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Antigenic variation and immune evasion in *Plasmodium falciparum* malaria

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Summary *Plasmodium falciparum* malaria is responsible for 2 million deaths each year. Even in endemic regions, immunity to malaria builds slowly and is rarely complete. Strategies such as antigenic variation and antigenic diversity are critical to a parasite's ability to evade the host immune response and infect previously exposed individuals. In this short review, the phenomenon of antigenic variation is discussed in relation to immune evasion and its impact on parasite pathogenesis. Recent advances in the understanding of the underlying molecular mechanisms of antigenic variation are examined and questions posed for future research.

Key words: antigenic variation, immune evasion, malaria.

Introduction

Malaria is one of the major global public health problems. Some 400 million people are infected annually and up to 2 million people die of the disease, mainly from infection with *Plasmodium falciparum*, the deadliest of the four human malaria species. Immunity to malaria is slow to develop and relatively unstable. Inhabitants of malaria endemic regions suffer many symptomatic infections in the first few years of life and the majority of deaths occur in children under 5 years old. In areas of unstable malaria transmission, there is substantial morbidity in older age groups and in areas of intense transmission. Even clinically immune adults have parasites detectable in their blood, a condition referred to as concomitant immunity. These features have led to the conclusion that individuals must be infected with a large number of antigenically diverse parasite strains in order to develop clinical immunity and that immunity needs to be boosted regularly. Furthermore, they highlight the importance of parasite immune-evasion strategies in malaria infection, disease and transmission.

The immune response to malaria

The processes involved in natural immunity to malaria are not well understood. The complexity of the human response to infection by *P. falciparum* reflects the multiple stages of the parasite life cycle and the many and varied antigens presented to the host. Antibody clearly plays an important role in anti-malaria immunity, and the protective value of maternal antibody in the first 3–6 months of life has long been recognized, as has the protective capacity of antibody transfused from immune adults to non-immune children.[1] Effector T cells are also known to be of importance to the anti-parasite response[2] but, with the majority of studies being performed in animal models,[3] it is still far from clear exactly how important they are in human malaria. Anti-parasite immunity must represent a combination of these elements, with genetic factors on both sides contributing to the final balance between host and parasite.

To understand the interaction of *P. falciparum* with the host immune system it is important to review briefly the parasite life-cycle (Fig. 1). The feeding of an infected female Anopheline mosquito can result in the intravenous inoculation of spindle-shaped sporozoites, which quickly disappear from the blood and invade hepatocytes. Over the next 10 days or so they divide and form thousands of merozoites which are released into the circulation when the hepatocyte ruptures. The merozoites then invade erythrocytes and the asexual (erythrocytic) cycle commences. During this cycle there is an amplification of the parasite population every 48 h; the invading merozoites mature inside the erythrocyte and develop through the ring and trophozoite stages into dividing schizonts which form other merozoites. The erythrocyte then ruptures and the cycle begins again with the invasion of fresh erythrocytes. Alternatively, a minority of parasites mature into the sexual gametocyte stage which can infect a feeding mosquito and continue transmission of the parasite.

The major arms of the immune response against *P. falciparum* are illustrated in the context of the parasite life cycle in Fig. 1. During their brief journey to the liver, the sporozoites can be rendered non-infective by antibodies against the circumsporozoite (CS) protein, blocking hepatocyte invasion.[4] If the sporozoite does gain entry to the hepatocyte, it may become the target of a cell-mediated response as antigens are processed and presented at the hepatocyte surface, leading to recognition by T cells.[5]

The brunt of both the humoral and cellular response to *P. falciparum* infection is borne by the erythrocytic cycle. The merozoites are the main extracellular component of the asexual cycle, circulating for a short time before invad-

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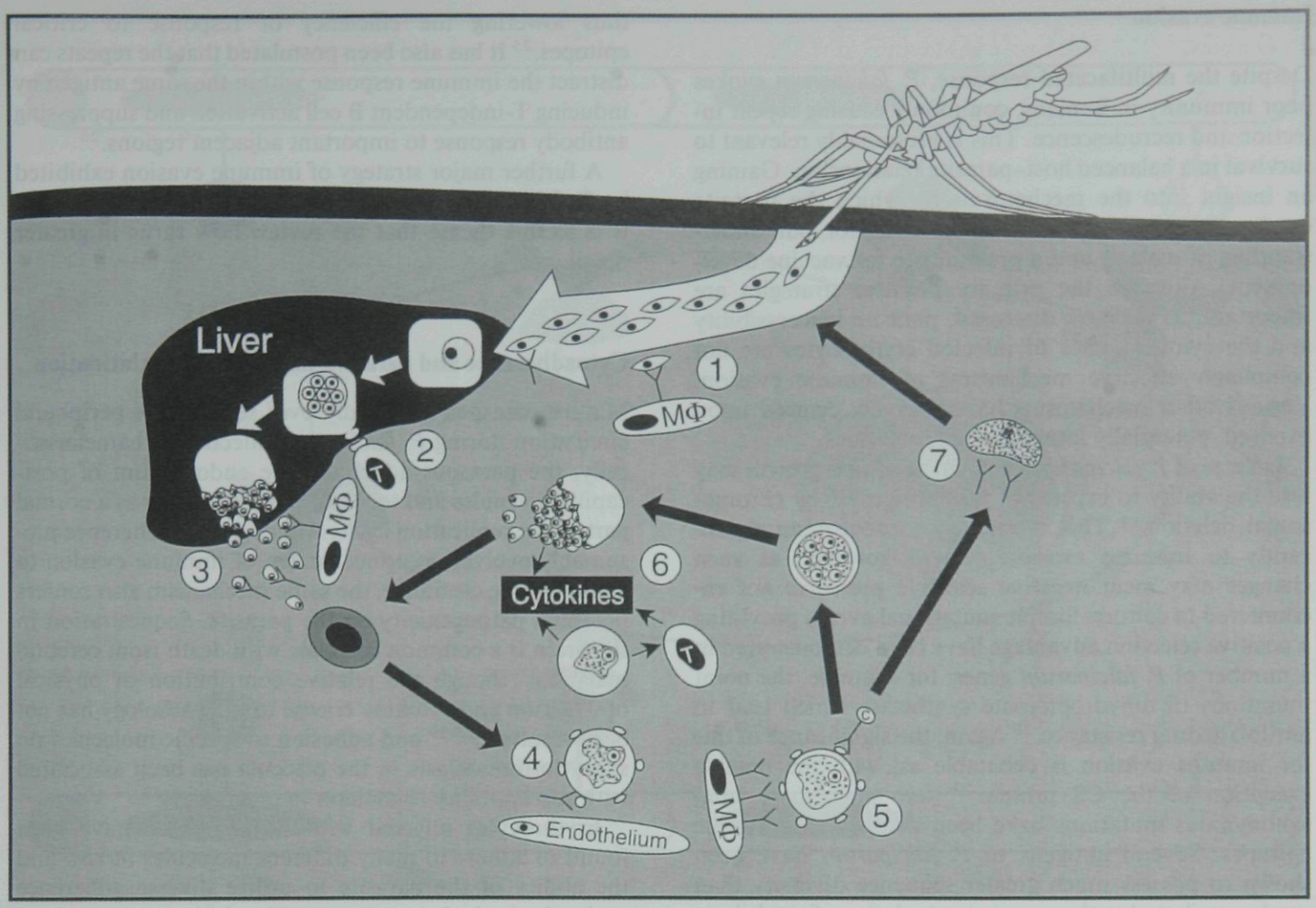

Figure 1 Potential immune responses in the life-cycle of *P. falciparum*. (1) Opsonization of sporozoites and blocking the invasion of hepatocytes. (2) Cell-mediated response to processed antigens at the surface of infected hepatocytes. (3) Blocking of merozoite invasion of erythrocytes. (4) Blocking sequestration of parasite infected erythrocytes. (5) Opsonization and phagocytosis/complement lysis of parasite infected erythrocytes. (6) Cytokine response to parasite toxins and soluble antigens. (7) Transmission blocking anti-gametocyte antibodies.

ing fresh erythrocytes. Antibody-mediated protective responses are directed against surface proteins of the merozoite and can directly block cell invasion,[6] neutralizing essential molecules and preventing antigen processing, or promoting secondary effects such as agglutination and phagocytosis. Such antigens, for example merozoite surface antigens 1 and 2 (MSA-1 and MSA-2) and apical membrane antigen 1 (AMA-1), are the prime candidates for an asexual malaria vaccine.[7] Parasite exotoxins and soluble antigens, such as S-antigen,[8] are released at the time of cell rupture and merozoite escape. The exotoxins can stimulate the production of pro-inflammatory cytokines, particularly TNF-α, IL-1 and IL-6,[9] either directly or via T cell and macrophage interaction. Though the identity of the toxins and the specifics of antitoxic immunity is currently the topic of hot debate as discussed by Schofield and Tachado.[10] Parasite derived glycosylphosphatidylinositol (GPI) has already been shown to stimulate macrophages to produce TNF-α and IL-1.[11] Factors such as TNF-α are known to contribute significantly to the induction of fever[12] and possibly cerebral pathology,[13] suggesting that antitoxic responses play an important part in protection from disease.

Once inside the erythrocyte it would seem that the parasite is in an ideal position, shielded from antibodies and multiplying in a cell unable to process and present antigens to T cells. However, as the parasite matures a number of changes occur at the erythrocyte membrane[14] and circulating parasitized cells are then recognized and destroyed in the spleen. To avoid this fate, mature trophozoites of *P. falciparum* have evolved the capacity to cytoadhere and thus sequester away during cell division, sticking to the endothelium of the small blood vessels in a variety of organs via neo-antigens exported from the parasite and inserted into the erythrocyte membrane.[15] These antigens advertise the presence of the parasite and provide a target for the immune system. Antibodies against the erythrocyte surface antigens can block cytoadherence,[16] and also opsonize the cell for phagocytosis or complement lysis.

A final target of the immune response is not directly protective to the host, yet anti-gametocyte antibodies may well have a role in controlling the transmission of malaria in endemic areas. Antibody to surface proteins on the gametocyte have been shown to interfere with the parasite development in the mosquito, but their importance in the natural cycle of *P. falciparum* transmission has yet to be proven.[17]

Immune evasion

Despite the multifaceted response, *P. falciparum* evokes poor immunity in humans, commonly causing repeat infection and recrudescence. This is presumably relevant to survival in a balanced host–parasite relationship. Gaining an insight into the mechanisms by which the parasite evades the immune system is essential for our understanding of malaria and a prerequisite for vaccine development. Although the primary physical strategies are important, as we have discussed, parasite inaccessibility and the cytoadherence of infected erythrocytes are not completely effective mechanisms of immune evasion. Various other mechanisms have evolved, centred upon exposed, potentially immunogenic molecules.

Isolates of *P. falciparum* adapted to *in vitro* growth may lose the ability to express certain antigens[18] by chromosomal deletion.[19] This is unlikely to contribute significantly to immune evasion *in vivo* however, as such changes may incur negative selective pressures not encountered in culture. Simple mutational events providing a positive selection advantage have been demonstrated in a number of *P. falciparum* genes; for example, the point mutations in dihydropteroate synthetase which lead to antifolate drug resistance.[20] Again, the significance of this for immune evasion is debatable as, with the notable exception of the CS protein,[21] few single base non-synonymous mutations have been mapped to antigenic epitopes. Several antigens of *P. falciparum* have been shown to possess much greater sequence diversity than can be attributed to the recent accumulation of single base mutations. This major sequence diversity has been observed in a number of vaccine candidate antigens and appears to be one of the principal mechanisms of immune evasion.

Antigenic diversity is defined as the expression of antigenically different alleles of a gene in different *P. falciparum* populations. The phenomenon of antigenic polymorphism and diversity has been well reviewed elsewhere[22,23] and will be discussed only briefly here. Immunodominant epitopes containing regions of short sequence repeats in tandem arrays are a common feature of many Plasmodium proteins, and serum from individuals who have had repeated infection have high concentrations of antibodies to these regions. Whereas the repeats in some antigens, such as CS protein and the ring-infected erythrocyte surface antigen (RESA), are conserved, others such as S-antigen, MSA-1 and MSA-2 are highly variable. The production of further antigenic diversity has been shown to be generated through intragenic recombination during meiosis in the (sexual) mosquito stage of the life cycle.[24] Together, these mechanisms generate a wide range of allelic variants to which the immune system must build a protective antibody repertoire.

Tandem repeats may also serve a broader function in immune evasion. The presence of repeats in antigens which are not obviously the target of protective response and the network of cross-reactivities that is apparent between repetitive regions of different antigens, has led to the hypothesis that polymorphic repeats can function as a 'smokescreen', affecting antibody affinity maturation and thus lowering the efficiency of response to critical epitopes.[25] It has also been postulated that the repeats can distract the immune response within the same antigen by inducing T-independent B cell activation and suppressing antibody response to important adjacent regions.[26]

A further major strategy of immune evasion exhibited by *P. falciparum* is that of clonal antigenic variation and it is to this theme that the review now turns in greater detail.

Cytoadherence and parasite-infected cell agglutination

Mature parasites are hardly ever seen in the peripheral circulation during *P. falciparum* infection. Characteristically, the parasites adhere to the endothelium of post-capillary venules and sequester in deep tissues as a normal part of the replication cycle. Although cytoadherence presumably evolved as a mechanism of immune evasion to avoid splenic clearance, the same mechanism also confers potential pathogenicity on the parasite. Sequestration in the brain is a common correlate with death from cerebral malaria,[27] though the relative contribution of physical obstruction and cytokine release to the pathology has not been resolved,[28,29] and adhesion to specific molecules on syncytiotrophoblasts in the placenta has been associated with susceptibility to malaria in pregnancy.[30]

Erythrocytes infected with *P. falciparum* have been found to adhere to many different molecules *in vivo* and the ability of the parasite to utilize diverse adherence molecules in the host will extend its ability to avoid clearance. Unfortunately, this versatility also extends its spectrum of pathogenicity. The major adherence molecules described so far are CD36,[31,32] intercellular adhesion molecule 1 (ICAM-1),[33] chondroitin sulfate A (CSA),[34] thrombospondin,[35] vascular cell adhesion molecule 1 (VCAM-1) and E-selectin.[36]

The parasite-derived molecule on the infected erythrocyte surface which has been implicated as the adhesion mediator is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1).[37] This is a high molecular weight protein, accessible to surface labelling and soluble in SDS, yet insoluble in Triton X-100.[38,39] PfEMP1 is usually present at the infected erythrocyte surface in association with knob like structures[40,41] (Fig. 2), but the details of this association and its function have not been unravelled.

Exposed to the host immune response, PfEMP1 is the target of isolate-specific agglutinating antibodies which are detectable in the convalescent serum of malaria patients.[42,43] Studies in The Gambia measuring a number of potential indicators of immunity to blood-stage antigens, showed that the agglutinating antibodies were the only predictive index for protection against clinical malaria.[44] Whilst this is not to suggest that these antibodies are the only protective response, it does suggest the importance of this target to both parasite and host. Field studies examining agglutinating antibodies in the serum of convalescent and immune individuals have revealed extreme diversity. For example, when we examined *P. falciparum* isolates from 20 Papua New Guinean children by performing agglutination assays with a panel of adult

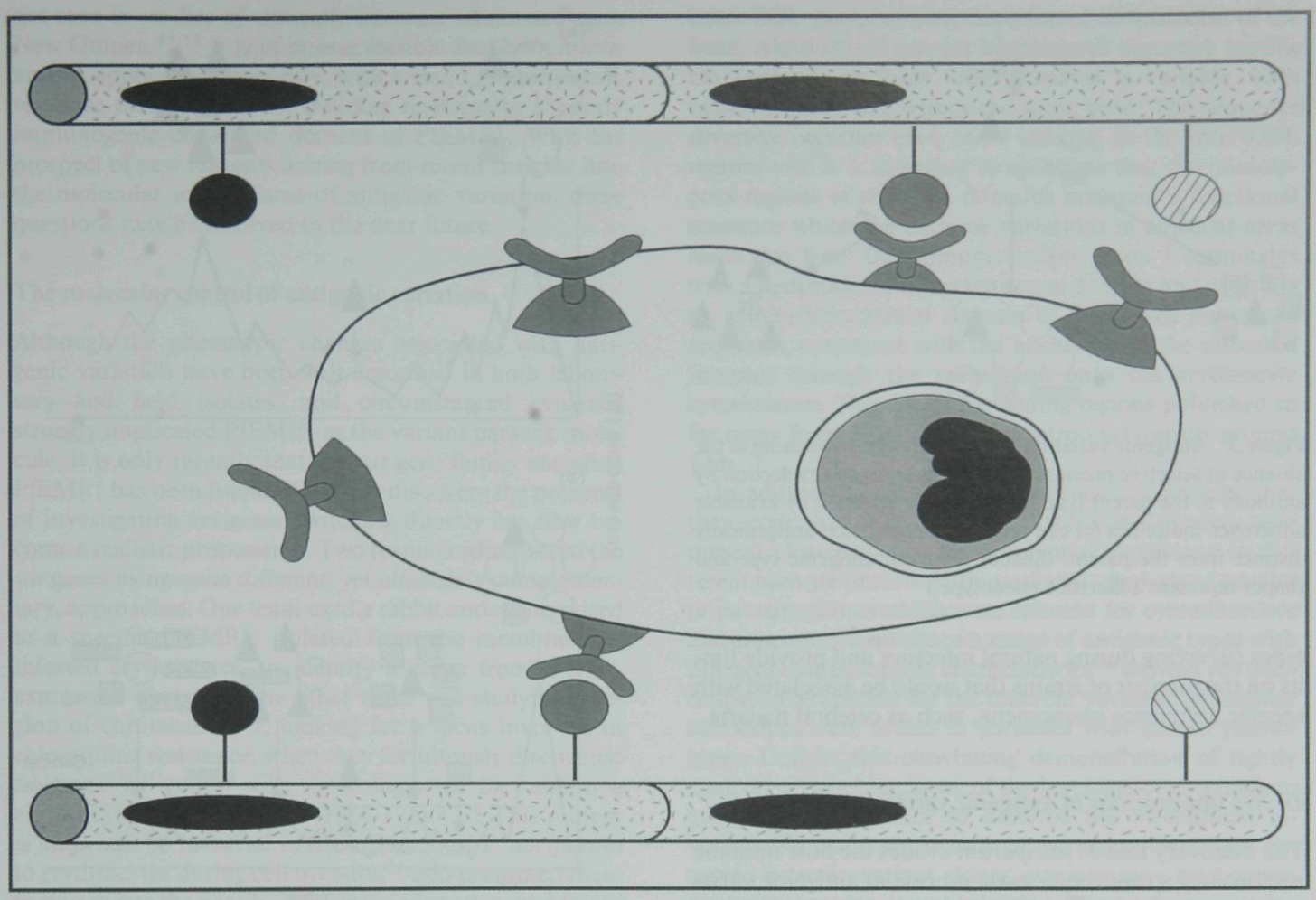

Figure 2 Infected erythrocytes adhere to molecules expressed on venous endothelium via the parasite derived protein PfEMP1, presented on knob like structures on the erythrocyte surface.

immune sera and the children's own sera, no two parasite isolates had the same antigenic type.[45] Furthermore, only two children appeared to have had previous encounters with any of the other 19 isolates. Considering the strong likelihood of each child having multiple past infections, this hints at a huge pool of different antigenic types in the community. This degree of diversity reflects a major mechanism of parasite immune-evasion, acting through variation of the exposed antigen.

Antigenic variation

In contrast to the definition of antigenic diversity given earlier, antigenic variation is the process by which a clonal parasite population can switch its antigenic phenotype. This important mechanism of immune evasion has been extensively studied in other parasites, particularly the African trypanosome where different surface coat glycoproteins are expressed during the course of infection,[46] but antigenic variation has only recently been demonstrated in *P. falciparum*.[43]

The suggestion that antigenic variation occurs in human malaria arose from studies on animal Plasmodium species. The first demonstration of the phenomenon was in the mid-1960s by Brown and Brown.[47] First they used a schizont-infected cell agglutination (SICA) test to show that if a rhesus monkey was infected with a single strain of *Plasmodium knowlesi* and drug cured, serum collected 2 weeks later caused specific agglutination of erythrocytes infected with this parasite. When they reinfected the monkey with the same strain, a parasite population was recovered which was not recognised by the serum from the first convalescent bleed. Antigenic variation was subsequently also demonstrated in *Plasmodium chabaudi* infection of mice[48] and *Plasmodium fragile* in torque monkeys.[49]

Proof of antigenic variation of *P. falciparum* was achieved *in vitro* by demonstrating that, in the absence of selective pressure, parasites could be cloned from a single-strain culture which were serologically distinct from the parent line.[43,50] Spontaneous emergence of antigenic variants was estimated to occur at the surprisingly high rate of over 2% per generation (Fig. 3). The antigenic switching observed was accompanied by size changes in PfEMP1, as demonstrated by immunoprecipitation.[43]

Another interesting aspect of this work was the demonstration that antigenic switching was accompanied by changes in cytoadherence properties, suggesting a link between dominant antigenic type and binding phenotypes. The question of comodulation was addressed in greater detail by selecting cloned lines for adherence to venular endothelium and by demonstrating that this selected particular antigenic variants.[51] These results led to the speculation that *in vivo* requirements for cytoadherence might restrict the number of antigenically distinct

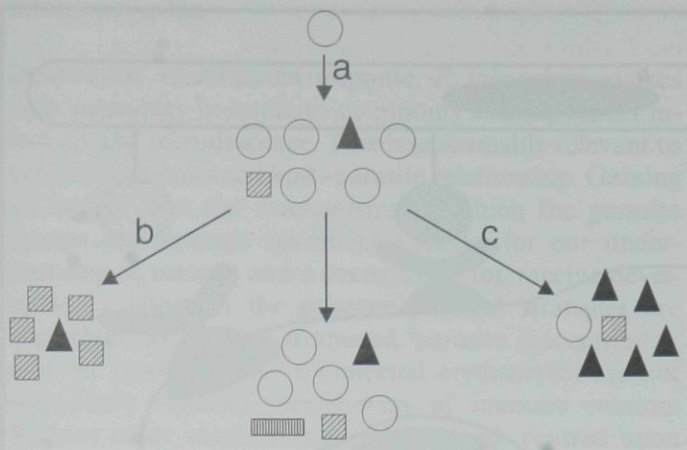

Figure 3 Antigenic variation causes phenotypic switching in the absence of selective pressure (a), and either negative selection by antibody to the parent type (b) or positive selection by available adherence molecules (c) can result in a population antigenically distinct from the parent. (Shades represent antigenic type and shapes represent adherence phenotype.)

types surviving during natural infection and provide limits on the number of strains that would be associated with specific adherence phenomena, such as cerebral malaria.

In vivo implications of antigenic variation

The discovery that *P. falciparum* evades the host immune response by a rapid, stochastic process of antigenic variation has many implications for the *in vivo* growth of parasites. In the host, the parasites will be subject to a number of constraints absent from the *in vitro* experiments, particularly the production of antibody and the relative levels of expression of adhesion molecules on the host cells (Fig. 3). As antibody is produced to a new infecting strain, the survival of the parasite will be determined by the rate at which new variants, not recognized by pre-existing antibody, are generated; the ability of the new variant to adhere, thus avoiding entrapment in the spleen; and the rate at which new antibody is produced. The relationship of these rates is critical in determining the course of infection. In Fig. 4 for example, it is postulated that a variant surviving pre-existing and primary antibody response, may go on to cause either a recrudescence of illness or a chronic asymptomatic infection, depending on the effectiveness of secondary and subsequent antibody responses.

As the negative selective pressure of antibody is applied to the parasite population, a positive selective pressure may also be exerted because of the link between antigenicity and cytoadherence. The main determinant of which variant predominates is the availability of specific adhesion molecules on the host tissue. After a few infective episodes during which the most common or highest affinity adhesion molecules are utilized, rarer variants will begin to be selected which target less widely distributed host molecules and their appearance could determine the relationship between exposure and specific disease outcomes such as cerebral malaria. Another level of complexity is added by the ability of cytokines to up-regulate a

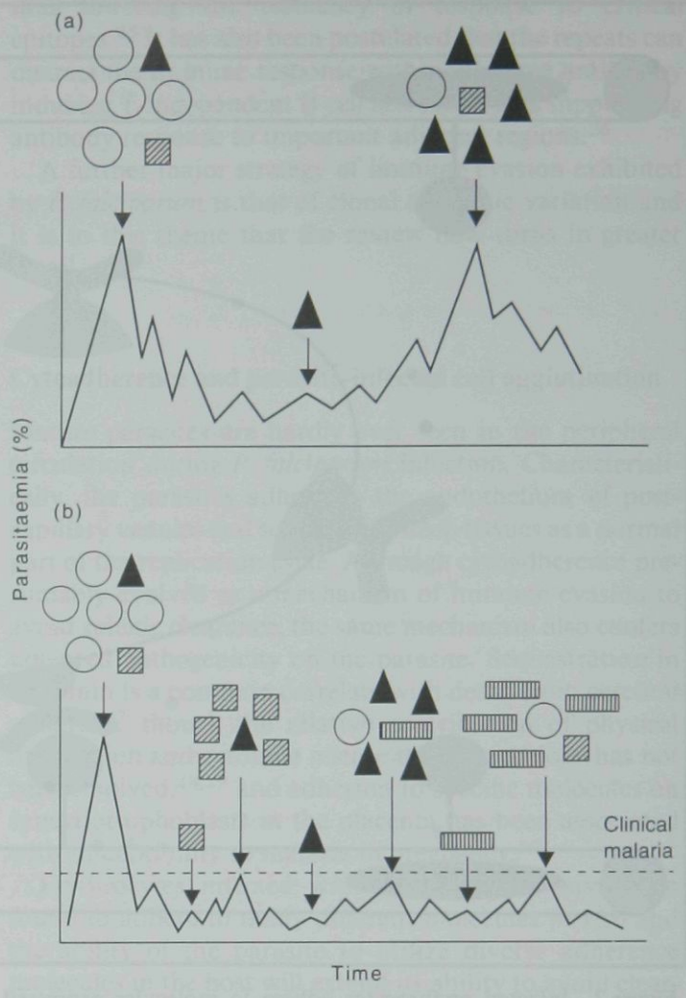

Figure 4 (a) Antigenic variants may escape the primary antibody response and multiply to cause recrudescent malaria. (b) When the generation of novel antigenic variants and antibody response is balanced chronic sub-clinical infection may result. (Shades represent antigenic type and shapes represent adherence phenotype.)

variety of host-cell adhesion molecules.[52] Positive selection forces may then vary independently of antibody response. Thus, it seems likely that the parasite possesses quite distinct cell-surface phenotypes and adherence properties at different time points during malaria infection. Intriguingly, if this proposed dynamic is a true reflection of the host parasite interaction, it suggests a mechanism by which the host might acquire antibodies of several specificities during infection with a single strain.

How natural immunity to malaria is acquired in the face of the potentially huge number of variant antigenic types generated by the parasite is the subject of much speculation and more than a little controversy. The main point of argument is whether immunity is strain-specific and achieved by exposure to a finite set of antigenic variants,[53] or strain-transcending and reliant on immunity to a common, poorly immunogenic epitope. The strain-specific theory of immunity is an attractive paradigm, though field studies in Africa have contradicted this a little by suggesting the presence of a so-called 'pan-agglutinating' antibody,[54] capable of recognizing virtually all strains. However, the pan-agglutinating response was

not seen in studies of clinically immune adults in Papua New Guinea.[45,55] It is of course feasible that both mechanisms apply simultaneously, with a rapid strain-specific response preceding a slow-building response to a poorly immunogenic conserved domain of PfEMP1. With the prospect of new reagents arising from recent insights into the molecular mechanisms of antigenic variation, these questions may be resolved in the near future.

The molecular control of antigenic variation

Although the phenotypic changes associated with antigenic variation have been well described in both laboratory and field isolates, and circumstantial evidence strongly implicated PfEMP1 as the variant parasite molecule, it is only recently that the *var* gene family encoding PfEMP1 has been found. With this discovery the prospect of investigating antigenic switching directly has now become a realistic proposition. Two teams co-discovered the *var* genes using quite different, yet ultimately complementary, approaches. One team used a rabbit antiserum raised to a specific PfEMP1, isolated from the membrane of infected erythrocytes, to identify a clone from a DNA expression library.[56] The other team was studying a region of chromosome 7, looking for a locus involved in chloroquine resistance, when they fortuitously discovered a series of genes with homology to the adhesive erythrocyte-binding protein (EBA-175).[57,58] This protein is known to be involved in the adhesion of *P. falciparum* to erythrocytes during cell invasion[59] and prompted them to investigate the possibility that the genes they had found may also be involved in cell adherence.

The picture that emerges from this long-awaited discovery is of a large and varied family of *var* genes distributed throughout the genome. The number of genes present was estimated to be 50–150, accounting for 2–6% of the genome. This huge genetic investment certainly supports the supposition that antigenic variation is a vital mechanism for the parasite. Although there was considerable variation in the handful of genes sequenced, certain family characteristics emerged, consistent with a variable cellular adhesion molecule (Fig. 5).

A major feature of the two-exon *var* gene is the presence of a variable number of cysteine-rich extracellular domains with homology to the glycophorin A binding molecule EBA-175 of *P. falciparum* and the Duffy blood group antigen-binding receptor of *P. vivax*. The first Duffy binding-like (DBL) domain and the adjacent cystine-rich inter-domain region (CIDR) form a head structure which is the most conserved region between different *var* genes. This relative conservation hints at a functional role for the head, possibly adhesive specificity. Between one and three other DBL domains have been found downstream of the head. Although all possess homologous signature motifs, the sequence of these DBL domains is variable, both between genes and within the same gene. The sequence diversity becomes even more striking in the inter-DBL regions and it is tempting to speculate that the homologous regions of the DBL domains maintain a functional structure whilst the extreme variations in adjacent areas mask this from the immune system. Exon 1 terminates with a hydrophobic transmembrane domain and exon 2 is an acidic, intracellular domain of fairly well conserved sequence, consistent with the anchoring of the adhesion receptor through the membrane onto the erythrocyte cytoskeleton. The size of the coding regions published so far range from 8–10 kb and the intron is typically around 1 kb.

In addition to cloning the *var* genes and proving that they code for PfEMP1, the three initial papers also present clear evidence for differential expression in different parasite lines.[56–58] In particular, analysis of a series of parasite clones which were selected for cytoadherence and subcloned to produce a range of antigenic types with different cytoadherence characteristics[58] showed *var* transcripts to be specific for the different variants, yet similar transcripts were found in parasites with shared phenotypes. Despite this convincing demonstration of tightly controlled switching, none of the investigators was able to advance any evidence to describe the mechanism involved. No major chromosomal rearrangements were observed between variant clones, nor was gene duplication apparent, making it unlikely that Plasmodium imitates the Trypanosomes with their well documented mechanism of duplicative transposition.[46] However, with many alternative possibilities for pretranscriptional, transcriptional and post-transcriptional control described in other systems,[60] this does not narrow the field down appreciably. A single outstanding feature observed was the presence of many 'sterile' transcripts related to exon 2, which may have some involvement in gene rearrangement or expression.

Whilst the secret of the switching mechanism remains intact, some insight into the generation of the great diversity seen amongst the family has been gained by the chromosomal mapping of *var* genes.[61] In studies concentrating on a contiguous yeast artificial chromosome (YAC) map of *P. falciparum* chromosome 12, it was shown that *var* genes could be present in either central or subtelomeric regions. The subtelomeric genes were found to be interspersed amongst repetitive elements, which are known to be a 'hotspot' for deletion, healing and recombination events,[62] leading to speculation that this would generate considerable gene mixing and diversity. The further find-

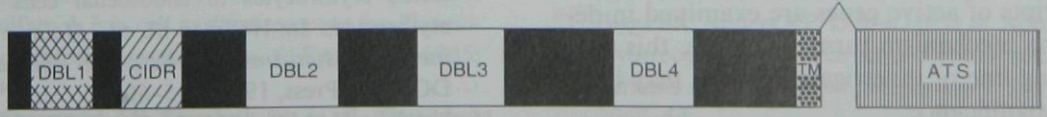

Figure 5 A schematic representation of a *var* gene (Su *et al.* 1996). A head region comprising of a Duffy binding-like domain (DBL) and a cysteine-rich inter-domain region (CIDR) is followed by one to four DBL domains. A single intron is preceded by a transmembrane segment (TM) and followed by an acidic terminal segment (ATS).

ing that the subtelomeric chromosome 12 gene examined has close homologies with *var* genes at the other end of the same chromosome and the subtelomeric regions of two other chromosomes, gives additional weight to this theory of heterologous recombination. It remains to be seen whether both central and subtelomeric genes are capable of being expressed, as one would anticipate a degree of redundancy in such a variable gene family.

The future

Antigenic variation is a major component of the immune evasion strategy of *P. falciparum* and an important factor in the parasite/host balance. The comodulation of the antigenic and adherence phenotypes of the parasite-derived erythrocyte neo-antigen PfEMP1 has implications not only for the pathogenesis of individual infections but also for the acquisition of immunity and transmission of disease within communities. An understanding of the mechanisms and dynamics of variation is therefore essential if we wish to control the disease and develop new therapies for its prevention and treatment.

Much of the field work done to this point, valuable as it has been, has concentrated on reporting the phenomenology of variation. Even the most basic questions about the rates of switching in human infection, relationship of variants to disease and acquisition of a protective repertoire of agglutinating antibodies remain unanswered, because of the lack of appropriate reagents to directly investigate them. With the identification and cloning of the *var* gene family the opportunity to create such reagents is becoming practicable.

A research priority is to clone genes expressed in parasites of characterised phenotype and identify specific binding motifs. Whether such motifs will lie in the DBL domains or in cysteine rich regions such as the CIDR, and if they will even be identifiable until the secondary structure of the molecule is solved, cannot be predicted. What is certain is the usefulness of phenotype specific probes for investigating many of the outstanding questions raised by field studies and the great therapeutic potential of adhesion blocking antibodies and peptides. A particular priority is the search for conserved, cross-reactive determinants, which may form the basis for an adhesion blocking vaccine.

The mechanism of gene switching is another pressing area for research. Genes which are differentially expressed when particular phenotypes are selected need to be studied in detail, and compared to the parent lines, to look for changes related to expression. At this stage, the upstream promoter regions will be of particular interest as transcriptional control is still a distinct possibility. Clues toward post-transcriptional modification may also become apparent when transcripts of active genes are examined in detail. Whatever the mechanism turns out to be, this promises a fascinating line of investigation which may have broad biological significance.

Other research questions of interest include further study of the generation of diversity in the *var* gene family and its relationship to location in the genome. Many questions are also raised about the trafficking of the protein from the parasite to the erythrocyte surface, its localisation and relationship to other molecules present in this region.

The study of antigenic variation of *P. falciparum* has recently passed a watershed with the discovery of the *var* gene family. We now look forward to an exciting period in malaria research, as the tools become available to tackle the fundamental questions of this important parasite phenomenon.

Acknowledgements

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