Natural immunities

P. Druilhe and H. Bouharoun-Tayoun

Parasitologie bio-médicale, Institut Pasteur, 75724 Paris Cedex 15

Research on malaria vaccines was recently compared to "A Game of Chess" (Mendis, 1991). This would suggest that it is a difficult and intellectually challenging occupation, but also that we know the rules of the game and the function of the various pieces. Is this really the situation? To us, it rather appears as a puzzle from which we can gather, after years of efforts, a very limited number of pieces, this preventing the completion of the whole picture. Moreover, and surprisingly, this game is frequently played using pieces from different puzzles. This is best illustrated by reviews, another favorite game of many malariologists, or discussions found in original articles, in which a result from one experiment using a given host-parasite system is put together with another piece (result) from a different host-parasite combination. All plasmodia obviously have a lot in common; however, depending on the species or for a given species depending on the host used, the whole picture, *i.e.*, the host-parasite interaction and the outcome, may differ greatly. Some pieces may eventually seem to fit more or less together, but rarely very well, and the construction becomes very fragile when one attempts to combine more than 2 or 3 results (pieces)...

Whatever way one looks at it, most researchers who have spent some time in the field of malaria would generally convene that "complex" is an appropriate term to define the subject of their studies.

How "complex"?

The degree of complexity of plasmodial antigens can be most easily summarized by recalling that the genome of plasmodium is about 250 times larger than that of viruses. Though complexity is obviously not in direct proportion to the genome size, this gives an idea of the multiplicity of plasmodial antigens which may be thought to be a target of one or another means of defence, or "vaccine-candidates" as they are frequently called. Even in the field of viruses, in which one has to deal with 50 to 100 times fewer molecules, it has sometimes been proven difficult to design vaccines even when the proper epitopes were defined (usually rather rapidly by B or T cells from convalescent individuals and *in vitro* neutralization assays). Considering that the design of vaccines against viruses presenting a polymorphism of some of their molecules, *e.g.* influenza or AIDS, is currently out of reach, it is worth recalling here that within plasmodium species we know that there is considerable genetic diversity. In particular, there prevails a very high degree of antigenic polymorphism, better defined by recent molecular studies.

It is thus a sign of outstanding optimism that, since the very beginning of research on molecular vaccines against malaria, when only a very limited number of molecules were identified, it was proposed that these were suitable vaccine candidates.

In our opinion, one of the main clues to a global understanding of the very subtle host-parasite interplay, and therefore to the choice of a suitable candidate, lies in the observation that the parasite can survive in natural host-parasite systems. This differs from most experimental systems in which either the host or the parasite is killed and in which long-term chronicity is seldom reached. In contrast with many viruses, most parasites and notably plasmodia never induce sterile immunity in their natural host. The type of immunity they induce enables the persistence of low numbers of parasites for years and does not prevent superinfection or re-infection after cure.

Such a pacific interplay between a highly evolved organism, man, and a highly complex microorganism immediately suggests that, amongst the large number of antigenically active molecules of the parasite, 99 % of them have been specifically selected so as not to induce defence mechanisms that would be fully effective, this allowing parasite survival. Each molecule may have its own means of escape, with some being poorly immunogenic (*e.g.* because of structural similarity with host components), others inducing biased, ineffective categories of cells or antibodies, still others being immunomodulatory, shed, or proteolytic to antibodies, etc. plus other strategies as yet unidentified. What is important to us is that we recognize that almost all of them must induce little or no responses.

It is a difficult task for the parasite to protect all its components from the immune system, and thus such protection cannot be achieved in all hosts. It is understandable that it is best achieved only in the natural host, and poorly in unusual experimental hosts.

From these considerations, it can be predicted that successful vaccination in artificial hosts should be achievable using a greater number of molecules than would be the case in the normal host. In the latter case, 99 % of parasite proteins, sugars or lipids bear epitopes which are not proper targets for defence mechanisms and thus are probably not suitable vaccine candidates.

At least some parasite components which would be proper vaccine candidates must exist, since in the natural host, immunity does occur and develop with exposure to the disease (particularly slowly in the case of *P. falciparum*, and probably faster in the case of *P. vivax* or *P. malariae*).

The induction of natural non-sterile immunity is part of the survival strategy developed by the parasite, since it needs a surviving host. It should be regarded as a normal mechanism capable of down-regulating parasite loads to a great extent, although never to zero. Natural immunity is better known and well established for the asexual phase occurring in red blood cells, but probably extends to all stages of development. Recent studies have demonstrated that regulatory mechanisms take place in humans which influence the development of gametes and other sexual stages in the mosquito for *P. vivax* and possibly also for *P. falciparum* (Mendis, 1991). To the extent that *in vitro* assays can be relevant to *in vivo* situations, recent studies also suggest that immunity which develops to several antigens located on the sporozoite surface of *P. falciparum* have a profound effect on the number of parasites transforming into liver stages (Mellouk *et al.*, 1990). More work will be needed before we can establish whether regulatory mechanisms take place or not when *P. falciparum* lies inside the hepatocyte.

On the basis of the above considerations, it is our belief that we lack the main part of the knowledge required to distinguish the potentially good or acceptable vaccine candidates from the poor ones amongst the multitude of epitopes dispersed in hundreds of molecules. Even if we were to achieve this distinction, we also lack an acceptable host in which to assess the validity of our results. The lack of reliability of models in turn impedes us from drawing any firm conclusions from vaccine experiments performed in those models which are precisely aimed at defining whether a candidate is good or not. This constitutes a vicious circle. The results from such assays are further complicated by the difficulty of making a good immunogen from an identified antigen.

Thus it seemed to us a more realistic and practical goal to rely on human studies to identify the basis of naturally occurring regulatory mechanisms. Thereafter, one can attempt to reproduce artificially what is known to occur in nature and therefore to determine what can be achievable through immunization. The possibility also remains of raising an artificial type of immunity to molecules which do not normally induce protective mechanisms, though it is our belief that we lack the information and the proper models required to buildup a rational strategy towards this goal. Obviously, the amount of data which can be gathered by investigations in humans is less than that derived from models (both *in vivo* and *in vitro*), but it has the advantage of being more reliable, thus enabling us to proceed to the next step on stronger grounds.

Assessing the basis of naturally occurring immunity has required the setting up of specific tools for *in vivo* investigations under ethically acceptable conditions. Antisexual-stage immunity can be assessed *in vivo* relatively easily in the normal host, the *Anopheles*, through membrane feeding experiments. At the level of the pre-erythrocytic stages, we introduced an indirect approach some years ago. In groups of subjects cured with blood schizonticides and subsequently exposed to natural challenges by infected mosquitoes, the speed of re-occurrence of parasites in the blood could be followed up on blood smears; this enabled us to assess the speed of passage of parasites through the liver and revealed that most of the sporozoites inoculated were, in fact blocked at some place prior to invading RBC (red blood cells). Preliminary results from such studies and their correlation with either antibody prevalence and level or with the prevalence of a T-cell response indirectly indicated which of the antigens from these phases could be involved in inducing regulatory mechanisms (unpublished results).

At the blood stage level, the way was paved by the pioneers of the 60's (Cohen *et al.*, 1961; Edozien *et al.*, 1962; McGregor *et al.*, 1963). Apparently, much more work was done (Cohen and Butcher, 1971) than actually published and more was published than usually quoted. Detailed results were available for at least 31 individuals who received IgG from immune subjects. We long ago planned and recently (in 1987) performed another set of passive transfers of human IgG in humans with our Thai colleague Pr. Tan Chongsuphajaisiddhi and his group. This experiment was not an end in itself, but was only one step in a global scheme. Since the true goals could have been misunderstood, it is probably worth recalling here why and in what perspective it was performed.

There was no reason to have doubts about results obtained in the 60's indicating that immunity to *P. falciparum* asexual blood stages was, at least in part, mediated by antibodies, this in at least 24 of the 31 recipients. There were nevertheless many other reasons for repeating such an experiment; among these were the following.

1) This was a key experiment which was the ba-

sis for numerous further *in vitro* studies. Such was also the case for other essential experiments, such as, for example, vaccination of humans using whole irradiated sporozoites; the stakes here are high, and confirming their outcome could provide further information on the reproducibility of results. For example, in the case of blood stages which exhibit a high level of antigenic polymorphism, it was important to assess whether the few failures recorded in passive transfer of antibodies in the past resulted from technical problems (storage, dose of Ig, etc.) or from antigenic mismatches (especially since the highest failure rate was recorded when West African Ig were used upon East African isolates).

2) A major goal was to obtain biological material which was not and could not be collected in the 60's. The preservation of parasite isolates, DNA, antibodies and cells was essential in order to perform the numerous biological investigations and *in vitro* assays developed more recently, and therefore to assess their relevance in a situation in which the protective value of the antibodies used in such assays was perfectly defined *in vivo*, both clinically and parasitologically.

3) More important, even in our opinion, was the need to define the conditions, with respect to the ethical constraints of today, for safely performing Ig transfer in man. In view of the questionable relevance of both *in vitro* and *in vivo* models, it is likely that a definitive answer will only result from human *in vivo* assays. However vaccination trials, even in humans, may not yield unequivocal results. Because of the difference between the antigenicity and the immunogenicity of a given molecule, it is predictable that negative results obtained with a given vaccine would not lead to its unequivocal rejection. In the case of a stage which preferentially induces an antibody-dependent type of immunity, the passive transfer of antigen-specific human antibodies therefore appears to us as one means for circumventing difficulties linked to poor immunogenicity. This, in turn, requires that acceptable and reproducible conditions for such assays can be defined.

Thus the basis of our work is to define natural immunities, *i.e.* existing regulatory rather than killing mechanisms toward various stages and to attempt to reproduce them artificially. Whether they can be scaled up to full protection or not is an interesting question, but of secondary importance. The scheme of the work we have tried to set up includes *in vitro* studies based on material well defined by *in vivo* experiments, along with further *in vivo* assessment of the conclusions derived from the laboratory assays.

The first step, passive transfer of whole IgG, was deliberately limited to a maximum of 8 receivers, 3 of whom received a second treatment. This enabled us to reach the above goals and to obtain several secondary benefits. Full results have been presented in detail elsewhere (Bouharoun-Tayoun *et al.*, 1990; Sabchareon *et al.*, 1991) and will only be recalled here to stress some aspects of particular interest.

Information of major importance for the future is that conditions for the safe assessment of the effect of human antibodies in humans were defined. The laboratory assays performed to assess safety would need to be explained in detail; in fact, safety relies on (i) the quality of modern means of purification of IgG for i.v. use, (ii) the particular type of clinical situation chosen, *i.e.* a recrudescence after drug failure at the RI level; this allows the follow-up of a progressively rising parasitaemia in humans prior to the occurrence of major symptoms, and (iii) the observation that, during the trial, i.v. IgG proved to be fast-acting; because of this, a 24-h period of observation proved to be enough to decide the parasitological consequences of a given antibody. Future assessments can thus be made without any delay in drug administration.

That African adults can develop immunity which protects them against all and any *P. falciparum* parasite was indicated some long time ago by epidemiological surveys; passive transfer of IgG from West Africans into Thai patients confirmed this important data in 8 out of 8 geographically remote isolates. This ruled out doubts caused by (1) 4 failures out of 9 East African isolates treated with West African Ig (McGregor *et al.*, 1963), (2) only partial effects recorded with the same type of Ig upon Asian parasites in *Aotus* (Diggs *et al.*, 1972) and chimpanzees (Sadun *et al.*, 1966), and (3) the absence of an effect of Asian Ig on African parasites (Cohen, McGregor, Seave, unpublished, quoted in Cohen *et al.*, 1971). The 8 strains we studied indeed exhibited polymorphism in several molecules, as shown by Western blot patterns of these strains revealed by a given polyclonal serum (Bouharoun-Tayoun *et al.*, 1990).

After a first inoculation of IgG followed by a decrease in parasitaemia, the clearance of recrudescent parasites by a second inoculation of antibody in 3 out of 3 cases treated provided grounds for optimism, since it was feared that recrudescent parasites may have been selected by the transferred antibodies. This indeed has been found to be the case in monkeys immunized against a *P. knowlesi* antigen (David *et al.*, 1984). In our studies, the recrudescent populations of parasites responded at least equally well to the same pool of antibodies (fig. 1) despite the fact that they later proved to be phenotypically different from the original population (unpublished results).

The same IgG pool also proved very effective in clearing parasites from infected Saimiri monkeys (J. Gysin and P. Druilhe, unpublished), therefore validating to some extent this model and its future

use for modelling transfer experiments of defined antibodies.

From a therapeutic point of view, IgG may also have valuable applications. When administered i.v., this pool was found to act more rapidly than drugs. On the other hand, antibody titres were found to be extremely low in those individuals with severe malaria who died in spite of receiving adequate treatment, thus raising the possibility of a synergism between the action of drugs and antibodies (Brasseur *et al.*, 1990). In view of the very high fatality rate recorded in cerebral malaria cases (20 to 50 %) it now seems

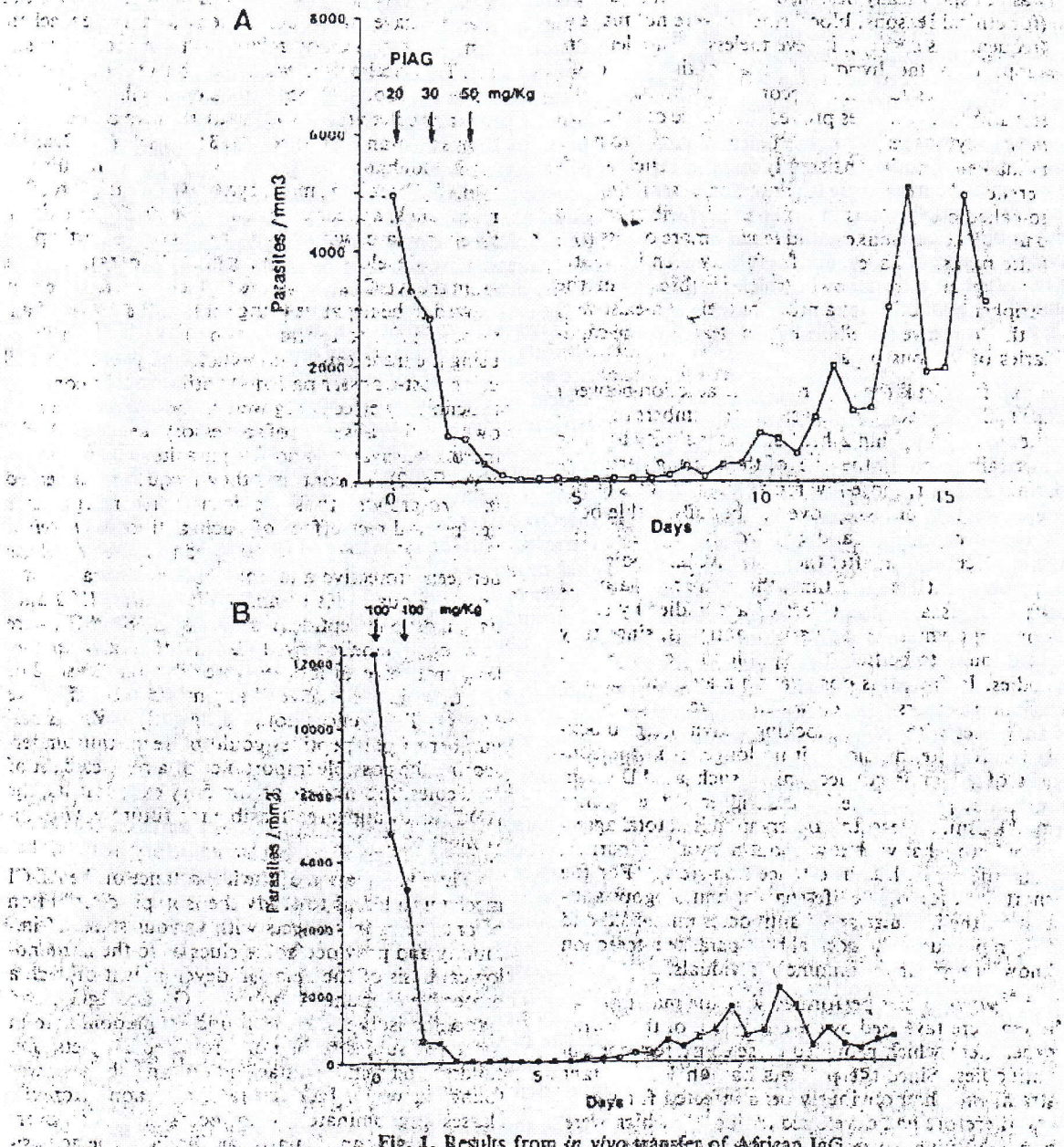

Fig. 1. Results from *in vivo* transfer of African IgG.
A) Mean parasitaemia in 6 patients receiving 100 mg/kg of PIAG over 3 days (20, 30, 50 mg/kg);
B) mean parasitaemia in 3 recrudescent cases receiving a second 200-mg/kg PIAG treatment.

important to investigate whether IgG treatment, together with fast-acting drugs, may improve the prognosis of severe malaria.

Incidentally, our experiment did not show a reversal of sequestered cytoadherent schizonts. Not a single mature form was seen by extensive screening of over 1,000 blood smears performed during the follow-up of 11 treatments. Though the experiment was not specifically designed to answer this question (for ethical reasons, blood smears were not made as frequently as desired), it nevertheless did not lend any support to the hypothesis that antibodies could reverse sequestration. In accordance with *in vivo* observations, antibodies proved incapable of reversing *in vitro* cytoadherence of the patients' strains to melanoma cells and inconsistently inhibited such an adherence. We did not see anything resembling the so-called crisis forms. These were described *in vivo* only in Rhesus monkeys and *in vitro* more or less pycnotic parasites have been frequently seen in poorly growing cultures. However, their absence in the peripheral blood is not a proof of their non-existence, as they could very well have been cleared in the capillaries of various organs.

Of interest is the apparent contradiction between the ability of IgG to clear very large numbers of parasites (2×10^{10} within 2 h...) and its inability to clear them all, whatever the level of the starting parasitaemia. However, those few remaining parasites (1 per 100,000 RBC or less) proved to be fully viable both *in vitro* by culture and *in vivo* as shown by the recrudescence seen after the protective antibodies disappeared. At the same time, they were not parasites who had escaped the effect of antibodies by selection of polymorphic or variant antigens, since they were found to be fully susceptible to the same antibodies. In fact, this observation may seem surprising in the perspective, which prevails today, of antibodies with direct blocking activity (*e.g.* blocking merozoite invasion); it no longer is in the context of an indirect mechanism such as ADCC in which the parasite itself is the trigger of the defence mechanism. At least this observation is in total agreement with what we know about naturally occurring immunity, which is in essence non-sterile. For the short duration of the life-span of homologous antibodies, the inoculation of antibodies mimicked and fully reproduced the normal host-parasite interaction known to occur in immune individuals.

In vitro studies performed with the material collected were favoured by the conditions of the *in vivo* experiment which provided close to perfect control antibodies. Since the patients had an R1-resistant strain, they had obviously been infected for weeks and therefore had developed antibodies which were non-protective, since the parasites recrudesced. In contrast, antibodies transferred on the same date proved protective against the same parasites. On day 0 of transfer, we thus simultaneously collected from each patient his/her parasites, antibodies which proved non-protective (before antibody transfer) and protective antibodies (after antibody transfer), and the same during recrudescences. For asexual blood stage research, this set-up appears unique in the possibility it offers to match *in vitro* assays with *in vivo* findings.

Up to now, *in vitro* studies performed with this material have mainly designated one defence mechanism as the best to correlate with the protective status. This mechanism, which relies on the cooperation between blood monocytes and cytophilic antibodies, is explored *in vitro* by an assay that we called ADCI (Khusmith and Druilhe, 1983; Lunel and Druilhe, 1989; Bouharoun-Tayoun *et al.*, 1990). In this system, IgG armed monocytes, when triggered by merozoites, appear able to release an as yet unidentified mediator which blocks intraerythrocytic plasmodial development (unpublished data). Results from the ADCI assays were very clear-cut, since they proved to be negative using each of the patients' antibodies prior to Ig transfer, positive for each strain using the transferred antibodies, and positive as well using post-transfer patients' antibodies. In contrast, the clinically effective Ig were non-inhibitory on their own in the absence of accessory cells, both for merozoite invasion and for parasite maturation assays (fig. 2). In contrast, they frequently increased *in vitro* growth, thus providing further arguments against a direct effect of such antibodies *in vivo*. IFAT and Western blots revealed that the difference between protective and non-protective sera did not lie in the quantities of antibodies. At a 1/100 dilution, most polypeptides resolved by SDS-PAGE were as strongly labelled by the 2 sets of sera. Nonetheless, protective antibodies were diluted at least 10 or 100 times in the receivers' serum, yet were effective *in vivo*. Differences concerning only a very small number of polypeptides could be seen, thus underscoring the possible importance of a restricted set of molecules. From these preliminary experiments, the ADCI assay appeared feasible for future *in vitro* investigations.

The confirmation of the importance of the ADCI mechanism led us to study the isotypic distribution of antibodies in subjects with various states of immunity and provided some clues as to the immunological basis of the delay in development of such a protective immune response. IgG1 and IgG3, two cytophilic isotypes, were found to predominate in protected subjects. In non-protected subjects, *i.e.* children and primary-attack adults, an imbalance was found, in which IgG2 or IgG4, two non-cytophilic classes, predominated, with, occasionally, an overall low level of antimalarial antibodies. The acquisition of protection appeared to correlate with the ability to develop increased levels of IgG3 and IgG1

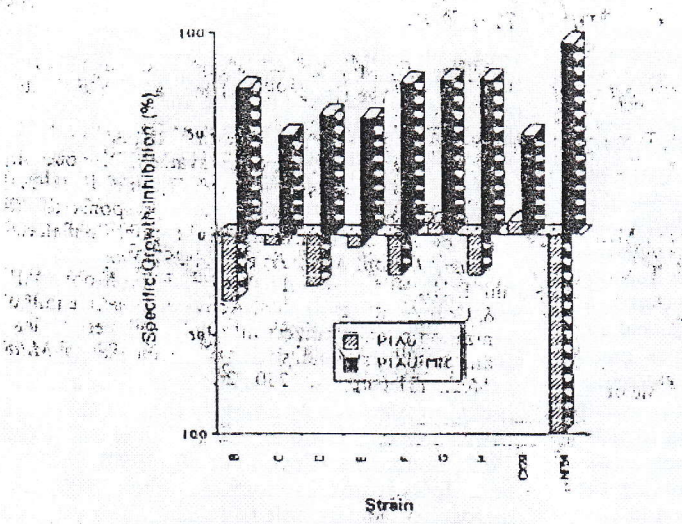

Fig. 2. Summary of 48-h ADCI assays performed with 9 *P. falciparum* isolates and African IgG (PIAG).

Results are expressed as the specific growth inhibition (SGI) in percent which takes into account possible inhibitory or improved growth effect of either cells or antibodies alone.

Results obtained by ADCI appear as black bars (monocytes + antibodies). Controls in the presence of antibodies alone appear as hatched bars (the negative SGI represents an increase of *P. falciparum* growth as compared to control IgG).

B to H are Thai strains isolated from patients B to H before PIAG transfer. C×29 is the strain isolated from Thai patient C on day 29 after transfer upon recrudescence of parasitaemia. NF54 is an African strain used as control in each experiment.

MN = monocyte.

antimalarial antibodies and to decrease the production of IgG2 antibodies. *In vitro*, IgG from protected subjects cooperated efficiently with blood monocytes, while IgG from non-protected groups did not; moreover, the latter inhibited the *in vitro* effect of the former in competition assays. Whole IgG from primary attack cases with increased IgG2 content, as well as IgG or IgM from children from endemic areas, competed with IgG from immune adults, thus suggesting that non-protected subjects had indeed developed antibodies to epitopes critical for protection, but that these antibodies were non-functional. More work remains to be done to identify the epitopes targeted by such antibodies.

In vitro studies performed with the same assay against a large range of strains or isolates using unpooled immune subject IgG, as well as competition assays using IgG from non-immune subjects, seem to indicate that the targeted epitopes of antibodies involved in ADCI may not be polymorphic. These results contrast with current concepts which postulate that immunity results from the acquisition of a series of immune responses towards highly polymorphic molecules. If confirmed, this would be a major reason to revive optimism about the future of a malarial vaccine.

In conclusion, we have tried to bring forth evidence that, despite difficulties, limited *in vivo* experiments for research purposes are achievable in humans. They can provide information of a type difficult to gather in any other way. In our opinion, such information could be of critical importance in orientating future research.

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