biopsy.

Bone marrow aspiration may be useful in showing the presence of malaria pigment in macrophages (Fig. 2) if a retrospective diagnosis of malaria is required in a treated patient. Similarly, postmortem examination of the bone marrow may be useful in patients who may have died of undiagnosed malaria, and pigment will also be found in other tissues such as the liver or spleen.

Hematopoiesis in Malaria

Introduction

The anemia of malaria cannot be entirely explained by destruction of red cells (see Chapter 3). Early observers noted that there was also a suboptimal

reticulocyte response to the anemia, and this has been variously attributed to toxic depression of bone marrow (1, 16), hematinic deficiencies, hypersplenism and ineffective erythropoiesis (17–19). Bone marrow dysfunction is an important factor in the anemia of falciparum malaria, and to a lesser degree of vivax malaria, accounting for the severity and prolonged duration of anemia disproportionate to the degree of parasitemia. The type of dysfunction is dependent on whether the clinical presentation of malaria is acute or chronic, the age and previous exposure to malaria and the state of immunity (20, 21; see also Chapter 3).

Of particular importance is the severe and prolonged anemia found in children in areas of high malaria endemicity in Africa without overt symptoms of malaria and usually with low levels of circulating parasites (19, 22, 23). Ineffective erythropoiesis has been shown to be one of the most important causes of slow recovery from anemia in these children, but the mechanisms by which dyserythropoiesis is produced still remain unclear.

Bone Marrow Cellularity of Patients with Malaria

A summary of the major studies on the bone marrow of patients with malaria is shown in Table 1. In adults with acute falciparum or vivax malaria, a decrease in bone marrow cellularity is a common finding. This has been found in nonindigenous nonimmune subjects who were mainly adults, including Europeans (17, 24) or immigrants from nonmalarious areas of the same country (7, 16, 25). By contrast, bone marrow cellularity is increased in children with acute malaria in malaria-endemic areas and in those with chronic malaria and severe anemia (19, 20).

It has been postulated that the reduced bone marrow cellularity in acute malaria in nonimmune subjects is due to a direct suppressive effect of malaria on hemopoiesis (16). More recently, overproduction of proinflammatory cytokines or cytokine imbalance has been proposed to be the cause of bone marrow suppression (see Chapter 6).

Erythropoiesis

In acute malaria there is a reduced number of erythroblasts and a relative increase in the myeloid/erythroid (ME) ratio. The proportional reduction of

Table 1. Major bone marrow studies on patients with malaria.

Author	Number of patients	Parasite species	Category of patients and number in each category	Main conclusions
Thonnard-Neuman, 1944 (24)	34	*P. vivax* *P. falciparum*		Anemia in part due to lack of release of reticulocytes
Knütgen, 1963 (17)	119	Mainly *P. vivax*	Adults; nonimmune Europeans, semi-immune Liberians	Variable active proliferation of different cell lines, marrow dysfunction "dyspoeiesis"
Srichaikul *et al*., 1967 (16)	49		Mainly adult Thais	Variable degree of suppression of erythropoiesis, usually in acute malaria
		P. vivax *P. vivax* *P. falciparum* *P. falciparum* Mixed Mixed	25 acute 7 chronic 3 acute 5 chronic 4 acute 5 chronic	
Abdalla *et al*., 1980 (19)	20	*P. falciparum*	Gambian children 6 acute 7 subacute 7 chronic	Dyserythropoiesis confirmed as a major cause of anemia, especially in chronic malaria
Phillips *et al*., 1986 (25)	23	*P. falciparum*	Thai, mainly nonimmune adults Uncomplicated Acute $n = 22$ Convalescent $n = 19$	Dyserythropoiesis also seen in acute malaria
Wickramasinghe *et al*., 1987 (7)	9	*P. falciparum*	Mainly adults Cerebral malaria	Confirms dyserythropoiesis in CM; sinus packing with parasitized RBCs; possible loss of sinus wall integrity
Wickramasinghe *et al*., 1989 (26)	9	*P. vivax*	Mainly adults	Dyserythropoiesis also seen in vivax malaria
Abdalla, 1990 (20)	89	*P. falciparum*	Gambian children 14 acute malaria 35 acute on chronic 40 chronic	Confirms relationship between dyserythropoiesis and malaria

erythropoiesis is most marked in Gambian children with acute malaria (mean erythroblasts 13.7% (19), 18.2% (20)), in Thai adults with acute vivax or falciparum malaria (13.6% (16)), and in Thai patients with cerebral malaria (15.9% (7)). Normal mean numbers of erythroblasts were observed in nonimmune Europeans, Gambian children with acute on chronic malaria (26.3% (19), 31.4% (20)), and Thais with vivax malaria (26). In Thai adults with predominantly vivax malaria, there was an increase in the proportion of erythroblasts in convalescent BM (mean 32.2%) even when there had not been an improvement in hemoglobin levels (16).

The reduction in the percentage number of erythroblasts in acute malaria may be seen in a setting of a hypoplastic marrow and may represent a generalized suppression of hemopoieisis. In other cases, as in Gambian children with acute malaria, although the bone marrow cellularity is increased, erythropoiesis appears relatively suppressed (19). The lack of erythropoietic response in acute malaria may be interpreted as either being due to the acuteness of onset of the anemia, or to suppression of erythropoiesis, or to diversion of stem cells into myelopoiesis in reaction to the acute infection.

In hyperendemic areas, patients with chronic malaria show a different pattern of erythropoiesis. Erythroid hyperplasia is observed in Gambian children with chronic malaria (19, 20). These patients often improve clinically and hematologically when malaria is treated even without additional therapy, indicating that the clinicohematological picture is due to malaria. Erythropoiesis in these patients is often hyperplastic, sometimes markedly so, but without an appropriate reticulocyte response (17, 19, 20, 24).

Qualitative Changes

In acute and chronic malaria, erythropoiesis is predominantly normoblastic. Morphological abnormalities of erythropoiesis, termed dyserythropoiesis, have been described in the bone marrows of patients with acute vivax or falciparum malaria, and in chronic malaria (17, 19, 24, 27, 28). The changes are listed in Table 2, and various terminologies have been used to describe these abnormalities. The changes are nonspecific and may be found in other conditions but what is striking about them is the severity in some cases.

Megaloblastic changes indistinguishable from those of B_{12} or folate deficiency have also been described in erythroblasts of patients with malaria (3, 17, 20, 27; Fig. 8). It has been postulated that megaloblastosis in this situation

Table 2. Dyserythropoietic features seen in patients with malaria.

(1) Cytoplasmic vacuolation and incomplete hemoglobinization (Fig. 3).
(2) Cytoplasmic bridging. Two distinct cells that are joined together by a thin strand of cytoplasm. These are cells at a very late stage of mitosis that have not yet completely separated (Fig. 4). Up to 5% of erythroblasts in normal bone marrow show this feature.
(3) Markedly irregular nuclear outline (Fig. 5).
(4) Karyorrhexis: fragmentation of the nucleus (Fig. 5).
(5) Multinuclearity: there is lack of cytoplasmic division after the nucleus has divided, leading to bi- or sometimes multinuclearity (Fig. 6).
(6) Internuclear bridging. This is rarely seen. There is a thin strand of chromatin connecting the nuclei of two cells that appear to have almost completely divided but for the nuclear bridge.
(7) Ring sideroblasts: iron loading of mitochondria.

may be due to folate deficiency caused by increased demands of hemolysis in patients with malaria or may also be related to the use of antifolate antimalarials (see Chapter 4). An alternative explanation is that the megaloblastosis is another manifestation of dyserythropoiesis in malaria. This second hypothesis is supported by a study showing the coexistence of severe dyserythropoiesis and megaloblastic changes in both white and red cell precursors, with normal levels of serum B_{12} and RBC folate (20, 29) and a normal dU suppression test in six out of seven bone marrows from patients with malaria (30). Although megaloblastosis due to folate deficiency may not be contributory to the anemia in malaria under normal circumstances, it may be of importance during pregnancy where there are increased demands for folate (see Chapters 2 and 4).

Knüttgen first noted dyserythropoietic features in malaria in 1963 (17). He termed myelopoeisis "*dyspoetische*" (German for "dysplastic"). In addition to the dyserythropoiesis, Knüttgen also recognized dysplasia in other cell series. Earlier, Bianchi (27) and Thonnard-Neuman (24) had described appearances in the bone marrow that with hindsight can also be interpreted as dyserythropoiesis. Thonnard-Neumann, in particular, commented on the lack of appropriate reticulocytosis in these anemic patients, despite erythroid hyperplasia, and suggested that there was a block in the release of reticulocytes from the bone marrow.

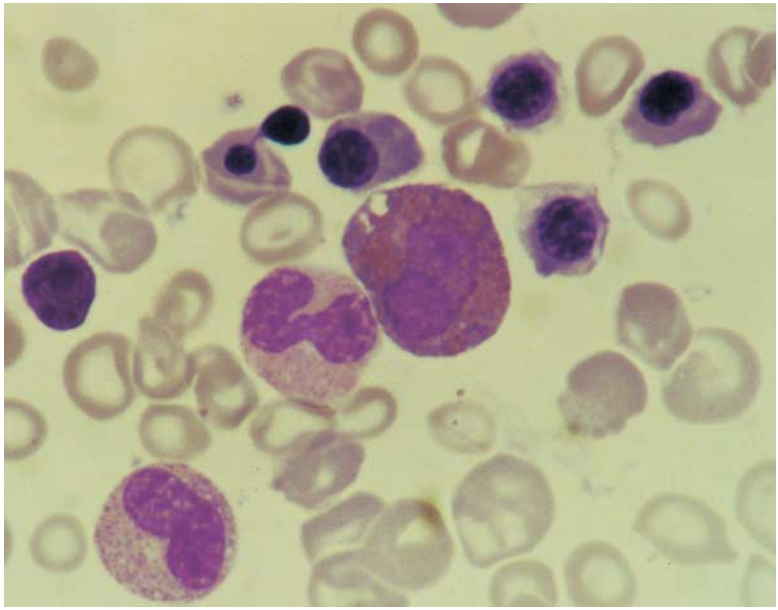

Figure 3 Dyserythropoietic changes in chronic malaria: some erythroblasts show cytoplasmic vacuolation and there is also extrusion of the dumbbell-shaped nucleus.

Knüttgen in 1963 noted the significance of dyserythropoiesis in relation to the anemia of malaria. This observation was further highlighted in 1980 by a study on Gambian children with severe malarial anemia (19). The presence and importance of dyserythropoiesis were also confirmed in adult Europeans with acute malaria (28), in patients with cerebral malaria (7), and in patients with severe vivax malaria (26).

Dysplastic erythroblasts are most frequent in the bone marrows of patients with chronic malaria. Changes were most severe in chronic malaria where all patients with severe anemia had more than 10% of erythroblasts showing dyseryrthopoietic features and 30% of patients with more than 40% of erythroblasts showing dyserythropoietic features (19, 20). In patients with acute malaria the dyserythropoitic features were less marked, 57% showing less than 10% dyserythropoietic cells and 37% showing less than 20% dyserythropoietic cells (20). In patients with vivax malaria, dyserythropoiesis is generally mild

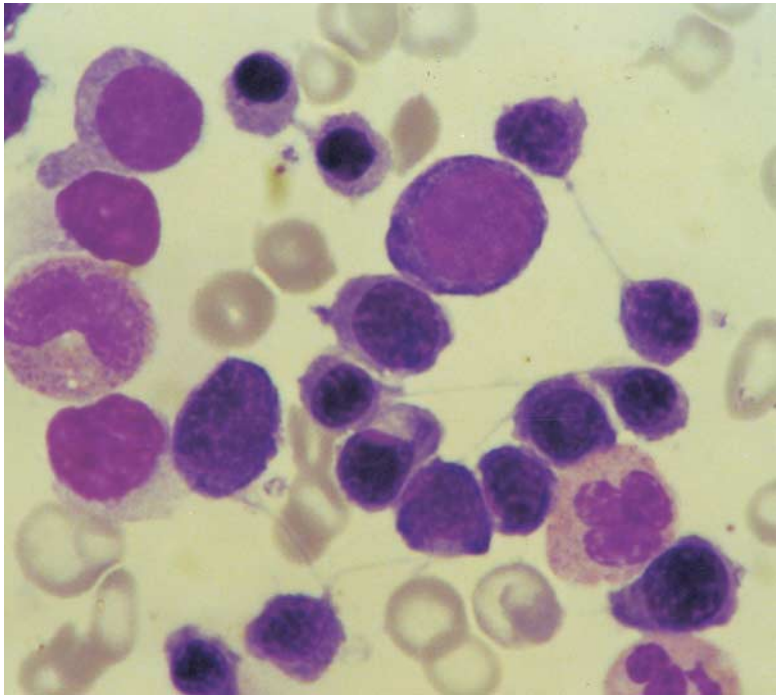

Figure 4 Dyserythropoiesis in chronic malaria: several erythroblast pairs show intercytoplasmic bridges.

(less than 5% dysplastic erythroblasts), but in two cases with more severe anemia the dyserythropoiesis was more marked (above 10%) (26).

Dyserythropoietic changes may persist or first appear in the bone marrow, especially after treatment of acute malaria (25, 28). In the latter study, the prevalence and severity of dyserythropoiesis were increased when repeat bone marrow sampling was carried out between 4 and 25 days after treatment of the malaria.

Distribution of Hemosiderin

The storage iron in the aspirated marrow particles and macrophages is usually increased in patients with acute vivax or falciparum malaria and in chronic malaria (16, 19). In acute malaria, especially in nonimmune adults, hemosiderin

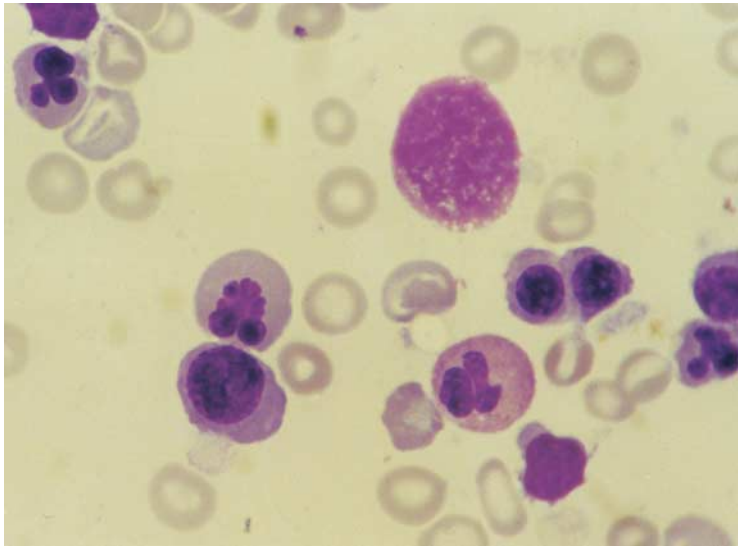

Figure 5 Dyserythropoiesis in chronic malaria: nuclear lobulation and karyorrhexis.

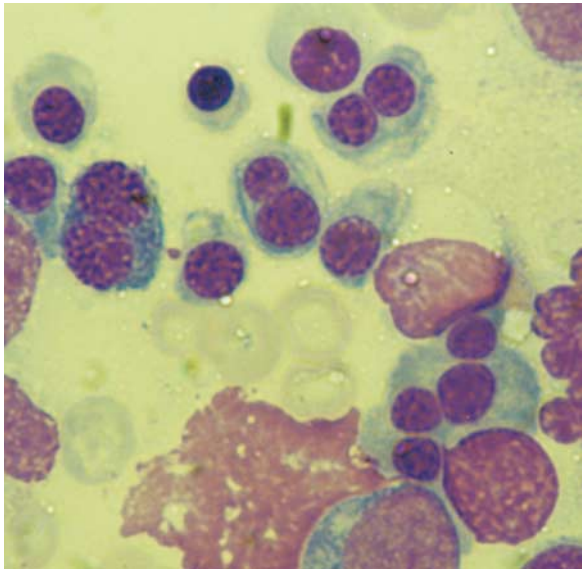

Figure 6 Dyserythropoiesis in chronic malaria: multinucleated erythroblasts.

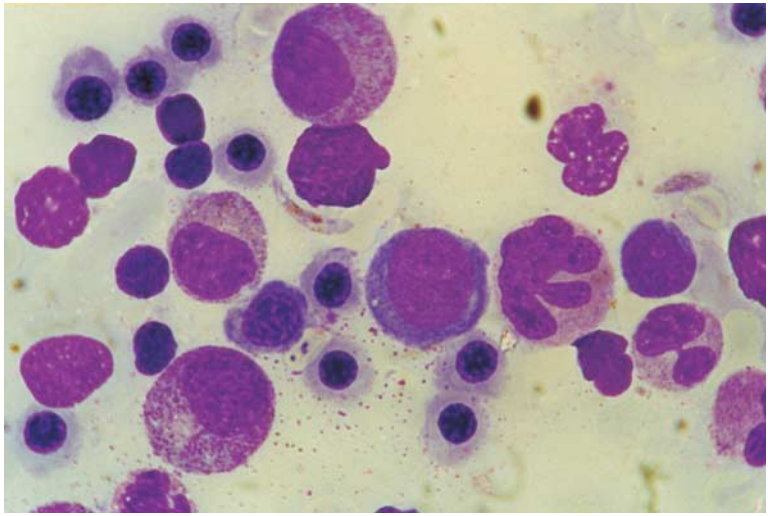

Figure 7 Bone marrow in chronic malaria showing immature gametocytes, erythroid hyperplasia and a giant metamyelocyte.

Table 3. Theoretical differences between patterns and effects of cytokine release in acute and chronic malaria.

	Acute	Chronic
Release of inflammatory cytokines and anti-inflammatory cytokines	Released in bursts High circulating levels Systemic effects	? Continuous low level release Low circulating levels Local effects Imbalance
Effects on erythropoiesis	Suppression of erythropoiesis Low EPO (dyserythropoiesis)	Dyserythropoiesis High EPO

distribution is similar to that seen in the anemia of chronic disease; an increase in macrophage iron and a reduction in sideroblasts are often seen (19, 25). In Gambian children with acute and subacute malaria, a lower percentage of sideroblasts than normal (means 3.3% and 5.5% respectively, versus above

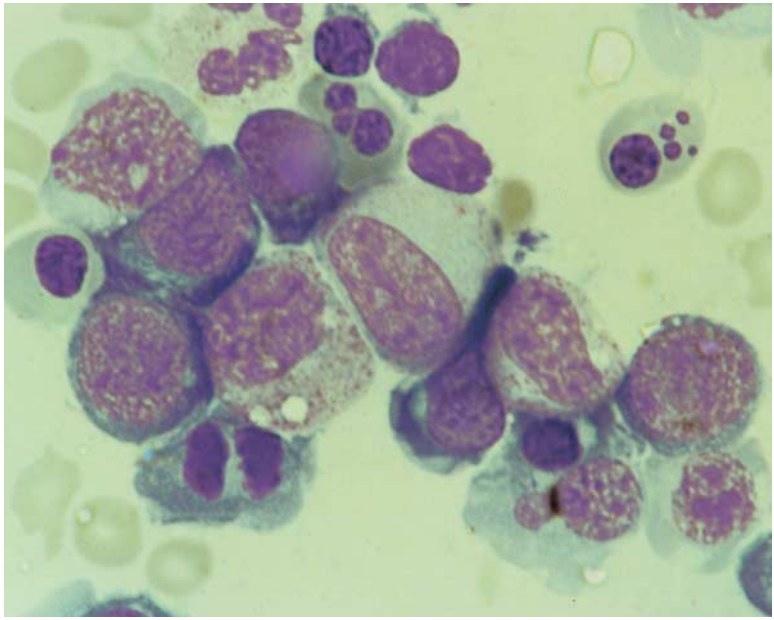

Figure 8 Bone marrow in chronic malaria showing nuclear fragmentation and occasional megaloblasts.

20%) is seen (19). Children with chronic malaria show a different pattern of iron distribution, with an increase in storage iron as well as an increase in siderotic granules in erythroblasts. In a minority of patients a small number of ring sideroblasts were seen. In two patients where a repeat bone marrow was performed, these changes were not found after recovery.

Myelopoieisis

This is fully discussed in Chapter 5; only a summary is presented here.

Numerical Changes

There is often a reduction of the ME ratio, presumably reflecting erythroid hyperplasia in the bone marrows of patients with chronic malaria and relative lymphocytosis in those with acute malaria. The percentage of lymphocytes

appears to be inversely related to that of erythroblasts, with an increased proportion in those with acute malaria and a relative reduction in those with chronic malaria, and an intermediate value in the intermediate cases (19, 20).

The percentage distribution of the various cell types of neutrophil granulocyte series is normal. In bone marrows of patients with acute malaria, the proportion of eosinophils may be increased.

Plasma cells are normal in number but monocytes appear to be increased in bone marrows from some patients with acute malaria.

Morphological Features

The myeloid cells of patients with malaria show some dysplastic features, noted in both Gambian children and nonimmune European adults (5, 20, 31). The features observed include ring neutrophils and giant metamyelocytes (GMMCs). A semiquantitative assessment of GMMCs in malaria showed that they are more commonly seen in the bone marrow of patients with chronic malaria and rarely seen in patients with acute malaria (20). The appearance of GMMCs reflects the severity of dyserythropoiesis, but was shown not to be due to B_{12} or folate deficiency (29), which were excluded by normal levels of serum B_{12} and red cell folate and by normal deoxyuridine suppression test results (30). It is concluded that these changes are due to dysplasia in the myeloid series similar to that seen in the erythroid series.

Phagocytosis

A full discussion on this topic is found in Chapter 5.

Megakaryocytes

Thrombocytopenia is common in acute falciparum or vivax malaria (see Chapter 8). The thrombocytopenia does not appear to be related to underproduction of megakaryocytes, as these are usually increased in number, as in other conditions where there is an increased peripheral platelet turnover.

The majority of the megakaryocytes in malaria appear normal in size and morphology, but Knüttgen observed that occasional megakaryocytes may show some dysplastic features and suggested that thrombocytopenia was in

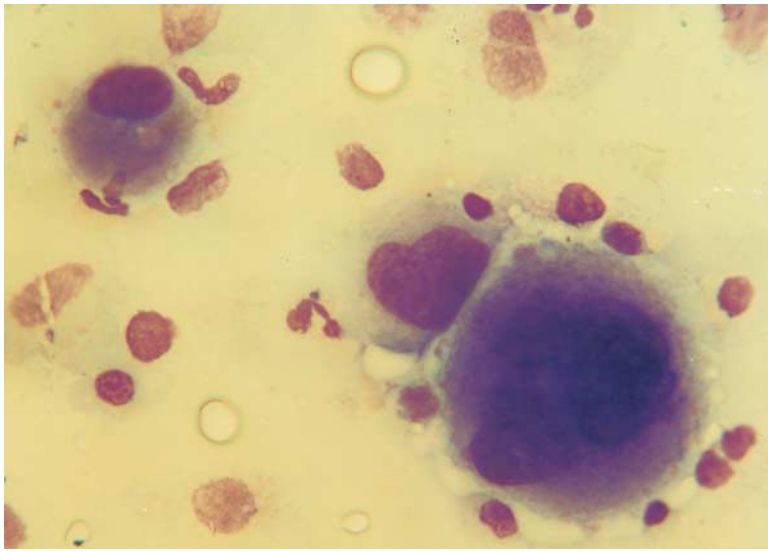

Figure 9 Megakaryocytes in acute malaria: there are increased numbers of megakaryocytes and some are hypolobulated, suggestive of increased platelet turnover.

part due to ineffective megakaryocytopoiesis (5). Mild dysplastic features were also seen in the megakaryocytes in the bone marrows of Gambian children with malaria (20). The significance of these findings is not clear, as some of these changes are also seen in patients with ITP (personal observation) and may reflect a certain amount of dysmegakaryopoiesis due to accelerated megakaryocyte development.

Ultrastructural Changes Affecting Human Bone Marrow in Malaria*

The study of the ultrastructure of the bone marrow in malaria has confirmed and added to characterization of the dyserythropoiesis in malaria. Abnormalities were seen in both acute and chronic malaria. The cytoplasmic abnormalities include unusually large autophagic vacuoles, intracytoplasmic circular double

*Figures 10–13.

membranes, varying degrees of iron loading of mitochondria, an increased frequency of intercellular spindle bridges, and a marked reduction in the electron density of the cytoplasmic matrix with a scarcity of ribosomes. No malaria parasites were identified within erythroblasts. The nuclear abnormalities included the presence of bi- and multinucleated erythroblasts, intranuclear clefts, irregularities in the shape of the nucleus, myelinization or loss of part of the nuclear membrane or dilatation of the space between the two layers of the nuclear membrane and karyorrhexis (19) (Fig. 10). These changes were found to varying extents in all patients in this study but were more common in children with chronic malaria. Ultrastructural changes in erythroblasts similar to those in Gambian children were also seen in Thai adults with cerebral malaria and in those with vivax malaria (Fig. 13) (7, 26).

In the bone marrows of patients with cerebral malaria, some bone marrow sinusoids were packed with knobby parasitized red cells (Fig. 11(a)). These cells appear to attach to the endothelial cells and to each other through the surface knobs, with connecting strands seen at sites of separation (Fig. 11(b)). Complex interdigitations were sometimes seen between the parasitized cells and endothelial cells, and occasional cells appeared to enter the bone marrow parenchyma through this route, thereby potentially allowing newly released merozoites to infect reticulocytes (7).

The ultrastructure of erythroblasts in vivax malaria showed dyserythropoietic features similar to those seen in bone marrows of patients with falciparum malaria, but these were usually milder (26).

Ultrastructural observations on peripheral blood red cells in patients with malaria showed changes suggestive of damage to uninfected red cells (32).

Figure 10 Ultrastructural changes in bone marrow malaria. Dyserythroipoietic features in acute and chronic malaria: (a) erythroblast nucleus showing duplication of the nuclear membrane ×4660; (b) erythroblast with a karyorrhectic nucleus ×8775; (c) multinucletaed erythroblast with unequal nuclei; (d) two erythroblasts incompletely separated, showing an internuclear bridge; (e) erythroblast showing cytoplasmic vacuolation and iron laden mitochondria; (f) higher magnification showing electron dense material between the mitochondrial cristae.

(a), (b): Reprinted from *B. J. Haem* (**46**, 1980, p. 171), Abdalla *et al.*; with permission from Blackwell. (c)–(f): Reprinted from *Bailliere's Best Practice, Clinical Haem* (13.2, 2000, p. 277), Wickramasinghe and Abdalla; with permission from Bailliere Tindall.

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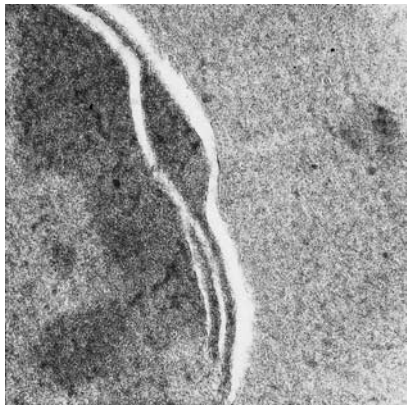

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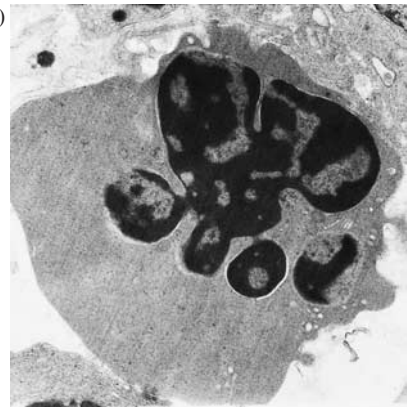

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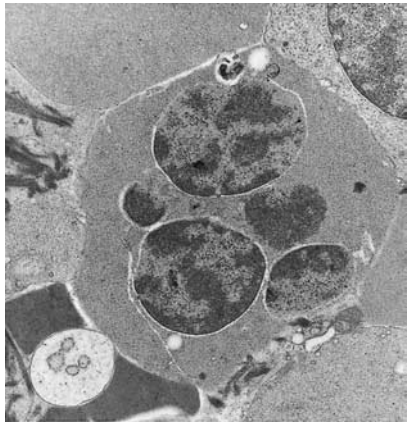

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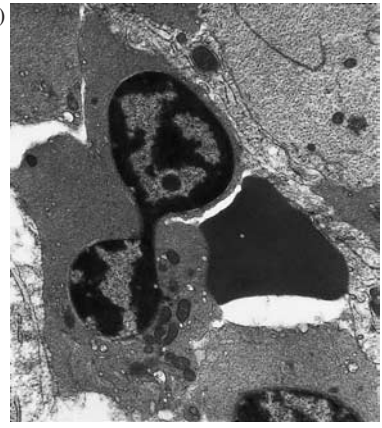

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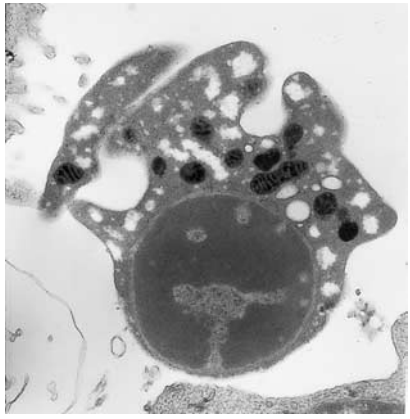

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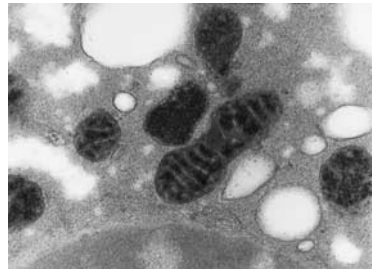

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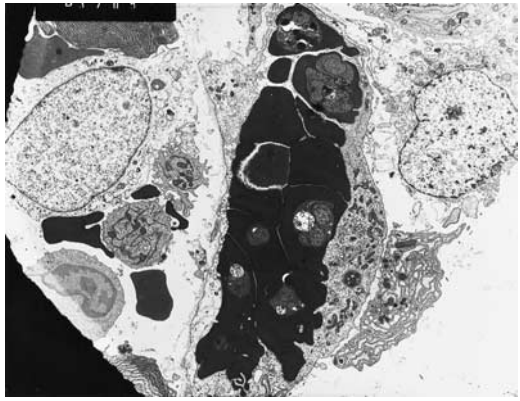

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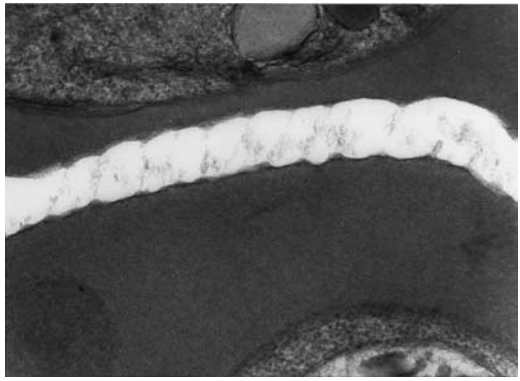

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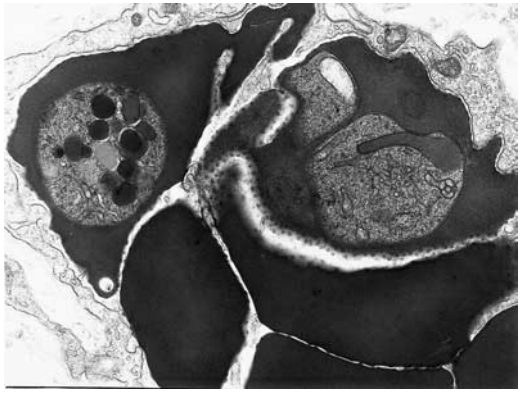

These included cells with multiple ragged processes with and marked cytoplasmic vacuolation and vesiculations (ropalocytes). Other cells showed autolytic vacuoles containing hemoglobin-like particles in association with mitochondria, and some of these appeared to be extruded through the cell membrane. In some cases this process appeared to end in collapsed cells with empty cytoplasm. In addition, some erythrocytes displayed electron dense membrane-associated knobs similar to those seen in parasitized red cells. The significance of these findings remains unclear.

Functional Changes Affecting Bone Marrow Cells in Malaria

Cell Cycle Studies

There are two published studies on the kinetics of erythroblasts in patients with falciparum malaria. In the first study, on six Gambian children with malaria, the technique of combined Feulgen microspectrophotometry and ^{3}H-TdR autoradiography showed evidence of cell cycle perturbation. In chronic malaria there was an increased proportion of cells in the G_2 phase (patient range 10–15%; normal 6–8%) and an increased number of cells with an intermediate amount of DNA but without thymidine incorporation (U cells) (patients 0.4–2%; normal ~1%). These results confirm that ineffective eryrthropoisis, inferred from the dyserythropoietic changes, is an important cause of anemia in malaria (33).

Figure 11 Ultrastructural changes in the bone marrow in acute malaria. (a) Bone marrow sinusoid tightly packed with parasitized red cells; ×4900. At higher magnification all of the intrasinusoidal red cells were found to be knobbed. (b) Higher magnification showing electron dense strands connecting the knobs of two adjacent parasitized erythrocytes within a bone marrow sinusoid; ×61 600. (c) Bone marrow sinusoid packed with parasitized RBCs at a higher magnification. Note that many knobs on the surface of the red cells form attachments between the parasitized cells to each other and to the endothelial cell surface.

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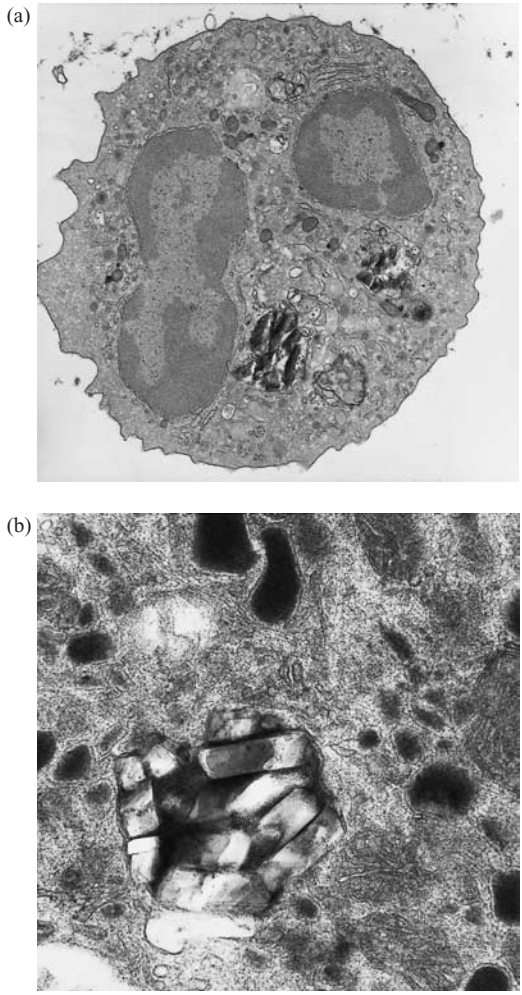

Figure 12 Ultrastructure of the bone marrow in acute malaria showing some white cell changes. (a) A granulocyte containing two aggregates of hemozoin in its cytoplasm; ×13 950. (b) Collection of hemozoin crystals within the cytoplasm of a macrophage; ×35 200.

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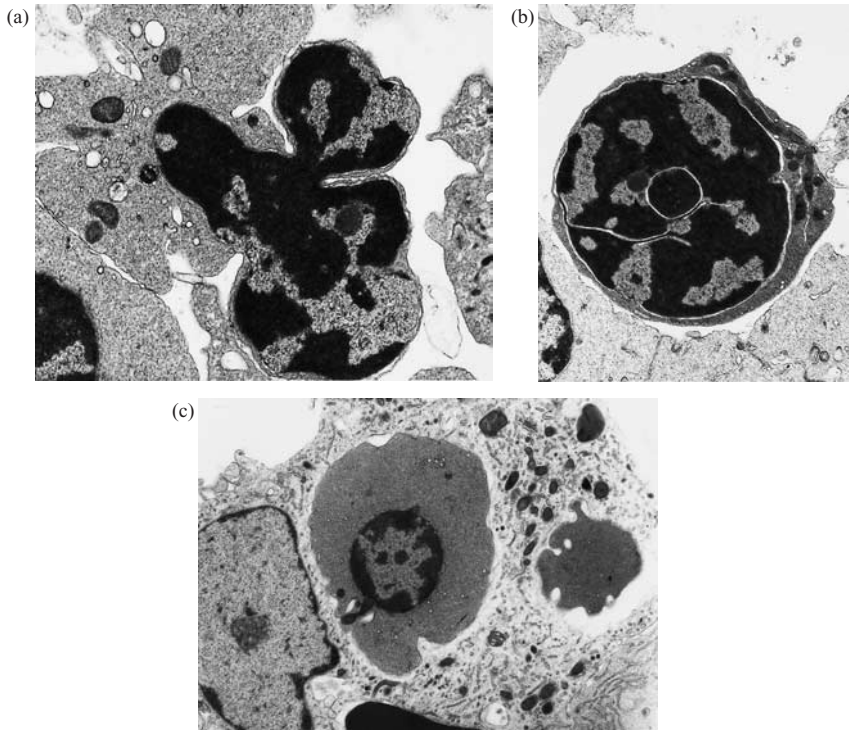

Figure 13 Ultrastructural changes in the bone marrow of patients with *P. vivax* malaria and severe anemia. (a) Erythroblast with a markedly deformed nucleus; (b) erythroblast with multiple intranuclear clefts; (c) macrophage showing phagocytosed erythroblast. Reprinted from *Bailliere's Best Practice, Clinical Haem* (13.2, 2000, p. 277), Wickramasinghe and Abdalla; with permission from Bailliere Tindall.

The second study utilized quantitative ^{14}C autoradiography in five cases of acute falciparum malaria in nonimmune Europeans and showed a marked relative loss of polychromatic erythroblasts in relation to basophilic and proerythroblasts. There was an improvement but not a complete return to normality after successful treatment of the malaria. The authors concluded that ineffective erythropoiesis also occurs in acute malaria. They also argued that erythroid hyperplasia in bone marrows of patients with malaria may be due to accumulation of erythroblasts in the bone marrow rather than to an increase in erythropoiesis (28).

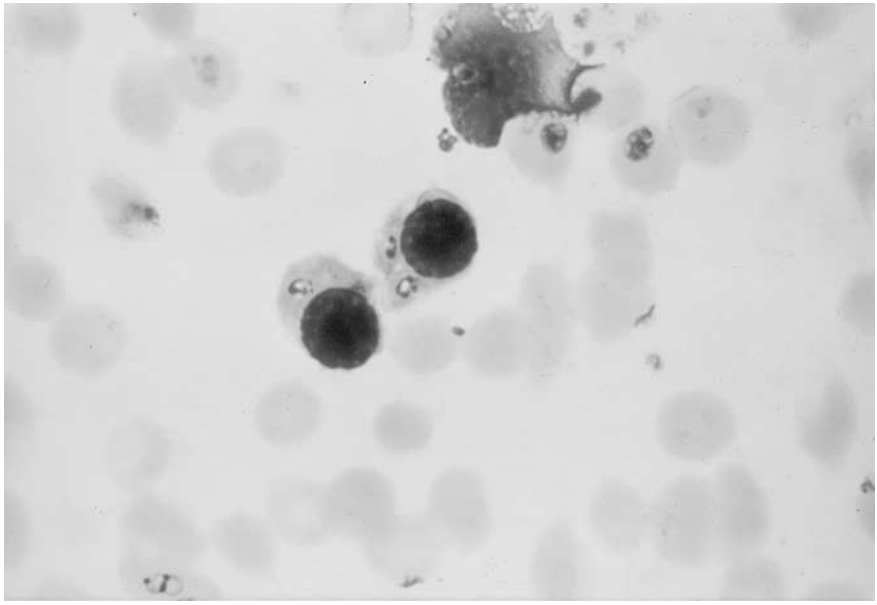

Figure 14 *In vitro* parasitization of erythroblasts by malaria parasites. This phenomenon has not been observed *in vivo*.

The Deoxyuridine (dU) Suppression Test

The dU suppression test is an *in vitro* test performed on bone marrow cells to detect tissue deficiency of vitamin B_{12} or folate. In normal bone marrow cells, preincubation with dU causes increased synthesis of thymidylate within the cells and suppression of uptake of tritiated thymidine. Suppression is less marked in these deficiency states because of reduced formation of intracellular thymidylate.

The dU suppression was found to be normal in six out of the seven Gambian children with severe anemia and malaria, the exception being a patient who also suffered from protein-energy malnutrition. All the others gave normal results despite the presence of GMMCs in five out of the seven bone marrows. These results confirm that the morphological features seen in the bone marrow, including in a few cases GMMCs and mild megaloblastic features, are not due to vitamin B_{12} or folate deficiency, and that these changes are part of the deserythropoietic picture (20, 30); see also Chapter 4.

Ferrokinetic Studies

Srichaikul and colleagues performed ferrokinetic studies *in vivo* (18) and *in vitro* (34) in order to study erythropoiesis in patients with malaria. 38 *in vivo* studies were performed on 36 patients: 5 with acute or primary malaria (2 *P. vivax* and 3 *P. falciparum*), 14 with chronic or secondary malaria (3 *P. vivax* and 11 *P. falciparum*) and 17 at the postparasitemic phase (5 primary and 12 secondary). The red cell iron incorporation curve showed reduced radioactive iron uptake throughout the period of the study, indicating that hemolysis is unlikely to be the cause of this reduction. The authors concluded that the reduced iron incorporation is due to either impaired production of erythroblasts or impaired release of reticulocytes from the bone marrow of these patients. The combination of data on iron incorporation and erythroid activity in the bone marrow indicates that two mechanisms operate at different stages of the disease. Thus, in acute malaria there is reduction of iron incorporation and reduced percentage erythroblasts, indicating suppression of erythropoiesis. In secondary malaria there is normal or increased erythroid activity but also reduced iron incorporation and lack of a reticulocytosis, suggesting that a defect in release of reticulocytes may be responsible for the continued anemia. The latter observations can also be interpreted as indicating ineffective erythropoiesis (18, 34).

In vitro ferrokinetics studies performed on bone marrow cells of patients with malaria by the same authors showed that there is a reduced rate of erythroblast proliferation, and also confirmed the reduced rate of iron incorporation into these erythroblasts. These effects appear to be independent of the presence of autologous malaria serum. The authors concluded that these abnormalities in ferrokinetics were caused by malaria, as shown by their reversal after malaria was treated (34).

Progenitor Cells

A study on 24 Gambian children with malaria using a semisolid culture system showed no deficiency or suppression of bone marrow erythroid burst-forming units (BFUe) or colony-forming units (CFUe) of patients with malaria and anemia. However, there was a significant negative correlation between the number of BFUe with parasitemia of above 1%. There was no suppression of CFUe and usually there was an increase in their number. CFUe were observed to produce colonies in the absence of added erythropoietin when autologous

serum was used in the system. There was also a statistically significant negative correlation between the number of BFUe and CFUe in the marrows of these patients (35). The conclusions from this study are:

(1) There is no demonstrable suppression of BFUe in patients with malaria.
(2) The inverse correlation between CFUe and BFUe and fewer BFUe in patients with higher parasitemias may be interpreted as being due to increased recruitment of CFUe from the BFUe pool.
(3) There is an increase in erythropoietin (EPO) or a similarly acting cytokine in the sera of children with anemia and malaria, leading to increased production of erythroid colonies in the absence of added erythropoietin in the culture system.

In another study, on 21 Thai adults with acute malaria, including 14 with mild-to-moderate anemia, bone marrow BFUe and CFUe were assayed during and after parasitemia (36). The authors compared progenitors assayed during parasitemia and after treatment, and the effects of sera at presentation and during convalescence, with those of AB serum. They concluded that there was no suppression of CFUe and BFUe in the presence of acute sera in uncomplicated cases but a relative suppression in those with complications. This effect was seen on acute and convalescent bone marrows. The postparasitemia sera, on the other hand, had no suppressive effects on both acute and convalescent bone marrows. The numbers of progenitors were fewer in convalescent bone marrows than in acute bone marrows. The authors' conclusions were that these findings may indicate a suppressive factor in sera during acute complicated malaria or that the effects may be due to EPO deficiency (36). It should be noted in interpreting these findings that the numbers of progenitors in this study were higher than those reported in normals in other studies, and a normal range in the Thai population was not quoted.

Taken together, therefore, the variable data provide no convincing evidence of an absolute suppression of BFUe in acute malaria. The presence of large numbers of CFUe without the addition of exogenous EPO, but in the presence of autologous sera from Gambian children with chronic malaria, suggests the presence of erythroid-colony-stimulating activity in these sera.

Erythropoietin Production During Malaria

Measurements of immunoreactive erythropoietin levels in the sera of patients with malaria and anemia have shown apparently conflicting results. Studies on African children with severe anemia in areas of hyperendemic malaria suggest that erythropoietin levels are appropriately increased to the degree of anemia (37, 38). In one study the levels were also measured 28 days after treatment of malaria, and it showed a reduction in levels correlating with hematological recovery (37).

Studies on Thai adults with complicated malaria (39) and on Sudanese adults with anemia (40) have, on the other hand, found that the levels of circulating erythropoietin are inappropriate for the degree of the anemia when compared to levels seen in Caucasian adults without malaria and corresponding levels of hemoglobin.

These results confirm that responses to malaria are different in children and adults where suppression of erythropoeisis appears to be a more important factor in bone marrow dysfunction than dyserythropoiesis (see Chapters 3 and 7).

The Postulated Causes of Dyserythropoiesis in Malaria

There is overwhelming morphological and functional evidence that dyserythropoiesis and consequently ineffective erythropoiesis play a major part in increasing the severity and the duration of anemia in patients with chronic malaria. To summarize: the dyserythropoietic features in the bone marrow of these patients are highly suggestive of ineffective erythropoiesis; cell cycle studies confirm perturbation of erythroblast proliferation; reticulocytosis is inappropriate to the degree of anemia despite erythroid hyperplasia; and there is no apparent progenitor or erythropoietin deficiencies.

The processes involved in the development of anemia have to be seen to operate as a continuum with different mechanisms dominating at different stages; many cases with chronic malaria may have an initial acute attack or several sequential attacks, possibly with decreasing clinical severity. The initial drop in hemoglobin is mainly due to hemolysis of parasitized and nonparasitized cells (Chapter 3). Bone marrow suppression and ineffective erythropoiesis may also play a transient role in accentuating the anemia of acute malaria, but ineffective erythropoiesis is the main mechanism by which

recovery is delayed and the duration of anemia is prolonged in the more protracted forms of malaria.

There is little doubt that the dyserythropoiesis and ineffective erythropoiesis observed in the bone marrows of patients with malaria are related to the presence of malaria parasites. Evidence for this includes the observation of brisk reticulocytosis after treatment of malaria with antimalarials alone in the majority of patients, and the improvement in morphological features of dyserythropoiesis as well as in the cell cycle perturbation in patients after treatment of malaria (20, 25, 33).

Various theories have been proposed to explain the perturbation of erythropoiesis in malaria. Originally hematinic deficiencies were considered to play a role (27). Knüttgen commented that the morphology of erythropoiesis was not typical of that of pernicious anemia but that there might be a defect in folate metabolism accounting for these abnormalities (17). Later studies have excluded hematinic deficiencies as a cause of dyserythropoiesis, as these have not been a constant finding in these patients (19, 20, 30). (See also Chapter 4.)

A direct effect of parasites on bone marrow has been postulated to cause bone marrow dysfunction. Direct parasitization of erythroblasts has not been demonstrated *in vivo* (19), presumably because there is normally no contact between merozoites and erythroblasts, though it may be induced by incubation of erythroblasts with malaria parasites *in vitro* (Fig. 14). Other postulated mechanisms include parasitization of reticulocytes and packing of bone marrow sinusoids leading to hypoxic damage to the marrow (7), or inhibition of the release of reticulocytes (7, 24). Although sequestration of more mature forms of parasites leading to sinus packing is seen in the bone marrow of patients with cerebral malaria (7), the marrow is not normally a preferred site of sequestration in noncerebral malaria (see the earlier section "Parasite Stages in the Bone Marrow"). Parasite sequestration in marrow sinusoids is not seen in cases of chronic malaria where the parasite numbers are very low. It is, however, possible that this mechanism may play a part in the dyserythropoiesis observed in bone marrows of patients with acute malaria.

The most attractive theory regarding the mechanism of dyserythropoisis in malaria is that the perturbation is due to parasite products and the effects that the release of these products have on host defence mechanisms, leading to release of a number of bioactive molecules that may upset the homeostatic balance in normal erythropoiesis.

Parasite Products That May Result in Dyserythropoiesis

During the process of schizogony, numerous parasite products are released in the microvasculature. This process is also accompanied by the febrile paroxysm which is characteristic of malaria and is now thought to be related to stimulation of host cells in particular monocytes and macrophages, to produce TNF and other pyrogenic cytokines (reviewed in Chapter 6).

The main particulate products released during schizogony include merozoites, malaria pigment and also the host's red cell membrane. A number of soluble antigens are also released, including the family of soluble antigens that are heat-stable (S antigens) and other molecules, including a glycosylated phosphotidyl inositol (GPI) anchor. More recent work has revived the concept of a malaria "toxin" and a putative molecule has been identified as a GPI-like molecule. The effects of these products on host cells have been studied *in vitro* and *in vivo* in a number of situations. Malaria soluble antigens, which are also heat-stable, can be detected in the circulation for a few weeks following an acute infection (41). The release of these antigens has several effects on the host, leading to some of the pathological features of malaria (see Chapter 6).

The details of studies involving the effects of malaria parasite products are fully discussed in Chapter 6. In summary, parasite products may lead to release by host cells of proinflammatory cytokines. Some of these may then have a deleterious effect on the host if overproduced and if not balanced by the appropriate anti-inflammatory response.

Host-Generated Molecules

The effects that parasite products may have on host cells may be mediated either directly, by a toxic action on target cells, or indirectly, via the release of proinflammatory cytokines. The role of cytokine release in malaria is discussed in Chapter 6. In summary, it is thought that in acute malaria, several proinflammatory cytokines are produced by T cells (usually but not exclusively γ/δ cells) in response to release of parasite products during schizogony. In acute malaria, and especially in severe malaria, TNF and IL-1 are important in pathogenesis, but IL-6 and IFNγ may also play a part. After the initial production of proinflammatory cytokines, there is a secondary release of inhibitory anti-inflammatory cytokines provoked by the release of the proinflammatory cytokines such as IL-10 and IL-4. The amount of cytokines, the timing and the

balance are all important in the pathogenesis of severe malaria, the types of complications and the subsequent development of immunity.

It has been suggested that TNF-α is an important cytokine in the anemia of malaria because of its suppressive effect on erythropoiesis (42) and because some dyserythropoiesis was seen in mice given TNF (43). TNF may well play a part in bone marrow suppression in acute malaria but is unlikely to be a major factor in the anemia of chronic malaria, as the clinical and the hematological picture are not those seen in other conditions such as sepsis and acute malaria where high TNF levels are seen.

More recently, it has been suggested that IL-10 underproduction may be an important cause of dyserythropoiesis. IL-10 is an anti-inflammatory cytokine usually produced in response to production of TNF and leads to downregulation of TNF production. It was found that in Ghanaian children low IL-10 levels and a high TNF/IL-10 ratio significantly correlated with severe anemia but not with other manifestations of malaria, such as CM (44). Similar results were found in Kenyan children (45). In Thai adults with severe malaria, low IL-10 levels and high IL-6/IL-10 ratios correlated with a fatal outcome (46).

Recently, the underproduction of another cytokine, IL-12, has been suggested as a possible explanation for severe protracted anemia. A study on Ghanaian children found that low levels of IL-12 correlated with severe anemia (47), and experimental work on mice with deficient IL-12 production has shown that these animals are unable to control parasitemia with overproduction of TNF and underproduction of IFNγ (48). Although IL-12 injected experimentally leads to anemia in various animals, including man (49), its effects on erythropoiesis are complex and depend on the amounts and types of other cytokines being produced. IL-12 is responsible for release of IFNγ, which in turn leads to activation of iNOS and production of NO. Both IFNγ and NO have been implicated in the pathogenesis of severe malaria and also protection against malaria.

It is unlikely that one cytokine is responsible for the dyserythropoiesis in malaria, but cytokine dysregulation and imbalance appear to be more likely explanations. Molecules such as NO and ROI produced locally by macrophages in proximity to erythroblasts may also be candidates for production of dyserythropoiesis, and a study of their role in this disorder should be made.

Is Dyserythropoiesis Advantageous in Malaria?

Severe malarial anemia (SMA) has been identified as one of three major causes of morbidity and mortality in African children with malaria, the other two being CM and severe acidosis (50). SMA usually occurs at a younger age and is associated with higher and more sustained malaria transmission, compared to CM, which is associated with a less stable pattern of malaria transmission (51). This clearly suggests that early exposure to malaria leads to acquisition of a different type of antidisease immunity which has been thought to be advantageous.

It can be argued that first exposure to malaria at an early age may produce an advantageous immunological response, even though some of these immunological processes may themselves contribute to severity of anemia (51). In addition, increased susceptibility to anemia in these young children may theoretically protect against more severe manifestations of malaria by reducing the number of cells available for rosetting, parasitization and sequestration, and therefore prolonging exposure to malaria at a low level and enabling quicker acquisition of immunity.

In one study, anemia as a single severe complication was not found to be associated with increased mortality (52), and in the context of the untransfused child with severe anemia and chronic malaria there is certainly less morbidity than expected from the severity of anemia provided the parasitemia is low (personal observation, SA, 53).

Severe anemia is easier to treat than other complications of malaria. Dyserythropoiesis is easily reversed and the anemia corrected when malaria is treated and parasites are cleared. As the bone marrow is primed with high levels of EPO and there is erythroid hyperplasia, recovery follows quickly. Observations confirm the easy reversibility of dyserythropoiesis, as often there is a marked reticulocytosis within three days of treatment and in some cases extremely high levels of reticulocytes (19).

Reduced malaria transmission may also theoretically result from dyserythropoiesis. Paul and colleagues found that the natural and induced stimulation of erythropoiesis in the vertebrate host with malaria leads to a shift in favor of male gametocyte production (14). This may cause a reduced reproductive success of the parasites and possible reduction in transmission of malaria. Because of the sustained erythropoietic drive associated with dyserythropoiesis seen in children with chronic malaria, prolonged increase in EPO

production may cause this shift to occur in these children, thereby reducing the efficiency of malaria transmission and providing protection to the community.

Conclusions

In this chapter the changes in the bone marrow, mainly with regard to erythropoiesis, have been discussed. The major findings in acute malaria are suppression of erythropoiesis, although a certain amount of dyserythropoiesis occurs; this is most commonly seen in nonimmune adults but is also seen, to a lesser extent, in children with acute malaria from endemic areas. The clinical context and the findings of a number of studies strongly suggest that this suppression of erythropoiesis is mediated by proinflammatory cytokines. The candidates for the acute proinflammatory cytokines include TNF, IL-1 and IFNγ, and possibly also IL-6, and those for the underproduced anti-inflammatory or regulatory cytokines are IL-10 and IL-12.

In chronic malaria, on the other hand, the picture is dominated by dyserythropoiesis and ineffective erythropoiesis, and the likelihood is that dyserythropoiesis may be produced by one or more factors, including the sustained local release of proinflammatory cytokines, NO and ROI, or imbalance between pro- and anti-inflammatory cytokine production, or macrophage dysfunction caused by these cytokines or by ingestion of malaria pigment. The dyserythropoiesis is present as long as the parasites persist even at low levels, but clears rapidly on clearance of the parasites (see Fig. 2, Chapter 3).

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Chapter 8

Platelets and Blood Coagulation in Human Malaria

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Thrombocytopenia is a frequent finding in acute falciparum malaria infection. It results from a combination of platelet activation, splenic pooling and reduced life-span due to antibody and cellular immune responses. A subclinical coagulopathy is common with activation of the intrinsic pathway, but clinically significant disseminated intravascular coagulation (DIC) is very rare. Platelets themselves or mediators derived from platelets and the coagulation cascades may effect the interaction between infected erythrocytes and the endothelium and be important in the pathophysiology of severe malaria. Very occasionally, thrombocytopenia may result from antibodies, produced against the anti-malarial drugs quinine and quinidine, interacting with platelets.

Introduction

Although malaria is an infection of red cells, the key pathological event in the development of severe falciparum disease is the interaction between the infected cells and the microvascular endothelium. Platelets and coagulation factors are important components of the extraordinarily complex environment surrounding flowing and sequestered parasitized red cells and the enclosing tubular vascular endothelium. These two components are affected by malaria infection and may be involved in the pathogenesis of disease. Indeed, thrombocytopenia and abnormal blood coagulation are common features of human malaria. Profound thrombocytopenia is associated particularly with severe *Plasmodium falciparum* infection. In the past platelets and coagulation factors were considered to be of key importance in the pathogenesis of severe malaria, through disseminated intravascular coagulation (DIC). Although subsequent studies demonstrated that clinically important DIC and lethal hemorrhage are very rare, recent research suggests that mediators linking platelets and the

coagulation cascades with the endothelium and the erythrocyte may have a role in the pathogenesis of severe malaria. In this chapter, we will review platelet and coagulation abnormalities in human malaria, their pathophysiological and clinical significance, their management and the influence of antimalarial drugs. The investigation of platelet and coagulation function in human malaria is carried out using blood taken from large peripheral veins. It should therefore be borne in mind that the results may not fully represent the focal function of these components deep in the microvasculature of organs, such as the brain, where the pathology is occurring.

Platelet Abnormalities in Malaria

Changes in the Platelet Count

When platelets are stimulated, they change shape, aggregate and release factors from their granules which stimulate a cascade of events resulting in platelet aggregation and adhesion to damaged endothelium, and activation of the coagulation pathways. In addition to their hemostatic function, platelets are also involved in the repair of endothelium and adjoining tissue, in inflammation, host defence and the control of vascular tone (1–3).

At the turn of the 19th century malaria was thought to be associated with high platelet counts (4). However, in 1924 reduction in peripheral blood platelet concentration was described in man (5) and has since been observed consistently during infection with all human malaria parasites species (6–8). The definition of thrombocytopenia as $<150 \times 10^9$/L (9), conventionally applied to Caucasians, may not be applicable to tropical populations, where a more appropriate cutoff may be $<100 \times 10^9$/L (10).

Estimates of the frequency of thrombocytopenia ($<150 \times 10^9$/L) in acute falciparum malaria range widely but recent estimates suggest an average of about 80%. The frequency of thrombocytopenia ($<100 \times 10^9$/L) amongst 431 adults in Oman with falciparum malaria was 56%, whilst 11% had thrombocytosis ($>500 \times 10^9$/L) (11). In a recent series of 156 adults hospitalized with falciparum malaria on the Thai–Burma border, the median (range) admission platelet count was 41 (2–254) 10^9/L and 92% had a count of $<150 \times 10^9$/L (White *et al.*, unpublished data). Although apparently never evaluated prospectively, it is likely that thrombocytopenia has poor predictive value for malaria as there

are many other common causes of fever and thrombocytopenia in the tropics, such as dengue and HIV disease (1, 12).

The frequency and degree of thrombocytopenia is similar in uncomplicated falciparum and vivax malaria (6, 11, 13). Some studies, but not others, have suggested a negative relationship between peripheral falciparum parasitemia, severity of the disease and platelet count (8, 14–16). A significant negative relationship between APACHE II scores of disease severity and platelet counts amongst Thai patients with falciparum malaria has recently been described (17). However, there is no clear relationship between platelet count and prognosis. In the series of adult Thai patients with falciparum malaria (above), there were no relationships apparent between the admission platelet count and admission parasitemia, hematocrit, white count, serum bicarbonate, creatinine and total bilirubin, plasma lactate and parasite clearance time (White *et al*., unpublished data). Platelet counts increase with the clearance of infection, taking approximately 4–10 days to rise into the "normal" range (13, 14, 18).

Mechanisms of Thrombocytopenia in Acute Malaria

Platelet production in acute malaria infection, as gauged by the density of megakaryocytes in bone marrow aspirates, is normal or increased (19). In contrast, platelet lifespan *in vivo*, measured using the ^{51}Cr allogenic platelet transfusion technique, has been shown, in Caucasians with falciparum malaria, to be reduced from the normal range of 7–10 days to 2–3.7 days. The daily platelet turnover was approximately quadrupled (20). Pretreatment serum concentrations of thrombopoietin, the key growth factor for platelet production, are raised in patients with falciparum malaria, and thrombopoietin concentrations fall as the platelet count recovers (17).

A variety of mechanisms have been proposed for malaria-associated thrombocytopenia. Below we consider factors which may be responsible, except for DIC, which is discussed in a later subsection.

Splenic Pooling

Pooling of platelets in the spleen is a normal physiological phenomenon, with up to one third of normal circulating platelets transiently residing on or near the surface of the splenic sinusoids (21). The size of the pool increases with

the size of the spleen, and in massive splenomegaly severe thrombocytopenia may result.

Splenomegaly is a common feature of acute malaria infection and is also a frequent finding in healthy subjects exposed repeatedly to malaria. For example, in a series of 169 patients with cerebral malaria, the spleen was palpable in 53% (22). The recovery of radiolabeled platelets one hour after injection into humans with falciparum malaria is greatly reduced, from a normal recovery of 62%, to 12%, reflecting pooling of platelets in the enlarged spleen (20). There is no apparent relationship between spleen size, as determined by palpation, and the degree of thrombocytopenia on admission (6), perhaps in part because, when palpable, a spleen is already three times the normal size, and platelet life expectancy is already markedly reduced.

Platelet Aggregation and Activation

Platelet aggregation and activation, in response to proaggregative factors such as adenosine diphosphate (ADP) and adrenaline, has been demonstrated in most, but not all, *ex vivo* studies (23–26). Part of this variability may be because such tests only examine surviving platelets, which have not aggregated or may be "exhausted" (26). In addition, *ex vivo* determination of platelet aggregation is highly dependent on techniques during the preparation of platelet-rich plasma and the extrapolation of these results to *in vivo* pathophysiology is open to question (27). Although no human postmortem study seems to have specifically addressed the issue quantitatively, doubt as to the relevance of platelet aggregates to human malaria is raised by the apparent paucity of platelets, or aggregates, in the brain microvasculature at postmortem (28, 29). Using the platelet count ratio technique, evidence was found that platelet aggregation does occurs *in vivo* in patients with *P. falciparum* and *P. malariae* infection, and that there is a positive relationship between peripheral parasitemia and the degree of platelet aggregation (30).

Platelet activation is suggested by reports of elevated plasma concentrations of platelet granule products such as β-thromboglobulin (βTG) and platelet factor 4 (PF4), elevated plasma and intracellular ADP concentration, elevated thromboxane A_2 and changes in platelet morphology (13, 23–25, 31, 32). Examination of the ultrastructure of surviving platelets in acute malaria infection has demonstrated evidence of activation, such as glycogen depletion, the formation of pseudopodia and degranulation. Abnormalities of the open canicular

system and microtubules have been noted (25, 33) and enlarged platelets, up to 7.2 μm in diameter, have been described in patients with both vivax and falciparum malaria (34).

However, the techniques used for the measurement of platelet proteins are critical and doubt has been cast on the validity of results (27). The finding of normal levels of βTG and PF4 in severe malaria but elevation of the βTG : PF4 ratio, suggests that malaria does induce mild platelet activation but because the platelet count is low, the absolute levels of plasma α-granule products are low, i.e. the (TG & PF4 : platelet count ratio is probably raised (27). The supernatant from falciparum malaria cultures enhances ADP-induced platelet aggregation *in vitro* (35).

Elevated plasma P-selectin, which may be derived from damaged or activated platelets, has recently been described in severe malaria (13). However, as P-selectin is also derived from damaged endothelium, the elevated levels may represent endothelial injury rather than platelet activation. If platelet microparticles, derived from activated platelets (36), are found in malaria, they may provide an important measure of platelet activation. The *in vivo* mediators of platelet activation and aggregation are unknown but may include ADP, factor VIIIR : Ag (the von Willebrand factor—vWF), and the platelet-activating factor.

Antibody-Mediated Mechanisms

Negative correlations between antiplatelet serum IgG antibody concentration and peripheral platelet count have been described for both vivax and falciparum malaria (25, 37). However, this result was not confirmed using a different technique (15)—no relationship was found between platelet count and antiplatelet antibodies, both in serum and bound to platelets. This does not discount an immune-mediated mechanism of thrombocytopenia, as only the surviving platelets can be assayed and the more heavily antibody-coated platelets may have been removed. *In vitro* experiments suggest that IgG binds to platelet-associated malaria antigens through the Fab terminus, but whether platelets absorb malaria antigens *in vivo* is unclear. Immune complexes are unlikely to be involved (37). Antiphospholipid antibodies occur in patients with falciparum and vivax malaria and it has been suggested that they may be, at least partially, responsible for platelet activation and thrombocytopenia (38).

Antiplatelet antibodies may activate platelet membranes, resulting in their removal by the hyperplastic reticuloendothelial (RE) system, particularly the

spleen (20, 25, 37). There are insufficient platelet count data from patients with malaria who have had a prior splenectomy to gauge the importance of the spleen in platelet removal (39, 40).

There is evidence that the splenic clearance of once-parasitized "pitted" red cells, experimentally heated (rigidified) and IgG-coated red cells are all enhanced during and after malaria attacks (41–43). Damaged or antibody-coated platelets may have analogous fates.

Erythrocyte ADP Release

It has been suggested that ADP, released by the hemolysis of erythrocytes, activates platelets, which are then removed by the spleen (44). Surviving, damaged platelets (with reduced sialic acid concentrations) may be hypersensitive, enhancing the hemostatic response and reducing the incidence of bleeding, despite thrombocytopenia (44). However, if ADP released from erythrocyte destruction was a key factor in platelet activation and subsequent removal, those patients with severe hemolysis should also have severe thrombocytopenia (45). Contrary to the ADP hypothesis, however, malarial black water fever, which is associated with massive acute intravascular hemolysis, is not characterized by severe thrombocytopenia (45, 46).

Parasite Invasion of Platelets

There is a single case report of *P. vivax* parasites observed within platelets (47). This has not been confirmed subsequently (25, 33) and, if it does occur, it is only an extremely rare contributory cause of thrombocytopenia.

Platelet Phagocytosis

A significant negative relationship between platelet count and plasma macrophage-colony-stimulating factor (M-CSF) concentrations in Thai patients with both vivax and falciparum malaria has been described recently (13). Severe malaria was associated with the highest plasma M-CSF levels. As has been proposed for autoimmune thrombocytopenic purpura, it was suggested that platelets are removed by activated macrophages in the spleen and liver. This hypothesis is supported by observations of macrophage phagocytosis of

platelets and increased macrophage density in the spleen, liver and bone marrow in human malaria (48, 49).

Platelet Adhesion to Erythrocytes

The peripheral blood smear of a Thai patient with an 80% falciparum parasitemia demonstrated extensive binding of platelets to both parasitized and unparasitized erythrocytes, raising the possibility that such platelet–erythrocyte interactions could contribute to malaria-associated thrombocytopenia (50) (see the section "Malaria, Pathogenesis, Platelets and Coagulation").

Oxidative Stress

Oxidative stress may, though lipid peroxidation, cause premature platelet death, leading to malaria-associated thrombocytopenia (51). Platelet superoxide dismutase and glutathione peroxidase activities were lower and platelet lipid perioxidation levels were higher among Turkish patients with vivax malaria than age-matched healthy controls. A significant negative correlation was found between the platelet count and the extent of platelet lipid peroxidation (51). These relationships have not been investigated in patients with falciparum malaria.

Hyperreactive Malarial Splenomegaly

Hyperreactive malarial splenomegaly (HMS; also known as the tropical splenomegaly syndrome) occurs amongst adults and older children in areas of intense falciparum malaria transmission. It is characterized by gross, firm, chronic splenomegaly with polyclonal hypergammaglobulinemia, from uncontrolled B cell activation, and elevated serum IgM (52). The resultant hypersplenism leads to thrombocytopenia but the details of platelet kinetics and function have not been investigated (see Chapter 10).

Coagulation Abnormalities in Malaria

Pro- and Anticoagulant Mediator Abnormalities

In falciparum malaria, synthesis is reduced and consumption increased of most procoagulant factors. Mild prolongation of the prothrombin time and partial thromboplastin time may occur (14, 18, 26, 53, 54).

In severe malaria, factor XII and prekallikrein levels are significantly lower than in controls and levels rise in those who survive, suggesting activation of the intrinsic coagulation pathway in acute disease (18). The depletion of factors X and VII in severe disease (53) also implies activation of the extrinsic pathway. Factor V levels may be low, normal or raised (53, 55). Factor VIII R : Ag (vWF) is raised in patients with cerebral malaria (22). Positive correlations between parasitemia and factor VIIIR : Ag plasma concentration has been described (8, 54) but whether the factor arose from platelets or endothelium is uncertain.

Concentrations of plasma fibrinogen, the precursor of fibrin, may be elevated, normal or reduced. High concentrations are more common in both uncomplicated falciparum and cerebral malaria. This variability is explained by the acute phase response which increases fibrinogen production, initially masking accelerated fibrinogen consumption (22, 53, 56–60). In severe malaria fibrinogen consumption may sometimes outstrip production, leading to hypofibrinogenemia (61). High plasma concentrations of fibrinopeptide-A (FpA), which is cleaved from fibrinogen by thrombin, occur in patients with falciparum malaria. FpA concentrations were significantly negatively correlated with the fibrinogen concentration, suggesting that thrombin activation and subclinical procoagulant activity occurred (62).

The occurrence of fibrinolysis *in vivo* is indicated by the presence of fibrin degradation products (FDPs) in plasma. FDPs are slightly raised in a minority of patients with uncomplicated falciparum malaria and severe malaria, but most studies suggest that their concentration is not related to disease severity (22, 56–58, 63–65). Plasma tissue plasminogen activator levels were significantly reduced and plasminogen activator inhibitor (PAI-1) levels increased in both vivax and falciparum malaria, suggesting dampening of fibrinolysis (54). Overall, these results suggest mild secondary fibrinolysis in malaria.

The extent of actual microvascular fibrin deposition in severe malaria has been controversial (66). Current evidence suggests that, although it occurs, it is not widespread or of pathological importance (28, 29, 56, 66–70). In life, fibrin

deposition is probably visible as the pale center of retinal Roth spots (71), which may occur in patients with severe malaria (72).

Factor XIII is an important mediator between the coagulation and fibrinolytic systems through catalyzing the cross-linking of fibrin, increasing the clots' resistance to fibrinolysis. Factor XIII subunits A and B and factor XIII activity are reduced in falciparum malaria and levels are correlated inversely with disease severity, parasitemia and polymorphonuclear (PMN) elastase activity (73). The low levels of factor XIII are unlikely to be due to coagulation activation by thrombin, as only subunit A is a substrate in this process. However, plasma PMN elastase concentrations are elevated in severe malaria (73, 74), and through degradation of both the subunits A and B, it may be responsible for low factor XIII levels (73). This finding may help explain the existence of a procoagulant state in malaria but a concurrent low incidence of fibrin deposition. Fibrin deposition may be precipitated by the malaria-induced coagulopathy, but if the resulting clots are unstable due to inadequate cross-linkage, they will be readily dispersed and degraded. This hypothesis predicts that FDPs derived from unlinked fibrin would predominate over FDPs from linked fibrin, such as D-dimers.

Antithrombin III (AT-III) is an *in vivo* anticoagulant which binds irreversibly to thrombin, preventing further coagulation activation. AT-III is consistently reduced in patients with malaria, and levels are particularly low in those with severe disease (14, 18, 22, 54). Elevated plasma thrombin : AT-III complexes are associated with low AT-III levels, suggesting that the low levels of AT-III are due to increased consumption rather than reduced synthesis (18, 60, 73). AT-III concentrations are correlated positively with platelet count and negatively with parasitemia and disease severity (14). The concentrations of the naturally occurring anticoagulants, protein C and protein S, are also reduced in falciparum malaria (14, 18, 54, 60). Thrombomodulin is an endothelial cell surface protein that enhances the interaction between protein C and thrombin. Plasma thrombomodulin levels are raised in both falciparum and vivax malaria (75, 76), probably reflecting endothelial cell damage, and may enhance the anticoagulant activity of the reduced levels of protein C.

Therefore, in adults with severe malaria there are multiple, systematic, interconnected disturbances of pro- and anticoagulant factors and related mediators that shift the balance toward activation of the coagulation cascade and mild secondary stimulation of fibrinolysis. As fibrin may be poorly cross-linked, the

increased fibrinolytic activity may be sufficient to lyse the clots, preventing extensive fibrin deposition.

It is uncertain how malaria precipitates activation of the coagulation pathways. Five possibilities have been suggested. Firstly, *in vitro* experiments have shown that *P. falciparum*–infected erythrocytes and monocytes from patients with both vivax and falciparum malaria have procoagulant activity, although the mechanisms involved are unclear (77, 78). Secondly, microvascular endothelial damage, particularly as a result of parasitized erythrocyte sequestration in falciparum malaria, may stimulate coagulant cascades. The tissue factor is the key endothelial initiator of coagulation, and serum from patients with falciparum malaria increases endothelial cell procoagulant activity and tissue factor mRNA levels (60). Platelets, the coagulation cascade and the endothelium are interlinked through a mesh of complicated negative and positive feedback loops; activation of one component has potentially wide ramifications throughout the microvasculature. Thirdly, cytokines, such as TNFα, which have procoagulant activity, are found at high concentrations in patients with both vivax and falciparum malaria. However, the importance of TNF in the pathogenesis of severe malaria remains unresolved (54, 79–81). Fourthly, plasma concentrations of polymorphonuclear leucocyte (PMN) elastase, which may lead to the proteolysis of coagulation factors and inhibitors, are raised in falciparum malaria. PMN elastase release is probably stimulated by TNF and inactivates the AT-III receptor site involved in thrombin inhibition. PMN elastase levels are positively correlated with severe disease and parasitemia, and negatively correlated with AT-III, factor XIII levels and the platelet count (73, 74). Fifthly, platelet and granulocyte microparticles, which are procoagulant, have been demonstrated in a wide range of diseases with coagulopathy, including meningococcal sepsis (36). If they also occur in malaria, they may contribute to procoagulant activity and DIC.

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (DIC) is a complex, variable, feared disorder in which there is widespread intravascular coagulation induced by procoagulants, with resultant fibrin deposition, consumption of platelets and procoagulant factors with secondary increased fibrinolysis, bleeding and ischemia. It is characterized by thrombocytopenia, prolonged prothrombin, activated partial thromboplastin and thrombin times, raised FDP and

D-dimers, decreased concentrations of fibrinogen and clotting factors, and fragmented erythrocytes visible in a blood film. The pattern of laboratory abnormalities is variable and reflects the balance between coagulant consumption and secondary fibrinolysis. As DIC represents a continuum of hematological abnormalities, with different time courses and etiology, it is difficult to produce a precise numerical definition (82).

During the 1960s and 1970s, DIC was thought to be an important contributor to the pathophysiology of severe malaria in adults. Indeed, case reports and small series have demonstrated that it does occur in severe malaria (53, 56, 61, 82–84). However, larger scale, prospective studies indicate that it is not a common clinical feature in severe malaria (18, 22, 31, 55, 86, 87) or that it occurs at all in patients with uncomplicated malaria (57–59). Similarly, DIC and hemolytic–uremic syndrome have been reported in patients with vivax malaria, mostly in south Asia (88–91), but coincident occult falciparum malaria has not been excluded through techniques such as detection of blood *P. falciparum* histidine-rich protein-2 (HRP-2).

Four lines of evidence argue against an important role of DIC in adults with malaria. Firstly, in prospective studies, life-threatening bleeding is uncommon. Amongst 169 Thai patients with cerebral malaria, clinically important bleeding occurred in only 4% of patients (22). In a series of 560 adults with strictly defined severe malaria, but without tests for coagulopathy, studied in Vietnam, 15% developed gastrointestinal bleeding (92). Amongst 532 patients with "severe and complicated malaria" in Rajasthan, hematemesis or malena were noted in 3% of patients and spontaneous bleeding in 10%, although retinal hemorrhages were included in the latter estimate (93). Secondly, there is no clear relationship between the severity of laboratory measures of coagulation, mortality and clinically important systemic or retinal bleeding when it occurs (22, 94). The retina may be a useful "window" to the brain, showing pathology and hemorrhage during life. Retinal hemorrhages occur in ~15% of patients with cerebral malaria but their occurrence, although significantly associated with reduced plasma AT-III, raised serum creatinine, low hematocrit, high parasitemia and schizontemia, was unassociated with platelet count, plasma fibrinogen, factor VIIIR : Ag, prothrombin time or eventual prognosis (72). Thirdly, thrombus deposition in the macro- and microvasculature is characteristic of DIC. Although such deposits have been described in patients dying of malaria (95), they are conspicuously absent from most studies of postmortem specimens

(28, 66). Fourthly, fragmented red cells, thought to be formed in intravascular fibrin mesh and characteristic of DIC, have been reported very rarely in severe malaria (96). However, both the absence of fibrin and fragmented red cells in malaria-associated DIC would be expected if factor XIII levels were low and the resulting unlinked fibrin readily destroyed. Measuring the proportion of plasma FDPs emanating from un-cross-linked and cross-linked fibrin could further test this hypothesis.

The available evidence suggests that coagulopathy and DIC are also not important clinical features in children with falciparum malaria; most reviews do not mention it as a feature of severe childhood malaria (97–100). In a prospective study of 1843 African children with severe malaria, only 2 developed spontaneous bleeding (101). There are case reports of peripheral gangrene, mostly in Asian children with falciparum malaria, without evidence for DIC or a vasculitis (95, 102, 103).

The controversy over the clinical importance of DIC in malaria arose from imprecision and variability in the definition of DIC, often based only on raised FDP concentrations, and of the severity of the malarial infection, lack of exclusion of other concurrent causes of DIC, and by extrapolation from case reports, which are records of rare events. As reduced platelet counts are common in malaria, and occur independently of DIC, thrombocytopenia, except when profound (e.g. 20×10^9/L), should not be included in the case definition of DIC in the context of malaria. The possible cooccurrence of bacterial infection leading to DIC (12) has not been considered in many studies. For example, 9% of Thai adults with cerebral malaria had septicemia (22). DIC may also be precipitated during pregnancy by a retained dead fetus and obstetric complications arising from severe falciparum malaria.

Other potential causes of overt bleeding in malaria, without DIC, include uremia, liver dysfunction, vitamin K deficiency and the use of drugs such as aspirin and heparin. Whether there is an association between falciparum parasite cytokine release induction, cytoadherence or genotype and pathogenicity to platelets and coagulation factors is unknown.

Malaria Pathogenesis, Platelets and Coagulation

The human microvasculature is an extraordinarily complex organ and during its infection with falciparum malaria parasites a myriad of different

interconnecting immunological, biochemical and structural components are disrupted. Trying to tease out the important causal factors in hematological and endothelial dysfunction has been difficult and much remains to be dissected.

Interest in platelets and coagulation mediators as factors in the pathogenesis of severe malaria, after being dismissed with the conclusion that DIC was relatively unimportant, has returned. This has occurred because of an increased understanding of the role of these factors in pathogen–endothelium–cytokine interactions in infectious disease. For example, human platelets may kill the protozoan *Toxoplasma gondii* and bind to *Staphylococcus aureus*, perhaps promoting abscess formation (2). Vivax malaria also results in cytokine, platelet and coagulation abnormalities, but sequestration is thought not to occur in this infection and there has therefore been little investigation into interconnecting microvascular dysfunction in this nonlethal malaria.

Within the platelet and coagulation systems, CD36 (platelet glycoprotein IV), P-selectin, platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31), thrombospondin, integrin α_v β_3, bradykinin, PMN elastase, platelet-activating factor, AT-III, factor XIII, thromboxane and phospholipase A_2 have all been implicated in the pathogenesis of severe malaria. Although some of these factors bear the name "platelet," this does not necessarily imply that platelets are involved in their action in malaria, as knowledge of their location and function within the body has broadened since the molecules were first named. However, as many endothelial molecules responsible for parasitized erythrocyte sequestration are also found in platelets, it is possible that platelets, or platelet microparticles, do have a role in the pathophysiology of severe malaria.

Platelets and platelet products may be involved in the sequestration of parasitized erythrocytes to the endothelium of capillaries and venules—the key pathological process in severe malaria (81, 104). Platelets may be important in the pathogenesis of murine cerebral malaria through the fusion of platelets, bearing the integrin leucocyte function antigen-1 (LFA-1), with endothelial cells (105). However, the pathophysiology of murine cerebral malaria is fundamentally different from that found in humans and the relevance of these findings to cerebral malaria in man is very doubtful. P-selectin, PECAM and thrombospondin (TSP) are found on both endothelium and platelets and, *in vitro*, parasitized erythrocytes "roll" on these molecules. This rolling action may

assist the more adhesive endothelial CD36 in immobilizing infected erythrocytes (104, 106, 107). The receptor for TSP on the parasitized erythrocyte may be band 3 (108). TSP is also released into the plasma and concentrations are low in patients with severe malaria. However, as other α-granule products are raised in malaria, this finding probably arises from reduced endothelial cell production or increased consumption during cytoadherence (27). The integrin α_v β_3 (vitronectin receptor), found on both platelets and microvascular endothelial cells, has recently been shown to act as an adhesin for erythrocytes parasitized with falciparum malaria (109).

One of the main endothelial surface molecules thought to be responsible for the adhesion of parasitized erythrocytes to human vascular endothelium is CD36 (104, 110). This molecule is also expressed on platelets, which have been demonstrated *in vitro* to adhere to parasitized erythrocytes via this glycoprotein (107, 111). As mentioned above, platelets have been reported adhering to parasitized and unparasitized red cells in the peripheral blood smear of a patient with 80% falciparum parasitemia (49). Recently, the *ex vivo* autoagglutination, or clumping, of parasitized erythrocytes has been associated with severe falciparum malaria amongst Kenyan children (112) and with cerebral malaria in Thai adults, and is probably mediated by the binding of parasitized erythrocytes to platelet CD36 (113).

Rosetting, or the binding of uninfected erythrocytes to parasitized erythrocytes to form aggregates, may be important in the pathogenesis of severe falciparum disease (114). *In vitro*, low concentrations of heparin and a heparin fraction with low AT-III affinity and hence low anticoagulant activity, abolish rosetting by many, but not all, falciparum strains. The latter heparin fraction has been proposed as antirosetting therapy in human malaria (115). Rosetting *in vivo* may be prevented or disrupted by endogenous heparin, endothelial heparan sulfate and other glycosaminoglycans. A possible protective role of platelets has been suggested by the observation that the growth of falciparum parasites *in vitro* is inhibited by platelets (116). Whether this observation has relevance to *in vivo* human malaria is unknown.

Disturbances of the coagulation system may also be involved in the pathogenesis of severe malaria. It has been suggested that the potent hypotensive agent, bradykinin, released by the action of kallikrein on kininogen, might be a factor in the intractable hypotension often found in severe malaria (18). Bradykinin may also increase endothelial cell expression of P-selectin (117)

and stimulate IL-8 release—bradykinin antagonists may therefore be of value in severe malaria. It has also been suggested that thromboxane A_2, through its action on vascular tone and platelet aggregation, is involved in the pathogenesis of severe malaria (118). However, there are very few data on bradykinin and thromboxane A_2 concentrations in human malaria.

PMN elastase, which is found at high concentrations in severe malaria, may be involved in malaria pathogenesis, through the inhibition of AT-III and lysis of factor XIII (see the subsection "Pro- and Anticoagulant Mediator Abnormalities," p. 234). PMN elastase levels may also damage endothelial matrix proteins and lead to endothelial cell detachment and increased microvascular permeability (73, 74). The platelet-activating factor (PAF) is a platelet, PMN and monocyte activator, which also effects vascular tone, as well as endothelial, hepatic and neuronal function. It is produced by endothelial cells and is rapidly metabolized to the more stable lyso-PAF, which may be converted back to the short-lived PAF. Synthesis is stimulated by factors such as thrombin, IL-1, TNFα, bradykinin and phospholipase A_2 (PLA_2). The PLA_2 is a proinflammatory enzyme involved in the reformation of PAF from lyso-PAF. Plasma PLA_2 is massively elevated in acute falciparum malaria and positively associated with plasma TNF, parasitemia and death (119). In a small series of patients with severe falciparum malaria, markedly reduced plasma lyso-PAF levels were found, which were correlated negatively with parasitemia (120). Whether this result represents enhanced conversion of lyso-PAF to PAF is uncertain. Reduced unparasitized red cell deformability is strongly associated with severe malaria, anemia and prognosis. Rigid uninfected erythrocytes probably compound microvascular obstruction due to the adherence of parasitized erythrocytes to the endothelium (121). There is *in vitro* evidence for an as-yet-unidentified product of *P. falciparum* parasites that decreases red cell deformability (122).

Antimalarial Drugs and Platelets

The antimalarial drugs, quinine and its diastereomer quinidine, can cause antibody-mediated thrombocytopenia. Although this adverse effect is rare, these compounds are probably the commonest cause of iatrogenic thrombocytopenia (123). *In vitro* experiments suggest that the parent compounds, rather than their metabolites (although the most abundant quinine metabolite in man,

3-hydroxyquinine, was not tested), are responsible for the drug-dependent antibody and complement-mediated platelet destruction (124).

Antibodies to quinine and quinidine bind to platelets through their Fab domains to platelet membrane glycoproteins GPIb/IX and GPIIb/IIIa—the fibrinogen receptor (123). Whether quinine and quinidine antiplatelet antibodies cross-react does not appear to have been determined. Multiple quinine-dependent antibodies to platelets, neutrophils, lymphocytes and erythrocytes, resulting in neutropenia, thrombocytopenia and the thrombotic thrombocytopenic purpura/hemolytic–uremic syndrome (TTP-HUS), have been described (125). Of 225 patients with TTP-HUS in a series from the USA, the syndrome was associated with quinine in 8%, and quinine, taken for leg cramps, was the commonest TTP-HUS-associated drug (126). Another Cinchona bark alkaloid, cinchonine, is a component of the antimalarial drug Quinimax®, which is used particularly in Francophone Africa (127). It is a potent inhibitor of platelet aggregation, mediated through inhibition of calcium and protein kinase C pathways in platelets (128).

Chloroquine is taken up by platelets and released on their activation. This is the reason why chloroquine concentrations are higher in serum than in plasma. Although chloroquine has been associated with thrombocytopenia (123), normal pharmacological doses in volunteers and *in vitro*, it was found to have minimal effect on platelet function (129). There is evidence that the related compound hydroxychloroquine prevents postoperative deep vein thromboses, by an unexplained mechanism (130). The antimalarial sulphonamides and diaminopyrimidines, such as sulphadoxine and pyrimethamine, may also cause thrombocytopenia (59). Other possible causes of thrombocytopenia in association with malaria are adjunctive drugs (e.g. thiazides, penicillin, heparin [even at doses used to maintain the patency of intravascular cannulae], paracetamol, frusemide and ranitidine), massive stored blood transfusion, posttransfusion purpura and any cause of DIC. In Africa aspirin is widely used as an antipyretic and sold as fake chloroquine (131). Small doses exert a profound and long-lasting effect on platelet function by irreversible inhibition of thromboxane synthase. Salicylate poisoning has been shown to be a factor in the genesis of metabolic acidosis in Kenyan children with severe malaria (132). Presumably, the high plasma salicylate levels also result in severe irreversible platelet dysfunction, prolonging the bleeding time (133).

Clinical Management—Malaria, Platelets and Coagulation

In the practical management of severe malaria, thrombocytopenia, coagulopathy and DIC are rarely important. Indeed, the prognostic importance of DIC in malaria even as a very rare event may be questioned; there are very few documented reports on the death of patients with falciparum malaria, generalized bleeding and laboratory evidence for DIC (82–85). The little information available on platelet and coagulation systems in childhood malaria suggests that, like renal dysfunction, they are relatively unimportant in the young.

The only intervention shown to be beneficial in reducing the mortality of severe malaria is antimalarial therapy. In the early 1970s intravenous and intramuscular heparin was recommended in the treatment of cerebral malaria, based on the premise that cerebral malaria is caused by cerebral intravascular thrombosis and from experiments with malaria-infected nonhuman primates (85, 134, 135). Enthusiasm was increased by the apparent antimalarial activity of heparin in experimental primates (136). Others (6, 59) cautioned against this therapeutic approach, and indeed in those studies where heparin was used in severe malaria, the incidence of serious bleeding was very high (67, 85). A more recent large study found no beneficial effect of intravenous or subcutaneous heparin on parasite and fever clearance or TNF α serum concentrations, but the sample size was insufficient to test whether mortality was affected (16). Intravenous streptokinase and antithrombin III have been given to at least one patient each with falciparum malaria and DIC, with apparent success (95, 136).

There is no clear evidence to guide clinical decisions in the face of worsening severe thrombocytopenia, possibly due to quinine antibodies, and the need to continue quinine therapy. Options include testing for quinine-dependent platelet antibodies and replacing quinine with another antimalarial, such as an artemisinin derivative. High dose intravenous immunoglobulin has been given to two patients with severe thrombocytopenia presumed to be secondary to quinidine, with apparent benefit (122, 137). Immunoglobulin has also been given to a child (who survived) with severe thrombocytopenia and falciparum malaria, although the low platelet count may have been secondary to sulphadoxine and pyrimethamine therapy (138). A cumulative effect of quinidine with aspirin on platelet aggregation *in vitro* and on bleeding time *in vivo* has been demonstrated (133).

For those rare patients who develop DIC, antimalarial therapy and vital organ support should be instituted. If bleeding and factor deficiencies are

evident, fresh frozen plasma and cryoprecipitate (if there is hypofibrinogenemia) may be given (82). Transfusion of fresh blood is recommended for spontaneous systemic bleeding in severe malaria (31). Stored blood is of no value in the replacement of platelets, as they are depleted within 48 h of storage, and of limited value in the replacement of coagulation factors (139). The efficacy of fresh blood is controversial (140) and has the potential for infecting the patient with pathogenic contaminants, such as hepatitis B, the human immunodeficiency virus, or more malaria parasites! However, with the low availability of platelet concentrates and fresh frozen plasma in the tropics, the risk–benefit ratio of fresh blood transfusion may be decreased. Drugs that may effect platelet function or numbers should be avoided. Vitamin K can be injected intravenously if deficiency is suspected, for example, in a malnourished or alcoholic patient.

Invasive procedures, such as central vein cannulation and lumbar puncture (LP), are commonly required in severe malaria but are potentially dangerous in patients with coagulopathy and thrombocytopenia (31). There is little evidence to guide clinical decisions. By extrapolation from patients with thrombocytopenia and coagulopathy of other causes, the main factor reducing the risk of central venous access is the experience of the physician with the procedure, rather than the attempted correction of the hemostatic defect (141, 142). If possible, access should be obtained by the internal jugular or femoral veins, rather than the subclavian vein, as bleeding from the latter cannot be controlled by manual pressure. Similarly, there is little evidence to judge the risks, such as spinal subdural and subarachnoid hematoma, and benefits, such as detection of coincident bacterial meningitis, of LP in patients with cerebral malaria. It has been recommended that platelet transfusions, often unavailable in tropical hospitals, should be given before performing lumbar punctures in cancer patients if the platelet count is $<20\times10^9$/L (12, 143). Whether this is appropriate in severe malaria patients is unknown. It is logical, but unproven, that the use of a narrow gauge, and probably bullet-tipped (Whiteacre), LP needle would reduce the risk of iatrogenic bleeding. Intramuscular injections, often required in severe malaria for adjunctive treatment (e.g. phenobarbitone), can be given except in overt coagulopathy. If only intramuscular antimalarial treatment is available, it should be given even in the face of severe coagulopathy.

Despite many clinical trials, no adjunctive therapy has been shown to be of benefit in reducing the mortality of severe malaria (144). Many therapeutic

interventions, with effects on the platelet and coagulation systems, are currently undergoing clinical trials in patients with bacterial sepsis (145). Of these, AT-III, activated protein C and the platelet-activating factor inhibitor have possible future applicability in reducing the high mortality of severe malaria.

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Chapter 9

Innate Resistance to Malaria Conferred by Red Cell Genetic Defects

Ronald L Nagel

Introduction

Humans exhibit variable susceptibility to infection and most of this resistance is either genetic or acquired through previous exposure to such agents. Most red cell genetic variants represent, in effect, polymorphic genetic resistance to the most significant, frequent and devastating disease of red cells that has affected mankind, namely malaria. This disease is responsible for an estimated 2–4 million deaths a year, mainly of children and pregnant women.

Among the four species of malaria that afflict man, *P. falciparum* has the highest mortality—hence its strong selective pressure. *P. falciparum* infection may lead to death, particularly in the first five years of life. Since mortality occurs well before the reproductive age, carriers of variants that confer resistance and increased survival in the face of this disease contribute such gene(s) to the next generation more often than those more susceptible, increasing the variant gene frequency in the population. Genetic polymorphism, a concept generated by EB Ford (1), states: "Polymorphism is the occurrence together in the same habitat of two or more distinct forms of a species in such proportion that the rarest of them cannot be maintained merely by recurrent mutation." Ford went on to explain that the model required that the rarest form should have increased fitness. Balanced polymorphism is one in which increased fitness of the heterozygote is compensated, in part, by the decreased fitness of the homozygous state. The Ford concept was first applied to hemoglobinopathies by Haldane (2) (see below), and explains the high frequencies of seemingly disadvantageous red cell defects found in humans. In this chapter, the present knowledge of

the effect of the hemoglobinopathies, hemoglobin (Hb) F and certain red cell membrane variants on the life cycle of the parasite will be discussed.

Innate Resistance to Malaria Conferred by Hemoglobinopathies

In the last two decades, two important new methodologies have made major contributions to the study of innate resistance to malaria by red cells with genetic variants, namely *in vitro* malaria culture and transgenic mice. The *in vitro* method, pioneered by Jensen and Trager (3) for the culture for *P. falciparum*, allowed the testing of the effect of red cell genetic polymorphisms on the growth of this parasite, including both invasion of the red cell and development of the parasite once inside the cell. The advantages of this method are:

- The interaction of the red cell with malarial parasites can be studied separately from the role of conditions encountered *in vivo*, such as the liver, the spleen, antimalarial antibodies, complement, interactions with other cells and cytokines.
- Mixtures of red cells with different characteristics can be studied simultaneously.
- The confounding effects of stress reticulocytes and leucocytes released from the marrow are avoided.
- Red cells can be modified and nutrients individually suppressed or modified in quantity or quality.
- Red cells can be defined on the basis of age, and intrinsic properties of the cell membrane, cytoplasm and hemoglobin can be evaluated.

Shortcomings of the culture system should also be taken into account:

- Cultures fail to address the role of immunity, the reticuloendothelial system, the complement system, or the splenic/liver filtration system in parasite growth.
- The culture medium is excessively rich in a variety of nutrients, such as gluthatione and glucose: the effects of some red cell defects upon parasite growth might be apparent only when critical nutrients are in limited supply, for example in β-thalassemic red cells (see below).

The second methodology involves the use of transgenic animal models to study the mechanisms of resistance by genetically abnormal hemoglobins and other red cell variants (4–8), and test some of the current hypotheses derived from clinical or culture studies under controlled conditions *in vivo*. In addition, these models allow the testing of the contribution of organs such as the spleen, in the pathogenic process. Finally, use of transgenic animals with overexpressed or knocked-out genes is potentially capable of assessing the impact of these genes on pathogenesis. On the negative side, transgenic mice represent models using a different host and different malarial strains to those involving human disease.

P. falciparum Malaria and Sickle Hemoglobin (HbS)

The sickle gene is found in sub-Saharan Africa, the Middle East, the Mediterranean countries, among the "tribals" of India, and in other places worldwide to which these populations have migrated (gene flow).

Early epidemiological studies suggested that the heterozygote carriers of the β^S gene could acquire *P. falciparum* malaria but had a reduced relative risk of dying of the infection (9, 10). The geographical distribution of the HbS gene is virtually identical to the area of the world in which malaria is (or has been) endemic (10). Full elucidation of the mechanisms of protection by HbS remains lacking. Luzzato *et al*. (11) reported that the rate of sickling of parasitized sickle cell trait red cells was 2–8 times greater than that of nonparasitized cells. They postulated that malaria induces sickling in circulating sickle trait cells that normally do not sickle, which leads to increased removal from the circulation, thus destroying the parasite in the process ("suicidal" infection).

Studies by Roth *et al*. (12) used cultured parasitized red cells (PRBCs). The authors compared sickling curves on non-PRBCs in culture with those of early and late PRBCs, all at physiological levels of N_2-induced deoxygenation. Accelerated morphological sickling occurred in sickle cell trait cells containing ring forms of the parasite, lending support to the Luzzatto hypothesis.

"Suicidal" infection is not the only mechanism involved in the innate resistance of sickle cell trait individuals to malaria. Friedman (13) demonstrated that sickle cell trait cells, cultured at 17% oxygen, sustained the growth of *P. falciparum* normally. After two days of normal growth in 17% oxygen, reduction of the oxygen content to 3% resulted in parasite death after a few days. The effect of deoxygenation was even more pronounced in HbSC disease and

sickle cell anemia (HbSS) erythrocytes. This finding strongly suggested that parasites within sickle cell trait cells, even if they have survived invasion during their ring form stage, will be seriously hampered during the deep vascular schizogony, a stage that causes stasis, and hence hypoxia and further lower pH, release of TNF-α, etc. In red cells containing sickle hemoglobin, these conditions will favor sickling and failure of the parasite to thrive.

The actual mechanism of parasite growth inhibition or death in sickle erythrocytes has been explored. Friedman *et al*. (14), studying sickle cell trait cells, proposed two possible and not mutually exclusive mechanisms: (a) the loss of K^+, which accompanies sickling itself, could be detrimental to parasite growth, as had been suggested for *P. lophurae*, an avian malaria parasite (15); (b) the loss of water concomitant with the loss of K^+ would progressively increase mean cell HbS concentration and HbS polymerization, since this reaction is affected by the 20th power of the initial HbS concentration. The decrease in sickling in sickle cell trait cells suspended in high K buffers (16), leading to inhibition of K^+ efflux and concomitant loss of water, does not distinguish between these two possibilities. Nevertheless, ouabain-treated red cells, enriched in intracellular Na^+ but low in K^+, do sustain the growth of parasites normally (17, 18), suggesting that cell water loss and an increased tendency for polymerization of deoxyHbS (and not the level of intracellular K^+) is the most likely mechanism of growth inhibition.

Other work has suggested that the sickle polymer is an inappropriate substrate for the proteases of the parasite (19). Ultrastructural studies (20) suggested that after 6 h of deoxygenation, parasitized sickle cell trait cells showed vacuolization that was interpreted as metabolic inhibition, and parasitized sickle cell anemia cells showed disruption of the parasitophorous vacuole and other membranes. This membrane disruption is hard to interpret, but could be caused by mechanical puncture by spearlike polymers, or a profound metabolic disruption breaking down the membrane repair mechanism. The polymer, with its considerably right-shifted oxygen equilibrium curve, might kill the parasite by oxygen toxicity, when it releases O_2 during polymerization. Significant decrease in the invasion rate has been observed under these deoxy conditions (21, 22).

In summary, culture methods have revealed that the carriers of the sickle cell trait are partially protected from death caused by *P. falciparum* malaria through at least two mechanisms: a dramatic increase in sickling rates of parasitized

sickle cell trait cells makes them prone to be removed from the circulation and is a first line of defense; parasites that have escaped this defense find themselves incapable of prospering at the stage in which their host cells adhere to the endothelium of venules. This analysis is not contradicted by the frequent death of patients with sickle cell anemia affected by malaria in endemic regions. *in vitro* data clearly indicate that the sickle cell anemia erythrocyte is an inhospitable host for plasmodia—worse than sickle cell trait cells. But patients can die of the infection. They might not die of cerebral or hepatic malaria, as do individuals with normal hemoglobin, but rather of the other serious complications of malaria to which they are particularly susceptible—dehydration, acidosis, cytokine release, etc.—many of which directly or indirectly lead to increased sickling.

We turn now to the use of the transgenic mouse model [reviewed recently by Nagel and Fabry (23)]. New data have emerged from infection by many rodent Plasmodia species and diverse strains of mice (24). They include *P. chabaudi adami*, which preferentially invades mature erythrocytes and causes a self-limited infection in laboratory mice. *Plasmodium berghei*, in contrast, tends to invade reticulocytes and usually causes a lethal infection in laboratory mice. *P. yoelii* 17XNL is related to *P. berghei* and invades reticulocytes but causes a nonlethal infection, although another strain of *P. yoelii*, 17XL, is lethal.

Studies of sickle transgenic mice *P. chabaudi adami*, *P. berghei* and the *yoelii* strains were used in nonlethal and lethal infections. In these studies, a transgenic mouse line expressing both the human β^S and α globin genes was used (25). While these mice did not have anemia, they had compensated hemolysis. Thus, they appeared to represent a model intermediate between the sickle cell trait and sickle cell anemia.

Sickle transgenic mice and the two parental mouse strains, C57BL/6J and FVB, infected with 10^6 *P. chabaudi adami*–infected erythrocytes (24), produced a significant reduction in time of patency (appearance of parasites) and level of parasitemia. *P. berghei*–infected erythrocytes were also significantly protected, but less than *P. chabaudi adami*.

These experiments demonstrated the protective effect of the sickle gene *in vivo* but not the mechanism involved (inability of parasites to grow in HbS-containing cells versus host removal of infected cells before the parasites have a chance to mature ("suicidal" infection)). Diminished parasitemia was consistent with either hypothesis. To investigate the possibility, mice were

splenectomized and inoculated, postsurgery, with *P. chabaudi adami*–infected erythrocytes. The most striking difference was between splenectomized and intact sickle transgenic mice. Intact sickle mice had a shorter course of infection, whereas in the splenectomized sickle mice, the protective effect of the sickle gene was completely abolished. This experiment also demonstrates that the sickle erythrocytes were fully able to support the growth of *P. chabaudi adami*, suggesting that the protective effect must involve the spleen.

Finally, Hood *et al*. (8) studied the effect of three transgenic models, expressing 39, 57 and 75% β^S to challenge with the virulent strain of *P. yoelli* 17XL, which is lethal and appears to cause cerebral malaria. Protection was in direct proportion to the level of expression of the β^S gene, demonstrating that innate resistance is particularly active when dealing with severe forms of malaria, paralleling the human situation.

In conclusion, the transgenic mice studies confirmed and extended previous epidemiological and *in vitro* studies, indicating that the sickle cell trait phenotype is protective against *P. falciparum* malaria. They also established that the spleen plays a significant role in protecting sickle transgenic mice from malaria, validating *in vivo* the Luzzatto hypothesis of "suicidal" infection.

Malaria and the HbC Trait and HbC Disease

The β^C gene, at high frequency, is restricted to West Africa. It has its maximum frequency in northern Burkina Faso (formerly Upper Volta) and the incidence decreases concentrically in surrounding areas.

While conclusive epidemiological evidence exists on the protective role against severe malaria of HbS heterozygosity, conflicting results for the HbC trait have been reported and no epidemiological data exist on the possible role of the CC genotype. Recently, a large case-control study performed in Burkina Faso on 4348 Mossi subjects found that HbC is associated with a significant 29% reduction in the risk of clinical malaria in HbAC heterozygotes and a striking 93% in CC homozygotes (26). The authors argued that the very mild phenotype of HbAC and even CC, compared to the strongly disadvantaged SS and SC genotypes and the low β^S gene frequency in the geographic epicenter of β^C, support the hypothesis that, in the long term and in the absence of malaria control, HbC would replace HbS in central West Africa.

The previous data are in agreement with the work of Agarwal *et al*. (27), who performed a case-control study in the Dogon of Bandiagara, Mali. The HbC trait was present in 68 of 391 uncomplicated malaria control cases, whereas it was detected in only 3 of 67 cases of severe malaria—a significant difference. Also, the HbC trait was present in only 1 of 34 cases with cerebral manifestations, the most common presentation of severe malaria in this population. Episodes of uncomplicated malaria and parasitemias were identified in cases of homozygous (CC), which indicates that *P. falciparum* parasites are able to grow within HbCC erythrocytes *in vivo*. This is not in conflict with the data of Olson and Nagel (16), which found a reduction in growth, not its abolishment. Thus HbC does not appear to protect against infection or uncomplicated malaria but can protect against severe malaria in the Dogon population of Bandiagara, Mali (27). This, of course, is not surprising and no different from HbS: these hemoglobins do not protect one from acquiring malaria but from dying of this disease. The authors suggested that the protective effect associated with HbC may be greater than that of HbS in this population, which is in accordance with the more recent data of Modiano *et al*. (26). The differences between these two studies could be due to the much smaller numbers in the Mali study, but also the fact that the population of the Dogon is divided into an endogamic noble class and two endogamic servant castes (tanners and blacksmiths). The polymorphic frequencies of β^C and β^S are geographically (valley vs plateau) as well as social-status-dependent (noble vs servant castes). The impact of this unique social structure on malaria innate resistance needs to be investigated.

Our understanding of the interaction between HbC red cells and malaria is less complete than that of the HbS interaction. Friedman *et al*. (28) were the first to describe severely decreased growth curves of *P. falciparum* in oxygenated CC cells. This finding was confirmed by Olson and Nagel (16) using a synchronized culture system. They also showed that deoxygenation does not significantly modify *P. falciparum* growth in HbC disease cells and, unlike the case with HbS-containing erythrocytes, normal development is not restored when HbC cells are suspended in a high extracellular K^+ buffer.

PRBCs from homozygotes for HbC were resistant to lysis compared to parasitized normal cells. In synchronized cultures, degenerated schizonts were observed on day 4 after initiation of culture (second cycle), an observation compatible with the incapacity of parasitized HbC-containing cells to complete their schizogony. It appears, therefore, that HbCC cells, due to their dramatically increased osmotic resistance, have a much reduced ability to rupture and

release merozoites in a normal fashion. This interpretation is also compatible with the characteristic rather flat growth curve of *P. falciparum* in HbC cells, in contrast with the down-sloping shape of the growth curves observed with other abnormal red cells.

There is no explanation why HbC trait cells, when studied *in vitro*, sustain the growth of the parasite as well as normal cells. This is problematical since the established concept is that the high frequency of HbC in certain populations is the product protection against malaria. One possibility is that the malaria culture system is not sensitive enough to detect the advantages of the HbC trait cells, or that the advantage of these cells pertains to a portion of the malaria cycle not tested by the culture system, such as endothelial adhesion, interaction with the spleen, or phagocytosis. Alternatively, the selective pressure might not be operating on the HbC carrier at all (see below).

Malaria and HbSC Disease

The interaction of red cells from patients with HbSC disease (double heterozygous for HbS and HbC) and the malaria parasite is interesting. While oxygenated SC cells are indistinguishable from normal cells as a host for plasmodia, in partially deoxygenated SC cells the parasite rapidly dies. In other words, SC cells are almost as inhospitable as sickle cell anemia cells (28).

While the mechanism for this observation is unknown, possibilities include the lowering of intraeythrocyte pH caused by parasite metabolism, inducing more polymerized deoxy HbS, which is a poor substrate for the parasite, because of the high MCHC caused by the presence of HbC. Increased crystallization of HbC induced by the presence of HbS (29) could introduce another potentially inadequate substrate for the parasite's proteases. This hypothesis will be possible to test *in vivo* since an HbC transgenic mouse has already been produced (30), and a transgenic SC mouse is feasible.

It has been proposed that selective pressure might operate mostly on HbSC rather than HbC trait individuals (28). This interpretation, if correct, will be a qualitative extension of the balanced polymorphism paradigm. Supporting this notion, HbC has been found to coexist with HbS in all geographical locations, although it can be segregated according to caste, as in the Dogon regions in Mali (31). It is possible that individuals with SC disease have a significantly higher fitness than normal individuals because of associated resistance to malaria, but

an even higher fitness than individuals with the HbC trait, as demonstrated by the intensity of growth inhibition of malarial parasites *in vitro*.

A model of this type as exemplified by HbS and HbC would explain the facilitation of introducing another advantageous gene into a population that already has one or more genes that provide malaria resistance: double heterozygotes might compound the mechanisms of resistance, rendering individuals fitter in a malarious region. Almost all populations exposed to endemic malaria exhibit more than one red cell defect that resists infection. Further work on the state of equilibrium, or the lack of it, of HbC gene frequency, and interrelationships of the HbA, HbS and HbC genes in Africa, will have to precede the acceptance of this hypothesis.

Malaria and HbE

HbE is the most frequent hemoglobin structural mutation in Southeast Asia and perhaps the world (32). The highest frequency of this gene is observed in the "HbE triangle," the area where the frontiers of Cambodia, Laos and Thailand meet. HbE can be found in other malarious regions, like Bangladesh, the state of Assam in India, Madagascar, South China, Indonesia and the Philippines. Early epidemiological studies suggested a connection between this variant hemoglobin and malaria (32). In the central region of Indochina, red cell genetic variants including HbE, α and β-thalassemia, and G-6-PD deficiency, are so frequent that Sicard *et al*. (33) calculated that only about 15% of the population have "normal" red cells.

The first *in vitro* study of the relationship between *P. falciparum* and HbE-containing cells demonstrated a moderate decrease in growth of *P. falciparum* in HbE disease cells, but normal growth in HbE trait cells (34). A subsequent study appeared to contradict this finding (35); however, the growth curves in HbE disease and HbE trait cells were not generated with the same innoculum in this study, and hence variability in the number of parasitized cells from one innoculum to another is so great that it may obscure any real difference. This discrepancy was resolved by a third study (36), which confirmed and extended the results of Nagel *et al*. (33). Diminished growth of parasites both in the HbE trait and HbE homozygous, particularly marked with the latter, was maximized at 20% oxygen but present and decreased at 5% oxygen (36). The antioxidant vitamin C partially inhibited the decrease of growth in the 20% oxygen cultures. The latter is interesting, since HbE is somewhat unstable and

capable of generating free radicals and inducing oxidative damage to the red cell membrane (37). Significantly higher levels of antimalarial antibodies and lower parasitemias were found in carriers of HbE compared to normal individuals from the same areas, an exciting observation that needs confirmation and explanation.

Parasitized HbE disease and HbE trait erythrocytes are phagocytized to a greater extent by normal human monocytes than infected erythrocytes from normal individuals (38). Late trophozoites and schizonts were particular targets, suggesting that the surface of parasitized HbE-containing red cells is modified differently from normal red cells, probably due to free radical damage.

In conclusion, culture-based evidence has demonstrated that HbE interferes with the growth of the malaria parasite, which can render carriers partially protected from infection. The mechanisms involved are not well understood but oxidative damage to the parasite induced by the instability of HbE is a strong possibility. If this is confirmed, increased antimalarial antibodies in these individuals might signal frequent but benign infections, conducive to early immunological resistance and survival through a mechanism of double protection.

Malaria HbF and the Thalassemias

Neonatal Red Cells and Red Cells Containing HbF

P. falciparum growth is reduced in all red cells containing HbF, suggesting that the effect is produced directly by HbF and not by the age or morphological differences that exist among red cells containing HbF. Included among these cells are cord blood cells containing predominantly HbF, red cells from infants (F cells, or cells that contain on average about 20% HbF) and red cells from adults homozygous for hereditary persistence of fetal hemoglobin (HPFH) characterized by red cells containing close to 100% HbF (39). These studies were performed in a medium relatively deficient in reduced glutathione and the HbF effect disappeared when parasites were grown in RPMI-1640 and at a pO_2 of 5% (28, 40). This result is consistent with the HbF effect being mediated by an increase in intraerythrocytic oxidative stress. Invasion of *P. falciparum* is increased at least in neonatal cells relative to adult cells, presumably because of their younger age (39).

Shear *et al*. (41) used transgenic mice expressing human $^{A}\gamma$ and $^{G}\gamma$ and α mouse chains (40–60% $\alpha_2^M\gamma_2$) to examine the effects of HbF on survival. Animals were infected with three types of rodent malaria: *P. chabaudi adami*, which causes a nonlethal infection mainly in mature red cells; *P. yoelii* 17XNL, which induces a nonlethal infection, invading primarily reticulocytes; and *P. yoelii* 17XL, a lethal variant of *P. yoelii* 17XNL that almost invariably causes death in 1–2 weeks. Data indicate that this last strain may cause a syndrome resembling cerebral malaria caused by *P. falciparum* in humans (7). In these transgenic mice infected with *P. chabaudi adami*, the parasitemia rose more quickly, in agreement with previous data (39), but was cleared more rapidly. Mice infected with *P. yoelii* 17XNL showed a clear reduction in parasitemia, and splenectomy prior to this infection did not alter this protection. While control mice died between 11 and 23 days, transgenic mice cleared the infection by day 22 and survived; similar results were seen in splenectomized animals. These studies suggest that HbF has a protective effect *in vivo* against malaria infection which is not mediated by the spleen.

Light microscopy revealed that intraerythrocytic parasites developed more slowly in HbF erythrocytes, and electron microscopy showed that hemozoin formation was defective in transgenic mice. Finally, digestion studies of HbF by human recombinant plasmepsin II demonstrated that HbF is digested only half as well as HbA (41). It appears that HbF provides protection from *P. falciparum* malaria, by the retardation of parasite growth. The mechanism involves resistance to digestion by malarial hemoglobinases based on the data presented and by the well-known properties of HbF as a superstable tetramer.

In conclusion, resistance of normal neonates to malaria can now be explained by a double mechanism: increased malaria invasion rates, reported in neonatal red cells (see above), will direct parasites to fetal cells, as well as F cells, and less to the 20% of HbA-containing red cells, amplifying the antimalarial effects of HbF. These findings do not entirely exclude the participation of oxidative stress in this phenomenon (see below). HbF is not particularly prone to be a source of free radicals because of its stability; in effect it is resistant to methemoglobin formation as well as heme loss.

Impaired growth of *P. falciparum* in HbF-containing cells is important because this effect may explain the decreased parasitemia observed in the first six months of life (39, 40) and might be at least a contributing factor to the advantages of of β-thalassemia, since carriers exhibit a significant retardation of the HbF-to-HbA switch. This period is critical in the resistance toward the

infection, since the humoral and cellular defenses are often not fully effective until the age of five years.

Malaria and β-Thalassemia

JBS Haldane (2) invoked the concept of balanced polymorphism to explain the high frequency of some hemoglobinopathies, including β-thalassemia. Early population studies have been summarized (42, 43), supporting the selective advantage of β-thalassemia compared to other red cell polymorphisms. A survey in northern Liberia found an increasing frequency of the β-thalassemia trait with increasing age, suggesting that carriers had increased survival (44). Using the criterion of parasite density with parasitemia greater than 1×10^9/L, indicative of the probability of lethal infection, a relative risk for lethal infection of 0.45 in 1–4-year-old children was calculated, demonstrating clearly the protective effect of β-thalassemia in malaria.

In early culture studies, parasites grew normally in β-thalassemia trait red cells, but parasites were more susceptible to oxidants than were parasites growing in normal cells (45–47). Yet, some have found differences in *in vitro* parasite growth in β-thalassemia red cells by changing the conditions of culture and varying the composition of the culture media (47, 48), particularly the use of MEM instead of the usual culture medium, RPMI 1640. MEM reduces the concentration of amino acids indispensable to parasites which they normally obtain by digesting hemoglobin. If these nutrients are supplied abundantly in the medium, the limitations encountered by the parasite in hypochromic red cells could be masked.

Anderson *et al*. (49) have studied 18 β-thalassemia families from the Ferrara region of Italy, where the incidence of an inherited low flavin mononucleotide (FMN)-dependent pyridoxine phosphate (PNP) oxidase activity—a sensitive indicator of red cell FMN deficiency—is higher in related members of these families than in the unrelated spouses, controls, and subjects without a family history of thalassemia. There was a markedly higher incidence of red cell flavin adenine dinucleotide deficiency in thalassemia heterozygotes than in their normal relatives, suggesting an additive effect of thalassemia on red cell flavin adenine dinucleotide deficiency that resulted from the inherited reduction of riboflavin metabolism. Diversion of flavin adenine dinucleotide to other flavin adenine dinucleotide enzymes might be an important factor. Any impact of this finding on malaria remains to be investigated.

β-thalassemia erythrocytes infected with *P. falciparum* have a reduced tendency to cytoadhere or to form rosettes (50). Reduced expression of parasite antigens is found on the surface of infected β-thalassemia red cells in the trophozoite or schizont stage using inhibitory pooled serum, but not on the ring forms. Noninhibitory pooled immune serum did not show a difference between parasitized thalassemic red cells and parasitized normal red cells. If confirmed, this finding will suggest another, qualitatively different mechanism of protection by β-thalassemia.

Studies of the role of oxidant stress in mediating the protection against malaria in β-thalassemia red cells showed red-cell-age-dependent sensitivity to oxidant stress in thalassemia and normal red cells (51). Parasite invasion and growth deteriorated with red cell age, particularly in cells with late parasite maturation arrest or abnormal morphology. While there was a negative correlation between parasite activity and oxidant stress in both normal and thalassemic red cells, the relationship was weak.

Transgenic mice have contributed to the study of β-thalassemia and malaria. C57BL/6J mice with a homozygous β-thalassemia syndrome (52), as well as transgenic thalassemic mice which expressed the human β^A gene (53), were infected with *P. chabaudi adami*, *P. chabaudi* (strain 1309) or *P. berghei*. Thalassemic mice infected with *P. chabaudi adami* had a delayed and diminished rise in parasitemia compared with both normal C57BL/6J and transgenic thalassemic mice which expressed the β^A transgene (4). A 48 h delay in both detection of parasites and occurrence of peak parasitemia was observed in the thalassemic mice. Importantly, the level of peak parasitemia in the thalassemic mice was approximately 40% that of the normal and the β^A transgenic mice, while normal and β^A transgenic mice were not significantly different from one another. When a more virulent strain of *P. chabaudi* was used, similar delays in patency and peak parasitemia were observed, but the levels of parasitemia were similar to controls (4). Thalassemic mice were not protected from *P. berghei*. While parasitemia in the β^A transgenic mice was similar to that in control mice, parasitemia was greater than in normal mice (4). Thalassemic mice may not be protected from *P. berghei* due to the high level of reticulocytes in these mice. A small but significant increase in parasitemia in the β^A transgenic mice may therefore be due to the slightly elevated reticulocyte count that exists in these animals.

In conclusion, studies indicate that the thalassemia phenotype protects mice from *P. chabaudi adami* and *P. chabaudi* (strain 1309) infection. Correction of the thalassemia phenotype by the β^A transgene abolished this protective effect. It

is unlikely that the protection seen with *P. chabaudi* infection is due to reticulocytosis, because the number of mature red cells available for parasite invasion far exceeded the number of infected red blood cells.

Thus, a definitive mechanism for the inhibition of malarial parasites in β-thalassemia erythrocytes remains elusive. Among the possible causes are limitation in essential amino acids present in a low MCHC red cell and increased susceptibility to oxidant stress (54), increased vulnerability to cell-mediated damage (phagocytosis) (55), and elevation and persistence of HbF in infancy and early childhood (42, 56) and associated low pyridoxine-phosphate oxidase activity in thalassemias (57).

Since an important player in this phenomenon may be oxidative stress, it deserves particular attention. Unfortunately, this is complicated and the following assertions need to be made: the malaria parasite produces oxidants that can potentially damage the red cell, the red cell hosting the parasite responds with increased resistance to oxidation, and thalassemic red cells are likely to be already partially damaged by the presence of the red cell defect. Data that support the previous list of events are the following:

(1) The malaria parasitized red cell is under substantial oxidative stress. Good evidence for this has been provided by Atamma and Ginsberg (58), who demonstrated that normal red cells and isolated parasites have high reduced gluthatione (GSH) over oxidized glutathione (GSSG), and the ratio GSH/GSSG amounts to about 300 in both cases, indicating substantial antioxidant defenses. In the parasitized red cell this ratio falls to 30, a 10-fold effect, indicating a sizeable oxidant challenge.

(2) The infected red cell mounts a defense against the oxidant onslaught, recognizing its danger, and increases its α-tocopherol content (59). The depletion of plasma α-tocopherol, retinol and carotinoids in children with *P. falciparum* (60), and the previous observation of this in rodent malaria, suggest that vitamin E is obtained from plasma by the parasitized red cell. In addition, the hexose monophosphate shunt (HMS), which produces NADPH for reductive antioxidant protection, is 78 times more active in infected than in normal red cells, and the parasite contributes 82% of this activity, and that of the host cell is increased about 24-fold (61).

(3) Thalassemic red cells are oxidatively damaged and their red cell membrane suffers oxidative alteration in addition to the binding of globin chains, mostly in the form of hemichromes (62, 63).

Hence, a unifying hypothesis of the mechanism of protection will have to involve, among other things, an oxidative stress-dependent pathway, of the following sort: the thalassemias, i.e. α- and β-thalassemias, HbE and Hb Constant Spring, protect the carrier from dying of malaria, because the oxidative stress of parasite invasion to the red cell is added to the intrinsic oxidative stress of the thalassemic red cell, reducing their viability. This allows the selective destruction and removal of thalassemic PRBCs. The details of the mechanisms involved, and the differences between the different thalassemias, will need to be worked out. Nevertheless, the solution of this problem might indicate potential avenues of therapeutic intervention in malaria.

In vivo models will allow studies of host factors in the protection from malaria. These could involve increased phagocytosis, because of either increased binding of IgG (64) or intrinsic alteration of the thalassemic erythrocyte (65). However, splenectomized thalassemic mice had a further delay in the rise of parasitemia (4), a paradoxical effect needing further study, and might involve a different organ origin of the red cells with different properties.

Malaria and α-Thalassemia

Based on the diagnosis of α-thalassemia by DNA analysis, it was established that the high frequency and distribution of this condition in Melanesia were a result of natural selection by malaria (66). α-thalassemia frequency, but not the frequency of other unlinked DNA polymorphisms, exhibited an altitude- and latitude-dependent distribution which correlated with the presence of malaria endemicity throughout Melanesia. Previous studies in which α-thalassemia was detected by hematological indices were much less sensitive and accurate.

In culture, there was a significant decrease in the parasite growth rate in HbH disease red cells (cells lacking three α-globin genes) or cells from Hb Constant Spring carriers, but normal growth was found in the red cells of persons who lacked only one or two α-globin genes (67, 68). Infected α-thalassemia red cells were also found to be more readily phagocytosed than infected normal red cells. These findings underscore the possibility that variant red cells may impair parasite survival by extrinsic mechanisms which are not detected in culture. For example, variant red cells could offer protection to the host by selective destruction of PRBCs in the spleen, an effect undetectable *in vitro*.

Of particular interest are the findings of Williams *et al*. (69), who studied the epidemiology of malaria in Vanuatu, in the northeast Pacific. In a holoendemic

region of these islands, they found that the incidence of uncomplicated malaria (*P. falciparum* and *vivax*) as well as splenomegaly (commonly associated with malaria infection) was increased rather than decreased, among children less than five years of age carrying the $-\alpha/-\alpha$ genotype. No genotype effect was found in children greater than five years of age. This apparent contradiction of increased susceptibility in children with α-thalassemia is resolved by the following hypothesis: α-thalassemia might increase susceptibility to *P. vivax* in young children, which in turn represents a form of vaccination against the potentially fatal *P. falciparum* by cross-reactive cellular and humoral immunity. This ground-breaking hypothesis needs to be examined in more detail but offers the possibility of a final picture in which there is a mixture of innate and acquired immunity, coparticipants in the protection against malaria.

Enzyme Deficiencies

Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency and Malaria

Many epidemiological studies have associated the carrier state for G6PD deficiency with innate resistance to malaria. In an informative clinical study of 702 Nigerian children aged 1–6 years suffering from malaria, the morbidity rates and parasitemia of patients with different G6PD status provided evidence that in heterozygous females the gene for G6PD deficiency (GdA–/GdB) confers an advantage against malaria (70).

The first study on *P. falciparum* cultured in G6PD-deficient red cells was conducted by Roth *et al*. (47). This study of erythrocytes from male hemizygotes and female heterozygotes from the island of Sardinia tested their ability to support growth *in vitro* of *P. falciparum*. Parasite growth was approximately one-third of normal in cells from both hemi- and heterozygotes for G6PD deficiency. The data support the hypothesis that G6PD deficiency may confer a selective advantage in a malarious area; the female heterozygote may be at a particular advantage because the resistance to malaria equals that of male hemizygotes, but the risk of fatal hemolysis is less. However, more female heterozygotes must be studied to confirm this hypothesis. The relationship between the thiol status of the human erythrocyte and the *in vitro* growth of *P. falciparum* in normal and in G6PD-deficient red cells was investigated by Miller *et al*. (71). Pretreatment with the thiol-oxidizing agent diamide led to inhibition of growth of the parasite in G6PD-deficient cells, but did not affect parasite

growth in normal cells. Diamide-treated normal erythrocytes quickly regenerated intracellular glutathione (GSH) and regained normal membrane thiol status, whereas G6PD-deficient cells did not. Parasite invasion and intracellular development were affected under conditions in which intracellular GSH was oxidized to glutathione disulfide and membrane intrachain and interchain disulfides were produced. Hence an altered thiol status in the G6PD-deficient erythrocytes could be one of the factors that underlie the selective advantage of G6PD deficiency in the presence of malaria.

P. falciparum–infected red cells possess at least two pathways for the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH): (1) the glucose-6-phosphate dehydrogenase (G6PD) pathway and (2) the glutamatedehydrogenase (GD) pathway using glutamate as a substrate. Uninfected erythrocytes lack the GD pathway. The NADPH generated can be used to reduce oxidized glutathione (GSSG), which accumulates in the presence of oxidative stress. In red cell G6PD deficiency, the first pathway is reduced or absent, and the host cells as well as the parasites within them are vulnerable to oxidant stress.

P. falciparum, during its intraerythrocytic development, produces its own G6PD, which has properties different from those of human G6PD. O'Brien *et al*. (73) isolated the G6PD gene from *P. falciparum*, which consisted of a single open reading frame without introns, coding for a protein of 910 amino acids, almost twice as long as any previously sequenced G6PD molecule. The mRNA is 5.1 kb in size and has an exceptionally long 5′ untranslated region of some 1000 nucleotides. The G6PD gene was mapped to chromosome 14 of the parasite. The C-terminal portion of the predicted protein has 39% homology with human G6PD, with a number of characteristic, fully conserved peptides. The N-terminal portion of the predicted protein has no homology with human G6PD, but contains a peptide in which 7 out of 12 amino acids are identical to the putative glutathione binding site of human glutathione S-transferase.

Luzzatto *et al*. (74) have previously shown that the parasite, when passaged serially through G6PD-deficient red cells, undergoes adaptive changes that gradually improve its ability to multiply in these deficient cells. To explain the above paradox, data demonstrate that this adaptive process is associated with and may consist in the induction of synthesis of a novel G6PD coded by *P. falciparum* that may serve its own metabolic needs. However, the host red cells, and hence the parasite itself, remain vulnerable to oxidant stress (75).

Because of variable X-chromosome inactivation, heterozygote females have different proportions of red cells with G6PD deficiency, depending which of the two X chromosomes (the normal or the variant) predominates. Roth *et al*. (76) investigated the effect of heterozygosity for G6PD deficiency on parasite growth. Blood obtained from 8 female Sardinian G6PD-deficient heterozygotes contained G6PD normal cells ranging from 13% to 60%. Blood from a G6PD-hemizygous male, containing 100% deficient red cells, was mixed in different proportions with compatible normal blood. In both experiments, parasite growth was inhibited by the presence of deficient cells. In both cases, it was found that the inhibition could be explained by a simple dilution of normal cells by G6PD-deficient cells. Thus, the typical female heterozygote is also protected to a significant extent. When one is considering the "malaria hypothesis" as it relates to G6PD, protection of the female heterozygote as well as the male hemizygote must be taken into account.

Roth *et al*. (77) studied the enzymes of the glycolytic pathway in normal red cells parasitized with *P. falciparum* in culture at varying parasitemias as well as in isolated parasites. The levels of all enzymes except diphosphoglycerate mutase, G6PD, and adenylate kinase were elevated. Extreme elevations of hexokinase, aldolase, enolase, pyruvate kinase, and adenosine deaminase concentrations were noted. In most cases, electrophoretically distinct bands of enzyme activity were also seen. These findings partly explain the previously noted 50- to 100-fold increase in glucose consumption of infected red cells.

G6PD-deficient erythrocytes are abnormally vulnerable to oxidative denaturation, which may include the release of ferriheme, a known cytolytic agent. Janney *et al*. (78) found significantly more ferriheme in G6PD-deficient erythrocyte membranes than in normal membranes. After incubation of erythrocytes with menadione, an oxidant drug, the values increased significantly more in G6PD-deficient membranes than in normal membrane, indicating increased hemoglobin denaturation. [^{14}C]-chloroquine binding to intact erythrocytes was measured to verify that hemoglobin denaturation in G6PD-deficient erythrocytes releases ferriheme in a form available to interact with ligands. Results indicated that ferriheme becomes available to interact with endogenous ligands and, thus, to mediate menadione-induced hemolysis in patients with G6PD deficiency. Furthermore, the increase in ferriheme may mediate the selective toxicity of menadione for *P. falciparum* parasites growing in G6PD-deficient erythrocytes. Ferriheme release in response to the intraerythrocytic oxidant stress

introduced by malaria parasites may also account for the resistance to malaria afforded by G6PD deficiency.

In view of the presence of the G6PD pathway in parasitized red cells and the description of a parasite-derived G6PD enzyme (79), the question is whether the pathways for the reduction of GSSG provided by the parasite can substitute for the host G6PD in red cells deficient in G6PD activity. The reduction rate of GSSG, monitored in the presence of buffered infected or control red cell lysates and substrates, is a sensitive test and shows that only parasitized red cells can reduce GSSG via the GD pathway. In parasitized G6PD-deficient Mediterranean red cells (completely G6PD-deficient), there is detectable GSSG reduction via the G6PD pathway, not found in uninfected lysates from the same individual. In G6PD A- (African type, featuring partial deficiency), a small increment in the G6PD-dependent reduction of GSSG can also be detected. However, when compared to G6PD normal red cells, the activities from the parasite-derived pathways are too small to substitute for normal host enzyme activity. Hence, while the plasmodium provides additional pathways for the generation of NADPH that may serve its own metabolic needs, the host red cells and hence the parasite itself remain vulnerable to oxidant stress (79).

In contrast with the findings of Roth *et al*. (76), Cappadoro *et al*. (80) found that, in using five different strains of *P. falciparum*, there was no significant difference in either invasion or maturation when the parasites were grown in either normal or Mediterranean G6PD-deficient red cells. They were also unable to detect differences in the amount of *P. falciparum*–specific G6PD mRNA in normal versus deficient parasitized red cells. Also, the rate of ^{14}C-CO2 production from D-[1-^{14}C] glucose, reflecting intracellular activity of G6PD, contributed by the parasite was very similar in normal and deficient red cells. By contrast, phagocytosis of PRBCs by human adherent monocytes of parasitized red cells at the ring-parasitized erythrocytes (RPEs) demonstrated a difference: G6PD-deficient RPEs were phagocytosed 2.3 times more than normal RPEs, while no difference was observed at the more mature trophozoite stage (TPEs). Phagocytic removal markers (autologous IgG and complement C3 fragments) were significantly higher in deficient RPEs than in normal RPEs, while they were very similar in normal and deficient TPEs. Reduced glutathione was much lower in deficient RPEs versus normal RPEs. The authors concluded that impaired antioxidant defense in deficient RPEs may be responsible for membrane damage followed by phagocytosis. Because RPEs, unlike TPEs, are

nontoxic to phagocytes, the increased removal by phagocytosis of RPEs would reduce maturation to TPEs and to schizonts, a potentially highly efficient mechanism of malaria resistance in deficient subjects.

The discrepancy between the Roth *et al*. (76) and Cappadoro *et al*. (80) results could be the consequence of the type of red cell sample used in each study. Roth's samples had been shipped from Sardinia and were two days old (as stated in the paper), while Cappadoro's were fresh. It is likely that Roth's samples suffered oxidative stress in transit, because of the G6PD deficiency, while the normal control did not. Nevertheless, the combined data can be interpreted now, to indicate that oxidative damage of G6PD red cells (by the parasite or external causes such as drugs, infection and fava beans) might reduce the growth of the parasite.

In summary, our knowledge of the interactions between malaria and G6PD deficiency has advanced considerably in the last two decades, but we do not have a definitive picture of the mechanisms of innate resistance involved in this system. There are data supporting the presence of three interrelated mechanisms. It is clear that G6PD-deficient red cells lack the capacity to resist sustained oxidative stress adequately and hence the free-radical-producing parasite is a challenge to such cells. It is also possible that this situation makes the red cell more susceptible to phagocytisis, as discussed above in the case of β-thalassemia. Also, oxidative stress induced by the parasite, plus the normal red cell oxidative stress (methemoglobin formation, etc.) partially unquenched by the enzyme deficiency, results in an environment in which normal parasite growth is limited. The individual weight of all these phenomena remains to be determined.

Red Cells with Cytoskeleton Abnormalities

Southeast Asian Ovalocytosis

Invasion of red cell by merozoites is complex process, involving several stages, including initial nonspecific attachment, reorientation of the polarity of the merozoite, red cell membrane flapping and the zipper-type introjection of the merozoite into the red cell (81). Given this complexity, it is not surprising that cytoskeletal proteins could be active participants in this process and that their mutants might generate red cell resistance to *P. falciparum*.

Melanesian or Southeast Asian ovalocytosis is the sole highly polymorphic red cell cytoskeleton abnormality. Present in about 30% of the Melanesian population of Papua New Guinea and other aboriginal populations of Southeast Asia, ovalocytosis is characterized by red cells with axial ratios smaller than that of elliptocytes (82, 83). Hemolysis is absent. Deformability of these cells is diminished, deformation by temperature (thermal deformation) of these cells is abnormal, and the expression of surface blood group antigens is altered (84–86).

Epidemiological evidence suggests that ovalocytosis confers resistance to high levels of parasitemia with *P. falciparum*, *vivax* and *malariae* (87). Ovalocytes are highly, but not absolutely, resistant to invasion by *P. falciparum* merozoites (88). These cells are also resistant to invasion by *P. knowlesi*, which binds a receptor distinct from that used by *P. falciparum* (89).

This observation is complicated by the fact that the presence of malaria infection might involve the generation of ovalocytes. In individuals infected with *P. falciparum*, $6.3 \pm 8.4\%$ of erythrocytes were ovalocytic while only $0.46 \pm 0.4\%$ ovalocytes were seen in controls. Ovalocytes contained significantly fewer parasites than discocytes. These findings could reflect a response of the malarious patient to malaria multiplication in their circulation (90).

In culture studies, hereditary ovalocytosis appeared protective to the red cell (91). Interestingly, higher ovalocythemia (75–100%) was found in malarious patients while their ovalocytosis parents, without malaria, had 25–50% ovalocythemia. This suggests that the nonovalocytes were being removed from the circulation preferentially. The invasion index was 1.52 ± 0.91 in ovalocytosis, while it was 4.45 ± 1.51 in normal individuals. Parents had an infection index of 1.81 ± 0.81, which also indicated significant protection. The same was true for the red cell deformability index. This finding has been interpreted to indicate that it is not the red cell shape but decreased deformability that limits parasite invasion.

In contrast to studies mentioned above, in Madang, Papua New Guinea, the prevalence of ovalocytosis did not differ between children with and without acute falciparum malaria or according to the α-globin gene haplotype (92). Two or more linear or irregularly shaped pale regions in the red cells had high sensitivity and specificity (over 94 and 99% respectively) for the morphological diagnosis of ovalocytosis. In contrast, the presence of ovalocytes had high specificity (100%) but low sensitivity (69%). In acute malaria, hemoglobin levels and red cells counts were lower in patients with ovalocytosis than in normal children.

Contrary to the findings of Bunyaratvej *et al*. (91), O'Donnel *et al*. (92) found that ovalocytosis patients had lower ovalocythemia, suggesting that a selective loss of ovalocytes might contribute to the anemia in the presence of malaria.

Some of the Southeast Asian ovalocytosis is due to heterozygosity for an eight-amino-acid deletion (400–408) in erythrocyte band 3 (93). This mutant protein is anomalously attached to the membrane cytoskeleton and is highly aggregated with ankrin, forming stacks containing bands of intermembrane particles (IMPs), which are resistant to alkaline pH and trypsin. Affected cells have a marked decrease in membrane rotational mobility, contributing to their increased rigidity. In effect, these membranes are incapable of cytoskeletal extension, which is indispensable for deformation. Why the merozoite is unable to invade the ovalocytic cell is unknown.

Naturally occurring anti-band-3 autoantibodies bind to erythrocytes infected with the FCR-3 "knobby" variant of *P. falciparum* (94). (Knobby variants are those parasites which demonstrate the presence of knobby excresences on the surface of mature parasite-infected red cells when viewed by electron microscopy.) These autoantibodies recognized a larger-than-240 kD protein in extracts made from surface-iodinated infected erythrocytes. The antigen was present only in erythrocytes infected with a knobby variant, and was removed by trypsin treatment of intact infected cells. Two-dimensional peptide map analysis demonstrated that the antigen was structurally related to band 3. These observations raise the question of whether band 3 is modified by the infection: is this a subclass of band 3, and are there genetic mutants of band 3 that confer innate immunity to malaria? *P. falciparum* invasion and growth in the band 3 variant Memphis (56 Lys → Glu) (95) was normal (96).

Hereditary Elliptocytosis

Another cytoskeletal defect that has been found to resist malaria invasion by both *P. knowlesi* and *P. falciparum* is elliptocytosis, due to either the absence of glycophorin C or band 4.1 (97). These erythrocytes have cytoskeletal abnormalities with reduced interactions that stabilize the anchoring of the cytoskeleton core to the protein imbedded in the bilipid layer and also exhibit alterations in deformability. That invasion is inhibited for two types of plasmodia suggests that this defect alters a later stage of invasion than receptor recognition.

Hereditary elliptocytosis due to protein 4.1 deficiency and structural variants that increase the content of spectrin dimers also exhibit parasite abnormal

growth (98), a pattern distinct from that of hereditary spherocytosis (see below).

Hereditary poikilocytosis due to the αI/74 mutant spectrin was associated with significantly reduced parasitemia. Hereditary elliptocytosis due to the αI/65 variant spectrin also had reduced invasion. Decreased invasion correlated with the percentage of spectrin dimers present within the membrane of variant cells. In contrast, in a partial deficiency of protein 4.1 (HE/4.1+) that had a normal percentage of spectrin dimers, invasion was unchanged or increased (99). These data suggest that the αI domain variants might interfere with the merozoite–receptor interaction at the surface of the red cell and/or the mechanism of endocytosis. Membrane proteins may become modified during intracellular growth of *P. falciparum*. An 80 kD phosphoprotein associated with the membrane in normal red cells may be a phosophorylated form of 4.1 forming a complex with the MPI antigen of the parasitized red cell or be generated by the intracellular parasite. Erythrocytes from a homozygote for 4.1(−) hereditary elliptocytosis, whose red cells were completely devoid of protein 4.1, also lacked the 80 kD phosphoprotein, demonstrating that this protein is indeed a phosphorylated form of protein 4.1(100). Whether this protein alters the red cell membrane and affects parasite growth and survival remains to be determined. A similar mechanism could operate in other mutations of protein 4.1.

Homozygotes for hereditary elliptocytosis, with an absence of protein 4.1 and the Leach phenotype due to glycophorin C absence, were similarly resistant to parasite invasion, but only the protein 4.1 abnormality had reduction in parasite growth (Christi, 1996; 101). Since the p55 protein is deficient in both of these abnormal red cells, this protein could be involved in the invasion abnormality but not the parasite growth defect.

Hereditary Spherocytosis

Hereditary spherocytosis has not been reported at polymorphic frequencies in any region of the world. Nevertheless, since the underlying molecular defect leads to a cytoskeleton protein abnormality, it is of great interest to investigate the fate of the *P. falciparum* malaria parasites in these cells and to explore the possibility that red cells membrane proteins are utilized by the parasite, beyond the initial encounter during invasion.

Parasite growth was decreased in direct proportion to the extent of spectrin deficiency in hereditary spherocytosis (Schulman, 1990). This abnormality was

unique and characterized by normal growth during the first two-days, followed by abnormal growth with a delay proportional to the decrease of spectrin. In hereditary spherocytes that exhibited 46% decrease in spectrin, abnormal growth was immediate after day 2. Hereditary spherocytosis cells with 23–32% decrease in spectrin had apparent abnormal growth, beginning at day 3 or 4. Hereditary spherocytosis cells lacking spectrin deficiency had normal growth. Preincubation of hereditary spherocytosis cells in a culture medium had no effect on the results. It seems unlikely that hereditary spherocytosis itself is responsible for the abnormal growth. Abnormal invasion was excluded as a cause of the growth abnormalities observed. Ankyrin/spectrin-deficient mice showed a dramatic decrease in invasion by *P. berghei* and *P. chabaudi* (5). These experiments suggest that skeletal membrane proteins may play a role in both invasion and growth, according to the defect present, and raise questions regarding erythrocyte innate resistance. These include the mechanisms of the growth impairment and delay, and the role of host cytoskeleton proteins in the construction of the parasite's own set of membranes, e.g. parasitosphorous vacuolar membrane.

Studies of hereditary spherocytosis and elliptocytosis suggest that, in the absence of invasion defects, spectrin and other interacting proteins, either modified or in the native form, play important roles as constitutive elements of the parasite membranes. Alternatively, the host cytoskeletal proteins may be involved in the events that follow invasion—generation of the parasitophorous vacuolar membrane, without direct incorporation into the parasite membranes. Finally, cytoskeleton or membrane proteins are known to be critical in the insertion of parasite proteins into the red cell surface (4.1 and PfEMP1) (102), and mutations in these proteins could interfere with this adaptive process, as was suspected earlier by Schulman *et al*. (98). Although the lack of polymorphic frequencies of the cytoskeleton abnormalities does not support a selective advantage, Delauney (personal communciation) has reported that a variant of Band 3 (Band 3 Montefiore) might exist in frequencies higher than 1% in Africa, suggesting a polymorphic frequency.

Mice with abnormalities of erythrocyte membrane spectrin have been utilized to further characterize the roles of spectrin and ankyrin in malaria infection. Homozygous mice expressing the nb mutation synthesize normal amounts of spectrin, but no ankyrin. Because of the ankyrin deficiency, only 50% of the normal amount of spectrin is bound to the erythrocyte membrane (103). Homozygous WBB6F_1 (nb/nb), heterozygous (nb/+) and homozygous

(+/+) mice were infected with *P. chabaudi*–parasitized erythrocytes. Controls (+/+) displayed a course of nonlethal parasitemia which was patent on day 3, peaked on day 10, and was cleared by day 14 after infection. Heterozygous animals had a lower peak of parasitemia, which cleared by day 17. Strikingly, nb/nb mice did not display a patent parasitemia. The nb/nb mice were also refractory to *P. berghei* (5). In contrast, nb/+, +/+ and parental C57BL/6J mice all succumbed to this lethal parasite. Parasites can invade erythrocytes of nb/nb mice. However, there was no development of the parasites within their erythrocytes (5). Anemic W/W^v mice were completely susceptible to *P. berghei*. Mice with the sph/spherocytosis mutation synthesize ankyrin but do not synthesize the K-chain of spectrin (104). Mice carrying the sph/sph mutation synthesize only 20% of the β-chain of spectrin and are not susceptible to infection with *P. chabaudi adami* or *P. berghei*.

Blood Group Antigen Polymorphisms and Malaria

Duffy Blood Group and *P. vivax* Malaria

P. vivax is the most prevalent malaria parasite worldwide. While mortality is low, the relapsing behavior and the impact that morbidity has on economic activities are significant. Recent resistance to antimalarials has contributed to making its control difficult.

Serology, Protein Chemistry and Ethnic Distribution

The Duffy blood group consists of three genotypes identified initially in Caucasians: Fy(a+b), Fy(a−b+) and Fy(a+b+) (107). In contrast, West Africans and 68% of African Americans do not express Fya or Fyb, and the absence of both corresponds to the Duffy negative phenotype, denoted as Fy(a−b−). In individuals of the Fy(a−b−) phenotype, this sequence remains silent in erythroid cells but is transcribed and expressed on endothelial cells of postcapillary venules. Fy is produced in endothelial cells of capillary and postcapillary venules, epithelial cells of kidney collecting ducts, lung alveoli and Purkinje cells of the cerebellum. The Duffy protein plays a role in inflammation and in malaria infection. The protein is a member of the superfamily of chemokine receptors and is the receptor to which certain malarial parasites, notably *P. vivax*, bind to invade red blood cells. The parasite-specific binding site, the binding site

of chemokines, and the major antigenic domains are located in overlapping regions at the exocellular N terminus of the Duffy protein (108).

In Papua New Guinea, in a *P. vivax*–endemic region where the resident Abelam-speaking population is characterized by an extremely high frequency of α-thalassemia, they also exhibit the mutation responsible for Fy[a−b−]. Emergence of the FY*A (null) genotype in this population suggests that *P. vivax* is involved in selection of this erythroid polymorphism, and might potentially compromise α-thalassemia/*P. vivax*–mediated protection against severe falciparum malaria (109).

The Duffy blood group has been studied in Thai and Indonesian populations (110), revealing the presence of Fya antigens showing weak reactivity to anti-Duffy antisera, as well as a discrepancy between the genotype shown by the PCR-RFLP study and that predicted by the phenotype. The PCR-RFLP study also suggested the presence of an alternative genetic basis for the Fy(a−b−) phenotype, which differs from the African type.

Innate Resistance

Red cells from the Duffy-negative phenotype are resistant to invasion by merozoites of *P. knowlesi*. This mechanism of innate resistance is highly effective, as demonstrated by the near-lack of *P. vivax* in the areas with high prevalence of Fy(a−b−). Anti-Fya and anti-Fyb specifically inhibited invasion of *P. knowlesi* merozoites into human red cells of the Fy(a+b−) and Fy(a−b+) phenotype. Miller *et al*. were the first to propose that the innate resistance of some African Americans to *P. vivax* malaria might be related to Fy(a−b−). Fy(a−b−) erythrocytes cannot be invaded by *P. vivax in vitro* (111). An anti-Fy6 monoclonal antibody inhibits the invasion of Duffy-positive erythrocytes by *P. vivax*.

P. vivax prefers reticulocytes for invasion, although these cells correspond to less than 2% of the red cell population (112). *P. vivax* merozoite surface protein-1 (Pv−MSP−1) is believed to have an important role in attachment and invasion of merozoites to red cells.

Duffy and Var Genes and Adhesion

Serendipitously, defining Duffy binding ligands provided a clue to the identification of genes encoding variant endothelial cytoadherence proteins of *P. falciparum*. Parasite chromosomes contain gene sequences that are similar

to region II of the erythrocyte binding proteins of *P. knowlesi*, *P. vivax* and *P. falciparum* (113, 114). This family of related sequences, the ≈80+ variant (Var) genes, contain multiple domains homologous to Duffy binding proteins, which had been shown to have cytoadhesive properties. One of them, PfEMP1, a very important member of the adhesion protein family in *P. falciparum*, is a member of the Var gene family and part of the parasite strategy to diminish the number of individual exposed antigen sites so as to inhibit the immune reaction of the host.

Fusion proteins containing Duffy-binding-like alpha (DBL-alpha) domains of PfEMP1 have been used to detect antibody activity in the plasma of patients exposed to *P. falciparum*. A strong correlation between the immune status of the patients and reactivity with recombinant proteins was observed, which was interpreted as a reflection of the number of infections acquired over time (115). This finding opens the door for potential inmunization with these types of fusion proteins.

Complement Receptor 1 Knops Blood Group, ABO Blood Groups and Malaria

In a study in Gabon, Lell *et al*. (116) found that whereas the sickle cell trait (AS) was significantly associated with mild malaria, blood group A was associated with severe malaria. Hence, the severity of malaria is partly determined by the presence of blood group A and the sickle cell trait.

The ability to bind to multiple receptors, as well as the ability to form rosettes and giant rosettes, is significantly more frequent among isolates from children with severe versus mild malaria. Rosettes and giant rosettes are significantly more frequent for children with severe malaria, and the cell aggregates are larger and tighter than for those with mild disease. Binding of immunoglobulins and of heparin to infected erythrocytes was very common, and binding to heparin and blood group A was associated with severity of disease. These results support the idea that isolates that bind to multiple receptors are involved in the causation of severe malaria and that several receptor–ligand interactions work synergistically in bringing about severe disease (117).

Complement receptor 1 (CR1) is a ligand for the rosetting of *P. falciparum*–infected red cells with uninfected cells. Moulds *et al*. (118) studied three known polymorphisms of CR1, in European Americans, and African

Americans and Malians, to determine if genetic differences existed in an area endemic for malaria that could involve innate resistance. The Knops blood groups are located on CR1. The frequencies of Knops blood group phenotypes McC(b+) and Sl(a−) were greatly increased in Africans compared to Europeans. McC(b+) frequency was similar in African Americans and Malians. In contrast, the Sl(a−) phenotype was significantly higher in Malians (39% vs 65%, respectively). There was an increased frequency of the largest size (250 kD) of CR1 in individuals from Mali, but this did not differ significantly from African Americans in the USA. Both cohorts of Africans had higher expression of red cell CR1 than European Americans, but there was little difference between the African Americans and the Malians. Thus, the most important CR1 polymorphism relevant to rosetting of malaria-infected cells appears to be the Knops blood group (12).

Rosetting of cells containing mature trophozoites and schizonts is believed to be an important factor in the development of cerebral malaria. The ability of red cells from healthy donors to form rosettes in culture has been found to be significantly greater in the cells of groups A and B than in those of group O. Interestingly, quinine, artesunate or artemether *in vitro* reduced rosetting significantly, but mefloquine and pyrimethamine had no effect. Uninfected red cells from patients with falciparum malaria exhibited a lower rosetting ability than red cells from healthy donors. The rosetting of uninfected red cells of all blood groups from patients with uncomplicated/severe malaria decreased significantly within 2 h of the patients of treatment with artesunate or artemether, but the mechanism is unkown (119).

Glycophorin Deficiency

Glycophorin A is the major transmembrane sialoglycoprotein of red blood cells and contributes to the expression of the MN and Wright (Wr B) blood group antigens. Glycophorins play an important role in the invasion of human red cells by *P. falciparum*. En(a−) cells deficient in glycophorin A and S−s−U− cells deficient in glycophorin B are partially resistant to invasion, while trypsin treatment of S−s−U− cells (which removes most of the remaining sialoglycoprotein) are almost totally resistant to invasion. Once parasites enter these glycophorin-deficient cells, they develop normally. Furthermore, the observed increased invasion of young red cells compared to older red cells is not related quantitatively to the presence of glycophorins on the cell surface. Hence, while

Table 1. Points of the parasite life cycle that are affected by RBC genetic variants that may lead to protection.

Life cycle	Method of protection	Variants
Invasion	Reduction or inability to invade	Blood group antigens Duffy and vivax malaria Glycophorin B deficiency SEA ovalocytosis
Growth retardation	Variant Hb poor substrate for digestion	HbS, HbF
	RBC size	Thalassemias IDA
	Oxidative damage	Thalassemias G6PD HbF
	Mechanical damage	HbS
Schizogony	Reduced ability to rupture during schizogony	HbC

glycophorins are both specific and important in the invasion of cells by *P. falciparum*, it is only part of a complex process (120).

There is interaction between glycophorin A and band 3, another transmembrane protein in the red cell. In transgenic mice where the band 3 expression was knocked out (band 3 $-/-$), red cells contained protein 4.1, adducin, dematin, p55 and glycophorin C, but not glycophorin A as detected by Western blot and immunocytochemistry techniques. Nevertheless, PCR detected the presence of glycophorin A mRNA. Pulse label and pulse chase experiments show that glycophorin A is not inserted in the membrane and is degraded in the cytoplasm. These findings suggest that band 3 plays a chaperone-like role, which is necessary for the insertion of glycophorin A (GPA) (121).

The extent of participation and changes of glycophorins (GPs) and membrane proteins in the merozite–human erythrocyte interaction during the invasion of *P. falciparum* have been studied by immunoblotting techniques. Polyclonal antisera to glycophorin A and to its C-terminal fragment (residues 82–131), as well as M and N specific monoclonal antibodies, were used. The MN blood group system resides in GPA. In addition to the normal glycophorin pattern, a band with a molecular weight of 77.5 K (band 1′) was found in the

Table 2. Protective effects against malaria of RBC genetic variants.

Complication	Pathology	Stage in life cycle	Method of protection	Variants
CM ARF ARDS Hepatitis (Anemia)	Small vessel blockage Ischemia Local cytokine release	Rosetting Sequestration and adhesion	Poor rosetting Poor cytoadhesion	Thalassemias IDA HbS Blood group "O" HbC HbE SEA ovalocytosis
CM ARDS SMA Hepatitis	Cytokine release	Schizogony	Immunity: Antitoxic Antischizont Impaired rupture	HbC
Hyper-parasitemia Anemia	Multiplication	Reinvasion	Immunity: Antimerozoite	Thalassemias HbE

parasitized erythrocytes and their saponin pellets. Moreover, several GP bands ranging from 28 K to 54 K were present in saponin pellets when probed with anti-GP and anti-peptide-C sera. This reflected the disintegration of erythrocyte membrane GPA through the invasion of *P. falciparum*. GPA in the saponin pellets of the parasitized MM erythrocytes surprisingly cross-reacted with the N MoAb, suggesting that they may have come from the intermingling of the host GPA in MM erythrocytes. The saponin pellets of parasitized erythrocytes preserved a considerable amount of ankyrin, band 3, protein 4.1 and 4.2, while the GP bands were mixed with the parasite proteins and the disintegrated products of membrane proteins (122). This finding opens up the possibility that mutation in the host MN blood group system could affect parasite invasion.

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Chapter 10

The Spleen and Malaria

Imelda Bates

Introduction

The spleen plays a pivotal role in the pathophysiology of malaria. One of its major functions is to remove infected and damaged cells from the circulation, and it can therefore exert a direct effect on the ability of the malaria parasite to multiply and cause severe disease.

Splenomegaly in Malaria

Incidence of Splenomegaly in Malarious Areas

Palpable enlargement of the spleen is present in 50–80% of residents in malarious areas (1). Both the "spleen rate" (i.e. the prevalence of splenomegaly) and the "parasite rate" (i.e. the prevalence of parasitemia) are useful indicators of the intensity of malaria transmission in a community. In areas of stable malaria, where intense transmission occurs for at least 6 months of the year, children gain substantial immunity to malaria by the age of 5–6 years, but there is a significant mortality in younger children. Spleen rates of 75% and parasite rates of over 90% are typical in children aged 2–6 years in areas with stable malaria. Adults in the same area have much lower spleen rates but have a high parasite rate with low levels of parasitemia, reflecting their development of both antiparasitic and antitoxic immunity. In areas of unstable malaria the period of disease transmission is short and may fluctuate widely from year to year. Individuals do not have the opportunity to develop immunity to malaria, so when outbreaks occur all ages are equally affected. As the prevalence of

malaria rises, both the spleen rate and the average spleen size in the population increase (2).

In areas that are endemic for malaria, women tend to have higher spleen rates than men (3), possibly as a result of the relative immune suppression and their increased attractiveness to mosquitoes during pregnancy (4). Genetic factors also influence the spleen rates in a population. In coastal Madang province in Papua New Guinea, two genetically distinct groups of women with the same malaria exposure were shown to have significantly different mean spleen rates. Women with highest spleen rates also had larger and more persistent splenomegaly. This is likely to reflect genetic differences in their immune responses to malaria (5).

Incidence of Splenomegaly in Acute Malaria

Malaria infection is always associated with a degree of splenomegaly (2, 6–8), but in order for the spleen to be palpable on abdominal examination, it must be enlarged to 2–3 times its normal size. 70–80% of individuals with acute malaria have palpable splenomegaly, often accompanied by hepatomegaly (9).

The spleen begins to enlarge within 3–4 days of the onset of symptoms of acute malaria and regresses over several days or weeks after the start of treatment (1). It is clinically useful to monitor the spleen size during the course of malaria infection, because if the spleen fails to regress as expected after treatment, there is a greater likelihood of relapse (10).

Hackett's Classification of Splenomegaly

For epidemiological studies it is useful to document not only the spleen rate, but also the magnitude of splenic enlargement in a community. The "average enlarged spleen" is a useful malariometric index (8), which is calculated from the frequency distribution of five classes of enlarged spleens. These spleen classes were first described by Hackett (11) and range from "not palpable on deep inspiration" (class 1) to "lowest point palpable in right iliac fossa" (class 5) (see Table 1). Although this classification is useful for cross-sectional population surveys, it is not helpful when the spleen size of an individual patient needs to be monitored prospectively to establish whether it is enlarging or regressing.

Table 1. Classification of splenic size according to Hackett (8).

Class of spleen	Description
0	Normal spleen not palpable on deep inspiration.
1	Spleen palpable below the costal margin on deep inspiration.
2	Spleen palpable below the costal margin, but not below the horizontal line halfway between the costal margin and the umbilicus, measured from a vertical line from the left nipple.
3	Spleen with the lowest projecting margin below halfway between the costal margin and the umbilicus but not below.
4	Spleen with the lowest margin below the umbilicus but not below a horizontal line halfway between the umbilicus and the symphysis pubis.
5	Spleen with the margin below that of class 4.

Measurement of Splenic Enlargement

Bedside Measurement with a Tape Measure

The length of an enlarged spleen can be simply measured using a flexible tape measure. If the method is carefully standardized, it can reliably be used to monitor changes in spleen size. Care should be taken to measure from the junction of the anterior axillary line and the left costal margin to the tip of the spleen. Grossly enlarged spleens expand sideways as well as downwards, so it is important to follow the contour of the spleen with the tape measure (Fig. 1). This is a particular problem in the later stages of pregnancy when the uterus displaces the spleen into the left flank (12).

Splenic Ultrasound

In adults the spleen length measured by ultrasound is a reasonable representation of splenic size for routine use in individual patients and correlates well with spleen size measured by single photon emission computerized tomography (13). However, in adults the spleen length decreases with age and increases with rising body weight. This needs to be taken into account when comparing splenomegaly between individuals (13). Ultrasound can also be used to calculate the volume of the spleen from measurements of the areas of

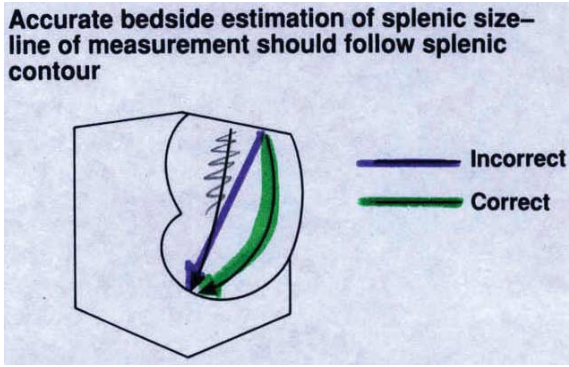

Figure 1 Measurement of an enlarged spleen using a tape measure. (From: Bates I (1991), "Hyper-reactive malarial splenomegaly in pregnancy," *Tropical Doctor* **21**: 101. With permission from the publishers.)

parallel serial sections (14). A formula for the "splenic volumetric index" has been devised which enables splenic volume to be calculated from ultrasound measurements of spleen breadth, thickness and length. This method correlates well with single photon emission computerized tomography measurement of spleen volume (15).

Other Methods

Splenic area and splenic volume can be measured using ^{99}mTc-labeled tin colloid isotopic studies (16) and single photon emission computerized tomography (17), respectively. Both are useful techniques for demonstrating nonpalpable splenomegaly.

Histology of the Spleen in Malaria

In acute malaria the spleen becomes hyperemic and congested, making it appear more red than usual. Expansion of the splenic tissue stretches the splenic capsule, which becomes thin and friable (1). Microscopy of histological sections reveals that the splenic enlargement is mostly due to massive expansion of macrophages and reticular cells, the stromal cells which form the splenic filtration beds. There is phagocytosis of malaria pigment and red cells, and marked infiltration with both parasitized and nonparasitized red

cells. Scattered thrombi with focal necrosis can lead to infarction and make the spleen more susceptible to hemorrhage and tears.

In contrast to the soft texture and hyperemic microscopic appearance of the spleen in acute malaria, when the spleen is chronically enlarged, as in hyperreactive malarial splenomegaly, it appears dark gray and firm (2, 8, 18, 19). The capsule is tough and thickened, and scars from old infarcts may be visible (20). Microscopically, connective tissue is increased and this may be associated with a degree of fibrosis (8, 18, 21). The splenic sinusoids are dilated and infiltrated with an expanded population of lymphocytes and occasional lymphoid aggregates (20, 22–24). In one study the lymphocytic infiltration was severe enough to be compatible with a neoplastic lymphoproliferative disorder (23).

Splenic Complications of Malaria

Rupture of the spleen

Worldwide, spontaneous splenic rupture is most commonly associated with malaria (25, 26), but rupture is also a recognized complication of infectious mononucleosis, splenic congestion and leukemia (27). Figures on the true incidence of splenic rupture in naturally acquired acute malaria infections are difficult to obtain because of underdiagnosis. Estimates suggest that rupture occurs in less than 2% of cases of acute malaria (1). It is almost exclusively confined to individuals with limited or no prior exposure to malaria and has been reported in acute congenital malaria (28). Subcapsular hematomata, which may not necessarily be associated with complete splenic rupture (25), can occur after minor trauma such as the increased intra-abdominal pressure associated with coughing or vomiting. Splenic rupture seems to be particularly associated with *Plasmodium vivax*, perhaps because this parasite causes more pronounced splenomegaly than other malaria species (2).

Many of the older reports of splenic rupture in acute malaria were based on outcomes in patients in whom malaria infection had been induced therapeutically as treatment for syphilis. There are several factors which make spleens which have enlarged as a result of induced malaria, rather than naturally acquired infections, more likely to rupture. Observations from early studies therefore cannot necessarily be extrapolated to natural malaria infections. The syphilis patients with iatrogenic malaria tended to be older than those with naturally acquired infections and were therefore more likely to have

underlying splenic pathology. Antimalarial treatment was withheld for several days to allow the parasitemia to have the desired effect on the spirochetes and, because they were not living in an area where malaria was transmitted, the syphilis patients had very little chance to develop any malarial immunity (27).

In chronic splenomegaly the spleen is much tougher than in acute malaria and it is doubtful whether these spleens, although they may be massively enlarged, are any more likely to rupture than normal spleens (27, 29). Even when splenic rupture does occur it is essential to attempt to preserve some splenic tissue because of the well-known risks of overwhelming infection with encapsulated organisms in patients without a functioning spleen. The Papua New Guinea Splenic Injury Study Group managed to treat over 50% of ruptured spleens in adults conservatively and to preserve splenic tissue in a proportion of those who required laparotomy (30). Even in children with ruptured, enlarged spleens, repair or partial resection, rather than total splenectomy, is feasible (31).

Hypersplenism

Only a minority of patients with splenic enlargement have the syndrome of hypersplenism. This is characterized by splenic enlargement and reduction in any, or all, of the cellular components of blood—red cells, leucocytes and platelets.

Anemia is an inevitable consequence of malaria infection. The mechanisms causing anemia are complex and include an increased breakdown and dysfunctional production of red cells (32). Chronic splenomegaly adds yet further complexity to the etiology of malaria-associated anemia. Both intact and damaged red cells are phagocytosed by the expanded macrophage pool in the spleen (33) and up to 40% of the total red cell mass may be sequestered from the circulation in grossly enlarged spleens (29). Both these mechanisms stimulate a vigorous erythropoietic marrow response which is partially able to compensate for the increased red cell destruction (34). Plasma volume expansion and consequent dilution of the circulating cells is the major factor in producing anemia in hypersplenism. Hemodilution occurs in splenomegaly irrespective of the underlying cause (34) and is proportional to the size of the spleen (35). In hyperreactive malarial splenomegaly the dilutional anemia is compounded by the oncotic effect of markedly increased amounts of circulating polyclonal immunoglobulins. The overproduction of immunoglobulins has been estimated to account for up to 70% of the total plasma volume increase (36).

The thrombocytopenia in hypersplenism is due to destruction of antibody-coated platelets exhibiting malaria antigens on their surface (7, 37, 38) and to sequestration within the substance of the spleen. A grossly enlarged spleen may contain up to 90% of the total body platelet mass. The leucopenia of hypersplenism is predominantly due to an absolute reduction in total circulating neutrophils. This is thought to be the result of increased margination within the splenic vasculature. In hypersplenism, anemia and thrombocytopenia, but not usually neutropenia, may be severe enough to cause symptoms, and to contribute significantly to morbidity (39). Splenic sequestration of T lymphocytes has also been described in Gambian children with acute malaria (40).

The hematological consequences of hypersplenism secondary to malaria are not necessarily the same as those in which the underlying cause is not malaria. In portal hypertension the spleen size shows a negative correlation with the white cell count but not with the hemoglobin or platelet count (41), whereas in Gaucher disease the exact opposite appears to be true (42).

Other Splenic Complications of Malaria

Splenic Cysts

Cysts are an unusual complication of malaria and are generally the result of hematoma formation following splenic infarction or congestion. Occasionally they may be consequent on conservative management of traumatic splenic rupture (30).

Ectopic Splenomegaly

Ectopic spleens that become chronically enlarged, for example due to hyperreactive malarial splenomegaly, may cause atypical abdominal or pelvic pain. In ectopic splenomegaly there is a significant risk of splenic torsion with subsequent infarction and rupture (43), so this condition warrants management by splenectomy.

Reticuloendothelial Function in Malaria

The spleen has a pivotal role in the host defence system against malaria by clearing micro-organisms and opsonized or damaged cells. It is also an

important site of antibody synthesis, particularly against soluble antigens, and a reservoir of exchangeable platelets (30% of platelets are normally held in the spleen). At rest the spleen receives 4–5% of the cardiac output (44) and the whole of the blood volume circulates through the spleen in about 4 min.

Structure of the Spleen

Immunological activity in the spleen is located in the white pulp with T cell clearance concentrated round the periarterial lymphatic sheath and B cell clearance in the lymphatic nodules. The spleen has a unique open vascular circulatory system with filtration beds between the end of the arteries and the beginning of the veins. This bed is made up of a network of stable, contractile reticular fibers and cells. Macrophages, interdigitating cells and follicular dendritic cells are interspersed throughout the filtration beds. These cells are responsible for clearing the blood of damaged cells and foreign particles by direct filtration and after opsonization with antibodies and/or complement components (45, 46).

Associated with the basic reticulum framework are mobile, contractile barrier cells (Fig. 2). These are developmentally related to fibroblasts and are highly responsive and flexible, enabling the filtration beds to rapidly alter their filtration capacity in response to splenic activity. The barrier cells may exert a controlling influence on the capacity of the spleen to selectively shunt blood through the filtration beds or directly into splenic veins (47). Antimalaria drugs have the potential to alter the dynamics of the splenic microcirculation by causing relaxation of the reticular cells, thereby enhancing splenic filtration capacity (48, 49).

Malaria infection is a potent stimulus for the splenic stromal cells to rapidly expand and mobilize. It induces an increase in mononuclear cells, expansion of the white pulp and development of germinal centers (47) with consequent splenic enlargement. There is a marked increase in nonspecific reticuloendothelial activity in the spleen and although this is initiated by malaria infection, it is not specifically directed against malaria. The reticuloendothelial hyperactivity has been demonstrated experimentally by an accelerated rate of clearance of radio-labeled albumin aggregates (50). Clinically this is manifested as enhanced phagocytosis of both parasitized and normal red cells which contributes to the etiology of malarial anemia (2, 50, 51).

The spleen uses several different systems for filtering the blood. It can completely remove damaged cells or organisms ("cull"), selectively remove only a

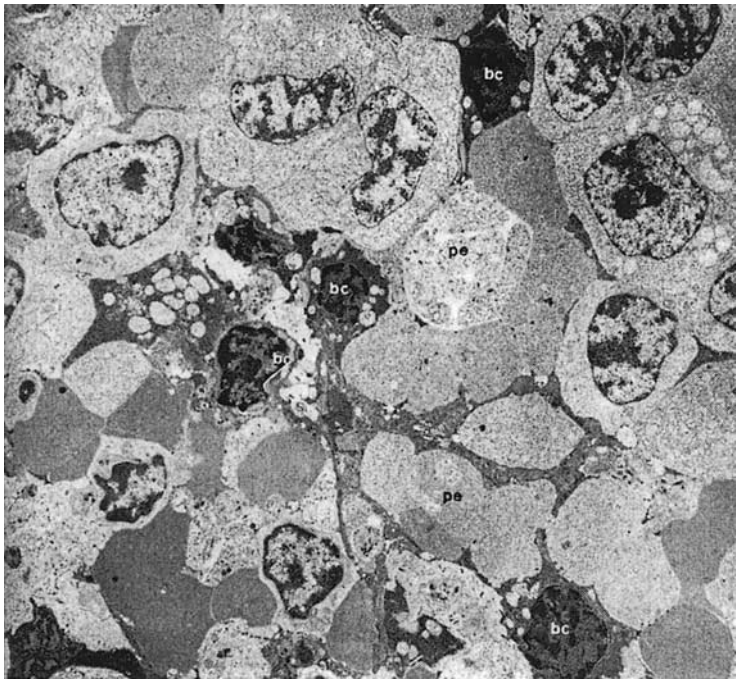

Figure 2 Association between barrier cells (bc) and other cells in murine spleen red pulp infected with *P. yoelii* (×3100). (From: Weiss (1990), *Immunology Letters* **25**: 165. With permission from the publishers.)

part of the red cell contents, such as inclusions or parasites ("pit") and sequester reticulocytes so that they can mature in the splenic tissue ("remodel"). Normal red cells are pliant enough to be able to squeeze through the slits between endothelial cells and pass through the filtration beds to enter the draining veins. Red cells that have become rigid and inflexible as a result of malaria infection are held up in the splenic microvasculature. Depending on the degree to which the cells are abnormal, they are either culled from the circulation or pitted of their inclusions, leaving an indentation in the surface of the red cell which can be visualized by phase contrast or electron microscopy (52). The pitting of malaria parasites from red cells has been shown to have a role in controlling malaria infection (53, 54). However, the process of pitting alters the membrane of the red cells, making them more spherical and less deformable; so they are susceptible to removal by the stromal cells. The rate of clearance

of heat-damaged red cells can be used as a marker of splenic filtration capacity and this method has been used to show that in adults with acute malaria and palpable splenomegaly, the filterative capacity of the spleen can increase eightfold (49).

In addition to the enhanced filtration of cells by the spleen, the removal of antibody-coated red cells by splenic macrophages is also upregulated in acute malaria (55). The extent of upregulation appears to be independent of splenic size. The degree of opsonization of red cells increases as the parasite matures within the cells, and this not only increases the likelihood that the cell will be removed by the spleen, but also that it will escape the splenic clearance mechanism through cytoadherence and sequestration within other organs. In severe malaria the splenic capacity to clear parasitized cells from the circulation can be overwhelmed. The reasons for this may be related to blockage of Fc-receptor-mediated interactions and compromised phagocytic activity (56). It has been estimated that a "typical" patient with severe malaria will lose about 2% of their packed cell volume through destruction of nonparasitized cells alone. This is likely to be a significant contributory factor in the development of late anemia and may be exacerbated by a depressed reticulocyte response (57).

Hyposplenism and Malaria

General Features of Hyposplenism

Patients without spleens or with functional hyposplenism are at increased risk of severe infections, particularly those caused by encapsulated organisms. Such organisms, especially *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*, are prevalent in areas where malaria also occurs. These infections are most common in the first two years after splenectomy and the risk is greatest in children but can persist into adulthood (58). It is therefore recommended that patients without a functioning spleen should be vaccinated against *Streptococcus pneumoniae*, *Haemophilus influenzae* type b (Hib) and meningococcal meningitis.

Functional Hyposplenism and Susceptibility to Malaria

In view of the critical role played by the spleen in combating malaria infection, it is perhaps surprising that individuals who have developed a degree of

natural immunity to malaria prior to splenectomy do not seem to be at particular risk of developing severe malaria (59). Humoral and cellular immune responses, and the severity of parasitemia, appear to be similar in splenectomized patients and local inhabitants with normal splenic function (60, 61). The apparent protection that asplenic patients living in malaria endemic areas exhibit against developing severe malaria may in part be due to the fact that many are prescribed lifelong malaria prophylaxis (62–64).

In contrast, there are many reports of severe malaria occurring in individuals without functioning spleens and with no immunity to malaria (e.g. 65, 66). In the absence of a spleen all the developmental stages of *P. falciparum* appear in the peripheral blood, including schizonts and immature gametocytes (66, 67). Nonimmune patients with hyposplenism are therefore generally advised to avoid travel to malaria-endemic regions (68).

Hyperreactive Malarial Splenomegaly

Hyperreactive malarial splenomegaly (HMS) is the commonest cause of massive splenic enlargement in many malaria-endemic areas. It was formerly known as *tropical splenomegaly syndrome*, but was renamed in 1983 (69) to reflect the improved knowledge about the role that malaria plays in its pathogenesis.

Causes of Massive Splenomegaly in Malaria-Endemic Areas

There have only been a few detailed studies of the causes of massive splenic enlargement in tropical areas (e.g. 24, 70, 71). The commonest causes are said to be HMS, portal hypertension, chronic myeloid leukemia, myelofibrosis, thalassemia, hemoglobin SC and, in endemic areas, schistosomiasis and visceral leishmaniasis.

The application of new investigative tools in tropical countries may result in refinements to the list of conditions associated with massive splenomegaly. The use of modern diagnostic methods in West Africa, such as polymerase chain reaction for the diagnosis of lymphoproliferative disorders, has indicated that lymphomas may be an underdiagnosed cause of massive splenomegaly (Bedu-Addo G and Bates I, 2003, *Lancet*). Unusual causes of massive splenic enlargement that have been reported from malarious areas include lymphoproliferative disorders (24), sickle cell (HbSS) anemia (70, 72), Gaucher disease

Table 2. Classical causes of massive splenomegaly in the tropics.

Hyperreactive malarial splenomegaly
Schistosomiasis
Kala-azar
Thalassemia major
Chronic myeloid leukemia
Myelofibrosis
Splenic cysts and tumors
Lipid storage diseases
Idiopathic nontropical splenomegaly

(73), amyloidosis (24), gross lymphoid hyperplasia, lymphosarcoma, brucellosis and tuberculosis (70).

Prevalence and Epidemiology of HMS

The highest prevalence of HMS in the world is in Papua New Guinea (74), where up to 80% of adults have massive splenomegaly which is predominantly due to HMS (34, 74). HMS has been reported from regions with both meso- and hyperendemic patterns of malaria transmission (74, 75). It is common in parts of sub-Saharan Africa and has also been reported from Brazil (76), India (77) and Indonesia (78).

The prevalence of HMS can be estimated from community studies of splenic enlargement in adults. Overall, about 20–30% of adults in areas where malaria is endemic have palpable splenomegaly, and in about 20% of these the spleen is massively enlarged. In Africa, 11–45% of patients with massive splenic enlargement in Senegal, Nigeria, Uganda, Tanzania and Zambia have HMS (reviewed in 24), giving an estimated overall prevalence of 1–2%. This estimate is confirmed by studies from West Africa which have formally documented the prevalence of HMS in the general population as 2% in Nigeria and 0.4–1.2% in Gambia (71). In the past the true prevalence of HMS was difficult to measure because of ambiguity over the diagnostic features. In a study from Nigeria only about half the patients initially diagnosed on clinical grounds as having HMS

actually had the condition (79). This prompted the publication of criteria for the diagnosis of HMS (80).

The criteria require that malarial immunity should have developed before HMS can be diagnosed, and for this reason the condition is rarely described in children under 10 years of age. There appear to be geographical differences in the rate at which men and women are affected by HMS. In Nigeria the ratio of men to women is between 1 : 2 and 1 : 3 (24, 70), whereas the proportion of men and women affected in Papua New Guinea is equal (81).

Pathogenesis of HMS

The pathogenesis of HMS is not yet fully understood. The underlying abnormality appears to be an aberrant immune response to repeated malaria infections, which results in overproduction of IgM. The high circulating levels of IgM are due to the expansion and activation of B lymphocytes, rather than a defect in IgM catabolism (82). There is no evidence of a failure to switch from IgM to IgG production in HMS, as IgG levels are not suppressed (83). Polyclonal activation of B cells is a well-recognized phenomenon in acute malaria and HMS may represent a chronic form of this acute phase response. Several suggestions have been put forward to explain the nature of this B cell proliferation. The expansion of the B lymphocyte pool may be facilitated by a lack of T suppressor cells in HMS patients, possibly due to a lymphocytotoxic IgM antibody (84–86). Alternatively, malaria-associated mitogens which are capable of stimulating B cells without recruitment of T cells may play a role in chronic polyclonal B cell overactivity in HMS (84, 87, 88). Recently, specific IgM overproduction has been described in animal models in response to a *P. falciparum* repeat peptide sequence (89).

Chronic overproduction of IgM in HMS has several consequences. By far the most clinically important is the formation of immune complexes which can be detected in the circulation (90, 91). In an attempt to enhance removal of these from the circulation, there is gradual expansion of the spleen and, to a lesser extent, the liver. The IgM produced in HMS is polyclonal, and only a small proportion is directed against malaria antigens, the rest complexing with a variety of other antigens including auto- and heterophile antibodies. (92, 93). IgM is also implicated in the formation of cryoglobulins in HMS (90, 91). Mixed cryoglobulinemia has been strongly associated with hepatitis C virus infections

(94) but there is no evidence of increased prevalence of hepatitis C in patients with HMS (133).

There is evidence that genetic factors may contribute to the development of HMS. For example, the condition is more common in Fulas in Gambia (71), Fulanis in Nigeria (70), Kamba and Luo in Kenya (95), Rwandan immigrants in Uganda (97) and non-Austronesians in Papua New Guinea (5).

Diagnostic Criteria for HMS

Criteria for the diagnosis of HMS were first published in 1979 (79) and modified shortly after to comprise both major and minor criteria (97) (Table 3).

Major Criteria

Measurement of spleen length has been shown to be a reasonable indicator of splenic volume (13), and this can be done simply by using a tape measure and following the contour of the spleen. Assessment of splenic volume by ultrasound may be difficult in areas where HMS is common, particularly as measurements need to be repeated in order to monitor disease progress.

It is important to know the local normal mean IgM value in order to apply the third major criterion, because normal IgM levels can vary widely even

Table 3. Criteria for the diagnosis of HMS.

Major criteria
(1) Massive splenomegaly ($\geq$10 cm) for which no other cause can be found
(2) Immunity to malaria acquired through long-term residence in a malarious area
(3) Raised serum IgM, at least 2 SDs above the local mean
(4) Clinical and immunological response to malaria prophylaxis
Minor criteria
(1) Familial occurrence
(2) Hypersplenism
(3) Lymphocyte proliferation
(4) High antimalarial antibody levels
(5) Hepatic sinusoidal lymphocytosis
(6) Normal lymphocyte response to phytohemagglutinin

within a small geographic area (81). Although, as a group, HMS patients do have markedly raised IgM levels, there is extensive overlap in the IgM levels of individuals with HMS and other conditions (98, 99). This means that although a high IgM may help to indicate a diagnosis of HMS, a normal IgM level does not exclude the diagnosis (95, 100).

Minor Criteria

Although lymphocytic infiltration of the liver sinusoids has been frequently described in HMS, the presence of normal liver histology does not exclude the diagnosis (101, 102). Furthermore, hepatic sinusoidal lymphocytosis is not specific for HMS. It has also been described in patients without a palpable spleen (103), chronic lymphocytic leukemia in Nigeria (70), Felty's syndrome (104), Epstein–Barr virus infection (105) and active hepatitis secondary to hepatitis B and hepatitis C infections (106, 107). The lymphocytic infiltration of the liver sinusoids in HMS comprises predominantly T cells and is probably caused by a cell-mediated response to an antigen present in liver cells (108). Because of the hazards associated with performing liver biopsy in patients with thrombocytopenia and in hospitals where prebiopsy coagulation studies and emergency blood transfusions may not be available, liver ultrasound, has been used as an alternative (102, 109). The majority of patients with HMS have a normal liver ultrasound so the main use of ultrasound in HMS is to exclude diffuse liver pathologies such as cirrhosis.

Raised antimalarial antibody levels are a consistent feature of HMS (110–112). Malarial serology can therefore be helpful in diagnosing HMS providing that the results are interpreted in relation to the local background levels of malaria transmission (95). Paradoxically, although antimalarial antibody levels are very high in HMS, there is less parasitemia and a paucity of malaria pigment in liver and spleen macrophages compared to the local population (23, 64, 70, 86, 113, 114).

In general, a combination of these criteria is useful for differentiating between HMS and other causes of massive splenomegaly apart from lymphoproliferative disorders. To make the criteria for HMS more stringent in this aspect, modifications have been proposed which include a reduction in spleen size of at least 40% with malaria prophylaxis and demonstration of the polyclonal nature of the lymphocytes (115).

Table 4. Modified criteria for the diagnosis of HMS.

(1) Persistent massive splenomegaly (≥10 cm) for which no other cause can be found
(2) No evidence of a monoclonal population of lymphocytes
(3) Slow (i.e. over not less than 12 weeks) reduction of ≥40% in splenic size with malaria prophylaxis
(4) Immunity to malaria acquired through long-term residence in a malarious area
(4) Raised total serum IgM levels
(5) Raised antimalarial antibody levels

Clinical Features of HMS

HMS is predominantly a disease of adults aged 20–40 years and it affects women more than men. Considering the degree of splenic enlargement that can occur in HMS, a surprising number of patients are asymptomatic (116). Others may have been aware of their enlarged spleens for some time but not sought medical attention. Symptoms are often related to splenomegaly which causes a feeling of dragging or fullness, rarely described as pain, in the abdomen. The spleen size may wax and wane, especially in association with fevers, but the general trend is for it to slowly enlarge. The frequency of other symptoms shows some geographical variation and they include malaise, weight loss, cough, headaches, menstrual irregularities and breathlessness. Physical examination reveals a firm, nontender, enlarged spleen which is almost invariably accompanied by hepatomegaly, and signs of anemia. Less commonly, jaundice and fever may be present.

The etiology of anemia, which is almost universal in HMS, is multifactorial. The massive splenic enlargement leads to hypersplenism with thrombocytopenia and, less commonly, leucopenia. The splenomegaly itself results in hemodilution (34, 35), which plays a major role in lowering the hemoglobin. A significant proportion of patients with HMS also experience episodes of acute hemolysis which are associated with jaundice, increasing splenomegaly, a reticulocytosis and raised levels of unconjugated bilirubin. The hemolysis may sometimes be preceded by a brief febrile illness, is more common in pregnancy (62) and can be severe enough to cause prostration and require blood transfusions (117). The cause of the hemolysis is unclear; both the direct Coombs test for IgG and complement, and screening tests for glucose-6-phosphate dehydrogenase deficiency are negative (74; personal observation). Nevertheless it

is often possible to curtail the hemolytic process with a short course of steroids (118), suggesting that the underlying mechanism might have an immune basis.

Management of HMS

Since proguanil was first shown to have a beneficial effect in HMS (113, 114), antimalarial drugs have been the mainstay of treatment. On continuous therapy with proguanil 100 mg/day the spleen slowly regresses, the hemoglobin rises and the IgM levels return toward normal. Other antimalarial drugs, such as chloroquine base 300 mg/week (77) and cycloguanil embonate 350 mg every 3–6 months (119, 120), have also been used successfully. The choice of drug needs to take into account local malaria drug resistance patterns. As HMS is a chronic disorder which will recur if treatment is stopped, the drugs should not be cumulative or toxic when used over many years.

The reason why HMS responds so well to malaria prophylaxis is not clear. Although antimalarial antibody levels are high in HMS, the levels of parasitemia are disproportionately low and there is little or no malaria pigment in the reticuloendothelial macrophages. Proguanil as a single agent is not a recognized treatment for malaria and the dose of proguanil used for HMS is less than that normally recommended for prophylaxis. The fact that several different antimalarials with differing modes of action have been shown to be effective in HMS implies that it is the antimalaria effect that is important rather than any other pharmacological activity.

Clinical Course

Despite the lack of symptoms in many patients and the seemingly innocuous nature of the condition, HMS is slowly progressive. Long-term studies have shown a surprisingly high mortality in HMS, with 57% of those with the largest spleens dying during a six-year observation period. Infection appeared to be the most serious hazard to health (121). It has been suggested that the enlarged spleens in HMS function poorly, thereby putting the patients at risk of overwhelming bacterial infection. In countries where HMS is prevalent, infections are a common cause of death even amongst previously healthy individuals, and it has been difficult to show whether HMS increases susceptibility to infections or not. Cook *et al*. (122) found that sepsis was common in HMS and associated with fluctuations in splenic size. Crane (117) also noted that secondary

infections were frequent and could be severe and prolonged, and contributed to the high mortality. An impaired neutrophil response to pyogenic infection has been observed in HMS patients (116), manifested by an abnormally slow reduction in resolution of temperature after treatment for pneumonia. However, this may have been due to inability of the immune system to respond in the early stages of the infection, because resolution of the chest X ray abnormalities was not delayed (123).

In contrast to those studies, Pitney (23) could not demonstrate any increased risk of infection in HMS despite some patients having leucopenia. Similarly, Ziegler (124) did not find any evidence of generalized impairment of humoral or cellular immunity in HMS patients in Uganda. In functional hyposplenism, surface pits, which are normally removed by the spleen, are increased and can be visualized using electron or phase contrast microscopy. An elevation in pitted red cell counts is strongly correlated with the incidence of severe infection (52). Ghanaian patients with HMS have red cell pits in less than 2% of circulating cells, indicating that splenic pitting ability is normal (personal observation).

Development of Lymphoma

The second-most-common cause of death in HMS appears to be lymphoma (62, 114, 119). The difficulty of distinguishing between HMS and lymphoproliferative disorders is well recognized and seems to be a particular problem in West Africa. There are several reports from Nigeria in which HMS has been confused with lymphoproliferative disorders, especially "African chronic lymphocytic leukemia," a condition characterized by female predominance and splenomegaly rather than lymphadenopathy, as in classical chronic lymphocytic leukemia (125–127).

The situation in West Africa is complicated because in this region HMS can be associated with a lymphocytosis severe enough to mimic chronic lymphocyte leukemia (108, 116, 128). Initially the response of patients' lymphocytes to mitogens such as phytohemagglutinin was incorporated into the diagnostic workup for massive splenomegaly in Nigeria. Lymphoproliferative disorders have a blunted response compared to the normal response of lymphocytes from HMS patients (129). However, later studies have shown that this technique is of only limited value because cells from patients with lymphoma do not necessarily exhibit impaired blastic transformation (70). In more recent studies,

Southern blotting and polymerase chain reaction have been used to determine the pattern of immunoglobulin gene rearrangements in DNA from lymphoma cells. Although they are not 100% specific these new methods have enabled a much clearer distinction to be made between neoplastic lymphoproliferative conditions and the polyclonal lymphocyte expansion of HMS (12, 130).

These techniques, in combination with immunoglobulin gene sequencing, lymphocyte immunophenotyping and evaluation of lymphocyte morphology in the peripheral blood, have recently led to the identification of a novel tropical splenic lymphoma which has many features in common with HMS (131, 132). This tropical splenic lymphoma predominantly affects women, and, like HMS, it is associated with much higher IgM and antimalarial antibody levels than are found in the local normal population (83). HMS and tropical splenic lymphoma cannot be distinguished by routine clinical and laboratory methods. Apart from molecular determination of lymphocyte clonality, the main difference between these two disorders is their response to antimalaria drugs. In HMS, by definition, there is a good response, with at least a 40% reduction in spleen size, whereas in the lymphoma there is little or no response (115).

Initially this tropical splenic lymphoma was thought to be a type of splenic lymphoma with villous lymphocytes because of the cytoplasmic projections on circulating tumor cells (131) and their CD20+/CD5− phenotype (132). However, analysis of the V_H gene sequences demonstrated that, unlike splenic lymphoma with villous lymphocytes, the tumor cells are derived from a naïve B cell.

Despite showing unusual serological reactions to the Epstein–Barr virus, there is no evidence that the B lymphotropic viruses, EBV, the hepatitis C virus or HHV8 are directly involved in the etiology of tropical splenic lymphoma (133). On the other hand, malaria may be implicated as tropical splenic lymphoma is closely associated with HMS and the two disorders have similar markedly raised total IgM and antimalarial antibody levels (83). These recent studies add credence to the observations made by earlier researchers that HMS may evolve into a lymphoproliferative disorder (63, 114, 119, 134) and highlight possibilities for investigating the multiple steps involved in the process of tropical lymphomagenesis.

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Chapter 11

Malaria and Burkitt's Lymphoma

Christine A Facer

Introduction

Endemic Burkitt's lymphoma (eBL), an aggressive monoclonal B cell tumor, is the most common cancer of children in the tropics. It was recognized more than 40 years ago by Dennis Burkitt as a distinct pathological entity with peculiar epidemiological characteristics (1). In a comprehensive epidemiological study, Burkitt showed that the tumor was geographically limited to the lowland areas of sub-Saharan Africa (the "lymphoma belt") and was suspected of having a viral etiology with malaria as an important risk factor. This hypothesis was strengthened by the isolation of the Epstein–Barr virus (EBV) from cultures of tumor cells (2) and, later, identification of viral DNA sequences in the same cells (3). At that stage Burkitt's lymphoma might have been considered solely as a virally induced cancer. However, progress in the fields of immunology and molecular genetics has shown that eBL has a complicated biology and molecular pathogenesis and that it represents the end point of a complex interaction between several cofactors acting in concert and, as such, has assumed considerable importance as a model of multistage carcinogenesis.

The purpose of this review is to present an update and reappraisal of our current understanding of the molecular pathogenesis of eBL, with emphasis on the role of malaria and EBV and their respective interactions with the immune system in the genesis of the lymphoma.

Infections and Malignancy

A large proportion of surprisingly diverse types of human cancer have infections as precipitants of neoplasia. There is now strong evidence that several viruses, bacteria and parasites (Table 1) are responsible for an estimated 15.6% of the worldwide incidence of cancer (4, 5). An important feature that these infectious agents have in common is the ability to either establish *latency*, i.e. for the viral genes to persist in a subset of cells following infection, or to become *chronic* infections under certain conditions (Table 2). Another characteristic of several types of oncogenic infections is that the risk of malignancy is related to the higher level of viral/bacterial/parasitic replication that is usually seen, raising the possibility of the organisms causing secondary genetic change to target tissues. This could be due to a *very early* (e.g. EBV, HTLV-1) or *severe primary* infection or disruption of normal immune function leading to a change in the level of latent infection. The impact of these infections is becoming increasingly evident, because they are responsible for a cascade of opportunistic infections in, for example, HIV infection. In global terms, the burden is the heaviest among populations in developing countries, reflecting the impact of very early infection with these agents and the subsequent risk of malignancy (see the subsection "Role of EBV in the Etiology of eBL," p. 319). Despite these observations, there remains the fundamental question as to why cancer develops in a few people when many are infected.

The Epstein–Barr Virus

The Epstein–Barr virus (EBV) is a ubiquitous, potentially oncogenic B lymphotropic DNA herpes virus of humans and subhuman primates which establishes lifelong latency within its host. It is estimated that 95% of the adult population worldwide is infected with the virus, but chronic disease due to EBV is relatively rare. EBV is associated with several malignancies: eBL, AIDS-related lymphoma, posttransplantation lymphoproliferative disease, Hodgkin's lymphoma and rare T lymphocyte lymphomas (6). The following summary on the virus is not intended as a fully comprehensive account of the subject and the reader is referred to several excellent reviews on the virus (7–11).

The restricted tropism of the virus relates to the distribution of the virus receptor, an epitope on the complement receptor 2 (C3dR, CR2, CD21) found on

Table 1. The major potentially oncogenic infections and their associated malignancies.

Organism	Malignancy	Age
(i) ***Viral***		
	eBL (95%); sBL (25%)	<16 years (early infection)
	Hodgkin's lymphoma (35–50%)	Young adult (late infection)
EBV	Non-Hodgkin's lymphoma	Adult
	Nasopharyngeal carcinoma	Adult
	Gastric adenocarcinoma	Adult
	Breast cancer	Adult
	Testicular cancer	Adult
HTLV—1 & 2	T-cell leukemia/lymphoma	Adult
HBV	Hepatocellular carcinoma	Adult
HCV	Hepatocellular carcinoma	Adult
HGV	Non-Hodgkin's B cell lymphoma	Adult
HPV	Cervical cancer	Adult
HHV-6	BL?	Adult
HHV-7	BL?	Adult
HHV-8	Kaposi's sarcoma	Adult
(ii) ***Bacterial***		
Helicobacter pylori	Gastric adenocarcinoma Mucosa-associated lymphoid tissue lymphoma (MALT)	Adult
(iii) ***Parasitic***		
Plasmodium falciparum	eBL	<16 years
Schistosoma haematobium	Squamous cell carcinoma of urinary bladder	Adult
Schistosoma mansoni	Abdominal BL?	
	Colonic carcinomas	<16 years
Fasciola hepatica	Liver cancer	Adult
Clonorchis sinensis	Cholangiocarcinoma	Adult
Strongyloides stercoralis	T-cell leukemia	Adult

HTLV = human lymphotropic virus
HBV = hepatitis B virus
HCB = hepatitis C virus
HGV = hepatitis G virus
HPV = human papilloma virus
HHV = human herpes virus

Table 2. Unusual and unexplained features of cancers with a viral etiology (e.g. EBV).

• *Long incubation* period between initial infection with the virus and appearance of the lymphoma/cancer
• *Ubiquitous* nature of candidate viruses and *rarity* of the cancer with which they are associated
• *Initial infection* with the virus is often *subclinical*
• Need for *cofactors*
• Different viral *strains* may have different oncogenic potential
• Human host plays a vital role in susceptibility to the cancer, especially the *age* at the time of infection, *genetic* characteristics and *immune* status
• Cancers result from a *multistage* process in which the virus may play a role at different points in the pathogenesis: host's immune system, oncogenes, chromosomal translocation and a variety of events at the molecular level

B lymphocytes (12). Following infection with EBV, the B cell rapidly proliferates in a continuous polyclonal expansion, a process known as immortalization (13). Interestingly, the B cells most susceptible to infection are those producing IgA immunoglobulin (14), although why this should be is unknown. In addition to B cells, the virus also infects epithelial cells of the oropharynx (15), cervix (16) and stomach (17); CD4+ T lymphocytes in patients with fatal T cell lymphomas (18) and peripheral blood CD4+ cells in a child with chronic active EBV infection (19).

Primary infection normally occurs in young children by horizontal transmission (virus in saliva) without any accompanying disease. Following this primary exposure, a polyclonal expansion of B cells occurs inducing strong humoral and cellular immune responses. Despite these responses viral genomes persist in a small number of B lymphocytes (estimated at around 1×10^5 circulating B cells) which can be reactivated under certain conditions such as severe immunosuppression. Latently infected B cells can also be induced to replicate *in vitro* by treatment with phorbol esters, corticosteroids and anti-immunoglobulin.

Socioeconomic class is an important factor in the age stratification of primary infection. Thus, in developed countries of the Western world, 90% of individuals are infected by adulthood, whereas in developing countries, 99% of children are already infected by 3 years of age. A delayed asymptomatic

primary infection to adolescence or beyond produces a severe but limited lymphoproliferative disease known as infectious mononucleosis (20).

Virus Cycle and Gene Expression

The EBV virus is composed of a linear DNA core, an icosahedral capsid and an outer envelope. Its genome is a double-stranded DNA molecule of approximately 172 k base pairs divided into unique internal repeat and terminal repeat domains (21). Following infection of a cell, the linear viral DNA joins by these terminal repeats at each end to form a circularized episomal genome. The number of terminal repeats so formed is maintained in the progeny, a feature which permits detection of clonality and origin of the virus (22, 11).

Approximately 80 proteins are encoded by the genome (23) and one of the surprises resulting from its molecular analysis is that there are three geographic subtypes of EBV (24) which are microheterogeneous in the number of tandem repeats of one of the proteins, EBNA-2 (25), associated with ability of the virus to immortalize and induce B lymphocyte proliferation (25). The EBNA-2 AC strains predominate in Asia; EBNA-2 AD strains predominate in the US; EBNA-2 B strains have all been identified in sub-Saharan Africa (24). Type AC has a greater capacity for immortalization of peripheral blood lymphocytes *in vitro*. whereas Type B is normally found in eBL tumor cells and poorly transforms B cells *in vitro* (25–27). A high frequency of EBNA-2 B is also observed in HIV associated B cell lymphomas, implying that this strain may possess greater potential oncogenicity than Types AC and AD. Type B strains may be enhanced by the immunosuppression following infection with HIV and tropical diseases such as malaria (see the subsection "Immunosuppression and Disrupted Immunosurveillance," p. 338) (28).

Following infection of susceptible IgA-positive B cells (80–90% of viral DNA is found in lymphocytes carrying sIgA in healthy individuals) (14), presumably at the oropharyngeal site, the cells undergo blast transformation, synthesize DNA, produce and secrete immunoglobulin (EBV is a potent B cell mitogen), and eventually become immortilized. This process is associated with viral latency which allows cells to proliferate indefinately *in vitro* into lymphoblastoid cell lines (LCL; 29). Circularization of the EBV genome results to form an episome present extrachromosomally in the nucleus (23). Up to 10 viral genes are expressed in latently infected cells and latency may be one of three types according to expression of these genes (Fig. 1) (reviewed extensively;

30–32). Briefly, Type I is characterized by a restricted gene expression: just two EBV-encoded nuclear small nonpolyadenylated RNA (EBER-1 and EBER-2) molecules and a single nuclear antigen essential for the maintenance of latency, EBNA-1. Type II shows, in addition, expression of latent membrane proteins, LMP-1 and LMP-2. Type III displays additional expression of the nuclear proteins EBNA-2 to EBNA-6 by virtue of differential promotor usage (33). BL cells usually display latency Type I but may convert to Type III in culture (34), the classic phenotype of LCL *in vitro*. More recently, expression of EBNA-2 and LMP-1 has also been described in tumor cells (33). Both are necessary for the immortilization of susceptible B cells *in vitro*, yet expression of these proteins is not observed in most BL (35).

Cells with a latency phenotype Type II and III are readily recognized by viral-specific cytotoxic T lymphocytes (CTLs; see below). The functions of the viral proteins associated with these phenotypes, EBNA-1, -2, -3, -5, -6 and LMP-1, are largely, but not completely, unknown. LMP-1 induces expression of cellular adhesion molecules (important for interaction with T cells) and induces the oncogene *bcl-2* (which inhibits apoptosis) (36). The function of the LMP-1 molecule is similar to those of the ligand-activated CD40 molecule and the tumor necrosis factor receptor (37). The *ebna-2* gene transactivates the expression of *lmp-1* and *lmp-2* (38). In addition, the genes *ebna-2*, *ebna-3* and *lmp-1* transactivate the expression of a number of B lymphocyte genes, including those encoding for the adhesion molecules ICAM-1 (intercellular adhesion molecule-1), LFA-1 (lymphocyte function associated antigen-1) and LFA-3 (lymphocyte function associated antigen-3), and the activation-related marker CD23 (39).

The virus in latently infected B cells can be activated to enter the lytic cycle by a variety of products, such as phorbol esters, anti-immunoglobulin and corticosteroids. One of the first genes, a most important one, is the viral *bzlf-1* gene. Its protein product, ZEBRA, plays a critical role in initiating the

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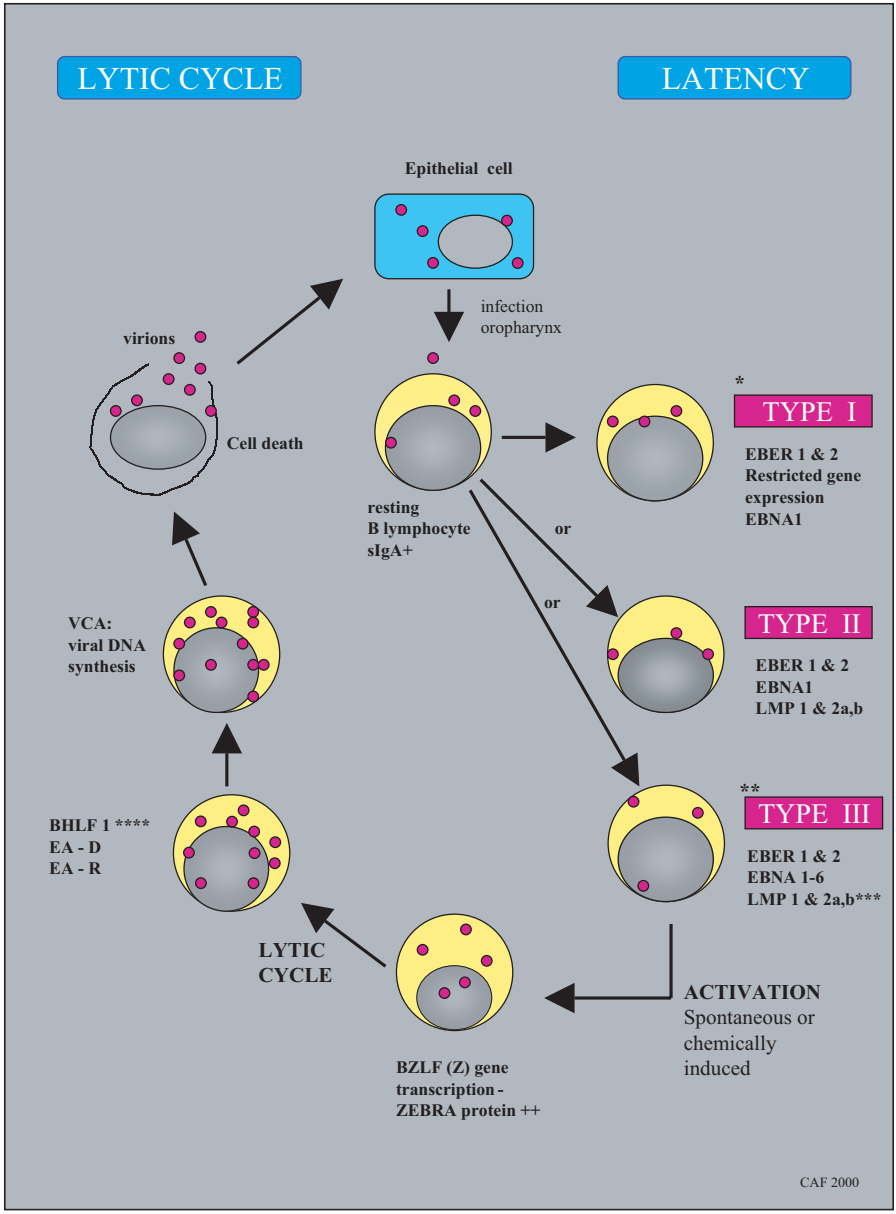
Figure 1 Schematic diagram of the life cycle of EBV following infection of a B lymphocyte. ∗ eBL phenotype (but occasionally EBNA2- and LMP1-positive). ∗∗ BL cells in culture normally convert from Type I to Type III latency. ∗∗∗ LMP is the target of the CTL response. ∗ ∗ ∗∗ BHLF gene is homologous to *bcl-2* protooncogene and inhibits apoptosis. ++ There is a significant correlation between ZEBRA expression and response to cytotoxic therapy *in vivo*. © CAF.


LYTIC CYCLE
LATENCY
Epithelial cell
infection
oropharynx
virions
Cell death
resting
B lymphocyte
sIgA+
*
TYPE I
EBER 1 & 2
Restricted gene
expression
EBNA1
or
or
TYPE II
EBER 1 & 2
EBNA1
LMP 1 & 2a,b
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TYPE III
EBER 1 & 2
EBNA 1-6
LMP 1 & 2a,b***
VCA:
viral DNA
synthesis
BHLF 1 ****
EA - D
EA - R
LYTIC
CYCLE
ACTIVATION
Spontaneous or
chemically
induced
BZLF (Z) gene
transcription -
ZEBRA protein ++
CAF 2000

Figure 1

switch from latent to lytic infection, so disrupting latency. ZEBRA functions as a transcriptional activator mediating the switch by binding to the promotor of many genes involved in DNA replication (40, 41). Its expression in BL tumor cells appears to make them sensitive to the cytotoxic effects of cyclophosphamide (42).

Activation to a full lytic cycle with cell surface expression of the viral capsid antigen (VCA) is observed only in a minority of cells and rarely in tumors.

Immunological Control of EBV and Tumor Cells

An understanding of what constitutes the immune response to EBV and lymphoma cells is necessary in order to interpret the etiology of eBL. The presence of EBV in epithelial cells and B lymphocytes provokes a strong humoral response directed against lytic as well as latent viral proteins. Initially, this response is aimed primarily at EBV antigens associated with the lytic cycle, such as early antigen and VCA. Antibodies to the viral envelope glycoprotein (gp350) are important for neutralizing viral infectivity, although only low titres are produced in a natural infection (43). These antibodies appear to limit the spread of the virus but have little effect on the oropharyngeal phase of replication (8). Eventually antibodies to latent proteins EBNA-1 and EBNA-2 reach detectable levels and IgG antibodies to EBNA-1 and VCA remain throughout life (44), implying continued or reactivated viral replication.

At the same time as the humoral response is developing, a substantial cell mediated immune response is mounted. Virally infected cells have the potential for unlimited growth, but this does not generally occur in normal individuals with an intact immune system due to potent natural killer (NK) and T lymphocyte responses (8, 11), both Class I (CD8+) and Class II (CD4+). EBV-specific cytotoxic CD8+ lymphocytes can be found in infected individuals in high frequency throughout life (as assayed by the *in vitro* T cell regression assay (see the subsection "Immunosuppression and Disrupted Immunosurveillance," p. 338), implying continued virus activity. T cells remove latently infected B lymphocytes following recognition of the latent membrane proteins, LMP-1 and LMP-2 (45). Despite this, some virally infected cells manage to escape CTL activity and a fine balance exists between the two unless this is subverted, for example, by immunodeficiency states (HIV infection and organ or stem cell transplantation).

In contrast, the BL tumor cell is not recognized and killed by CTL due to defective expression of certain EBV (no LMP-1/2), HLA and adhesion molecules, although sensitivity can be modified *in vitro* (46).

Finally, the importance of cytokines in the control of EBV-infected cells should be mentioned. Interferons inhibit EBV-induced lymphoproliferation and induction of immunoglobulin synthesis. Other cytokines may have a detrimental effect on the host. Thus the systemic release of tumor necrosis factor (TNFα) and lymphotoxin-α (LTα, formerly known as TNFβ) contributes to the severity of non-Hodgkin's lymphoma (NHL) including BL (47). Here the presence of the less common variant of the human TNFα allele, TNF2, in which individuals produce higher plasma concentrations of TNF than those with the TNF1 allele, constitute a risk factor for first line treatment failure of NHL. Thus patients with the TNF2/2 genotype had a higher rate of relapse and progression (47). The TNF2/2 genotype has also been associated with susceptibility to severe *P. falciparum* malaria in African children (48, 49) and might constitute an additional risk factor for the development of BL. A study of the TNF2 allele frequency in children with lymphomas compared to the general population should provide an answer.

Role of EBV in the Etiology of eBL

There is considerable evidence for an oncogenic role of EBV in eBL, as has been summarized, and reviewed elsewhere (50–53). The most compelling evidence of a causal association came from a prospective study some 20 years ago of 2000 healthy Ugandan children aged 1–8 years (54–56). Those children who developed BL had high titres of anti-EBV antibodies in pretumor blood samples taken up to 72 months prior to tumor diagnosis indicating possible reactivation of the virus prior to tumour development. This idea is strengthened by the observation that the IgG anti-VCA antibody titre was 8–10 times greater than the local mean in children who went on to develop BL (a 30-fold increase in risk).

A further risk factor in developing BL is the age at which EBV infection occurs: the younger, the greater the risk. The very early age of infection in children in developing countries represents a 200-fold increase in risk compared to children in developed countries (57, 51).

More recent evidence implicating a role for EBV comes from molecular studies on the interaction of viral and cellular genes (Table 3).

Table 3. Evidence linking EBV with eBL.

Seroepidemiological
• High antibody titres to EBV antigens in all children with eBL
• High titre (8–10 × greater than local mean) of IgG VCA antibodies in pre-Burkitt sera
Immunological
• EBV transforms B lymphocytes *in vitro*
• EBV transforms B lymphocytes *in vivo* to produce polyclonal tumours in subhuman primates and immunosuppressed individuals
Molecular
• 98% of cases of eBL show multiple copies of the viral genome in each tumor cell
• EBNA-1 influences *c-myc* expression by increasing immunoglobulin enhancer function
• C-terminus of LMP-1 may mimic cell signaling induced by TNF and constitutively promote cell growth and transformation
• LMP-1 induces elevation of cellular *bcl-2* expression (antiapoptotic)
• EBV *bhrf-1* gene encodes for viral *bcl-2* homologue
• EBV inactivates the tumor-associated suppressor genes, retinoblastoma and *p53* via EBNA-5, which colocalizes with both *in vivo*
• EBV may contribute to the breakpoint location on Ch8 and Ch14

Epidemiology of Endemic BL and Malaria Transmission

Epidemiological observations have revealed that eBL is mostly confined to an area 15° north and south of the equator, with a prolongation southward on the eastern side of Africa (1). The tumor is found restricted to lowland areas of the tropics where there is a consistently high temperature (>15°C), heavy rainfall and high humidity, namely sub-Saharan Africa, Papua New Guinea and parts of South America (Fig. 2) (58). In the Cote d'Ivoire it is more commonly found in wooded areas than in savannahs, by a factor of 5 : 1 (59). As originally noted by Burkitt, these are areas holoendemic for malaria transmission (splenomegaly and parasitemia found in 75% of children aged 2–9 years) and where infection with EBV occurs within the first few years of life. The suggestion that infection with *P. falciparum* somehow acted as a major cofactor helped explain several epidemiological observations: the age incidence of the tumor, its geographic clustering and its seasonal incidence in Africa. These associations will be discussed in detail later on in the chapter. In contrast, in those African countries with little malaria transmission because of high altitude

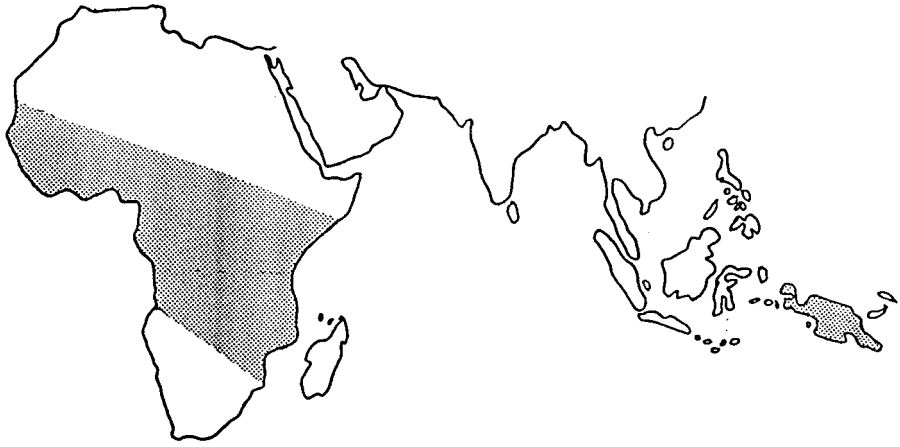


Figure 2 The geographical distribution of eBL is restricted to those areas where malaria is holoendemic.

(e.g. Rwanda), eBL is rare, and when it does occur it is normally abdominal in presentation (60). Endemic BL is reported to occasionally occur in time–space clusters (including multiple cases in one family) in certain parts of Africa (61) and Papua New Guinea (62). This has been taken by some to suggest that an additional environmental risk may be operating which could partly explain the familial associations. Alternatively, genetic predisposition to chromosomal aberrations has been proposed to underlie familial clustering of BL (55). However, the relative infrequency of familial eBL suggests that any genetic susceptibility would be strongly modulated by environmental factors, particularly malaria. An increased relative risk (3.7) associated with HLA-DR7 and BL has been reported in one African study (63), although the study was small and no DQ alleles were looked for. This is obviously an area that calls for reanalysis using the more sophisticated technologies available today for HLA typing, such as restriction fragment length polymorphism (RFLP) combined with PCR.

The estimated cumulative incidence of of eBL has risen almost 6-fold in the last 30 years, from 1.2 to 6.5 cases per 10 000 individuals per year (in Uganda), with the suggestion that the spread of AIDS and the accompanying immunosuppressive effects has been responsible (64). In neighboring northern Kenya a similar increase has been seen. Here the incidence of eBL has doubled from

5 to 10 cases per 10 000 individuals per year from 1989 to 1995 (Facer, unpublished observations), a familiar picture also found in Malawi (van den Bosch, personal communication). The environmental factor responsible for this substantial increase is unknown, although it does coincide with both the AIDS epidemic and the spread of multidrug-resistant *P. falciparum* malaria.

Other cofactors in addition to malaria have been proposed. A third infectious agent has been suggested to explain the shifting loci of cases in Malawi, as time–space case clusters coincided with epidemics of an arboviral infection causing Chikungunya fever (65). Two human herpesviruses, HHV-6 and HHV-8, may likewise be involved (66). The use of herbal medicines (in children) made from *Euphorbia* species (*E. tirucalli*?; 67) that grow extensively in Africa has also been linked to the pathogenesis of eBL (68). Some Euphorbias contain significant concentrations of the tumor promotor TPA (12-0-tetradecanoylphorbol-13 acetate). *In vitro*, plant extracts were found to enhance EBV transformation and reduce EBV-specific CTL function by downregulation of LMP-1 (69–71). However, one problem with this theory is that it does not explain cases of eBL in other countries, such as Papua New Guinea and Brazil, where *Euphorbia* species are not so prevalent and where local herbal medicines are unlikely to include extracts from this plant genus. Additionally, it fails to explain the marked increase in numbers of African BL cases in the last ten years, as mentioned above.

The incidence of eBL peaks between the ages of 5 and 10 years, with a consistent male predominance (boys are 2.5 times as often affected as girls), facts that could be of assistance in formulating hypotheses of lymphomagenesis.

The major proportion of childhood cancers in Africa are reported as eBL, with 45% in Kenya (72) and 67% in Ghana (73), and these have a distinct geographic pattern (lowland) and ethnic distribution (Luo in Kenya) but no apparent association with socioeconomic status or other variables (72).

One of the most interesting and complex features of Burkitt's lymphoma is that it occurs in two distinct forms: African endemic BL as described above, and sporadic BL (sBL) found outside the geographical limitations of eBL. The two forms are clinically and histologically similar, and surface membrane marker analysis of biopsied tumor cells indicates that they arise from cells arrested at a unique, recognizable stage of B cell differentiation (see the subsection "The BL Tumor Cell Has a Germinal Center Origin," p. 329). Both show uniformity in specific chromosomal translocations, although fine molecular mappings indicate differences in chromosomal breakpoint location (see the subsection

Table 4. Differences between eBL and sBL.

Feature	eBL	sBL
Geographic distribution	Climatically defined (Equatorial Africa; Papua New Guinea)	Not climatically defined (Europe, Middle East, Japan, US)
Average annual incidence (children <15 years)	5–10 per 100 000	0.2 per 100 000
Association with EBV	98% cases	15% cases
Common lymphoma sites (in decreasing order)	Jaw, abdomen, orbit, paraspinal	Abdomen, bone marrow, jaw, lymph nodes
Cell differentiation stage	Early B cells	Late differentiated B cells
Ch8 breakpoints	74% are upstream of *c-myc*	56% are within the first intron/exon of *c-myc*
Ch14 breakpoints	20% within Sμ region in 50% cases	30% within Sμ region in 28% cases

"Molecular Epidemiology of Translocations," p. 331), suggesting that eBL and sBL arise by different mechanisms. One clear difference is the association with EBV (Table 4). Endemic BL also shows a preferential jaw involvement and is also responsible for around 20% of the monoclonal EBV positive tumors diagnosed in AIDS patients (74, 75). An increasing number of Burkitt's lymphomas are now being reported in AIDS patients as a result of the prolonged survival of patients given new treatment regimens for HIV infection.

The codistribution of eBL and falciparum malaria is well documented. What is less well known is the distribution in Africa of non-Burkitt's/non-Hodgkin's lymphomas (NBNHLs); follicular lymphomas which display a similar, though less marked, geographical association (notably the higher grade lymphomas) with both malaria and eBL (72, 64). Like eBL, NBNHLs are associated with immunosuppression, a high B cell turnover, high EBV antibody titres, and B lymphocyte chromosomal translocations involving immunoglobulin loci (14;18 translocation in follicular lymphomas and diffuse B cell lymphomas (76)). The two may thus share, to a varying degree, a common pathogenetic origin (Table 5). It is less likely that malaria plays a direct etiological role in

Table 5. Comparison of eBL with follicular lymphoma.

Endemic BL	Follicular lymphoma
• Equatorial Africa/PNG	• Worldwide (high grade in equatorial Africa)
• 2–16 years	• Adults
• Nonrandom B cell translocation involving lg loci 8;14 (22 or 2)	• Nonrandom B cell translocation involving lg loci 14;18
• *c-myc* rearrangement—upregulation. Hypermutation—inhibits apoptotic effect *c-myc*. Proliferation	• *bcl-2* re-arrangement—upregulation. No apoptosis-prolonged cell survival
• Germinal center origin	• Germinal center origin?
• Frequency decreases with increasing age in malaria-endemic regions	• Frequency increases with age
• Associated with high B cell turnover and germinal center hyperactivity	• Associated with high B cell turnover
• Other genetic effects required for neoplasia	• Other genetic effects required for neoplasia
• EBV-positive	• EBV-negative
• Takes several years (4–6) to develop full complement of genetic errors required for malignancy	• Takes many years (>20) to acquire all genetic events necessary for malignancy
• t(8;14) B cells in peripheral circulation?	• t(14;18) B cells in peripheral circulation, frequency increasing with age
• t(14;18) B cells in peripheral circulation	

NBNHLs, since the major impact of malaria on the immune system occurs in infancy and most NBNHLs occur in adults. An essential feature of follicular lymphomas is the very slow accumulation over many years and into adulthood of abnormal genetic events in preneoplastic cells. Children are more likely to develop highly proliferative lymphoid neoplasms, possibly because the production of lymphoid cells is higher in the first decade of life.

Features of Burkitt's Lymphoma

Presentation

eBL is always extranodal and the most common sites of presentation include the jaw (50–60% of cases), followed by the abdomen (40–50% of cases) (77).

This appears to be age-related, with 100% jaw involvement in patients aged 3 years, gradually declining to 10% in those aged 15 years, with the tumors often multiple (78, 77).

Why should the jaw be the preferred site for tumor development? The presence of small foci of osteolysis and erosion of the thin plate of bone (*lamina dura*) surrounding unerupted teeth is the earliest radiological feature of jaw involvement (10). It therefore seems likely that factors in the environment of developing teeth may predispose to jaw tumor development and growth. BL is thought to develop against a background of reactive lymphoid tissue and it is possible that the tooth bud/lymphoid structure in the jaw permits an environment suitable for tumor progression (79), perhaps through local expression of the cytokines IL-4 and IL-10, both of which suppress macrophage and T lymphocyte functions, including the activity of CTLs.

Abdominal tumors involve the mesentery, retroperitoneum, and the omentum, often with ascites. Interestingly, in Brazil, where the incidence of BL is intermediate between eBL and sBL, abdominal tumors are the most common and then only in an older group of children (80). It is possible that gut-associated reactive lymphoid tissue is involved. Relevant to this suggestion is the recent observation that EBV-carrying tumor cells (with unusual Type III latency) can be found adjacent to small *Schistosoma mansoni* granulomas and scars (81, 80). As the intestinal wall is a common site for *S. mansoni* egg deposition (82), and is also the preferential primary location of abdominal lymphomas (80), the two well may be interrelated and detailed studies on the epidemiological association between schistosomiasis and abdominal BL are indicated. The geographical distribution of the two diseases overlaps and schistosomiasis is also more frequent in male children (82).

Clinical Features

Head and neck involvement in BL includes the facial bones, jaws and other extranodal sites, with jaw presentation the most common in Africa (Fig. 3, Table 4). This fast-growing tumor (doubling time of 24 h) is usually painless and rarely ulcerates unless traumatized. Central nervous system involvement may occur in 30% of all patients and may manifest as cranial nerve palsies and paraplegia. Peripheral lymph node involvement is rare and bone

involvement is more frequently seen during relapse than at admission. Macroscopically, the tumor is grayish-white in color, with areas of hemorrhage and necrosis.

The histologic classification of lymphomas has been a source of frustration for many years and the latest revised categorization is based on morphologic , immunologic and genetic techniques (83). More recently, classification of tumor cells according to their pattern of gene expression using "lymphochip" probes has helped in predicting lymphoma outcome and the designing of appropriate treatment (84).

The "lymphochip" technique has not yet been applied to BL, which is still described histologically as comprising small noncleaved undifferentiated lymphoid cells with numerous mitotic figures and areas of necrosis interspersed with many macrophages, giving a characteristic "starry sky" appearance. The neoplastic cells are usually monomorphic, with round nuclei and readily identifiable nucleoli resembling the small lymphocytes of germinal centers.

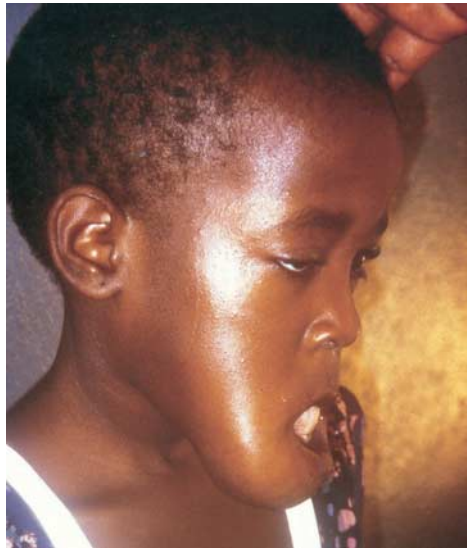
Management and Prognosis

BL is characterized by rapid progression, early dissemination and a propensity to spread to the bone marrow and CNS. Fortunately, it is normally very sensitive to drug therapy and is potentially curable by drugs alone. Surgery does have a place in reducing tumor mass, particularly in patients with abdominal tumors. However, surgery is not always practically and financially possible in most areas of the tropics, where treatment relies on drugs alone.

The single cheapest and most widely used drug for treating BL in Africa is multi-high-dose cyclophosphamide (85). This used to be one of the most effective drugs but in recent years relapsed cases have shown cyclophosphamide-resistant tumors, which curiously are sometimes found to be EBV-negative (B. Griffin, personal communication). The preferred treatment today is intensive combination therapy of short duration with methotrexate, vincristine, doxorubicin and ara-C (86, 87). Although these drugs are available in the main private hospitals in Africa, they are unaffordable and thus unavailable to the majority of African patients, even though as little as £120 will buy sufficient drugs to successfully treat up to ten children.

CNS prophylaxis has been achieved by intrathecal and systemic chemotherapy. Radiation therapy is limited to the treatment of overt CNS disease unresponsive to chemotherapy and in certain emergencies (88, 86).

(a)



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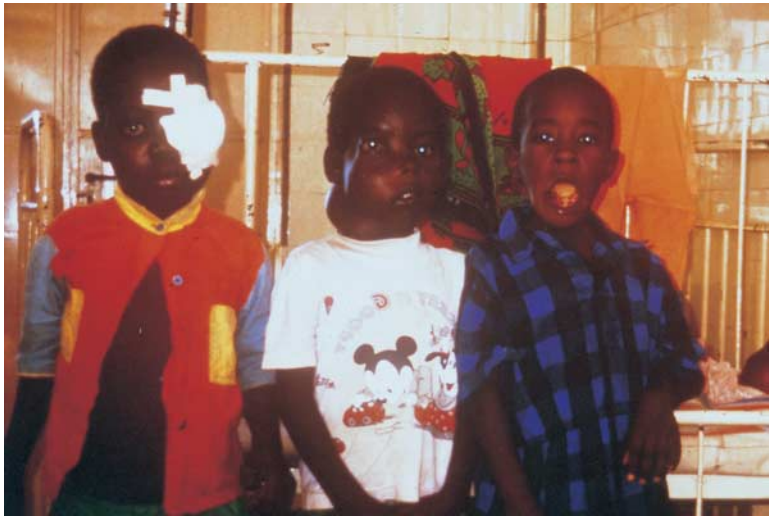

Figure 3 (a) A six-year-old Kenyan girl with eBL of the left maxilla and right mandible. (b) Three Kenyan children presenting with eBL of (left to right) the orbit, maxilla and mandible. © CAF

The ten-year survival rate varies from 30% to 85%, with prognosis dependent on the drugs used, the initial response to the drug regimen and the staging (higher survival for the early stages of the disease (I and II; 86). Most nonresponders will die within three months of diagnosis. Although some patients can attain long-term disease-free survival and therefore be potentially cured, a few may relapse even after 5–10 years, underscoring the need for continued surveillance even in those apparently cured.

Molecular Basis for Lymphomagenesis

Lymphomas, like all malignant tumors, arise as a result of the accumulation, within a single cell, of a set of genetic lesions that result in increased proliferation and clonal life-span. BL is no exception with its complicated biology and molecular pathogenesis, the definition of which continues to present a considerable challenge.

Among the non-Hodgkin's lymphomas, the most frequently observed genetic abnormalities are interchromosomal translocations, which appear to be lineage- and, to a large extent, lymphoma-specific (see below). Such translocations result in the increased or inappropriate expression of crucially important cellular proteins, many of which are transcription factors that regulate expression of other genes. However, these abnormalities *per se* are insufficient to induce a lymphoma and other genetic lesions are required. The likelihood of any given clone of cells accumulating a sufficient number of relevant genetic lesions to give rise to a lymphoma is probably a function of its life-span. Viral genomes (e.g. EBV and HTLV) can prolong survival of a cell clone by inducing the abnormal expression of cellular genes, such as *bcl-2*, which inhibit apoptosis. Amplification of the rate at which these genetic lesions occur is by interaction of inherited and environmental factors, the latter appearing to be the major determinant of incidence rates.

A set of at least eight genetic events is known to contribute to the genesis of eBL: the presence of EBV; the immunoglobulin/*c-myc* B lymphocyte translocation; mutations in *c-myc*, *p53*, *bcl-6* and *bcl-2*; moderate-to-high *bcl-*X_L expression; and, finally, somatic mutations in the immunoglobulin hypervariable regions. The following sections analyze these events in greater detail.

The BL Tumor Cell Has a Germinal Center Origin

Germinal centers are prominent histologic areas of lymph nodes comprising mostly B cells undergoing either extensive proliferation and maturation, or apoptosis (89). When these B cells are stimulated by antigen to proliferate, they undergo extensive somatic hypermutation of the variable regions of the Ig heavy and light chain genes.

The nature of the cell of origin in eBL is unclear but it is thought to be a memory B lymphocyte derived from a germinal center centroblast; a vulnerable and unstable cell prone to genetic error. Thus morphology and cell surface marker analysis show tumor cells positive for CD19, CD20, CD21 and surface Ig (all B cell markers) with a concurrent decrease in LFA-1/2 and increase in CALLA (common acute lymphoblastic leukemia antigen) and BLA (BL-associated) antigens (typical of germinal center centroblasts) (90). The paradox here is that eBL is almost always extranodal. Nevertheless, more recent analysis of immunoglobulin heavy chain variable (V_H) regions used by lymphoma cells confirms the germinal center origin (91). In the process of normal B cell differentiation, somatic hypermutation of V genes occurs in the germinal centers (92) and results in the introduction of point mutations in the variable regions of the gene sequence. If cells expressing the mutated sequences are then exposed to limiting antigen, then stimulation of only those cells that bind antigen most efficiently will result, leading to selection of V gene sequences of "best fit," with unstimulated cells undergoing apoptosis or programmed cell death (93). In the case of EBV-positive B cell tumors, considered as normal B cells "frozen" at a point of neoplastic differentiation, the mutational patterns of the V genes reflect the clonal history of the cell of origin (91). The accumulation of somatic clonal mutations in the V_H genes (94) deriving from the V_H3 family (95) and the pattern of mutation in the antigen contact domains, indicates that Burkitt tumor cells had (a) been exposed to hypermutation and had transversed the germinal center, and (b) been subject to antigenic (malarial?) selection, a situation also true for the follicular lymphomas (91).

Interchromosomal Translocations and Molecular Consequences

The characteristic marker of all Burkitt lymphomas is a nonrandom chromosomal translocation that involves a small segment of the long arm of chromosome 8 to one of three alternative sites on chromosome 14, 22 or 2: t(8;14),

Table 6. Chromosomal translocations in BL.

Translocation	Frequency (%)	Ig gene locus
t(8;14)	75	Heavy chain (V_H or J_H)
t(2;8)	9	κ light chain
t(8;22)	16	λ light chain

t(8;22) or t(2;8), with t(8;14) the most frequent (Table 6). The translocations juxtapose the proto-oncogene, *c-myc*, to one of the Ig loci (57, 50). As a result, *c-myc* becomes deregulated as it comes under the influence of the Ig promotor and the cell is maintained in a proliferative state (*c-myc* expression is associated with the ability of the cell to enter the cell cycle, i.e. to be "competent" to undergo DNA replication). Mutations in *c-myc* also appear necessary for tumorogenesis and around 65% of Burkitt tumor cells exhibit mutations (unrelated to the presence of EBV), mostly within the first 80 residues of exon 2 of the gene (96). These frequently occur at sites of phosphorylation, suggesting that the mutations have a pathogenetic role. The mutations are homozygous in all BL cells tested, implying that they occur *before* the *c-myc*/Ig translocation (96).

At this point it is relevant to mention briefly something about non-Burkitt, non Hodgkin's lymphomas (NBNHLs), in particular the follicular lymphomas, as the chromosomal translocations characterizing these will be discussed in relation to malaria later in the chapter. NBNHLs, notably the high grade lymphomas, show a strong geographical association with eBL in sub-Saharan Africa. The follicular lymphomas and B cell diffuse lymphomas, like BL and many other hematological neoplasms, have a characteristic chromosomal translocation. The t(14;18) (q32;q21) translocation is a complex process which juxtaposes the Ig heavy chain joining (JH) region on chromosome 14 with the *bcl-2* gene on chromosome 18, resulting in a dysregulated expression of *bcl-2* (97). Like the t(8;14) translocation, the t(14;18) is now thought to occur in a germinal center B cell during Ig gene rearrangement (98). The translocations in eBL and follicular lymphoma may thus share a common etiology, in that both have an early B cell origin which has been antigen-activated; a translocation involving the Ig heavy chain on chromosome 14, and a deregulated *bcl-2* gene. Indeed, t(8;14) or t(8;22) and t(14;18) can occur simultaneously within one tumor cell (99–101), a dual translocation associated with a particularly poor prognosis (101).

The clustered breakpoints in the t(14;18) have allowed development of PCR for the detection of the translocation in lymphoma biopsies, hyperplastic lymphoid tissue and peripheral blood lymphocytes (102, 103) to a level of detection of one translocated cell within approximately 10^5 normal cells (104), with surprising results. It was discovered that cells carrying *bcl-2* translocations persist for many years in patients with prolonged remissions following treatment of the lymphoma, with a continued demonstrable presence of the original t(14;18) clone without developing recurrent disease (105). In addition, cells with t(14;18) translocations can also be found in hyperplastic lymph nodes (98, 106), and in the circulation of healthy individuals, the frequency increasing with age (98, 107). This shows that 14;18 translocations are generated regularly in normal individuals and, alone, are insufficient to cause cell transformation, requiring additional oncogenic hits to establish the malignant phenotype (57, 98). It is reasonable to predict that B cells carrying other translocations, including t(8;14), might also occur in the peripheral circulation of normal individuals, with a greater frequency in those at risk of developing malignancy.

Molecular Epidemiology of Translocations

Although BL throughout the world contains the same 8;14 translocations, the breakpoints on chromosome 8 and the combination of breakpoints on the two partner chromosomes in the translocation, differ geographically. Magrath and colleagues, using Southern blotting, showed that whereas in sub-Saharan eBL the breakpoint on chromosome 8 is usually (in 74%) far upstream of *c-myc*, the American and North African sBL have breakpoints within the gene, in the first exon/intron (56%) (108). While ethnic factors cannot be completely excluded, the low incidence of eBL in American negroes, which contrasts with the high incidence of the tumor in black Africans, suggests that one or more environmental factors, acting via the immune system, are the primary determinants of both the incidence and the molecular subtype of the lymphoma. Interestingly, EBV is more often associated with tumors that have breakpoints far 5′ (>5 kb) of *c-myc*, suggesting that EBV might play a role in the production or maintenance of the malignant phenotype (57, 108, 109).

These differences (Fig. 4) have important implications. In tumors in which the breakpoint is in the first exon/intron of *c-myc*, the normal *c-myc* promoters, P1 and P2, become separated from the remainder of the gene and hence are not

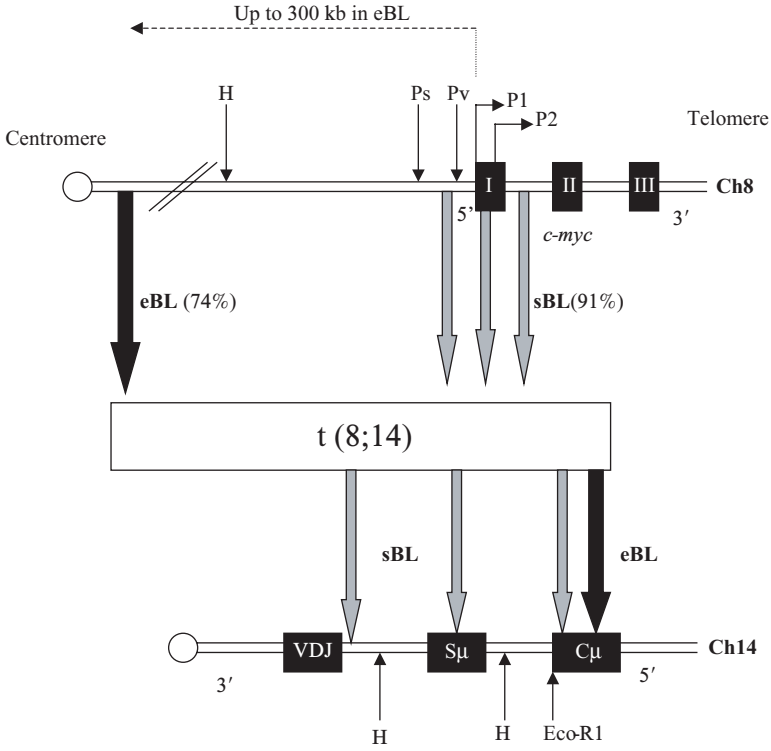

Figure 4 Schematic diagram of the differences between t(8;14) breakpoint locations in eBL and sBL. Most breakpoints in eBL (black arrows) are far 5′ of exon 1 of *c-myc* on Ch8 and translocate predominantly to a nonswitch region on Ch14. Conversely, most sBL breakpoints (gray arrows) on Ch8 are within or just outside exon 1 of *c-myc* and translocate to the switch μ region or just outside. I, II, III: exons of *c-myc*; H_1, P_S, P_V: endonuclease sites; P_1, P_2: predominant *c-myc* promoters. © CAF

available at transcription initiation sites. Moreover, the bulk of the regulatory region of the gene is deleted in such a translocation.

The distribution of breakpoints on chromosome 14 in eBL and sBL likewise differs. Around 30% of the breaks are within the switch ($S\mu$) region in sBL, compared to 20% in eBL (110) (Fig. 4). It is not known precisely how geographical location can influence both the site of chromosomal breakpoints and the presence or absence of EBV.

Accumulation of Multiple Genetic Lesions, Proliferation and Inhibition of Apoptosis

Lymphomagenesis requires that a lymphocyte should have a sufficiently long life-span to permit the accumulation of several genetic lesions; a single genetic accident such as a translocation is insufficient. Genetic lesions are also more likely to occur in cells that are actively proliferating. What do we know about these lesions in the eBL cell?

Although a number of genetic abnormalities have now been documented for BL, their order of appearance remains uncertain. A possible scenario for the evolution of eBL is discussed in the final section of this chapter. It is generally agreed that the life-span of a cell destined to become malignant must be prolonged by one of the first genetic lesions to occur. This may be a translocation event, as occurs in the follicular lymphomas, where the deregulated expression of *bcl-2*, a result of the 14;18 translocation, is clearly an essential component of the pathogenesis of this disease as an antiapoptotic agent. Translocation and mutation of *c-myc* in eBL may similarly lead to abnormal cellular proliferation (110).

Viruses such as EBV and HTLV can also cause persistence of the cell clones they infect, thus predisposing them to malignant change. One of the EBV latent membrane proteins, LMP-1, induces cellular *bcl-2* (36) and the virus encoded EBER RNA (p. 340) similarly upregulates expression of *bcl-2* protein to protect cells from any *c-myc*-induced apoptosis. More recently, a site on LMP-1 that constitutively associates with the tumor necrosis factor receptor 1 (TNFR-1)-associated death domain (TRADD), mediates NF-kappaβ, which is critical for long-term lymphoblastoid proliferation (112).

EBV-positive Burkitt tumor cell lines and fresh tumor isolates, though lacking LMP-1 (and thereby avoiding cell-mediated immune responses), are resistant to Fas-mediated apoptosis (113–115). Fas (CD95 or APO-1) is a cell surface receptor expressed in a broad variety of cells which, when triggered by Fas ligand (FasL) restricted to activated T lymphocytes, activates a cascade of downstream molecular events culminating in apoptosis (116). However, BL cells possess an intact Fas transduction pathway, i.e. Fas resistance is not due to downregulation of Fas as its transcription and protein levels are generally higher than in EBV-negative cell lines (114). An alternative explanation is that mutations in the proapoptotic protein, Bax, which is common to several apoptotic pathways, are involved in the resistance to apoptosis in BL cells (114).

Table 7. Properties of EBV and BL cells allowing establishment of persistence.

(1) EBNA-1 is not immunogenic (related to host protein).
(2) LMP-1 is absent or truncated in BL cells.
(3) *bcrf-1* gene product shares homology to IL-10: subsequent block of IL-2 and IFN-gamma and enhancement of CTL and NK cells.
(4) BL cells show downregulation of:
- HLA Class I
- HLA Class II
- Surface markers LFA3 and ICAM-1

(5) EBNA-2 blocks induction of IFN.
(6) Interaction with cellular oncogenes:
- LMP-1 and EBER RNA induce *bcl-2*: suppression of apoptosis
- *bhrf-1* homology to *bcl-2*: suppression of apoptosis

EBV-positive BL cells possess other mechanisms that permit resistance to apoptosis. For example, the EBV *bhrf-1* gene (Table 7) is homologous to the cellular *bcl-2* gene and, as such, has a similar antiapoptotic function (117). The consistent expression of *bhrf-1* in EBV-associated lymphomas suggests a pathogenic role for this gene product in a similar way to the translocated *bcl-2* protein.

The tumor suppressor antioncogene, *p53*, is necessary for apoptosis and its loss (by mutation or deletion) may lead to a prolonged cellular life-span. The *p53* point mutations found in EBV-positive and -negative BL cells (118) which prevent apoptosis could therefore increase the likelihood that additional mutations will accumulate (57). The proteins *p53* and EBNA-5 also form complexes *in vitro*, the functional significance of which is unknown, although it is noteworthy that EBNA-5 is essential for the B-lymphocyte-immortalizing function of EBV (119).

With this selection of antiapoptotic and proproliferation mechanisms in operation, it is not surprising that the EBV-positive B cell becomes a continuously proliferating one with an extended life-span.

A high frequency of other genomic changes in BL has also been described, notably in the *bcl-6* gene, with amplification up to 20-fold (120) and point mutations (121) in the regulatory regions of the gene, which suggests a role in lymphomagenesis. *Bcl-6* mutations represent a marker of germinal center B cells and thus corroborates the notion that the lymphomas originate from

germinal center lymphocytes (see the subsection "The BL Tumor Cell Has a Germinal Center Origin," p. 329). The assumption that *bcl-6* 5′ mutations may be related to Ig V gene hypermutation provides an explanation for the preferential association of these genetic lesions with eBL and AIDS-related BL (50% and 60% respectively) compared to sBL (only 28%) (121). Both childhood eBL and AIDS-related eBL are thought to be pathogentically related to antigen stimulation induced by chronic infection, which is known to be associated with a very high frequency of Ig V gene somatic mutations (95).

Malaria as a Cofactor

The preceding sections have shown that development of a lymphoma requires both inherited and environmental factors, with the latter being the major determinant of incidence within a given geographical region. What are the cofactors implicated in African BL and how do they operate? Extensive epidemiological and experimental data suggest that, in addition to EBV, other cofactors must be involved to create an accumulation of genetic accidents within the preneoplastic cell. The epidemiological clinical, immunological and other evidence linking malaria as one cofactor is circumstantial although persuasive when taken together (Table 8) (57, 122–124).

Established Geographical and Other Evidence

The role of malaria as a risk or cofactor, perhaps working in concert with EBV, is supported largely from the geographical coincidence of eBL and holoendemic malaria, as discussed earlier. This almost certainly relates to *P. falciparum* transmission; *P. ovale* and *P. malariae* may possibly play a role, although this is considered unlikely. *P. vivax*, although transmitted in East Africa, is of rare occurrence there and its absence in West Africa does not explain cases of eBL found on that side of the continent. The correlation with intensive malaria transmission is demonstrated not only on a macroscale in terms of worldwide distribution, but also on a microscale, as shown by an increased incidence of BL in those areas where there is a high malaria parasite rate (125). However, no marked difference between malarial parasitemia in eBL cases before diagnosis and in controls has been found (54).

An inverse association between eBL and hemoglobin S (HbS) would provide strong evidence for a role of falciparum malaria. Such an association has

Table 8. Summary of evidence linking malaria with the pathogenesis of eBL.

Epidemiological/serological

- eBL is only found in areas of holo- or hyperendemic *P. falciparum* malaria (the "lymphoma belt")
- Within an endemic area, eBL is not found (a) when there are pockets of no malaria, (b) in urban areas
- Within an endemic area, the distribution of eBL is similar to that of hyperreactive malarious splenomegaly
- Peak age incidence of eBL closely follows that of severe falciparum malaria
- Correlation between *P. falciparum* parasite rate and incidence eBL
- Hemoglobin genotype SS and AS may be underrepresented in eBL
- Prophylaxis with chloroquine decreases incidence

Clinical/experimental

- Postmortem specimens from eBL patients show heavy loading with malarial pigment
- Experimental malaria infection (mice) increases the oncogenic potential of tumour viruses

Immunological

(a) *Interaction with EBV*

- Malaria antigens stimulate DNA synthesis in established EBV+ lymphoblastoid cell lines
- Malaria antigens enhance normal lymphocyte transformation by EBV (increased VCA synthesis)
- Lymphocytes from malaria patients spontaneously transform into EBV+ lymphoblastoid cell lines
- Malaria patients show impaired CTL control of EBV
- Lymph nodes from children with malaria have increased numbers of EBER-1/2-positive lymphoblasts

(b) *Polyclonal B cell activation*

- Malaria antigens cause a persistent germinal center hyperactivity

(c) *Cytokine deregulation*

- Malaria stimulates production of cytokines (notably Th2 cytokines) that increase EBV+ tumor development

(d) *Genetic*

- Malaria in children in endemic regions causes increased lymphocyte genetic instability, as evidenced by a greater frequency of (14:18) translocations.

been described, although the study requires repeating with larger cohorts of children. Whether an inverse relationship exists with eBL and other red cell polymorphisms known to protect against severe falciparum malaria requires assessment.

Removing or protecting against a suspect environmental cofactor should, in theory, confirm or disclaim its role in the etiology of the tumor. A trial performed from 1978 to 1982 attempted to suppress malaria in the entire child population of the North Mara region of Tanzania. The aim was to see whether, by distributing minimal doses of chloroquine to all children aged <10 years, the incidence of eBL in the area would fall. Indeed, a decline in BL cases did occur over this period. The catch was that cases had already begun to decline prior to the onset of prophylaxis and that this fall was maintained in a control area where no prophylaxis was given. Measurement of malaria parasitemias showed that just after malaria prophylaxis had been implemented, the prevalence of parasitemia dropped markedly, but from then on climbed gradually despite continued prophylaxis. The reason for this was the progressive emergence of chloroquine-resistant *P. falciparum* (56).

The marked increase (from two- to tenfold, depending on the area) in the incidence of eBL in Uganda (64), Kenya (Facer, unpublished observations) and Malawi (van den Bosch, personal communication) from 1989 to 1995 interestingly mirrors the increasing spread of chloroquine resistance in these countries, tempting speculation that the two conditions are interrelated.

The apparent "eBL-protective" effect of malarial chemoprophylaxis in the early Tanzanian study contrasts with the recent report that chloroquine enhances several viral infections of experimental laboratory animals (126). From this it was proposed that the widespread use of antimalarials in malaria-endemic areas might predispose the population to viral infections (126). The relevance of this to EBV and eBL in Africa is questionable as few children in rural areas, where many cases of eBL occur, have access to antimalarials.

Initial seroepidemiological studies failed to show significant differences in titres of malaria antibodies in eBL patients and controls (125), although these studies need repeating using the more sophisticated immunological techniques available today for analyzing both humoral and cellular immune responses. It would also be of value to ascertain whether or not children with high anti-VCA antibody titres respond differently to defined malaria antigens than do children with lower titres.

Early experimental studies endorsed the theory that, somehow, infection with malaria activated latent viruses, increasing their potential oncogenicity, and the results were convincing. Concurrent infection with *P. berghei yoelli* greatly increased the early incidence of malignant lymphoma (thymoma) in mice infected with the Malony virus (127, 128). Of additional significance was the observation that the virus greatly worsened the malaria-related pathology (elevated parasitemia, anemia, splenomegaly). The combination of an oncogenic virus and a malaria parasite strengthened the pathological effect of each. In another early study, 64% of mice infected with *P. berghei* eventually developed malignant lymphomas of viral origin (129).

Immunological Clues

Immunosuppression and Disrupted Immunosurveillance

As discussed earlier, following a primary infection with EBV, specific HLA-restricted CTLs maintain lifelong surveillance of the viral carrier state. Failure of this cellular surveillance leads to uncontrolled polyclonal proliferation of EBV-immortalized B cells.

The marked immunosuppression typifying infection with malaria (49) extends to defective CTL control of EBV allowing expansion of EBV-infected B cells within the circulation of children (130) and adults with acute (131) and chronic (132) malaria. This expansion reaches five times the normal level to around 0.5×10^6 circulating B cells in Gambian children with acute *P. falciparum* malaria compared to convalescent controls (133). However, this expansion is not great when compared to conditions of severe immunosuppression, as occurs in HIV-positive individuals and transplant patients where the number of EBV genome-positive lymphocytes present in the circulation is respectively 25–45 times and more than 100 times respectively greater than that found in malaria (134). The depressed CTL response to EBV, combined with increased numbers of circulating EBV-positive lymphocytes, explains why lymphocytes from patients with malaria spontaneously transform into LCLs when placed in culture (123, 135). Obviously, during acute (and chronic) malaria, patients are in a state of disequilibrium with regard to host–virus balance, favoring poorly restrained polyclonal outgrowth of EBV-carrying B cells. Nevertheless, as mentioned above, the immunosuppression to EBV in malaria is not as profound as that which occurs in HIV-positive or transplant patients where polyclonal B

cell tumors are commonplace (8, 10, 53). The latter have never been reported in malaria.

Malaria Antigens Interact with EBV-Infected Lymphocytes

Given that lymphocytes from malaria patients spontaneously transform *in vitro*, it was interesting to discover that lymphocytes from healthy EBV-seropositive donors similarly transform when exposed to soluble malaria antigens *in vitro*. LCLs can be obtained from the culture of peripheral blood mononuclear cells (PBMCs) following pulsing with supernatants obtained from *P. falciparum* cultures (Wellcome and K1 strains) (136). This transformation was EBV-dependent since no LCLs were obtained following the addition of phosphonoformic acid (PFA), a viral DNA polymerase inhibitor, along with malaria antigen. Malaria antigens also appeared to stimulate viral replication and the lytic cycle as 4–5% of the cultured lymphocytes expressed the VCA antigen compared to <1% in controls (123, 136), an effect not dissimilar to that described for the tumor-stimulating properties of the phorbol esters. A variety of purified and recombinant malaria antigens failed to bring about transformation with the exception of MSA-1 from several falciparum strains, which repeatedly gave a significant degree of B lymphocyte transformation. This was not a mitogenic effect of the antigens, as control EBV-negative cord lymphocytes failed to transform. Cells from these immortalized cell lines were diploid with normal chromosomal G-banding patterns (136).

EBV in Lymph Nodes from Children with Malaria

It is now acknowledged that the BL cell has a centroblast origin (see the subsection "The BL Tumor Cell Has a Germinal Center Origin," p. 329), although there is uncertainty as to whether this cell becomes infected with EBV extra- or intranodally and whether infection with EBV is the first event in neoplastic transformation rather than a chromosomal translocation. A case for intranodal infection would be strengthened if EBV could be demonstrated within the germinal centers of lymph nodes taken from malaria patients. As normal individuals carry small numbers of circulating EBV-positive B cells, it is not surprising that they are encountered, albeit infrequently, in normal lymphoid tissue (50). However, during prolonged periods of B cell activation, as occurs in chronic infections, their number increases in the circulation and EBV can often be found

in hyperplastic lymph nodes, with the frequency of positive cells varying with the degree of lymphoid hyperplasia and underlying immune status (138).

The *in situ* detection of EBV in individual tumor cells and lymph nodes has been facilitated by development of suitable probes that hybridize with viral RNA. Within latently infected EBV-transformed B cells (and lymphoma cells), most of the EBV genome is either expressed at very low copy numbers (10–100 copies per cell) or not expressed at all. In contrast, the same cells contain very short non-protein-coding EBV transcripts, EBER RNA, that are expressed in great abundance (10^7 copies per cell), making them valuable targets for *in situ* detection of EBV in clinical specimens (139). Both EBER-1 and EBER-2 (whose function is unknown) exist complexed to cellular proteins (140).

Lymph nodes taken from children who had died of severe *P. falciparum* malaria were found to be markedly hyperplastic and were loaded with malaria pigment (141) (Fig. 5(a)). Hybridization with EBER-1 and EBER-2 probes showed only small numbers of extrafollicular positive lymphoblasts (Fig. 5(b) and Table 9), fewer than expected given the increase in numbers of circulating EBV-positive B cells in malaria (133). EBER-positive cells did not express the EBV oncogenic protein LMP-1, indicating that the cells were not in a transformed state. As no germinal center cells were positive for EBV, the results do not provide evidence for viral infection of a centroblast within a germinal center as a first step in the evolution of eBL.

Cytokine Dysregulation

Disruption of normal cytokine networks and predominant expression of Th2 cytokines is a key feature of malaria infections (see Kwiatowsky). In particular, numerous cytokines induced by malaria regulate B cell proliferation, notably IL-10 and IL-6. Serum levels of IL-6 are high in patients due to a combination of parasite IL-6 induction by macrophages and IL-6 production by EBV-infected B cells (142). The serum levels of IL-6 can be predictive of lymphoma development in AIDS patients (143) and one questions whether a similar situation exists for children with malaria.

IL-10, a potent B cell stimulator, sustains the continuous growth of a lymphoma once it becomes fully established (74). The cytokine (both human and viral origin) also suppresses the deletion of LMP-1-expressing tumor cells by CTLs, thereby permitting expansion of the lymphoma.

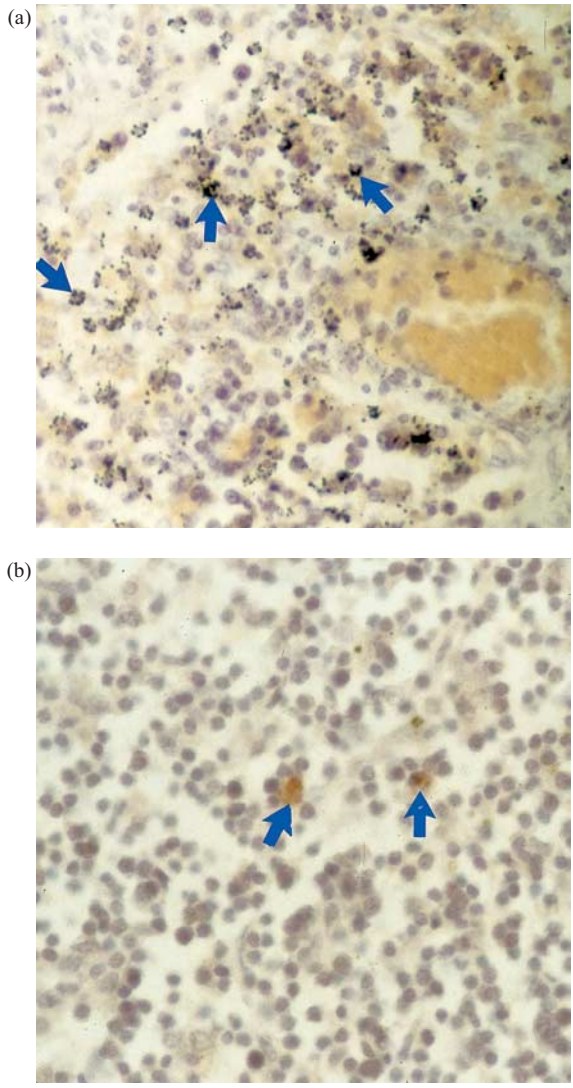

Figure 5 (a) Hyperplastic lymph node from an African child who died of acute *P. falciparum* malaria, showing extrafollicular accumulation of malaria pigment seen as a black deposit (arrows). (×250, haematoxylin) (b) Lymph node from an African child who died of acute *P. falciparum* malaria. *In situ* hybridization with EBER-1 and EBER-2 probes. Positive signal (brown stain) in two extrafollicular lymphoblasts (arrows). (×400) © CAF

Table 9. Number of EBER-1/2-expressing cells in lymph nodes taken at autopsy from African children with *P. falciparum* malaria.

	Number of EBER+ cells per 1 cm^2 section area			
Case	0	1–4	5–10	>10
M/174*		•		
M/175	•			
M/176		•		
M/177	•			
M/178			•	
HD (+ control)†				•
Tonsil (− control)	•			

* Also HIV-positive
† Reed–Sternberg cell staining only
60% (3/5) nodes positive
(20–30% hyperplastic normal nodes)
Extrafollicular
B lymphoblasts
LMP-1-negative

The importance of Th2-associated cytokines in lymphoma development cannot be overemphasized (144). Clinical evidence suggests that growth factors may influence the likelihood that a lymphoma will grow in certain anatomical locations. Thus it has been proposed that the Th2-cytokine-rich schistosome granulomas favor the development of abdominal tumors in Brazilian eBL (80). Similarly, the cytokine-rich reactive lymphoid tissue in the vicinity of the developing molar tooth buds of young children in Africa may predispose to jaw tumor development and growth (77).

In conclusion, the cytokine profile of malaria favors the outgrowth of EBV-infected B cells and is protumor (see Table 10 for summary).

Chronic Antigen Stimulation, Polyclonal B Cell Activation and Germinal Center Hyperactivity

Chronic, multiple antigenic stimulation, clinically manifested by hypergammaglobulinemia, polyclonal B cell activation (with induction of nonspecific and autoreactive antibodies) and germinal center hyperactivity, are postulated to

Table 10. Cytokines found elevated in falciparum malaria and their effect on EBV+ B cells and BL cells.

Effect	Cytokine elevated in malaria	Notes
Pro-EBV		
Pro-lymphoma		
• Suppression of CTL activity: both T cell proliferation and IFN-gamma production	IL-10, IL-8, IL-6, IL-4	EBV LMP-1 activates cellular IL-10 gene. Viral *bcf-1* produces viral IL-10: structurally and functionally homologous to human IL-10.
• Growth factor for EBV+ B cells	IL-10, IL-8, IL-6, IL-4, IL-1,	
• Increase in lymphoma growth	IL-10, IL-8, IL-6, IL-4	High levels of IL-6 in eBL sera. IL-10, IL-8 are autocrine growth factors. BL and LCL also secrete IL-12.
Anti-EBV		
Anti-lymphoma		
• Inhibition of EBV-induced B cell activation	TNF-alpha, IFN-gamma, IFN-alpha, TGF-beta	TNF-alpha: poorer prognosis in NHL for individuals with TNF 2/2 genotype. More severe malaria in African children with TNF 2/2 genotype. TNF-alpha and TNF-beta are both autocrine growth factors

be key elements in the pathogenesis of BL (74). Moreover, all can be found acting in concert during malaria infection (123, 136, 145). Both T-cell-dependent and -independent polyclonal B cell activation have been described. The former is stimulated by superantigen-like malaria products (i.e. those malaria antigens that interact with all T cells expressing a particular Vβ chain, thus stimulating a large number of T cells at any one time) (145); and the latter by antigens with repeated immunogenic epitopes. A feature of *P. falciparum* antigens is the marked number of cross-reactive tandemly repeated epitopes (146) which occur not only within repeats, but between repeats so forming a vast network of cross-reactivity (Fig. 6). The outcome of this is hyperactivity of germinal centers with a high turnover of immature B cells in the process of immunoglobulin gene rearrangement, a vulnerable stage for a genetic accident

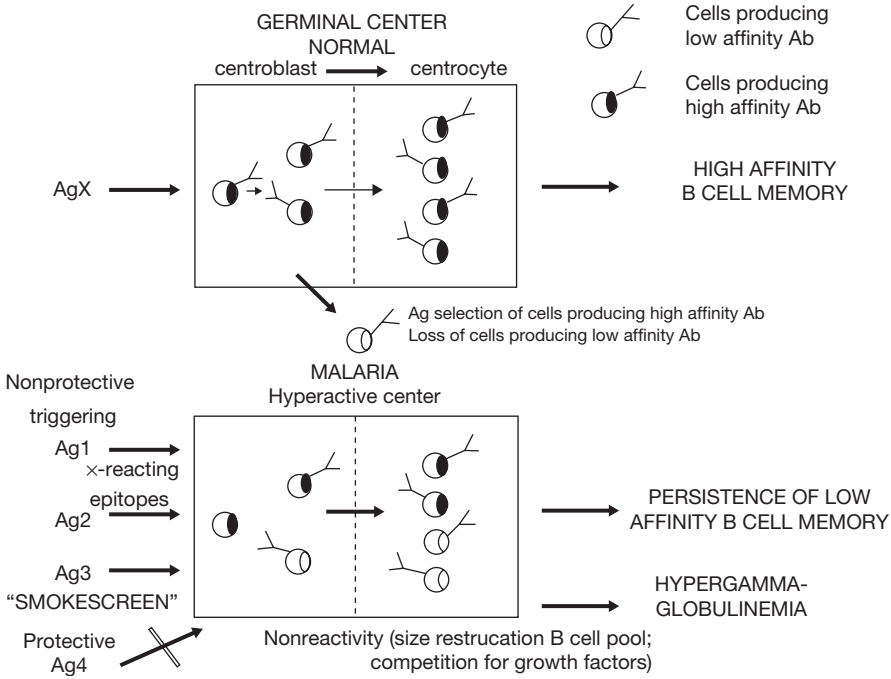

Figure 6 Polyclonal B cell activation and germinal center hyperactivity induced by malaria antigens. In the normal germinal center, an antigen X (AgX) will select those cells producing high affinity antibody (Ab). These cells are retained with the loss (by apoptosis) of B cells producing low affinity Ab. In malaria, parasite-derived antigens (many nonprotective) have repeated cross-reactive epitopes (Ag1, Ag2, Ag3). B cells not selected by Ag1 because of low affinity Ab might cross-react with greater affinity with Ag2 or Ag3 and be preserved. Hyperactive germinal centers result with persistence of low affinity B cell memory and hypergammaglobulinemia—all features of malaria. © CAF

such as a translocation to occur. If a cell carrying a translocation also has concurrent mutations in the *bcl-2* and *p53* genes, then the cell would avoid apoptosis, be preserved and finally be released into the circulation.

Chromosomal Translocations in Children with Malaria

If the hypothesis above is correct, then the premalignant B cells carrying translocations and mutations (but not yet infected with EBV at this stage)

should be detectable in the peripheral blood of malaria-infected children in endemic regions. Analysis of B lymphocytes for translocations is now technically possible using a variety of seminested, nested or long-distance PCR (103, 110, 149, 150). However, there remains a technical problem for the diagnosis of translocations in eBL. The t(8;14)(q24;q32) in eBL shows great variability of the breakpoint region, especially on Ch8, where the breakpoints lie far outside the HindIII fragment, up to 300 kb from the coding regions of *c-myc* (147). This makes a standard PCR ineffective for the detection of the translocation. Even the development of long-distance PCR (which employs more efficient DNA polymerases, so allowing amplification of genomic fragments many kilobases long) has only been successful for the detection of t(8;14) in sporadic cases of BL where the breakpoint on Ch8 is located preferentially within intron 1 or exon 1 of *c-myc* (Fig. 4) (147, 148). Thus it is still not possible to study African endemic BL by this technique, because the region involved is too wide.

Conversely, the great majority of chromosomal (14;18) translocations involve breakpoints within two cluster regions—the major breakpoint region (MBR) lying within the 3′ untranslated region of exon 3 of *bcl-2*, and the less common minor cluster region (MCR) in an intronic segment at least 20 kb 3′ to this (103). This remarkable clustering of breakpoints in *bcl-2* compared with other similar types of translocations, makes the rearrangement particularly suitable for detection by standard PCR in genomic DNA.

Common to the pathogenesis of t(8;14) and t(14;18) is persistent B cell hyperplasia and it is therefore not surprising that the two translocations can appear in the same cell (99–101). As technically it is not possible to screen lymphocytes for t(8;14) in eBL for the reasons given above, the following describes a survey that has been made of the frequency of t(14;18) in Kenyan children, which provides an indication of lymphocyte chromosomal instability in this population exposed to malaria (Facer, unpublished observations).

DNA purified from peripheral blood lymphocytes taken from 199 asymptomatic children (age range 1–10 years) living in the malaria-holoendemic region of Kisumu (Kenya); 65 age-matched children from nonmalarious Nairobi (as malaria controls); and 5 Kenyan children presenting with eBL, was compared for the background frequency of t(14;18) employing a replicate nested PCR (which improves the sensitivity of detection from 1 cell in 10^5 to between 1 in 10^6 and 10^7) with primers designed to hybridize to segments on either side of the translocation. Ch14 primers were one to the 5′ section of the MBR together with a primer to the repetitive consensus sequence that lies

3′ to each of the six joining segment exons (103). Ch8 primers were one to the 3′ section of the MBR together with a primer containing a heptamer from the sequence flanking non-rearranged diversity segments.

Individual t(14;18) clones were distinguished by speed of migration on agarose gels with a size variation of between 50 and 270 bp. Products using these primers had been sequenced earlier and found to be specific for the translocation.

The results are remarkable and relevant to a role for malaria in somehow triggering chromosomal translocations. Thus a large proportion (41.2%) of children with malaria harbor blood lymphocytes carrying t(14;18)—seven times that seen in controls (6.2%) (Fig. 7). The results are also significantly different

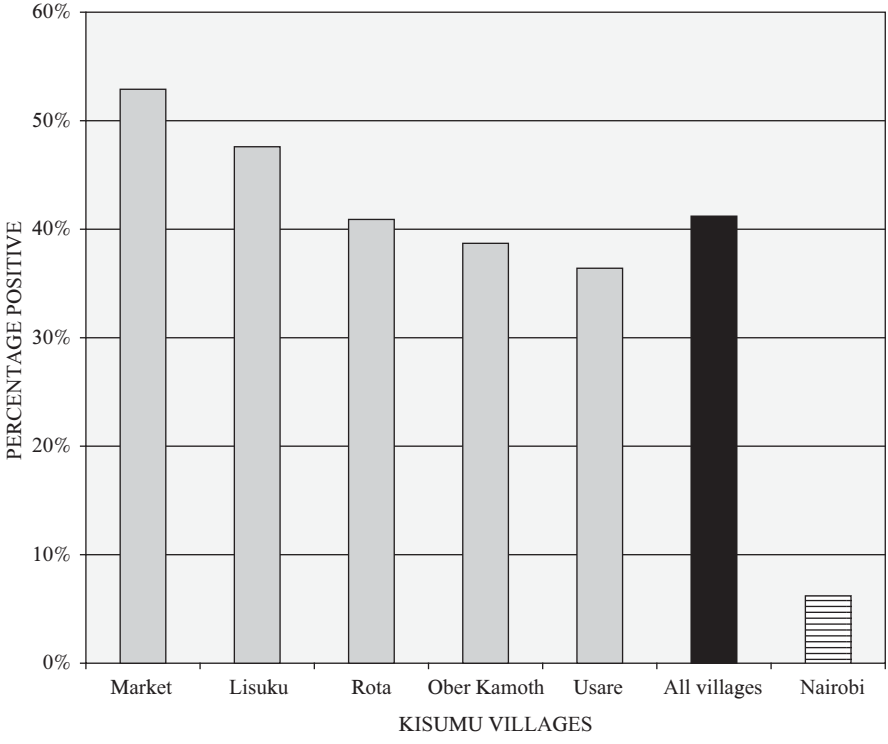

Figure 7 Comparison of the frequency of t(14;18) in peripheral blood lymphocytes from Kenyan children exposed (Kisumu villages) and not exposed (Nairobi) to malaria.

from the one study which looked for t(14;17) in peripheral blood lymphocytes in 12 normal American children aged 1 to 20 years and found 16% positive (107). Interestingly, 40% of eBL patients, albeit a small patient sample size, also carried this translocation (Facer, unpublished observations).

In addition, over twice as many children presenting with a positive thick blood film for malaria (predominantly *P. falciparum* but also some *P. malariae* and *P. ovale*) have t(14;18) clones compared with children who were blood-film-negative (57% vs 23%), supporting a role for malaria antigens in this translocation. The rise in the frequency of t(14;18) with age in 1–8-year-old children, peaking at 6–7 years (Facer, unpublished observations), interestingly mirrors the peak age incidence of eBL.

Just over half (51%) of the lymphocyte samples tested gave a single DNA band on gel analysis, indicating a lymphocyte clone with one breakpoint. A high percentage (49%) had multiple lymphocyte clones (2–10 clones), supporting previous reports that multiple independent t(14;18) events may occur in one individual (Fig. 8). Overall, a total of 41 different t(14;18) breakpoints (with PCR products ranging from 53 to 272 bp) were found in the 82 PCR-positive children from Kisumu (Facer, unpublished observations).

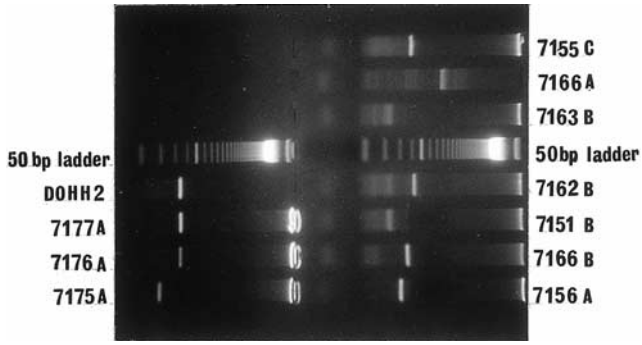

Figure 8 Gel electrophoretic analysis of PCR products for amplified *bcl-2/Ig* gene rearrangements. A nested PCR (MBR only) was performed on DNA from peripheral blood lymphocytes from Kenyan children with malaria. Positive control was a line originally derived from a patient with follicular lymphoma (DOHH2); negative control (not shown; no bands) was a sample of cord lymphocytes. Most samples show one predominant band; several bands in some lanes (7162B) indicate the presence of multiple rearrangements. © CAF

This study demonstrates that premalignant lymphocyte clones harboring the oncogenic (14;18) translocation are commonly present in the peripheral blood of African children exposed to malaria and in children with eBL. This makes the likelihood that other translocations involving Ig loci and associated with lymphoid hyperplasia, such as t(8;14), might also occur, so strengthening a role for malaria in this first genetic accident in the pathogenesis of eBL. Confirmation of this will follow when a more sophisticated long-distance PCR becomes available that will enable detection of breakpoints far 5′ of *c-myc*.

Scenario for the Evolution of eBL

Molecular Pathology

Burkitt's lymphoma, like all malignant tumors, represents the end result of the accumulation, within a single cell, of a set of genetic lesions that result in altered proliferation or increased clonal life-span. Preneoplastic clones must therefore survive sufficiently long enough to amass all the necessary genetic abnormalities for the emergence of a malignant tumor. This, in the case of eBL, is thought to be between two and five years. For a single cell to accumulate the full quota of genetic accidents, and presumably in the correct order following the first event, must be a fairly rare occurrence and might explain why the incidence of eBL is not higher in endemic regions.

To acquire multiple genetic lesions a cell must (i) have a sufficiently long life-span (prolonged by one of the first lesions, (ii) be actively proliferating, as genetic accidents are most frequent during mitoses, and (iii) be genetically unstable.

So what are these genetic events and what is their proposed sequence in an African child with a coinfection with EBV and malaria? The proposed scenario is viewed as a multistep process (Fig. 9).

The first step is the generation of lymphoid cells at high risk for the occurrence of translocations involving Ig loci. This is achieved in malaria by chronic antigen stimulation resulting in *persistent germinal center hyperactivity.* Superimposed on an already extremely high physiological turnover of B cells in germinal centers, this statistically increases the risk of a genetic abnormality occurring in such cells.

Malaria antigen (repeat epitopes) selects a centroblast at a time of Ig gene rearrangement (a genetically unstable cell) and activates that cell to cause the

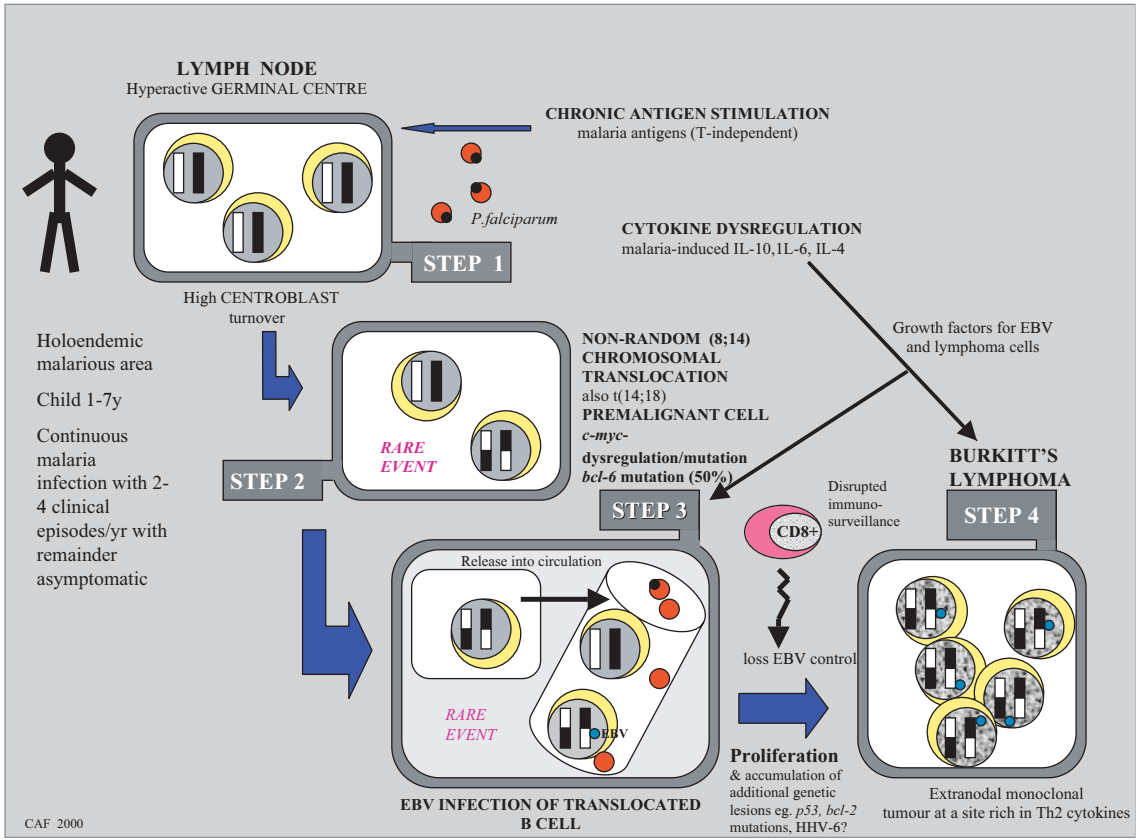

Figure 9 Scenario for the multistep evolution of endemic Burkitt's lymphoma. © CAF

second step and primary genetic abnormality (a relatively rare event), namely a *nonrandom 8;14 (or 8;22 or 2;8) translocation* with consequent expression of the *c-myc* gene (possibly already hypermutated). This effectively means continued expression of *c-myc* and maintenance of the cell in a proliferative state. Very recently, a study of the molecular mechanism involved in the t(14;18) translocation has indicated that the breakpoints contain uncorrected DNA misincorporations (153). This error-prone DNA synthesis has been proposed to be involved in the somatic hypermutation mechanism—an additional mechanism of diversification of Ig genes occurring within germinal centers. During this mechanism, point mutations are introduced in the rearranged V(D)J genes (and surprisingly also in the non-Ig gene, *bcl-6*). These observations raise the interesting possibility that, like the t(8;14) translocation, the t(14;18) translocation also takes place within germinal centers.

Seven times as many children with malaria as children without malaria have B cells carrying 14;18 translocations (Facer, unpublished observations), strengthening the notion that a high B cell turnover in this disease is likely to be responsible for the translocation. From this observation one assumes that the chances of an 8;14 translocation occurring in the same individuals would be fairly high. B lymphocytes carrying translocations are not malignant cells and, in the case of 14;18 translocations, can be found in the peripheral blood of normal individuals (107; Facer, unpublished observations) who never go on to develop malignancy.

Step 3 involves *infection by EBV of a B cell carrying a 8;14 translocation* either within a germinal center (considered unlikely, as no EBV-transformed cells were observed in malaria lymph nodes) or following release into the circulation. The risk of BL is related to the likelihood that a B cell carrying a translocation becomes infected with EBV, a second rare event in the scenario. The association between the breakpoint location and EBV in tumor cells (see the subsection "Molecular Epidemiology of Translocations," p. 331) does imply that EBV might co-operate in the translocation, although EBV is not capable of inducing translocations directly. No chromosomal arrangements are seen in lymphoid cells in infectious mononucleosis *in vivo* and only rarely in LCLs from normal individuals and then of a non-BL type (149). This would place EBV infection *prior to* the translocation event and presumably after the cell has left the germinal center (150). The EBV-driven polyclonal B cell proliferation that results would then cause a translocation. However, this theory does not

explain why the translocation is nonrandom. It is more plausible that the risk of an aberrant genetic recombination involving Ig loci in a B cell is related to the recombinational activity of these cells (i.e. germinal center B cells) rather than to their division (22, 149, 151) and that EBV infection *follows* a translocation event within a cell. There is no doubt, however, that virus infection precedes *malignant* transformation. Thus BL cells have clonal episomal forms of EBV, revealing that these cancers are clonal cellular proliferations that developed *after* viral infection of a single cell (10, 152).

The most recent analysis of the stepwise genetic changes lends further support to this conclusion. Cellular clones derived from the BL cell line (LAM) were characterized by the same *c-myc* rearrangement, had identical IgV gene sequences, yet possessed different EBV fusion termini. Other indications that EBV infection occurred late during lymphomagenesis were the mutations observed in both the *p53* alleles and the rearranged *c-myc* gene. The conclusion was that stimulation by antigen or superantigen initially favored the clonal expansion and accumulation of other cytogenetic changes which occurred prior to or during the germinal center phase of B cell maturation. EBV infection then took place on the exit of the cell from the germinal center, thereby promoting further lymphomagenesis (153–155).

Newly infected with EBV, the lymphocyte carrying the translocation undergoes perpetual proliferation unchecked by the malaria-induced disruption of viral CTL immunosurveillance. Further division increases the likelihood of *acquisition of further mutations* (*bcl-2*; *bcl-6*; *p53*; *ras*, retinoblastoma) and even, it has been suggested, infection with another oncogenic virus, such as HHV-6, all of which drive the cell toward malignancy (Step 4).

A cell that has accumulated all these genetic abnormalities fails to undergo apoptosis and continuously proliferates. Finally, it undergoes rapid clonal expansion into a tumor having found a suitable location, such as the developing molar tooth buds which are rich in cytokines (IL-6 and IL-10), capable of acting as growth factors. The result is an extranodal monoclonal EBV-positive lymphoma.

If eBL arises from a malaria-antigen-stimulated centroblast: (i) the lymphoma cells should, in theory, be expected to produce IgM antibody, (ii) the antibody should have specificity for a repeat sequence of a malaria antigen, and (iii) the antibody would be of low affinity (123). Unfortunately, attempts to examine these suggestions to date have not been successful. Only one third of

Table 11. Unanswered questions.

• Why is the incidence of eBL only 5–10 cases per 100 000 individuals?
• If EBV is essential in the scenario for the development of the lymphoma, why are there EBV-negative SBLs?
• If eBL has a centroblast origin, why is it always extranodal?
• If the immunosuppression of malaria is important, why is it that in other diseases with more profound immunosuppression, e.g. AIDS, the incidence of eBL is not higher?
• Why does malaria selectively predispose to EBV-positive eBL?
• Why does the immunosuppresssion seen in malaria not predispose to EBV-positive *polyclonal* tumors?

eBL cells in culture produce sufficient amounts of Ig and the apparent failure of this to react with *P. falciparum* schizonts (G. Bornkaam, personal communication) may relate to its low affinity.

Some Key Questions

This chapter has focussed on some recent findings that have made a significant contribution to our understanding of the stages involved in the multistep pathogenesis of EBV-positive Burkitt's lymphoma. However, there inevitably remain several unanswered questions that demand further study. These are shown in Table 11.

Perhaps the most difficult question to resolve has been, and still remains, the order and the timing of the oncogenic events that combine to generate the malignant BL cell. Nevertheless, major strides have been made in recent years in the understanding of the pathogenesis of lymphomas at the molecular level. Hopefully this new information, and that to come, will lead to an improved understanding of the neglected African Burkitt's lymphomas, their treatment and, eventually, their prevention with vaccination against the known cofactors, malaria and EBV.

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Chapter 12

Malaria and Blood Transfusion

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Prevention of Transfusion-Transmitted Malaria

History

The first case of transfusion-transmitted malaria (TTM) was reported in 1911, when intercontinental travel was an unattainable dream for most of the world's population, even those resident in affluent countries, and well before commercial air travel became established. Since that time there have been enormous changes in the ways people conduct their lives, such that most tourists are now within reach of highly malarious areas in a flight time of 12 h or less and inexpensive package holidays to the tropics are a reality. Yet there has been a failure to make a sustained impact on malaria transmission in a majority of malarious areas, most notably sub-Saharan Africa, which accounts for most of the world's deaths from malaria, currently estimated at between 1 and 2 million per year. Furthermore, the spread of chloroquine (and, later, multidrug)-resistant *Plasmodium falciparum* malaria, coupled with an ever-increasing number of travelers to its areas of endemicity, has led to an increasing proportion of malarias due to *P. falciparum* among international travelers returning to the temperate zone from trips to malarious areas. In 2000 there were 16 reported deaths in a total of 2069 cases of imported malaria in the UK.

Bruce-Chwatt (1, 2) reviewed worldwide data recorded from 1911 to 1979, during which the reported incidence of TTM rose from 6 to 145 cases per year. In the early years, *P. vivax* was the most frequently reported species, but by the 1950s *P. malariae* predominated, followed by *P. vivax*, *P. falciparum*, mixed infections and *P. ovale*. In the 1970s *P. vivax* was again the commonest, followed

by *P. malariae* and *P. falciparum*, though ominously the proportion of the last had risen substantially.

The last four cases of TTM reported by the UK Blood Services (all in London and southeast England, the last of which was a fatal case of cerebral malaria) were due to *P. falciparum* (3–5). The explanation for this can be seen in the figures for malaria imported into the UK in recent years. Before 1986 there were more cases of *P. vivax* than *P. falciparum*, but since that year *P. falciparum* has been the single most common imported species; indeed, the total for *P. falciparum* now exceeds that for the other three species combined (in 2000, 76% of imported malaria was due to *P. falciparum* (PHLS Malaria Reference Laboratory, personal communication). Figures for imported malaria from 1985 to 1995 reported by other European centers also show a substantial proportion due to *P. falciparum*, for example 82.2% in France and 59.4% in Italy, compared with 38.5% in the US over the same period (6).

In the US, there are approximately 1000 reported malaria cases per annum, compared with approximately 2000 in the UK (6), despite the US population's being approximately four times that of the UK; so one would expect a lower absolute number of TTM cases in the US. Yet, in the years 1990–1999, the absolute number of cases of TTM was higher in the US, with 14 cases, 10 (71%) of them being due to *P. falciparum* (7). Soldan (K. Soldan, personal communication) has compared TTM data for the UK over a 25-year period with that for the USA over 36 years. She calculated a rate of TTM per million donations of 0.055 for the UK and 0.221 for the US, and an incidence of TTM per million person years of 0.003 and 0.009 respectively. This may reflect differences in donor selection procedures or reporting bias.

Transmission of malaria has been reported from red cells, platelets, white cell concentrates, cryoprecipitate and from frozen red cells after thawing and washing (8).

Risk Factors

Risk factors for TTM can be identified from analysis of reported cases and include:

(1) Failure of history taking, such that a history of foreign travel was not elicited (3, 4).
(2) Administrative error in the blood transfusion system, leading to blood collected for plasma being inadvertently issued as whole blood (3). With

current computerized information systems this is now extremely unlikely in the UK.

(3) The use of adenine in preservatives used on stored blood may improve malaria parasites' survival. Thus, improved storage conditions in blood banks may have resulted in malaria parasites surviving in stored blood for longer periods (3). For example, *P. falciparum* was able to survive for 19 days in stored blood and to produce TTM in the recipient (3).

(4) The main risk of introducing malaria parasites into the blood supply comes from semi-immune individuals who are well and asymptomatic despite the presence of a low-grade malarial parasitema in their peripheral blood (9). Whilst such individuals were often born in and are former residents of malaria-endemic areas, it is important to note that expatriates resident for long periods of time in the tropics can also become semi-immune to malaria. For example, a case of TTM due to *P. falciparum* was thought to have arisen from blood donated by a man who had worked in Africa for 10 years, had a history of falciparum malaria, and failed to give a history of malaria or foreign residence when he donated (3). In contrast, those donors without significant malarial immunity are likely to be symptomatic and thus unlikely to attend a donor clinic or, should they attend, be rejected when questioned about their health (4). The potential role of semi-immune individuals as a source of TTM is further illustrated by Mungai *et al*.'s analysis (7) of the characteristics of donors implicated in cases of TTM in the US from 1963 to 1999. Whereas from 1963 to 1969, 45% of 11 donors, and from 1970 to 1979, 38% of 24 donors were former residents of malarious areas or visitors to their country of origin (itself malarious), the figures for these groups rose to 100% of 17 cases from 1980 to 1989 and 91% of 12 cases from 1990 to 1999.

Strategies for Prevention

It must be understood that complete prevention of TTM may not be possible (10). The strategy must be to minimize the risk of introducing malaria parasites into the blood supply, without excluding potential donors unnecessarily.

Identifying Potential Malaria Risk

In nonendemic areas, the criteria for identifying potential malaria risk depend upon screening candidate donors by history taking and, in some instances, by screening of donor serum for antimalarial antibodies.

Important questions are:

(1) The geographical location, i.e. whether or not a potential donor has visited or lived in a malarious area, defined by country. It is not a practical proposition to subdivide countries according to geographical zone within each of them. This would increase substantially the complexity of the assessment, which in itself would likely increase the risk of an error being made.
(2) The length of time an individual was resident in malarious areas; the longer the period of residence, the greater the risk of an individual becoming semi-immune and thus asymptomatic whilst parasitemic. The residency issue should apply whether the person concerned was resident as a child or as an adult, since long-term exposure as an adult can also result in an individual being semi-immune to *P. falciparum*.
(3) The length of time which has elapsed since the individual was last in a malarious area.
(4) Whether or not the candidate donor has a history of past malaria.

Action on deferral (excluding an individual from donating blood) is then based upon the risk factors identified and a knowledge of the time to presentation of malaria in individuals returning to the temperate zone from the tropics.

For example, since July 1995, the guidelines operating in Canada have required that donors reporting a history of diagnosis or treatment of malaria at any time in the past be permanently deferred from donating components for direct transfusion (10).

In the US prospective donors who have had a diagnosis of malaria will be deferred from donating blood for three years after becoming asymptomatic (7).

In the UK prospective donors who have had a diagnosis of malaria are permanently excluded from donation in the absence of a validated malaria antibody test (11).

It is recognized that such a policy will result in some unnecessary deferrals, as it is common to find a history of malaria in individuals from malaria-endemic areas, as fevers are often labeled as malaria on clinical grounds without laboratory confirmation (10). Nevertheless, permanent deferral does provide a useful margin of safety, as this group of potential donors is likely to contain some who are semi-immune to malaria and asymptomatic whilst parasitemic.

Furthermore, a three- or even five-year deferral would not totally exclude semi-immune persons, as cases of TTM have been linked to donations taken

more than five years after the last potential exposure of the donor to malaria. For example, in the US series covering 1963 to 1999 (7) the longest interval between travel to a malarious area and transmission of malaria via a blood transfusion was 5 years for *P. falciparum*, 2.5 years for *P. vivax*, 7 years for *P. ovale* and 44 years for *P. malariae*. Thus, it must be recognized that TTM will still occur, albeit rarely.

Although no set of guidelines can be perfect, current strategies will reduce risk to the minimum, provided they are properly applied. However, given the fact that donor selection still relies heavily on questionnaires and interview techniques, significant failures can still occur. Slinger *et al*. (10) commented: "Donors may give inaccurate information intentionally or unintentionally, or because they misunderstand the question posed, or because they are unaware or have forgotten that they previously have had malaria." Mungai *et al*. (7), in a series from the US, considered whether or not current guidelines were correctly implemented in 60 cases of TTM. Of concern is the fact that the guidelines had been correctly implemented in only 23 out of 60 (38%) cases, 4 of them due to *P. falciparum* and 15 due to *P. malariae*. In contrast, among the 37 cases in which the guidelines had not been correctly implemented, 22 were due to *P. falciparum* but only 3 to *P. malariae*. This suggests that the potentially very long persistence of *P. malariae* at low levels in the blood makes this a more difficult species to exclude, but that correctly followed time exclusion guidelines, even in the absence of an antibody screening test, are more effective in excluding *P. falciparum* as a source of TTM—*provided they are indeed applied*.

Antibody Testing

Draper and Sirr (12) examined sera from 415 known cases of malaria diagnosed in the UK. 88 sera were from UK residents who had traveled abroad and were suffering from their first attack of malaria, whilst 327 sera were from immigrants, who showed a wide range of malaria histories. One week after the onset of clinical symptoms, 78% of UK residents had antibodies to *P. falciparum* antigen by the Indirect Fluorescent Antibody Test (IFAT), but 100% of the immigrants were already seropositive. Furthermore, the immigrant patients also showed higher mean titres, longer persistence of antibodies and greater cross-reactions with other (nonfalciparum) malarial antigens. These findings were felt to be due to an anamnestic response following previous malaria (12), and this can be of value in serological screening of potential blood donors. Draper

and Sirr commented that "a visitor from a hyperendemic malaria area in Africa may have high titres of antibodies reactive to all antigens which may be associated with a low grade asymptomatic infection that is undetectable microscopically". Clearly, the presence of detectable antimalarial antibody could act as a useful marker for the potential presence of malaria parasites in the peripheral blood. Given the potential for malaria parasites to persist in certain patients for some years, as noted earlier, it is reassuring that in individuals who have suffered repeated attacks of malaria, antimalarial antibodies may be detectable for several years. For example, Draper and Sirr (12) studied sera from patients with neurosyphilis who had undergone malaria therapy for this condition, sometimes with several species, and then received radical treatment. 78% (14 of 18) patients still had detectable antibodies 11–20 years after their last attack of malaria, and 70% (12 of 17) were still malaria-seropositive after 20 years or more, one of them after 30 years. Such persistence of antibodies so long after radical cure of the malarial infection would lead to some individuals who are no longer parasitemic, being excluded as potential donors, but does provide a useful margin of safety if candidate donors who are malaria-antibody-positive are excluded from donating.

The IFAT, as used by Draper and Sirr, is still regarded as the "gold standard" for malarial serology, but is relatively labor-intensive and relies on individual observer skills to judge the end-point titre. An antibody detection method based on ELISA has therefore been evaluated as an alternative method, suitable for screening large numbers of samples, as required in transfusion microbiology laboratories. Chiodini *et al*. (4) examined the value of a malaria antibody ELISA with *P. falciparum* antigen on the solid phase in screening sera from potential blood donors for antimalarial antibodies. The ELISA was positive in 52 of 56 (93%) stored IFAT-positive sera. The positivity rate was 24/5311 sera (0.45%) from routine donors not exposed to malaria and 1.5% (15/1000) of donors potentially exposed to malaria in endemic areas. It was possible to test 10 of the 15 ELISA-positive sera by IFAT and 2 were positive. Thus, the ELISA was sufficiently sensitive and specific to screen at-risk donors and the authors estimated that use of the ELISA to test donors with a potential exposure to malarial infection could safely retrieve 40 000 red cell units discarded each year in Great Britain at the time of publication. It is important to note that use of the antibody detection ELISA is *not* recommended for the diagnosis of acute malaria—whilst the same ELISA detected 73% of acute *P. falciparum* infections and 56% of those currently infected with *P. vivax* with clinical symptoms, patients with

acute malaria would be excluded from donation as a result of their being clinically unwell. Furthermore, the vast majority of *P. falciparum* infections present within six months of leaving a malarious area, a time period when they would be excluded from donation by the criteria currently in force. Slinger *et al*. (10) expressed the opinion that malaria antibody detection tests lack both sensitivity and specificity and commented that although technically useful where the prevalence of malaria in donors is high, these tests would probably have a very poor positive predictive value in the Canadian setting and would lead to unnecessary rejection of donors with false-positive test results. In fact, the real situation is quite the reverse—in areas with a high prevalence of malaria, many donors would be malaria-antibody-positive, both from current and from past infection. Thus, given the higher overall prevalence of malaria, a much larger number of potential donors would be excluded unnecessarily (being parasite-negative, despite being truly positive for antimalarial antibodies) than would be the case in a very low prevalence area, such as a nonendemic area seeing only imported malaria. Although antibody from past infections would also lead to unnecessary donor exclusion in nonendemic areas, the absolute numbers would be very low; 1.5% repeatedly reactive by ELISA in the UK study of Chiodini *et al*. (4)—an acceptable level for blood transfusion services in the context of malaria screening to reinstate donors otherwise "lost" because of a possible malaria risk.

Antibody detection assays show high antibody levels and good sensitivity in individuals semi-immune to malaria—the very donors who are potentially at high risk of acting as a source of TTM by being asymptomatic but parasitemic.

In New Zealand, donors are eligible to *donate only plasma* for up to three years after leaving a malaria-endemic area (13). As a result, in the Auckland region alone, approximately 2300 units of red cells are lost each year, equating to approximately 4% of all collected blood. Davidson *et al*. (13) used the same commercial ELISA assessed by Chiodini *et al*. (4) to assess malarial risk blood donors in Auckland. The geographical exposure profile of a total of 515 donors was: Asia, 35%; Africa, 19%; India, 13%; Melanesia, 15%; and multiple geographical areas, 18%. Sera from 530 donors who had traveled to malarious areas tested by ELISA showed 1.7% to be repeatedly antibody-positive (similar to the 1.5% reported by Chiodini *et al*. (4) on UK "tropical area" donors). All 200 sera from random nonexposed donors were ELISA-negative (compared with 0.45% antibody-positive in the London study).

As Davidson *et al*. (13) have indicated, a positive malarial antibody result indicates that the donor has or has had malaria. Thus, it does not necessarily indicate that the person is harboring malaria parasites and so will err on the side of safety if malaria-antibody-positive individuals are excluded as blood donors.

On the other hand, a negative antimalarial antibody test cannot guarantee that the donor is not infected with malaria parasites; antibody may not be detectable in the first few days of malarial illness and infection with nonfalciparum species of the malaria parasite may not be detected by falciparum-antigen-based ELISA. Hence the need to retain a six-month exclusion period alongside antimalarial antibody testing.

Malaria Antigen Detection

Current malaria antigen detection assays are not sufficiently sensitive to exclude totally the presence of malaria parasites in a unit of blood destined for transfusion.

Nucleic Acid Testing

A case report of airport malaria in a French blood donor (14) raises the issue of nucleic acid testing for malaria in transfusion medicine. A worker at Roissy Charles de Gaulle Airport was hospitalized with fever which proved to be *P. falciparum* malaria, despite his never having visited malarious areas or having received a blood transfusion. However, he had worked on aircraft which had returned from Angola. Of concern was the fact that he had donated blood eight days prior to hospitalization, but fortunately the donation had not yet been used. Examination of the donated unit showed negative microscopy of the stained thick blood film and negative antimalarial antibodies. PCR was positive for *P. falciparum*. The authors estimated that the number of infected red cells present lay between 5 (theoretical limit of their PCR assay) and 5×10^3 (level of detection of their thick smears) per ml of pellet and that, if more than 1500 RBCs had been transfused, this level would have been sufficient to induce TTM.

Airport malaria is rare in Europe (less than 100 cases reported since 1977 (14)), so it is not appropriate to exclude blood donors who work in or live near international airports, but this case does raise some questions as to whether or not PCR should be used to screen donations for malaria parasites. PCR

testing of antimalarial antibody-positive donors could usefully demonstrate parasitemas below the level of blood film detection, such that PCR-positive individuals could be offered antimalarial chemotherapy. But a negative PCR test, with current levels of sensitivity, cannot guarantee that a whole unit of packed cells contains no parasites—the theoretical limit of detection for the PCR method used by Thellier *et al*. (14) was five parasites per ml of pellet, but a parasitemia tenfold lower, for example, although undetectable by PCR, would still be able to cause TTM in a patient receiving a whole unit of packed cells.

Comparison of Current Guidelines

UK Strategy

Criteria adopted from 31 July 2001 (11):

Obligatory:

(1) Permanently exclude anyone who has ever been diagnosed with malaria or who has had an undiagnosed febrile illness, which may have been malaria, while abroad or within 4 weeks of return to the UK from a malaria-endemic area.
(2) Defer "residents" from donating blood for 5 years following each return to UK from a malaria-endemic area.
(3) Defer all others for 12 months following each return to UK from a malaria-endemic area.

Definition:

A "malaria area resident" as applied to rule 2 above is someone who has lived in any of the countries of sub-Saharan Africa (except South Africa) or Papua New Guinea for a continuous period of 6 months at any time of life.

Exceptions:

Accept if a validated antibody test for malaria antibody is negative at least 6 months following the date of last potential exposure or the date of recovery from symptoms.

US Strategy (7)

Travelers who are residents of nonmalarious areas who have been in a malarious area may be accepted as donors one year after their return to the

nonmalarious area (irrespective of the use of chemoprophylaxis) if they have been free of malaria symptoms. Immigrants or visitors from endemic areas may be accepted 3 years after departure from the area if they have been asymptomatic. Former residents of malarious areas who now live in the US but who return to visit a malarious area may be accepted 3 years after their last visit. Prospective donors who have had a diagnosis of malaria will be deferred for 3 years after becoming asymptomatic (7).

Discussion of US policy has centered on the length of the deferral period for returning travelers. Writing in the US in 1991, Sazama (15) summed up the question being raised as "whether the current rate of occurrence represents an acceptable balance between risk to recipient and loss of donors, or whether a slightly greater risk may be tolerable". At that time the US was reporting 2–3 new TTM cases a year (0.5 per million transfusion recipients) and deferring 44 000 donors. Commenting on the work of Nahlen *et al*. (16), who advocated shortening the referral interval to 6 months after travel to a malaria-endemic area, irrespective of prophylaxis, Sazama (15) opposed the proposal for a 6-month donor referral interval on the grounds that in an era of demands for zero-risk blood transfusion, the balance had shifted, so that a rate of 0.5 cases per million transfusion recipients (equivalent to 2 or 3 new TTM cases per year in the US) was no longer an acceptable risk relative to the 44 000 donors deferred. Indeed, she felt that the issues at that time were whether the time of exclusion from malaria exposure should be extended, not reduced; whether persons ever infected with *P. malariae* should be permanently deferred (though she did not qualify the issue with respect to treated cases); and what more could be done during donor interviewing to reduce further the risk of TTM. The responses to these important questions must inevitably be tailored to the circumstances surrounding donor services in individual countries, taking into account the geographical origins of their TA donors, the proportion of TA donors in the whole donor pool and the availability or otherwise of malaria antibody screening of potential donors.

French guidelines to prevent TTM were first applied in 1986. Only one case has been reported since then and no cases of TTM have been reported in France since 1994 (14). French rules require the exclusion of donors for 4 months after their return from WHO-defined endemic areas, permanent exclusion of candidate donors with a history of malaria, and screening for antimalarial antibodies in donors presenting between 4 months and 3 years after their return from an endemic area (14).

The Council of Europe recommends exclusion of blood donors for 6 months after their return from an endemic area and they may or may not then be tested for the presence of antimalarial antibodies (14). As at February 2001, 10 European countries had adopted the recommendations (14).

Denmark requires a 1-year exclusion period, and a 3-year exclusion period operates in Ireland. Neither country requires an antimalarial antibody test (14).

Prevention of TTM in Malaria-Endemic Countries

Dodd (17, 18) has summarized strategies for the control of TTM in malaria-endemic countries. His view was that relatively little could be done to prevent transmission. Where appropriate, in countries with uneven malarial risk, attempts can be made to avoid using blood donations from malarious areas to treat patients from nonmalarious areas or areas of low malaria risk. Furthermore, it is possible to avoid collecting blood from individuals with a recent history of malaria. In areas of high endemicity, recipients may be routinely treated with antimalarials as a prophylactic measure (18). It is also important to recognize TTM and to treat infected recipients appropriately (17). Although Tegtmeier has commented that most recipients in endemic areas will be immune to the *Plasmodium* species present where they live, and therefore not adversely affected if they are transfused with blood from an infected donor (19), it must be remembered that those who need a blood transfusion will already be ill, and children receiving infected blood may not yet have become semi-immune to malaria, so lack of an adverse effect from transfused malaria parasites cannot be assumed.

Drug Treatment of TTM

P. malariae, *P. ovale* and most *P. vivax* (there is now some chloroquine-resistant *P. vivax* and one report of chloroquine-resistant *P. malariae* infection) can be treated with chloroquine according to standard treatment protocols. There is no need to follow chloroquine treatment with primaquine therapy in *P. vivax* or *P. ovale* infections as TTM, by definition, is not sporozoite-induced and thus does not involve any liver stages. *P. falciparum* infection is treated according to the likely sensitivity of the parasites in the geographical location from where it is thought to have come (20).

Exchange Transfusion for the Treatment of Severe Falciparum Malaria

Introduction

More than one million people per year are thought to die of malaria, almost all these cases being due to *P. falciparum* (20).

Severity of malaria is correlated with parasitemia. The classical studies of Field and Niven (21) showed a steep rise in the death rate from falciparum malaria at parasitemias above 100 000/μl (2% parasitemia), such that the death rate was greater than 50% with parasitaemias in excess of 500 000/μl. Although their work was published long ago (1937) and a lower death rate might be expected with modern therapy, the association of severe malaria with high parasitemias still holds. There are important caveats: children in areas of stable malaria endemicity may tolerate very high levels of parasitemia without severe symptoms (20) and severe malaria can occur even at low parasitemias, notably in nonimmune patients.

The aims of treatment for *P. falciparum* malaria are to eliminate the malarial parasitemia with antimalarial drugs, and to be alert for and treat appropriately any complications which develop. The rationale for the use of exchange transfusion (ET) in falciparum malaria (20, 22) is: (i) to reduce the parasitemia more rapidly than would be achieved by antimalarial chemotherapy alone; (ii) to remove parasitized cells from the peripheral circulation before they can sequester in the deep tissue capillaries; (iii) to remove parasitized cells from the peripheral circulation to minimize the likelihood of severe intravascular hemolysis and its attendant complications; (iv) to improve the rheological properties of blood by removing parasitized cells and introducing fresh red cells; (v) to reduce rapidly antigen load; (vi) (possibly) to remove circulating toxins or proinflammatory cytokines, immune complexes and free hemoglobin from red cell lysis (23); (vii) to increase systemic oxygen delivery and oxygen consumption (24); (vii) to correct anemia when present (simple blood transfusion could be used for this purpose). There is no doubt that the benefit or otherwise of ET needs to be determined by a randomized controlled study but, although this has been proposed more than once in the past (25), no sufficiently powered randomized controlled study has been reported to date (26). Indeed, given the logistical difficulties both in the tropics (access to intensive care and a safe blood supply) and in the temperate zone (too few cases even

at reference centers to achieve statistical significance), it is very unlikely to be undertaken.

Experience in the Use of Exchange Transfusion

Factors which need to be considered in assessing the pros and cons of ET are: (i) potential reporting bias, with authors more likely to report successful than unsuccessful outcomes following ET; (ii) variation in severity of cases considered for ET; (iii) differences in the amount of blood exchanged; (iv) variation in accuracy of parasitemia estimations—without a centralized reference facility it is impossible to gauge the degree of accuracy with which the percentage of parasitemia for each case was determined. Furthermore, from UK NEQAS parasitology data it is clear that microscopists tend to overestimate the percentage of parasitemia present, especially at higher levels of parasitemia (PL Chiodini, unpublished observations).

Thus, one is left with assessing the potential benefit from a series of case reports and from a few very small controlled studies.

ET plus antimalarial chemotherapy produces a more rapid fall in parasitemia than would be obtained from drug therapy alone. In severe falciparum malaria quinine can be expected to reduce the parasite burden by 50% in about 24 h, provided the parasite is sensitive. Artemisinin, and particularly its derivatives artesunate and artemether, act about twice as fast as this (27). Wilkinson *et al*. (22) developed a mathematical model of ET which relates volume of exchange to reduction in parasitemia and change in haemoglobin concentration. The predicted hemoglobin postexchange was not significantly different from the observed hemoglobin. For parasitemia, the "half" clearance was predicted to occur when 70% of the circulating volume had been exchanged. In fact, ET performed better in practice than was predicted, so the "half" clearance occurred after less than 70% of the circulating volume had been exchanged. The most likely explanation was felt to be the concurrent antimalarial chemotherapy which all the patients received.

Given the lack of an adequately powered randomized controlled study of ET, Riddle *et al*. (26) undertook a meta-analysis of comparison studies to determine whether ET as an adjunct treatment in severe falciparum malaria improves survival. Analysis of 9 studies, involving a total of 287 subjects, indicated that ET was not associated with increased survival compared with antimalarial chemotherapy alone (odds ratio 1.03, 95% CI 0.59–1.79). However, the authors

commented that significant problems in the comparability of treatment groups in the studies precluded a definitive answer.

It is worth examining some of the reported studies in more detail. Hoontrakoon and Suputtamangkol (28), working in Chumphorn, Thailand, undertook a retrospective analysis of 50 patients with severe falciparum malaria. Twenty one individuals who received partial ET as an adjunct to quinine therapy were compared with 29 who received quinine therapy alone.

2–10 units (median 6 units) of whole blood (500 ml per unit) were used in each exchange, depending on the admission parasitemia and the amount of blood donated by patients' relatives or friends. Exchanges usually took 2–3 h for 6–8 units.

The mortality rate of the ET group was 48% compared to 69% for quinine alone, but the difference was not statistically significant ($p = 0.3$).

There was a trend toward higher admission mean parasitemia in the ET group ($18 \pm 5\%$) than in the quinine group ($10 \pm 4\%$) (not significant: $p = 0.1$), and a significantly higher proportion (76%) in the ET group than in the quinine group (38%) had more than 10% parasitemia ($p = 0.03$). In 17 patients, parasite counts after the exchange were recorded and compared with admission levels. Mean admission parasitemia of $28 \pm 2.9\%$ fell to $1.7 \pm 5\%$ after ET ($p =< 0.001$). It is notable that 10 of the 29 quinine-treated patients developed ARDS after admission, compared to 3 of 21 quinine plus ET-treated individuals, and that ARDS was a significant risk factor for death (RR 16.3, 95% CI 1.4–194.2), but the use of partial ET as an adjunct to quinine therapy did not significantly reduce the risk of death compared to quinine alone (RR 0.4, 99% CI $-0.7 - 2.1$).

Many reports of ET in severe falciparum malaria describe its use in nonimmune individuals, treated in nonendemic areas on their return from overseas, for malaria contracted on a visit to a malarious area. Almost all of the patients reported are adults. Burchard *et al*. (29) were able to gather the records of 61 patients, all adult nonimmune Europeans, treated with ET in Germany, Austria and Switzerland, and compare them with data on 63 patients treated in hospitals where ET was generally not performed. They found that ET was applied according to the clinician's subjective impression rather than strict guidelines. The most commonly reported indications for ET were hyperparasitemia and multiorgan involvement. Levels of parasitemia varied from >5 to 70%. In 9 of 61 patients, high parasitemia in the absence of organ complications was

considered an indication for ET (range of parasitemias 6–70%). Organ complications were regarded as an indication for ET only in combination with hyperparasitemia. In the 59 cases for which the volume of the ET was available, the volume varied between 0.3 and 9.9 litres. Survival was not found to depend upon the volume exchanged ($p = 0.97$), but did depend on the level of parasitemia, in accord with previous work. The most important prognostic indicator for death was respiratory failure with artificial respiration, odds ratio 7.2 (CI 2.1–25.9). The authors compared survival in subgroups of patients with or without ET, using the subgroups in the guidelines advocated by Wilkinson *et al*. (22), and found that the probabilities of survival were approximately the same with or without ET. Although the number of patients in these subgroups was small, the authors commented that 3 of 4 patients with parasitemias greater than 30% survived without ET. They went on to say that in their opinion, the theoretical advantages of ET justify its use in extremely ill patients with falciparum malaria, but suggested that strict criteria be applied, for example on the basis of parasitemia (as suggested by Wilkinson *et al*. (22)).

There is no doubt that survival from hyperparasitemia is possible without ET; Fontes and Munhoz (30) described the case of a 16-year-old primigravid female at 32 weeks' gestation with a 46.6% parasitemia who survived with antimalarial chemotherapy, careful supportive treatment and infusion of a total of 4 units of packed red cells.

Mordmüller and Kremsner (31), working in Lambaréné, Gabon, studied 113 children enrolled in prospective chemotherapeutic and immunological trials who had falciparum malaria with more than 10% parasitemia. All the children were treated with chemotherapy alone; blood transfusions were given only when patients were severely anemic (hemoglobin less than 5 mg/dl or haematocrit less than 15%). Eighty-six patients with hyperparasitemia as the sole complication all made uneventful recoveries. A group of 27 patients had complications in addition to hyperparasitemia, but parasitemia levels as high as 81% showed a good response to chemotherapy alone. Only 2 patients died; both had cerebral malaria and other complications in addition to hyperparasitemia of 33% and 63%, with peripheral schizontemia in both cases. The authors concluded that any measure to reduce parasite load, apart from effective chemotherapy, is not indicated for the treatment of malaria, especially when it is potentially dangerous, expensive and labor-intensive. They stated that it would be unfounded to recommend ET for treatment of hyperparasitemia before a

prospective case-control study is performed. However, caution is required in attempting to extrapolate from this study. As Panossian (32) has pointed out, ET was not advocated for widespread use in malaria-endemic areas and the published reports of ET have predominantly concerned nonimmune adults treated in industrialized nations. Furthermore, pediatric patients in holoendemic regions can tolerate high parasite densities without developing cerebral or other end-organ complications. Panossian concluded that "in well-equipped facilities, there are theoretical advantages that justify the use of EBT along with monitored, parenteral chemotherapy for selected seriously ill patients, both non-immune and semi-immune, paediatric and adult, and including those with parasitemia levels as low as 5% as well as those whose parasitemia levels have reached the alarm threshold".

It is also well recognized that patients with hyperparasitemia may show significant clinical deterioration despite parenteral antimalarial treatment, and this is well illustrated by a report from Karnataka, India (33). An adult male whose blood film showed "numerous ring forms of *Plasmodium falciparum*" and who had fever, headache, jaundice and altered sensorium deteriorated after 24 h on parenteral quinine plus oral sulphadoxine pyrimethamine and oral tetracycline, lapsing into deep coma, with deteriorating renal function. Hemoglobin fell to 5.2 g/dl and an 80% parasitemia was recorded. A partial ET with 4 units of packed cells was performed over 3 h. The parasitemia fell to 4.4% immediately after exchange transfusion and the patient's sensorium improved significantly, such that he was fully alert 6 h after the ET. Another group from Karnataka (34) achieved rapid and significant reduction of parasitemia with a partial exchange transfusion of 40 ml/kg (half volume exchange), in 5 children (age range 3–8 years) with severe falciparum malaria. However, there was no comparison group and the experience of Mordmüller and Kremsner in Lambaréné, where children with hyperparasitemia did well on antimalarial chemotherapy without ET (31), must also be considered when deciding whether or not to use ET for children in malaria-endemic areas.

Indications for the Use of Exchange Transfusion

The WHO (20) suggests the following conditions for considering ET:

- Presumed nonimmune patient
- Persisting high parasitemia (i.e. more than 10% of circulating erythrocytes)

- Available compatible blood, screened for viruses (hepatitis, HIV, etc.)
- Facilities for safe exchange and monitoring
- Trained staff available
- Optimal antimalarial chemotherapy already started

The parasitemia-related criteria for ET for hyperparasitemia can be defined as follows (20, 22):

- Parasitemia >30% in the absence of clinical complications.
- Parasitemia >10% in the presence of severe disease, especially cerebral malaria, acute renal failure, adult respiratory distress syndrome, jaundice and severe anemia.
- Parasitemia >10% and failure to respond to optimal chemotherapy after 12–24 h.
- Parasitemia >10% and poor prognostic factors (e.g. elderly patient, late-stage parasites [schizonts] in the peripheral blood).

It is essential that strict criteria are indeed applied when taking the decision to use ET, as one of the factors which make it so difficult to draw firm conclusions as to the value of ET is the substantial variation in criteria for exchange utilized at different centers (26).

It is essential that ET is undertaken only if a safe (especially microbiologically) and fully compatible blood supply is available, and the procedure can be performed in an intensive care or high dependency unit staffed by appropriately trained individuals.

As with any treatment or procedure, the risks and benefits must be carefully evaluated in reaching a decision whether or not to perform ET. Recognized risks are iatrogenic fluid overload, transfusion reaction, transfusion-related infection and line sepsis (22). Cerebral hemorrhage has been reported from both manual ET and erythrocytapheresis. It seems to occur in association with concomitant DIC and high post-procedure hemoglobin >12.0 g/dl (35). The potential benefits from ET increase as the parasite burden increases, because each parasitized red blood cell will inevitably hemolyse, whether or not drug treatment is effective, and a proportion of infected red cells will sequester within hours (22). The benefits of ET are most obvious in nonimmune patients who have not responded to optimal chemotherapy (20).

Techniques for Exchange Transfusion

Wilkinson *et al*. (22) have described a suitable method for manual ET:

Preparation

The patient's hemoglobin concentration, platelet count, clotting times, hepatic function, renal function and blood glucose should be measured. Antimalarial drugs should have been started.

It is essential that the parasitemia should be independently verified by an experienced microscopist—given the tendency for nonspecialist microscopists to overestimate parasitemia levels, it is important to minimize the risk of ET being performed unnecessarily because the level of parasitemia has been overestimated.

Location

The patient should be in an ITU or HDU.

Technique

The patient should have central venous and radial arterial lines inserted. If there is any concern that fluid overload or respiratory distress is present, a pulmonary artery balloon catheter should be considered, giving full attention to the pros and cons of using the catheter in a critically ill patient.

A urinary catheter is essential, as are facilities for hemofiltration, acute hemodialysis or peritoneal dialysis.

Provided meticulous care is taken, ET is technically simple to perform.

Infusion and removal of blood should take place simultaneously to keep the circulating volume constant, with adjustments made to input and venesection rates so as to keep the central venous pressure in the low normal range. The infusion rate can be controlled by an infusion pump and blood withdrawn from the peripheral arterial line or a large-bore venous cannula. It is essential that ET is isovolumetric, so the venesection bag and packs of blood products for infusion should be weighed using a spring balance at the bedside.

In an adult, each 500 ml exchange can be performed over 20–30 min. It is so important that the ET is isovolumetric and that hemodynamic stability is

maintained that the exchange must not be rushed, so a rate of 500 ml every 60 min might be appropriate under some circumstances.

Whole blood, or red cell concentrates plus fresh frozen plasma can be used. The ratio of packed cells to other blood products used in the ET will affect the post exchange hemoglobin, and if the patient is severely anemic at presentation, an initial excess of red cells may be desirable. Platelet concentrates may be required to treat severe thrombocytopenia.

Regarding parenteral quinine, which is still used widely in many nonendemic areas, a small amount of drug will be exchanged and lost, but this is not significant in practice (36) because the volume of distribution of quinine (21/kg), although reduced in severe malaria (37), is much larger than the blood volume. On the other hand, free quinine levels may be expected to rise, since infused blood will be poor in α-1 acid glycoprotein, the main binding protein of quinine.

Immediately after ET, the percentage parasitemia should be rechecked to assess response to the procedure. This will help to decide whether or not additional volumes of blood need to be exchanged. If further ET is being contemplated, a new risk–benefit assessment must be undertaken (22).

Manual exchange has been deployed in almost all reported cases of ET for severe falciparum malaria and, ironically, was used in the treatment of a case of TTM with hyperparasitemia (PL Chiodini, unpublished observations and Ref. 3).

An alternative technique, worthy of consideration, is to use automated cell separator or apheresis hardware and software, which is usually deployed for leuco- or plasma-pheresis (38). In red cell exchange (erythrocytapheresis) the red cell fraction is removed, but the plasma, leucocyte and platelet fractions are returned to the patient along with donor red cells. The ET is thus undertaken as a single continuous-flow isovolemic procedure which is tailored to patient size, hematocrit and thus red cell volume. Macallan *et al*. (38) reported the use of erythrocytapheresis in addition to quinine and supportive treatment in five cases of severe falciparum malaria in travelers returning to the UK from the tropics. Two of them had initially received manual ET (volume exchanged unspecified in the first case, leading to a fall in percentage parasitema from 55 to 28%; 3 units removed, and 4 units of packed cells transfused, in the second case with a rise in percentage parasitemia from 21.5 to 23%). In all five cases, the authors aimed to exchange twice the patient's red cell volume, substantially more than is usually exchanged with the partial ETs described in most published series. The authors

reported dramatic reduction in parasitemia within 2 h in each of their patients, followed by complete clinical recovery. They stated that the response of parasitemia to erythrocytapheresis could be predicted from a single compartment exchange model modified from that of Wilkinson *et al*. (22) and found good agreement between observed and predicted values in most cases.

One patient had a brief episode of hypotension but it is unclear whether or not this was related to erythrocytapheresis. In two cases it was possible partially to correct anemia whilst maintaining isovolemia.

The report claimed significant advantages of erythrocytapheresis over traditional ET in efficacy, speed, hemodynamic control, and retention of plasma and cellular components. The latter point appears to be the major difference between manual ET and erythrocytapheresis. Macallan *et al*. (38) claimed that as plasma is returned to the patient, erythrocytapheresis is likely to lead to less metabolic, immune, coagulation and pharmacokinetic disturbance than is manual ET. On the other hand, some of the pathology of severe malaria has been attributed to circulating parasite-produced "toxins," immune complexes and proinflammatory cytokines. Resolution of the debate between these views will depend upon careful assay of the level of these molecules pre- and post-manual ET versus erythrocytapheresis and correlation of any changes with clinical outcome. However, given the comments already made about a controlled trial of ET, it is very unlikely that a study with adequate power will be possible, unless the clinical outcome of the two methods is dramatically different, which does not appear to be the case so far. Mainwaring *et al*. (39) have also reported successful use of automated red cell exchange for severe falciparum malaria.

ET, whether manual or automated, will remain somewhat controversial unless an adequately powered randomized controlled trial is conducted. Until such a time (and that appears increasingly unlikely (20)) the decision whether or not to use ET must be based on currently available evidence for and against. The WHO (20) comments that, despite the lack of randomized controlled trials, "there is an increasing impression that this technique can be beneficial in very sick patients."

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