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A Hypothesis about the Chronicity of Malaria Infection

P. Druilhe and J-L. Pérignon

It is generally accepted that malaria evolves as a chronic blood infection by escaping the immune responses directed against a series of antigens that express variable epitopes and/or by selecting parasite populations with distinct polymorphic antigens. However, exacting in vitro *studies, performed with clinically well-defined biological material, have correlated the state of protection of African adults (in whom low-grade infection persists) with an indirect defence mechanism where the antibodies are effective owing to their ability to cooperate with blood monocytes. Further studies showed that the antibody bridges the parasite (at the merozoite stage) with a monocyte and triggers the release of mediators which have a parasitistatic, reversible and non-antigen-specific effect. The fact that the parasite directly triggers the antiparasite effect leads Pierre Druilhe and Jean-Louis Pérignon to formulate here an alternative hypothesis for the chronicity of malaria infection, which would rely on conserved antigenic targets and, in contrast with direct mechanisms, would not select emerging mutated parasites. The above two mechanisms are discussed in the context of their fitness with clinical and parasitological observations. It is proposed that they are not mutually exclusive but, rather, may come into play successively as patients gradually evolve from high-grade symptomatic to low-grade asymptomatic parasitic infection.*

As are most other parasitic infections, human malaria is a chronic disease. Individuals exposed to repeated re-infection can develop a state of protection that prevents the occurrence of clinical symptoms and high parasite burdens. This type of acquired immunity known as 'premunition' is, however, nonsterilizing. No matter what the duration of exposure or the number of the parasite inoculations per month or per day may be, the type of immunity reached by adults living in endemic regions is such that, invariably, a few parasites remain circulating in the blood. Even in areas of the highest endemicity, cross-sectional surveys reveal a low-grade parasitaemia in about half of the adults protected against the disease[1]. Follow-up surveys show that nearly all of these adults will have a patent parasitaemia at one point or another over some period of time[2]. Cross-sectional surveys utilizing a sensitive polymerase chain reaction (PCR) technique confirm that more than 90% of adults consistently harbour parasite loads that are below the threshold of detection by microscopy[3]. As seasonal transmission is the most common situation in the majority of endemic areas in the world, the advantage for the parasite of such a chronic state is evident; it provides a parasite reservoir, which is essential when there is no transmission, since it allows the parasite to wait for mosquitoes to re-establish.

The question to which there has been no clear answer remains: 'By what immune mechanism can such a low-grade parasite load be maintained over months and years in individuals who have developed a protective immune response?' The paradox or immunological dilemma is appropriately expressed by McGregor[4] in his discussion of the results of the passive transfer of immunity by IgG:

> 'That gamma globulin of such potency may be obtained from the serum of normal African adults is astounding. While serum therapy effects a dramatic reduction in parasitaemia, in most instances parasites subsequently persist in low density. The reason for this phenomenon is not readily apparent for an immunity strong enough to overcome the great bulk of parasites might also be expected to deal effectively with the remaining few.'

Up until now it has been widely accepted that chronicity is a result of antigenic variation, rather than just antigenic polymorphism[5], in which case, the parasite, by expressing a new variant type, is able to escape the host response directed to the previous antigenic variant type. The recent cloning and characterization of the *'var'* gene family has added further support to this view. However, recent studies on the antiparasitic effect of antibodies *in vitro*, which were of proven protective efficacy *in vivo*, provide new insights into the mechanisms of defence that underlie premunition immunity against *Plasmodium falciparum* asexual blood stages.

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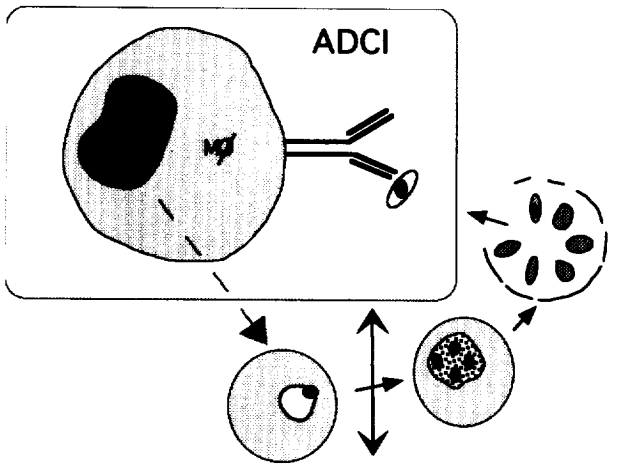

Fig. 1. Schematic representation of the antibody-dependent monocyte-mediated (ADCI) mechanism of growth inhibition of *Plasmodium falciparum*. Crosslinking of monocytes and merozoites by cytophilic antibodies leads to the release of monocyte-derived mediators (dotted arrow) which reversibly block intraerythrocytic parasites at the one-nucleus stage (double arrow).

The results of these studies offer a different view about how an antiparasite immunity can effectively protect the host and yet leave some parasites alive. They lead us to raise an alternative hypothesis (see below) about the nature of the chronicity of malaria infection. This hypothesis is discussed in the context of clinical and epidemiological data about malaria.

An indirect mechanism of action of antimalarial antibodies

Experiments of passive transfer *in vivo* have established that the main component of acquired immunity to blood stages leading to the state of premunition lies in the immunoglobulin G (IgG) fraction[4,6]. These experiments fully reproduced in non-immune receivers the parasitological picture observed in the donors of the Ig. For example, the injection of IgG induced a dramatic although incomplete effect in that parasitaemia decreased by a mean factor of 1000. However, this decrease did not ever reach a zero level, and some parasites remained in very small numbers close to the limit of microscopic detection. Just as the recipients still had remaining low level parasitaemias, the donors of the Ig were also still carrying parasites at the time of plasma collection.

Recent experiments[6] have also reproduced precise *in vitro* correlates of the *in vivo* observations[7]. These *in vitro* data clearly ruled out a direct effect of antibodies upon inhibiting invasion of red blood cells (RBC) by merozoites and supported a monocyte-mediated antibody-dependent effect, which we termed ADCI[8]. How the monocytes cooperate with antibodies to reduce parasite growth has subsequently been further defined[9] (see Fig. 1). This can be summarized as follows: when cytophilic antibodies, IgG1 or IgG3, crosslink merozoite surface antigens (of which one is MSP-3)[10] with the monocyte receptor for the Fc domain of IgG, Fc-γRII, (surprisingly the Fc-γRI receptor was not involved), the monocytes release soluble mediators (one of which is tumour necrosis factor, TNF-α), which are able to block the division of surrounding intraerythrocytic parasites at the early, one-nucleus stage of development[9].

As a result of this effector mechanism, it appears that parasites at the extra-erythrocytic, merozoite stage of development are able to trigger monocytes to release mediators that affect other parasites which are at the intraerythrocytic stage. This antibody-dependent monocyte-mediated effect is reversible (at least in part) in that it exhibits a parasitistatic rather than a parasiticidal effect because, *in vitro*, some parasites can start dividing again when the soluble mediators are removed[8]. Similarly, *in vivo*, the few parasites remaining after the passive transfer of IgG were found to be alive by *in vitro* culture[6].

One of the most remarkable features of this inhibitory mechanism is that the parasite is required to trigger the release of the mediators by the monocytes. In the absence of any merozoites released from bursting schizonts, antibodies are ineffective and the monocytes are not stimulated. Indeed, *in vitro*, antibodies alone were found to have the reverse effect and, instead of inhibiting invasion, often facilitated merozoite invasion in RBCs. Also, monocytes alone were ineffective and the simultaneous presence of monocytes and antibody also had no effect unless merozoites were present[9]. In classical ADCI experiments, starting at a very low parasitaemia, no significant inhibitory effect was found. When increasing numbers of merozoites were added, the blocking effect of the ADCI supernatant upon intraerythrocytic rings became proportionally larger[8,9,11] (F. Lunel and P. Druilhe, unpublished). Given the fact that the effect is mediated by soluble mediators which diffuse in the serum *in vivo*, or in the culture medium *in vitro*, it is to be expected that there is a threshold (ie. a minimum merozoite:monocyte ratio) required to obtain an effective local concentration of mediators.

Certain *in vivo* observations lend support to the above *in vitro* findings. Passive-transfer experiments have shown a direct relationship between the initial parasite load and the intensity of the decrease in parasite density following the injection of a fixed dose of IgG (Fig. 2). Ergo, humans who had a greater number of parasites present initially, experienced a stronger parasiticidal effect from the same amount of IgG than did individuals with lesser starting parasitaemias. Furthermore, in McGregor's classical Ig transfer experiments, it was observed in some patients that the antibody effect was delayed by 24–48 h. This observed delay was found to be related to the amount of time between Ig administration and schizont rupture. The decrease of parasitaemia started only when schizonts reached full maturation, ie. when merozoites were released and not when the Ig was given 24–48 h before[4] (I.A. McGregor, pers. commun., quoted in Ref. 9).

Thus, both of the above *in vivo* observations are in keeping with our *in vitro* findings that merozoites are crucial in the triggering of the antibody effect[9]. Our *in vitro* studies on the effects of passively transferred IgG would suggest that this decrease was not due to inhibition of merozoite invasion.

How is low-grade parasitaemia sustained?

Any immune mechanism for the state of premunition should fulfil two apparently contradictory criteria: it should be (1) very potent and, at the same

time, (2) exhibit an incomplete or partially effective (non-sterilizing) antiparasitic action.

If antibodies were to act by a direct mechanism, without accessory cells, by inhibiting RBC invasion or by inducing the removal of infected RBCs, the total disappearance of parasites should occur. It is difficult to imagine how an antibody effective enough to remove 99.9% of the parasite population would leave 0.1% alive. In the commonly accepted hypothesis, those 0.1% of parasites would bear, or switch to, a distinct antigenic phenotype. This situation has been studied in detail, and is well understood in the case of African trypanosomes. In trypanosomiasis, it was observed that when a variant parasite population emerges, it reaches high parasite densities (ie. 10^5–10^7 μl^{-1}). This sounds logical because this population was unaffected by the antibodies induced by, and specific to, the previous antigenic type. As the new antigenic type escapes the immune response elicited by the previous one, the immune mechanism can no longer maintain the parasitaemia at a low or medium density. Rather, it shows major fluctuations between very high (ie. 10^7 μl^{-1}) and very low (ie. undetectable) levels, as new variable surface glycoproteins (VSG) genes are switched on and new immune responses are raised. Essentially similar observations were made in *P. knowlesi*-infected Rhesus monkeys[12], with high parasite densities and the production of variant-specific antibodies. This is not the case with humans who have reached a state of strong immunity to malaria, in whom parasitaemias also fluctuate, although within a range of extremely low parasite densities. Parasitaemia never reaches the high levels that can be seen in non-immune subjects (eg. in primary attack cases and children in endemic areas). On the contrary, epidemiological studies consistently reveal that in immune adults, parasitaemia is maintained several orders of magnitude lower, close to the microscopic detection limit of about one parasite per 10^6 RBC 5 parasites μl^{-1})[2], or lower (5 parasites 100 μl^{-1})[3]. Thus, the two situations of African trypanosomiasis and of chronic malaria infection can be distinguished clearly by the range in which the parasitaemias fluctuate. This is why we are led to conclude that the different pattern of parasite densities in these two situations cannot be explained by a similar mechanism.

In the perspective of a monocyte-mediated, antibody-dependent and merozoite-triggered effect, we would like to propose the following alternative hypothesis. The invasion of RBC by liver-stage merozoites results in a very low-grade parasitaemia. At an initial stage, within the first schizogonic cycles, the number of new blood-stage merozoites released is too low, as compared with the number of blood monocytes in the body, to trigger any significant level of blocking mediators. As the antibodies have no direct effect, parasitaemia would increase rapidly to the point where it reaches a level high enough, yet very low in absolute numbers, to release a crucial number of merozoites. It is, in fact, the ratio of merozoites to monocytes that is crucial (as mentioned above). When monocytes are stimulated by 'enough' merozoites this would release sufficient amounts of mediators to destroy or block the division of parasites at the ring stage and, consequently, halt the rise

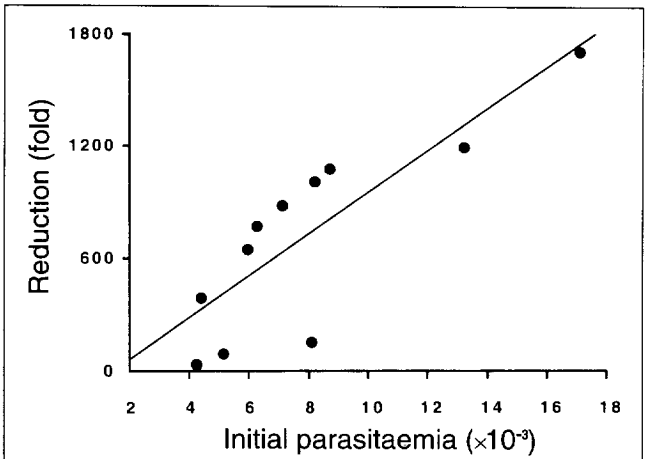

Fig. 2. Relationship between the initial parasite load and the intensity of parasitaemia decrease after administration of IgG in malaria patients (correlation coefficient $r=0.85$, $p<0.01$).

in parasitaemia. Schizogony being blocked, no new merozoites will be released and the monocyte will no longer be stimulated. After a short time, the concentration in mediators can be expected to decrease, as supernatants from stimulated monocytes loose their effect within 24–48 h and the lability of TNF-α is well-known. Therefore, parasitaemia would start increasing again up to the number of merozoites required to trigger a new wave of monocyte activation, and so forth. In such a scheme, despite very high levels of effective antibodies, parasitaemia would never be totally eradicated and would fluctuate within a narrow range, at a level close to the threshold merozoites:monocyte ratio crucial for triggering an effective concentration of mediators. Assuming an optimal ratio of one merozoite per monocyte (based on the *in vitro* conditions of ADCI), a quick calculation leads to a corresponding parasitaemia of two parasites per 10^6 RBC, or ten parasites per microlitre of blood, which is indeed within the range of parasitaemia harboured by protected asymptomatic individuals[2,13].

The full pattern of regulation of host–parasite interactions leading to the chronic low-grade parasitaemia characteristic of premunition is obviously more complex than that outlined above. For instance, the chronic presence of the parasite is likely to influence (eg. boost) the production of effective antibodies, to modify the balance of cytophylic to non-cytophylic antibodies, to influence the total number of monocytes and to result in lymphokine-dependent modifications in monocyte function. The relative amounts of antibodies, monocytes and merozoites can all be expected to influence the final range within which the parasitaemia is going to fluctuate.

Considering what is known of the epidemiology, the clinical picture and the biological features of the parasite, we defend the hypothesis that such an indirect mechanism, either the one described above or any other mechanism acting indirectly, fits better with *in vivo* observations than one directly affecting the parasite.

ADCI does not select for variant parasites

A most interesting consequence of such an indirect effect of antibodies is that, in contrast to a direct one,

it does not favour the selection of parasites expressing variant or polymorphic antigens.

In the context of a direct effect of antibodies, if a mutation or the expression of a novel *var* gene occurs, which results in a distinct antigenic type, this would lead to the fast selection of this new parasite population, since it would rapidly overgrow the wild type. This has been observed in monkeys immunized with a 140 kDa merozoite protein[14]. If the recent finding that there would be a 2% rate of emergence per cycle of new antigenic types is correct[15], it can be guessed that a direct effect of antibodies will always select for new, as yet unidentified, antigenic types. Again, this does not fit with *in vivo* observations because premunition appears to be one type of protection that extends to all parasites, despite their huge genetic diversity and mutation potential[6,16,17].

In the perspective of an indirect mechanism such as ADCI, as of any similar indirect mechanism, even when mutants are produced, there is no similar selecting force in favour of this new population: indeed variants, or mutants, can be expected to be quantitatively minor as compared to the wild-type population, at least at the time of their emergence. Therefore, there will be enough non-variant parasites to trigger the release of mediators by monocytes. The crucial issue, in such an indirect mechanism, is that only the afferent arm (the antibody bound to merozoite surface) is antigen specific, whereas the effector arm (the cytokines) is not: as the released mediators are not antigen-specific in their effect, they would be equally effective upon the wild and the mutant (or variant) types and, therefore, would not favour the latter.

If ADCI is a prominent mechanism underlying premunition, that is of protection against all strains an adult may encounter in an endemic area, it should be triggered by well-conserved regions of antigens. Indeed, this has been established for B-cell epitopes in MSP-3, a merozoite surface antigen identified as a target of cytophilic antibodies effective in ADCI.

Successive immunities based on distinct mechanisms

This hypothesis does not rule out the role of immune responses directed at variant or polymorphic antigens. It is becoming increasingly clear that there is not 'one' but a series of distinct immunities to malaria and, therefore, not just a single type of defence mechanism against blood stages. It is also clear that there are many polymorphic and variant malaria antigens that elicit specific immune responses. Antigenic polymorphism and antigenic variation are part of the strategy of the parasite to escape the direct effect of specific antibodies on the parasite (eg. the inhibition of invasion or the inhibition of adherence with further destruction in the spleen). As discussed above, the effect of these antibodies should lead to large variations of parasitaemias, which are not observed in premunition. This does not exclude the possibility that they play a role at an earlier stage in individuals who are not fully immune. In fact, Brown and Brown[18] suggested in 1970 that the predominant mechanisms of defence against plasmodia may be different during the first infections, with classical waves of parasitaemia corresponding to successive variants, and at the phase of chronicity. In the situation of chronic infection, they postulated 'a generalized nonvariant specific immunity to explain the gradual decrease in parasite numbers characteristic of chronicity'. They even hypothesized[19] that this 'additional immunity which is not variant specific' may involve cells and the production of cytophilic antibodies. Our considerations about the mechanisms underlying the chronic stage of malaria are consistent with their view, and may lead to a proposed general model of immunity against *P. falciparum* malaria.

Epidemiological findings involving partially immune subjects, as well as data on parasite genetic diversity and continuous genetic rearrangements, suggest that in the many years preceding the chronic phase, immunity against the parasite may mainly comprise defence mechanisms that depend on the direct effect of antibodies targeted to variant antigens (antigenic polymorphism and antigenic variation giving the parasite a means to escape the host's mechanisms of defence). At the other end of the scheme, the chronic stage of low-grade parasitaemia could be achieved by indirect mechanisms of defence such as ADCI, that actually depend on the presence of remaining parasites. Because this protective immunity does not seem to be strain-specific, it is reasonable to hypothesize that it may be directed at non-variant antigens. The progressive acquisition of the potent protective immunity resulting, after many years, in premunition, may involve the slow transition from one kind of defence mechanism to the other. This would mean, for example, the transition from the synthesis of non-cytophilic to cytophilic antibodies[20] or the elaboration of responses to conserved antigens emerging from the 'smoke-screen' of responses to variant antigens.

Conclusion

The apparent paradox of a chronic state of low parasitaemia remaining for months and years in individuals submitted to continuous antigenic challenges represents an example of a compromise between a parasite and its host. Examining the various defence mechanisms that may contribute to realize the *modus vivendi* named premunition, we were led to the conclusion that the general scheme of an indirect mechanism, in which an accessory cell has the effector role and the parasite load has a regulatory role, is one that fits better with epidemiological and clinical data than alternative defence mechanisms against blood stages previously proposed, which all rely on a direct effect of antibodies upon merozoites or infected RBCs[21,22]. In the same perspective, it can also be stressed that in other parasitic diseases which evolve as chronic infections (eg. schistosomiasis or filariasis) the main effectors of immunity identified to date also depend on ADCC type mechanisms involving accessory cells (for a review see Ref. 23). Whether our findings about the way antibodies can cooperate with monocytes correspond to a mechanism that operates *in vivo*, and whether this is the sole defence mechanism underlying premunition, is not so important because, in the above scheme, the reasoning would remain the same for any mechanism that functions in a similar feedback fashion (ie. where the parasite is required to trigger a parasititatic effect).

This can be viewed at least as an additional working hypothesis that merits investigation. Our aim was to stress the value of addressing the fitness of *in vitro*

observations with *in vivo* clinical data[24]: knowing the numerous defence mechanisms described *in vitro*, it is of interest to take into account clinical, epidemiological considerations, as well as the biological characteristics of both the parasite and its host, to try to determine which mechanisms fit better with *in vivo* observations in different subjects, according to the natural history of their encounter with the parasite.

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Presence of the Parasitophorous Duct in *Plasmodium falciparum* and *P. vivax* Parasitized *Saimiri* Monkey Red Blood Cells

B. Pouvelle and J. Gysin

Although the exchange of metabolites between the intraerythrocytic malaria parasite and the external medium has been studied extensively, the transport of molecules across the erythrocyte cytoplasmic membrane and cytoplasm and the parasitophorous vacuolar membrane needs to be investigated more fully to be completely understood. Recently, the concept of the parasitophorous duct, establishing a continuity between the environment and the vacuolar space surrounding the intraerythrocytic parasite, has been suggested to provide an explanation of how macromolecules can cross two membranes in a cell devoid of an endocytic system. This concept is highly controversial and has been suspected to be an in vitro *artefact. In this article, Bruno Pouvelle and Jürg Gysin present evidence of the existence of the parasitophorous duct in* Saimiri *monkey* Plasmodium falciparum- *and* P. vivax*-infected erythrocytes, with a series of* ex vivo *experiments showing stage and species dependent variations of the characteristics of this structure.*

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The existence of a novel membranous structure, the 'parasitophorous duct', has been described, giving macromolecules in the external medium access to the intraerythrocytic malaria parasite, *Plasmodium*

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