Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *87*, Suppl. V, 137-144, 1992

VIRULENCE AND THE IMMUNE RESPONSE IN MALARIA

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Many factors determine the virulence of a malaria infection. These include host innate resistance mechanisms and, with Plasmodium falciparum, *the ability to cytoadhere to endothelial cells, form rosettes, and induce release of cytokines. The effect on virulence of acquired immune responses can be determined by Class I and Class II MHC-antigens; levels of immunological responsiveness may be determined too in other ways. The structure of parasite surface antigens and their great diversity modulate the immune response and influence parasite survival and hence virulence, and transmission to the vector.*

Key words: malaria – virulence – innate immunity – cytoadherence – acquired immunity – MHC – antigenic diversity – transmission

Current views on virulence, a property of parasites that in comparative terms makes them more or less likely to cause severe disease, are very different from those of only a few years ago. Then, virulence was viewed as a consequence of a poorly developed host-parasite relationship; it was generally assumed that infections producing few clinical symptoms and with little risk of the host dying were the long established and best adapted associations. Conversely, severe disease and/or death as a result of parasitic infection reflected a much more recent and hence poorly developed association – a mismatch in some cases. According to this view *Plasmodium falciparum* is less well adapted to its human host than *P. vivax, P. ovale* or *P. malariae.*

This simplistic view was a consequence first of the recognition that many commensal and symbiotic associations occur and the belief that these represent evolution from an original parasitic stage. The idea that virulence *per se* is 'bad' also results from applying medical criteria to a biological situation. Every death from malaria, and there are probably two million each year, is a medical disaster but, in biological terms, two million hosts being killed out of a total perhaps four hundred million who are infected can be considered as highly successful parasitism.

Success for a parasite like *Plasmodium* is its ability to infect its new host (May & Anderson, 1990). In crude terms, it matters little to the parasite what happens to the host already infected as long as the consequence of that infection is that a large number of new hosts become infected (Read & Schrag, 1991).

We must not immediately then make the new mistake of believing the opposite situation that highly virulent parasites are inevitably the most successful; this clearly is not always so. Rather the level of virulence should be assessed in terms of how morbidity and mortality affect transmission.

Another common error is to take an incomplete view of immune responses. It is quite clear that the immune system has as its primary function resistance to infection. Its complexity is a consequence of parasitic infection (Mitchison, 1990) and, as a result of acquired immune responses, we recognize host protective mechanisms, immunopathological effects, and some responses with no demonstrable effects either beneficial or harmful. The parasites are seen too to be able to subvert or manipulate the immune responses but it is less readily appreciated that immune responses, like virulence properties, may serve to enhance transmission. In this review, I shall consider virulence, immunity and transmission in malaria to see how they relate to one another and what we can conclude in terms of attempts to control infection by immunological means.

VIRULENCE AND INNATE RESISTANCE

A number of factors have been proposed as giving some measure of innate protection against malaria and, consequently, as reducing the severity of the infection. These are largely based on epidemiological studies (Miller, 1988) but only in the protection provided by the sickle cell trait against *P. falciparum* is there any indication of how this is effective. The effect is not to prevent infection but to reduce the severity of disease, a result shown clearly in a recent study (Hill et al., 1991) where the prevalence of the trait in those with severe malaria was less than half that in patients with mild disease. The underlying mechanisms may be partly non-immunological – an impaired ability of the parasites to grow at low oxygen tensions in cells with reduced intracellular K^+ (Friedman et al., 1979) and partly immunological, the acquired immune response being more effective.

VIRULENCE OF THE PARASITE

It is still not clear to what extent the size of the dose of sporozoites inoculated by mosquitoes affects the subsequent severity of the disease. Recent studies would tend to indicate that this might not be very important since, even when mosquitoes have heavily infected salivary glands, only small numbers of the sporozoites are inoculated at each feed (Ponnudurai et al., 1991). Of course, in some areas, individuals may be exposed to several infective bites a night. Studies on the use of insecticide-impregnated bednets on the other hand do lead to the conclusion that dose of parasites can be important since, in The Gambia (Snow et al., 1988; Alonso et al., 1991), those who used the nets showed significantly lower mortality and clinical episodes than controls, but parasite rates were not different. The assumption is that a significantly smaller sporozoite challenge means a correspondingly smaller number of parasites subsequently released from the liver, giving the immune system one or two days more to respond before the parasites reach patent levels.

Cytoadherence, the binding of infected erythrocytes to endothelial cells is a natural feature of the development of *P. falciparum,* but not of the other human species of *Plasmodium*. Three of the well known cytoadherence molecues, CD36, ICAM-1 and thrombospondin, have been shown from *in vitro* studies to be receptors for *P. falciparum* – infected red blood cells (Berendt et al., 1990). In addition, *P. falciparum* – infected erythrocytes will bind uninfected red blood cells to form rosettes (Udomsangpetch et al., 1991). *P. falciparum* isolates vary in their ability both to cytoadhere and to form rosettes (Berendt et al., 1990) and this is likely to affect their virulence. Ho et al. (1991) showed that there was a correlation between the *in vitro* ability of *P. falciparum* isolates to bind to the CD36 receptor on C32 melanoma cells and development of severe malaria. However, there was no correlation between the cytoadherent property and occurrence of cerebral symptoms. The ability to bind to the cell adhesion molecules CD36 or thrombospondin was not associated with rosetting properties, and only 2 out of 18 African isolates of *P. falciparum* showed strongly positive rosetting (Hasler et al., 1990). However, when comparisons of isolates from children with cerebral malaria or with mild disease were made, there was a strong association between formation of rosettes and cerebral symptoms on the one hand, and lack of antibodies that could disrupt the rosettes on the other (Carlson et al., 1990). A monoclonal antibody to an epitope of a surface histidine-rich protein (Pf HRP1) had the same property of being able to disrupt the rosettes. While the ligands on infected erythrocytes that are required for cytoadherence and rosetting have not positively been identified, there is strong circumstantial evidence that they are of parasite origin (Newbold & Marsh, 1990). On the other hand, the binding properties might also involve modified host cell proteins such as band 3 (Sherman & Winograd, 1990).

Schizonts of *P. falciparum* mainly complete their development and rupture while sequestered. Rupture of schizonts is associated with release of soluble antigens and these have been shown experimentally to induce high levels of tumour necrosis factor (TNF) (Taverne et al., 1990). In *P. falciparum* infections, TNF, interleukin-1 (IL-1) and lymphotoxin (LT) are all increased (Kwiatkowski et al., 1990; Clark et al., 1991). Plasma levels of TNF and IL-1 were significantly higher in children with uncomplicated malaria than in children with other illnesses, higher still in children who survived cerebral malaria, and highest of all in those who died from cerebral malaria (Kwiatkowski et al., 1990) Clark et al. (1991) propose that cerebral malaria is a consequence of local induction of TNF and other cytokines by

rupturing sequestered parasites. The cytokines in turn induce release of reactive nitrogen intermediates, including nitric oxide (NO), from endothelial cells. NO is involved in neuro-transmission and this high exogenous source would disrupt neuronal function, and cause other cerebral symptoms such as systemic hypotension and intracranial hypertension.

VIRULENCE AND ACQUIRED IMMUNITY

Several recent vaccine studies on malaria have shown a marked MHC-associated restriction of the immune response. Most of these experiments have been in mice and have involved the use of synthetic or recombinant peptides and the responses to defined epitopes (reviewed by Riley et al., 1991) though not all (Good et al., 1988).

A recent elegant study has for the first time produced evidence to support the view that polymorphism in the immune system is a consequence of parasitic infection, and that specific HLA antigens are associated with protection against severe malaria. In a case control study, more than 600 children with severe *P. falciparum* malaria were compared with children of the same age and area of residence who did not have malaria but were suffering from other mild mostly infectious illnesses. From this study, a significant association was found with both a MHC class I and a class II antigen (Hill et al., 1991). There was association between the class I antigen HLA-Bw53 and protection from severe disease. It is presumed by the authors that this is mediated by cytotoxic T lymphocytes acting most probably against the liver stage parasites where from experimental studies they are known to be effective (Schofield et al., 1987; Renia et al., 1991). The class II antigen HLA Drw 13 was linked with protection against severe malarial anaemia, though not cerebral malaria. Both MHC antigens were shown to be represented more frequently in the African (Gambian) population than in non-Africans, giving support to the notion that this was natural selection driven by malaria infection. The results are perhaps surprising given, as we shall now see, the great diversity of antigen structure of *P. falciparum* and other malaria parasites and the evidence for strain or variant-specific immunity. The nature of the study made it impossible to equate protective effects with parasite density in the blood. However, effects on levels of parasitaemia were clearly not great yet the proposed mechanisms of protection are parasite related. It is difficult to envisage how this could be working either at the level of infection in the liver (Class I linked) affecting subsequent invasion of the blood, or in lessening the anaemia significantly.

Another study, this time looking at *in vitro* T cell responses to circumsporozoite protein (CSP) peptides showed differences in the peptides recognized by those from an endemic area (Gambian again) and Caucasians (Australian) who had been exposed to malaria. The Africans tended to respond to peptides that fell within the variant region of the CSP, in contrast to the Australians. Of interest too, those who had been exposed to infection but had not developed clinical malaria responded less well to the 29 peptides than those who had had clinical malaria *or who had never been exposed* (Zevering et al., 1990). We shall come back to this latter group.

Though MHC-linked immune responsiveness is now clearly shown (see also Riley et al., 1991), it is equally clear that non-responsiveness during natural infections or to native antigens has other explanations; even apparently MHC-restricted responses to defined peptides can be overcome by use of appropriate adjuvants (Alving & Richards, 1990). Thirty Gambian adults showed markedly different antibody and T cell responses (measured by proliferation and by IFNγ production) to a *P. falciparum* gamete surface antigen but these could not be MHC class II antigen linked (Riley et al., 1990). Moreover, the antibody response overall to this antigen was significantly lower in these clinically immune adults than in individuals recovering from clinical malaria (Ong et al., 1990; Targett et al., 1990). This implies some regulation of immune response leading to non-responsiveness as a result of repeated or lengthy exposure. In a study, this time to schizont antigens, that showed similar differences in responsiveness between adults of an endemic region and temporary residents who had had clinical malaria, it was shown that the non-responsiveness in proliferation assays could be reversed by addition of recombinant IL-2 (Chizzolini et al., 1990). A parallel study showed that the down-regulation of responsiveness was due to schizont-antigen specific $CD8^+$ suppressor T cells (Mshana et al., 1990). In lepromatous leprosy such $CD8^+$ cells were shown to be HLA-DQ restricted (Salgame et al., 1991). It should also be remembered that

lymphocyte sequestration and redistribution can occur (Hviid et al., 1991), all of which indicates that measured unresponsiveness is multifactorial; how this relates to parasite survival is not yet clear.

Two other major determinants of the immune response seen during natural infections and which may influence virulence and transmission should be considered.

Malaria parasites show extensive polymorphism and diversity in antigenic structure. To what extent this diversity, which is seen in both T and B lymphocyte epitopes, is a consequence of antigenic variation has still to be established with the human malarias but the large number of antigenically distinct forms occurring in *P. falciparum* and *P. vivax*, and the clear demonstration of clonally based antigenic variation in other *Plasmodium* species (Mendis et al., 1991) makes it likely that the two are related. If this ability to express single antigens in diverse forms is an evolved immune evasion process it will have a profound effect on virulence of the infections. Thus, natural isolates of *P. falciparum* show extensive polymorphism in the Th2R and Th3R immunodominant T cell epitopes of the circumsporozoite protein (Lockyer et al., 1989). Immune responses directed against this antigen include not only direct antisporozoite effects but also mechanisms acting only against the intracellular exo-erythrocytic schizonts. These include class I – directed cytotoxic T cell responses; the key peptides must therefore be presented with the MHC molecule on the surface of the infected hepatocyte.

Infected erythrocytes show extreme diversity in surface antigenic structure demonstrable serologically (Marsh & Howard, 1986; Forsyth et al., 1989; Day & Marsh, 1991). In addition, the best correlate of protective immunity was a variant-specific response (Marsh et al., 1989) giving clear evidence in support of the view the resistance in malaria is the summation of a series of variant specific responses. In *P. falciparum* infections, infected cell surface properties determine the ability to cytoadhere or rosette. Though the infected red blood ligands have yet to be identified specifically, there is a close association between ability to bind and surface antigen expression (Howard, 1988). In terms of virulence properties, coincident in large measure in *P. falciparum* with binding properties, it is of interest whether parasite populations mate randomly so that at any time there are likely to be a large number of distinct clones or whether the parasites tend to be clonal, presenting their repertoire of antigens in sequence and over a long period of time. The evidence for *P. falciparum* shows that the former panmictic situation obtains (Walliker, 1991) while, with *P. vivax*, which is equally polymorphic, inoculations are clonal (Udagama et al., 1990).

Many of the *Plasmodium* antigens that have been sequenced contain tandem repeat sequences, exemplified by the 40 repeats of the NANP sequence in the circumsporozoite protein of *P. falciparum* (Nussenzweig & Nussenzweig, 1989). Epidemiological studies show that the repeat regions are highly immunodominant. Of the various proposals made to explain the occurrence of such tandem sequences, two are of particular interest here. Anders (1986) proposed that the tandem sequences within different surface antigens formed a network of cross reacting epitopes. Because of this, and their immunodominance, the immune responses made would not mature but would remain as a large population of B cells generating low affinity antibodies. This would explain the inability of the immune system to eliminate the parasites completely. More recently, Schofield (1991) has proposed, with some good evidence, that the immunodominant repeat sequences induce T-independent immune responses. Molecules with such repetitive structures are well known to induce antibody by cross-linking immunoglobulin on B cells rather than by inducing T cell help. Such a response would again explain why infections are not neutralized and, above all, it would explain why immunity in malaria wanes quickly. It must be stressed that what is being proposed is T independent responses lacking memory to both sporozoites and asexual blood stages *during the course of natural infection.* All of the antigens containing the tandem repeat sequences have T cell epitopes but they are not immunodominant; indeed, one of the proposed functions of the repeat sequence is suppression of adjacent epitopes by its own immunodominance.

In contrast with this view is the finding that many uninfected individuals have a high level of reactivity to malaria (Pink & Sinigaglia, 1989). Moreover, in a detailed study of the subsets of T cells involved in response to *P. falciparum* schizont antigens (Jones et al.,

1990), responsiveness was found to reside in the CD45RO subset which contains predominantly memory T cells. The demonstration of reactive T memory cells in unexposed individuals can be explained if the proposal made by Beverley (1990) is correct, namely that memory is maintained by cross-reactive stimulation.

It is also difficult to reconcile the T independent response lacking memory with the high degree of polymorphism of the parasite and strain specificity of the immune response.

Finally, in looking at immunity in relation to virulence we should note that enhancement of parasite invasion by antibodies has been demonstrated. Thus, sporozoite invasion of hepatocytes can be facilitated by antibodies (Nudelman et al., 1989); this was determined by antibody concentration, high titres inhibiting invasion, low titres promoting it. Franzen et al. (1989) showed enhancement of invasion of erythrocytes by an antibody to an asparagine rich protein of *P. falciparum* and in this case the degree of enhancement increased with the concentration of antibody. It also induced a more rapid maturation of the parasites intracellularly. Both of these observations were made *in vitro* but, as we shall see, a similar observation has been made affecting transmission of the parasites from human to mosquito hosts.

TRANSMISSION AND THE IMMUNE RESPONSE

Gametocytes of *P. falciparum, P. vivax* and some non-human malarias induce antibodies during the course of natural infections that are sexual stage specific. Some of the target antigens have been identified and constitute potential transmission-blocking vaccines (Carter et al., 1988)

The anti-gametocyte antibodies can prevent fertilization in the mid-gut of the mosquito host since the antigens that elicit their formation are dominant molecules on the surface of micro- and macro-gametes. There is also some evidence that antibodies reduce the number of circulating gametocytes (Baird et al., 1991). On the other hand, membrane-feeding experiments with *P. vivax* (Pieris et al., 1988) and direct mosquito feeding on toque monkeys infected with *P. cynomolgi* revealed that antibodies to the sexual stages can enhance rather than block transmission. This effect was shown to be, like that with anti-sporozoite antibody, related to antibody concentration; low titre of antibody enhanced transmission while higher titres (of the same antibody) suppressed it. Data on other immune responses are more limited though T cells from some adults who had been exposed to *P. falciparum* over a long period showed proliferative responses or production of γ-interferon to Pfs 48/45, a gamete surface/gametocyte antigen of *P. falciparum* (Riley et al., 1990). To what extent cytokine production is induced naturally by gametocytes is unknown, but serum taken during malaria infection crisis was shown to kill intra-erythrocytic gametocytes, the killing being due to the presence of γ-interferon and tumour necrosis factor but not caused directly by them (Naotunne et al., 1991). In this case cytokine production was probably a consequence of the asexual parasitaemia. The effector mechanisms induced by the cytokines may include reactive nitrogen intermediates; recently Motard et al. (1993) have shown that nitric oxide is produced during *P. yoeli* infections and causes a significant reduction in the infectivity of gametocytes.

The demonstration of enhancement of transmission of *P. vivax* came from studies in Sri Lanka on the immunity of those exposed very little compared with that of individuals who had been infected frequently. There was little evidence that prolonged exposure produced a strong and sustained immunity. Rather the pattern was the same in everyone. The immunity waned quickly and was only maintained if the interval between infections was less than 4 months (Ranawaka et al., 1988). This implies either that immunological memory in the established sense is not developed – extending the opinion by Schofield (1991) on immunity to asexual and pre-erythrocytic forms – or there is some form of regulation of responsiveness occurring akin to that described above for *P. falciparum* (Ong et al., 1990; Riley et al., 1990).

Finally, one of the most fascinating recent studies has been that by Day and her colleagues showing first that the sequestration of *P. falciparum* gametocytes that occurs during their early development (Forsyth et al., 1990 and submitted for publication) is mediated by CD36 and thrombospondin, two of the receptors for asexual stage erythrocytic forms thus providing evidence of a link between transmission and virulence. This was further supported by

their demonstration that deletion of part of chromosome 9 caused the simultaneous loss of the ability to form gametocytes and to cytoadhere, implying again that they are closely linked. We still have no idea of the trigger for gametocyte formation but perhaps it is linked to the early stage of binding to endothelial cells.

Studies on immunity to sexual stages show that specific acquired immune responses can, during the course of natural infection, promote as well as depress the rate of transmission. In addition, the complex immunity to all earlier stages of infection can also be seen not just as response to a parasite trying to come to an accommodation with its host, but of one maximising its chances of being transmitted.

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