Chapter 34

Immunity to liver stages

Pierre L. Druilhe[1]
Laurent Rénia[2]
David A. Fidock[1,3¶]

[1] Laboratory of Bio-Medical Parasitology
Pasteur Institute
28, rue du Dr. Roux
75015 Paris, France
Phone: (33-1) 4568-8578
FAX: (33-1) 4568-8640
Email: Druilhe@pasteur.fr

[2] U445 INSERM
Institut Cochin de Génétique Moléculaire
Hôpital Cochin, Bâtiment Gustave Roussy
27, rue du Fbg Saint Jacques
75014 Paris, France
Phone: (33-1) 4046-9309
FAX: (33-1) 4407-1425
Email: Renia@icgm.cochin.inserm.fr

[3] Present address: Malaria Genetics Section, Laboratory of Parasitic Diseases, NIAID, NIH, Bldg. 4, Rm. B1-34, 9000 Rockville Pike, Bethesda, MD 20892-0425, USA, **Phone:** (301) 496-4023, **FAX:** (301) 402-0079, **Email:** dfidock@nih.gov

[¶]Corresponding author. To whom proofs should be sent.

1. INTRODUCTION

Until very recently, i.e. nearly fifty years after the discovery of liver stage (LS) forms (Shortt and Garnham 1948a; Shortt *et al.* 1948b), remarkably little attention has been paid to the events surrounding invasion of an hepatic cell by a sporozoite (Spz) and the further intrahepatocytic division of *Plasmodium* parasites. The first of many contradictions is that these stages were for so long forgotten, are the least known and studied, the most difficult to handle and yet arguably still hold the greatest promise in terms of vaccine potential.

It is indeed well established that the injection of irradiated sporozoites (IrrSpz) can induce complete, sterile protection against massive challenges both in experimental hosts and humans (Cochrane *et al.*, 1980). This is most uncommon and therefore striking in the field of immunity to parasites. What is less well appreciated but has become increasingly clear is that what has long been considered an "anti-sporozoite immunity" is in fact related to the development of liver-phase trophozoites and therefore should more appropriately be referred to as "liver-stage dependent immunity" (if not necessarily anti liver-stage immunity - see below).

As compared to the sexual stages whose development occurs in the mosquito, the mammalian host harbors two comparatively simple asexual modes of division of the parasite. However, the liver and blood schizogony share only the word "schizogony" as they differ in many respects:

1. *By their host cell*: the hepatocyte is one of the most complex and metabolically active cells of the body, able to express MHC class I and class II molecules, whereas the red blood cell is primarily a hemoglobin vessel with neither nucleus nor protein-synthesis machinery;

2. *By their numbers:* an infective mosquito bite is estimated to lead on average to the formation of a dozen or fewer liver schizonts which reside for only a few days, lost in about 3 kilogram of liver. In contrast, individuals can harbor billions or even hundreds of billions of erythrocytic schizonts, which in areas of heavy transmission can manifest as chronic infections for several decades, constituting a true parasitic septicemia;

3. *By their metabolism*: major differences in gene expression and metabolism between liver and blood schizogony are well demonstrated by the differential susceptibility of these stages to some anti-malarial drugs, e.g. the 4- and 8-aminoquinolines including chloroquine and primaquine.

4. *By their antigenic make-up*: among the ca. 50-80 antigens (Ags) characterized to date in detail, few appear to be shared between the two stages - further evidence of the profound changes which occur in terms of regulation of gene expression depending on the host cell;

5. *By their replication*: the 100 μm LS schizont resulting in the production of 10,000 - 30,000 liver merozoites shows profound replicative and morphological differences from the 12 μm blood stage schizont resulting in 8-24 erythrocytic merozoites;

6. *By the spectrum and efficacy of defense mechanisms*: an extremely wide range of immune effectors has been found experimentally to block the pre-erythrocytic phase, in contrast to the limited number implicated in immunity against asexual blood stages (ABS), of which none appears fully efficient;

7. *By their accessibility to investigators:* erythrocytic schizonts can be easily seen, cultured, collected and purified, whereas this is either extremely difficult or virtually impossible for liver schizonts;
8. *By their immunogenicity:* immunological studies indicate a remarkably high level of immune responses to pre-erythrocytic Ags, extraordinary given the vast differences in parasite loads of pre-erythrocytic and erythrocytic stages (see section 5.4).[1]
9. *By their medical importance:* the pre-erythrocytic stages are clinically silent while ABS infection is responsible for pathogenesis leading to an estimated 3 - 4 million deaths per year.

Despite significant progresses in the recent years, the paucity of biological tools, in particular the lack of animal models of clear-cut relevance to pre-erythrocytic stage immunity in humans, is and if nothing is done will remain, the most severe limitation to developing what could otherwise become the most successful malaria vaccine.

2. BIOLOGICAL FEATURES OF LIVER STAGES

The existence in malaria parasites of an exoerythrocytic stage was first uncovered in avian parasites, in which exoerythrocytic schizogony was described in reticuloendothelial cells (reviewed by Garnham 1966). The location of this mysterious "exoerythrocytic phase" in mammalian malaria parasites began to be solved by the great P. C. C. Garnham with the discovery of *Hepatocystis* (=*Plasmodium*) *kochi* in parenchymal cells of the liver of African monkeys (Garnham 1947). Soon thereafter, the LS location of primate Plasmodia was confirmed first for *P. cynomolgi* then for the human parasites *P. vivax* and *P. falciparum* (with the help of some gallant volunteers!) (Shortt and Garnham 1948c; Shortt *et al.* 1951). This indeed filled in a missing piece in the life cycle of mammalian malaria parasites while uncovering many as yet unanswered questions.

Why the hepatocyte was selected as the first stopover of Plasmodia in humans remains an enigma. The intrinsic usefulness of this cell is illustrated by the fact that all mammalian Plasmodia have chosen it for their pre-erythrocytic phase; this in spite of the probably divergent evolutions taken by these different species to arrive at their respective vertebrate hosts (Waters *et al.* 1993). One of the proposed hypotheses that attempts to explain this is that the very complex hepatocyte metabolism fulfills the (as yet

[1] Footnote : it might be of significance that sporozoite and liver stages were formerly called by the negative definition of "exoerythrocytic stages" (i.e. not belonging to erythrocytic). This led us a few years ago to rename them "pre-erythrocytic" stages.

unelucidated) needs of the dividing LS schizont. Another is that the hepatocyte was selected as a site favorable from an immunological perspective, either because of minimal immunological response (a theory not supported by recent immunological studies that indicate the contrary; see below), or as a means of inducing immune tolerance (Rajan 1997) thereby favoring parasite survival at the subsequent erythrocytic stage**.** Curiously, avian Plasmodia, from which *P. falciparum* is thought to have arisen (Boulard 1982, Waters *et al.* 1991), avoid hepatocytes altogether and instead target skin and lymphoid macrophages as well as endothelial cells in their "exoerythrocytic" cycle (Garnham 1966).

What is known is that the pre-erythrocytic phase is an extremely, perhaps the most, successful part of the life cycle of an ecologically successful parasite: Plasmodium, at least in terms of the high invasion and development rates in naïve animals. It is now generally agreed that a mosquito bite delivers on average 12-20 Spz (Rosenberg *et al.* 1990; Ponnudurai *et al.* 1991), which will either remain in the skin or enter the blood flow or lymphatic system (Meis and Verhave 1988; Sidjanski and Vanderberg 1997). Either way, the parasite must encounter a considerable number of cells reported as being able to ingest or kill Spz, or on numerous occasions find itself in an irrelevant site, making it extraordinary that as many as half of those Spz can successfully invade -and develop within - the hepatocyte (Bray 1962; Druilhe *et al.* 1984; Meis *et al.* 1990). How invasion occurs once the Spz reaches the liver has been a source of great debate (Vreden 1994). Some studies from *P. berghei* in rats suggest that Spz can transit safely via neighboring Kupffer cells before invading hepatocytes (Meis *et al.* 1983), an indication of the unusual interactions between phagocytes and Spz and possibly a vestige from once-related avian malaria parasites. Other studies however argue that Spz enter via the space of Disse in-between endothelial cells (Shin 1982) or are taken up directly after attaching to sulfated glycoconjugates present on hepatocyte surface microvilli or other hepatocyte surface components (see chapter 6/Frevert for an extensive review of the role of the CS and TRAP proteins in this process). An alternative possibility is that the Spz transits through the lymphatics from where it can gain direct access to hepatocytes, since the liver has a well-defined lymphatic system (Rouviere 1981). *In vitro* invasion, albeit occurring at a very low rate, provides evidence that Kupffer cell involvement is at the very least not essential (Hollingdale *et al.* 1984; Mazier *et al.* 1985). It is important to note that Spz infectivity rates are very dependent on the vertebrate host being studied. Available data show that in their normal host, humans for human plasmodia, or the tree rat *Thamnomys* for rodent plasmodia, about 50 % of the injected Spz transform successfully into maturing

liver schizonts and a blood schizogony can be obtained by injecting as little as 2 to 10 Spz. Such is not the case for more unusual hosts, such as laboratory mice, nor with *in vitro* conditions for production of LS, in which the success rate varies enormously (see section 4).

Looking back, it is striking that up until the mid-80's the only aspect of LS biology that received attention was that certain species are capable of arresting their development at this stage, only to "revive" at a later date and cause malaria relapses. While in the past relapses due to *P. vivax* and P. ovale were attributed to an imagined "exoerythrocytic" cycling schizogony in the liver, there are now convincing data that these relapses originate from parasites dormant in the liver and that no such cycling occurs (Collins *et al.* 1981; Krotoski *et al.* 1982a; Cogswell 1992). This is supported by findings of uninucleate "hypnozoites", dormant parasites that persist in the liver in *P. vivax*, P. simiovale and *P. cynomolgi* hepatocyte infections (Krotoski *et al.* 1980, 1982a, 1982b; Cogswell *et al.* 1991). This is in contrast to malaria recrudescences, for example observed with *P. malariae* and that result from chronic maintenance of very low-grade blood parasitemia (Vinetz *et al.* 1998). The biological basis remains totally unknown as to why a proportion of the parasites can remain dormant in their intra-hepatic development and why they can wake up later, for example when the temperature rises and vectors become more abundant.

Since the beginning of the 90's, there has been a much wider interest in LS biology, derived firstly from molecular and immunological studies aimed at developing pre-erythrocytic stage vaccines (see below) and secondly from the emergence and fast propagation of multi-drug resistant malaria, which has revived interest in "causative" chemoprophylaxis. At least theoretically the pre-erythrocytic stages represent a better target for preventive regimens than do the pathogenic erythrocytic stages. Given the effectiveness of a wide range of immune mediators (see section 6), they may in general be more susceptible targets. There are most probably numerous metabolic differences between liver and blood schizogony whose identification would be important both for drug and vaccine issues. For example, data suggests that there is a system for aerobically generating energy in the LS and a glycolytic metabolism system for the erythrocytic forms (Verhave and Meis 1984).

At the immunological level, many fascinating questions have arisen about the trafficking of cells to and from the liver, molecular trafficking within the host hepatocyte, the parasite's influence upon MHC class I and class II expression and the molecular differences between liver and erythrocytic merozoites, to name but a few.

Once inside, it should first be recalled that the development of the LS parasite shows unusual characteristics. There is a single generation of merozoites that progressively individualize among a large mass of cytoplasm, then separate as "packs " or islets in the process of pseudocytomerization of the aposchizogony, within which merozoite membrane formation happens shortly before schizont rupture. At an early stage of schizogony the cytoplasm contains numerous vacuoles which later migrate and empty their flocculent or granular material into the PVM space. This material, which participates in the process of merozoite individualization, might be of immunological importance as it appears to surround and adhere to individualized merozoites, potentially forming part of their membranes (Garnham *et al.* 1969; Meis *et al.* 1985; Meis and Verhave 1988). This material is also preferentially taken up by neighboring macrophages (Meis *et al.* 1985) and in immuno-electron microscopy (immuno-EM) studies many of the recently characterized LS Ags were found to be associated with it (Suhrbier *et al.* 1988, Atkinson *et al.* 1989; Fidock *et al.* 1994a; Bottius *et al.* 1996a). Given the morphological differences between liver and blood merozoites (Seed *et al.* 1974), and the profound differences in Ag expression, it is quite possible that the surface Ags of those two merozoite stages differ substantially.

Many studies have focused on the mechanisms of defense, but few have studied how the immune response is i) induced and ii) can target the infected hepatocyte. Priming of naive T cells by Spz can occur in lymphoid organs where they are activated by specialized presenting cells, macrophages, dendritic cells and B cells (Pape *et al.* 1997). Some studies suggest that Spz have developed a mechanism to avoid destruction by macrophages (Danforth *et al.* 1980; Seguin *et al.* 1989). Indeed, Spz are able to exit from and induce the death of macrophages (Vanderberg *et al.* 1990). Sporozoites are too large to be internalized by B cells, however released material (Stewart and Vanderberg 1992 and references within) might be taken up by specific B cells thereby initiating a T cell response (Link *et al.* 1993). Given the rodent data showing that cytolytic cells play a major role in immune defense, one important issue is that of protein or peptide trafficking from the LS parasite to the host cell membrane.

The parasite is separated from the host cell cytoplasm by the double membrane of its parasitophorous vacuole, which may constitute a barrier to the transport of malarial peptide epitopes to the host cell membrane by MHC class I or class II Ags. The possibility that infected hepatocytes can process parasite Ag and prime naive T cells was considered unlikely since hepatocytes are devoid of co-stimulatory molecules such as B7.1 and B7.2 (Vandenberghe *et al.* 1992). Nevertheless, recent studies involving brefeldin A, an inhibitor of protein trafficking to the golgi, indicate that malarial Ag processing and peptide presentation can indeed be mediated at least by rodent hepatocytes (Rénia *et al.*, manuscript submitted for publication). Another possibility is that the death of some infected hepatocytes may lead to Ag processing by neighboring macrophages or dendritic cells that could migrate to the closest lymph node and initiate a response. Another important issue concerns homing of T cells to the source of the LS infection. $CD8^+$ T cells have been found in the liver parenchyma of Spz-immunized animals (Hoffman *et al.* 1989a), although their specificity and anti-plasmodial activity was not confirmed. If LS Ag-specific T cells are induced outside the liver (either by immunization or during natural infection), then LS elimination depends on their ability to migrate to the liver. Adoptive transfer experiments using $CD8^+$ cytotoxic T cell clones have shown that while some T cell clones were protective, others completely failed to eliminate malaria LS parasites *in vivo*. The protective phenotype was found to be associated with the expression of adhesion molecules such as CD44 and VLA-4 implicated in homing (Rodrigues *et al.* 1992).

It should also be recalled that hepatocytes are not a homogeneous cell population. *In vivo*, hepatocytes are organized in a particular structure, the lobule, where a single terminal hepatic venule is surrounded by a circular zone of parenchyma with a number of terminal portal venules at the periphery. Hepatocytes on these two locations display different metabolism (Quistorff 1990), for example periportal hepatocytes have the enzymes for gluconeogenesis and sinusoidal hepatocytes have the enzymes for glycolysis. Moreover, hepatocytes are separated from the veins and arteries by the space of Disse (containing a dense extracellular matrix mostly composed of collagen, laminin and proteoglycans) (Wisse *et al.* 1985). There is ample evidence that the nature of the matrix regulates hepatocyte gene expression (reviewed in Bucher *et al.* 1990). Thus, parasites may survive better *in vivo* in a phenotypically distinct subset of hepatocytes.

This underscores the importance of understanding parasite and cell trafficking at the cellular or histological level, parasite Ag processing, priming of naive T cells, *in vivo* migration pathways of malaria-specific T cells and finally the recognition of infected hepatocytes during a protective immune response. Knowledge of these areas is of fundamental importance to the design of a vaccine against pre-erythrocytic stages of malaria.

3. SPOROZOITE- OR LIVER STAGE- DEPENDENT IMMUNITY?

The initial observation of Mulligan *et al.* (1941), that bites of infective mosquitoes exposed to UV radiation could confer protection to challenge, and the confirmation using X-irradiation and subsequent analyses by Vanderberg and Nussenzweig (Nussenzweig *et al.* 1967a, 1969a, 1969b), constitute the foundation upon which nearly 50 years of research on a Spz vaccine have been based (reviewed in Nussenzweig and Nussenzweig 1989; Druilhe and Marchand 1989; Hoffman *et al.* 1991). In hindsight, it appears that these initial observations may have been pointing in the wrong direction, the Spz stage.

In the field of "anti-Spz immunity", the past 10 years have seen a major and justified revival of interest in favor of the liver stages. The reasons for this important change of direction are two-fold: i) substantial evidence has accumulated that intrahepatocytic parasites, blocked in their development, are responsible for the immunity acquired following injection of IrrSpz (Mellouk *et al.* 1990a; Londono *et al.* 1991; Scheller and Azad 1995a; Chatterjee *et al.* 1996); and ii) investigations into the susceptibility of LS to immune effectors have been prompted by the relative lack of success of CS-based vaccines (see Doolan *et al.* 1998) and also by discoveries on the effect of cytolytic T cells and lymphokines on other hepatotropic micro-organisms such as viruses and bacteria (including Hepatitis B and Listeria; see reviews by Kaufmann 1993; Chisari 1997).

3.1 LS as inducers of protection

Indeed, scientific reports dating back many years have given indications that LS parasites were essential to the *induction* of the IrrSpz type of immunity whereas Spz were not (Druilhe and Marchand 1989). These indications were sufficiently important for us to reorient our work to the

study of the LS antigenic repertoire (Druilhe *et al.* 1984). Today, the evidence for the principal role of liver forms is 4-fold:

i) Protection was induced only when live Spz were injected by a route enabling liver infection. Although IrrSpz were strikingly immunogenic when injected intravenously, they gave no or very little protection when injected intramuscularly, intraperitoneally, subcutaneously or even orally, either with or without adjuvants (Nussenzweig *et al.* 1972a; Jakstys *et al.* 1974; Kramer and Vanderberg 1975; Alger and Harant 1976). It was also known that X ray-irradiated Spz needed to be alive: too low an irradiation dose led to blood stage infection while too high a dose led to absence of protection (Nussenzweig *et al.* 1972a; Scheller and Azad 1995a; Chatterjee *et al.* 1996). At the dose inducing protection but not resulting in a blood infection, Spz were found to transform into LS trophozoites that remained blocked in their development, unable to divide (Ramsey *et al.* 1982; Siegler *et al.* 1984; Nussler *et al.* 1989). It was also shown that live, non-irradiated Spz could achieve the same goal when injected in mice or rats treated with drugs that either block LS parasites (α-difluoromethylornithine) or prevent blood stage infection (chloroquine). These animals were protected against Spz but not ABS challenges (Beaudoin *et al.* 1977; Golenser *et al.* 1977; Meuwissen *et al.* 1978; Orjih *et al.* 1982; Francois *et al.* 1997). Conversely, evidence arguing against a critical role of Ags expressed solely at the Spz stage was provided by the demonstration that intravenous inoculation of Spz extracts or of Spz killed by multiple methods failed to confer any immunity (Cochrane *et al.* 1980; Alger and Harant 1976; and references within);

ii) Additional evidence pointing to young liver trophozoites came from the observation that following the injection of IrrSpz, the liver trophozoite could remain in its host cell for very long periods (up to half a year; Londono *et al.* 1991; Scheller and Azad 1995a), thereby creating prolonged antigenic stimulation. Strong support for the critical role of LS-blocked trophozoites was derived from precise *in vitro* correlates of *in vivo* clinical observations in humans and mice immunized either by high-dose IrrSpz and not protected, or lower-dose IrrSpz and protected (Mellouk *et al.* 1990a; Herrington *et al.* 1991). These studies demonstrated, for both humans and mice, a direct correlation between the ability of Spz to invade and their ability to induce protection;

iii) Confirmation of the essential role of LS parasites came with experiments in which the administration of primaquine, active against LS, was able to destroy remnant irradiated LS

trophozoites and simultaneously to abrogate the protection induced by IrrSpz (Scheller and Azad 1995a);

iv) Further support also came from experiments indicating that LS extracts (Scheller and Azad 1995a) or intact LS parasites (Rénia *et al.* 1994) could induce significant protection, whereas Spz extracts or inactivated Spz had consistently failed to achieve the same goal (Spitalny and Nussenzweig 1972; Alger and Harant 1976).

Thus today, there is a wealth of evidence concurring that the presence of an intrahepatocytic parasite is in fact the *critical* component in IrrSpz-induced immunity. This also suggests, though does not demonstrate, that Ags expressed by the LS parasite may be more relevant than those expressed solely at the Spz stage. The finding that reversal of protection occurred when primaquine was given at 7 days post-Spz inoculation but not at day 30 (Scheller and Azad 1995a) tends to rule out the possibility that this immunity could be of a "concomitant type" (i.e. concomitant to the persistence of the parasite) as described in schistosomiasis. Instead, it suggests that a certain delay of intrahepatocytic location is required for the induction of the critical immune effectors. Further studies are still needed to determine whether this immunity is related to the expression of novel Ags specific to LS, or alternatively whether the critical Ags are carried in by the Spz and the extended duration of the blocked liver-trophozoite constitutes an “Ag depot” which leads to improved immunogenicity, possibly by leading to optimized Ag presentation through the hepatocyte’s MHC pathway. In this context, it may be noteworthy that the expression of some of the recently characterized Ags shared between Spz and LS stages (see section 5) appears much more active during the latter.

3.2 LS as target of defense mechanisms

Pre-erythrocytic stage investigations have provided evidence that the intrahepatocytic location is the target for a very wide range of immune effectors, whereas the Spz stage is susceptible to mainly Ab-dependent mechanisms (see section 6). Indeed, it has been shown, at least in rodent models, that a very wide range of cell types can mediate the destruction or the blockade of LS parasites, either directly or indirectly by the release of lymphokine or other pharmacologically active substances. Antibodies can also act directly or indirectly on infected hepatocytes. One pertinent

example of the importance of cell-dependent defenses is that of inflammatory responses to infected hepatocytes. Following immunization of rats or mice with *P. berghei* IrrSpz, but not heat-killed Spz, challenge parasites were found to be surrounded by a granuloma consisting of macrophages, T cells, neutrophils and eosinophils (Khan *et al.* 1992, Hoffman *et al.* 1989a). It is probable that this cell migration is an important component of anti-LS immunity, though this lacks definitive demonstration. In this respect it is worth recalling that this resistance mechanism is highly prevalent in unnatural hosts (rats or mice) (Khan and Vanderberg 1991a, 1991b; Vanderberg *et al.* 1993, Meis *et al.* 1987) whereas it is rarely observed in the natural host *Thamnomys* (Bafort 1971). This innate immune response in unnatural hosts is also characterized by an infiltrate of mononuclear cells, neutrophils and eosiniphils around late-stage schizonts and emerging merozoites, and is likely to contribute to the lower susceptibility of mice to *P. yoelii* infection, as compared to *Thamnomys*, and of mice to *P. berghei* as opposed to *P. yoelii* (Khan and Vanderbergh 1991a).

Thus, considerable data now focus on the importance of LS, but one should distinguish in the literature those reports concerning the LS as a preferential *target* of defense mechanisms from those related to its potential importance as the crucial component in *inducing* protective responses.

4. TECHNICAL HURDLES ASSOCIATED WITH THE STUDY OF LIVER STAGES

The clinical silence of this phase of the cycle, its previously-presumed immunological silence, and above all the issue of accessing LS parasites in humans and their minuscule numbers in comparison to blood stage infections make it understandable that many researchers were discouraged from studying the liver stage. As a result, many of the necessary tools are presently missing or only partially adequate. The emphasis subsequently placed on hepatoma cell lines for *in vitro* work and rodent models for *in vivo* work, while facilitating research activities, may turn out to be detrimental as the possibility exists that a significant proportion of this data might be misleading.

4.1 Low output of human parasite LS production

In view of the unfeasibility of reproducing Pr Garnham's pioneering experiments in humans, we successfully attempted to obtain *P. falciparum* LS development in the South American monkey *Cebus appella* (Druilhe *et al.* 1984). This experiment led to the first demonstration of the expression of novel LS-specific Ags in *P. falciparum*. It also showed that no adaptation was needed for this stage and that the host specificity was wider for hepatic than for blood stages (to which this monkey species is refractory). Liver stage infections by *P. falciparum* and *P. vivax* were also readily obtained in *Saimiri*, *Aotus* (P. Druilhe, unpublished data, see Figure 1) and chimpanzees (Draper *et al.* 1971; Collins *et al.* 1973; Rossan and Baerg 1975; Szarfman *et al.* 1988a; Meis *et al.* 1990). However, despite the high Spz to LS transformation rates the resulting density of LS in the liver was low. Progress in the primary cultivation of hepatocytes subsequently enabled *in vitro* production of LS material for all four human parasites as well as many rodent and simian species (Doby and Baker 1976, Strome and Beaudoin 1979, Sinden and Smith 1980, Lambiotte *et al.* 1981, Hollingdale *et al.* 1981; Pirson 1982, Mazier *et al.* 1982, 1984, 1985, 1987; Smith *et al.* 1984; Millet *et al.* 1988a, 1988b). The very low output of both *in vivo* and *in vitro* methods nevertheless has prevented much needed access to both proteins and mRNA. Furthermore, among the many attempts to separate infected from uninfected hepatocytes, none have so far proven successful (personal communications and unpublished data). Combined with this is the significant difficulty of producing workable quantities of Spz (which for human malaria parasites is feasible only with *P. falciparum*) and the frequent presence of contaminating bacteria and yeast which severely affect *in vitro* investigations.

4.2 Measuring the efficacy of interventions against the liver stage.

One of the reasons behind the lack of efficient methods for producing LS material from human malaria parasites lies in the remarkable success of the IrrSpz vaccine. Since IrrSpz vaccines induced sterile protection, this set the highest possible standard for subsequent vaccine trials. Accordingly, their success was determined on the basis of the emergence - or not - of a blood infection (or sometimes delays in patent infections). This prevented any accurate quantitative assessment of the LS parasite burden as a result of immunization and therefore a refinement of the Ag choice and delivery. Another consequence was the lack of investigation of cellular and parasitological events taking place inside the liver, which could otherwise have supplied important clues as to the mechanism induced by immunization. The LS

remained an enigmatic black box. Only recently have techniques been developed to quantify the LS load, thus simplifying the assessment of protection (Ferreira *et al.* 1986; Arreaza *et al.* 1991; Li *et al.* 1991; Briones *et al.* 1996; Hulier *et al.* 1996). These techniques have however yet to be successfully applied to *P. falciparum* LS and microscopic investigations remain an essential tool (Figure 2).

4.3 Variability depending on host/parasite combinations

For reasons cited above, pre-erythrocytic stage research has focused almost exclusively on rodent malaria models, in spite of their debatable relevance (see below and section 6). For practical reasons this research was usually performed using laboratory mice, and not the normal *Thamnomys* host. However, mice are generally far *less* susceptible to Spz invasion and successful LS development than are rats, being themselves less susceptible than the normal host (Nussenzweig *et al.* 1966; Wéry 1968). There is also a wide degree of variability of susceptibility between congenic strains of laboratory mice, with some strains requiring the injection of Spz in numbers three orders of magnitude higher than others to develop a blood infection (Jaffe *et al.* 1990). These differences also depend on the parasite species, thus one should speak of host/parasite combinations. Many additional factors influence the outcome of this combination (Nussenzweig *et al.* 1966; Wéry 1968; Vreden *et al.* 1995; Scheller *et al.* 1994), including the host's age and hormonal status, e.g. estrogen levels, contributing to the lesser susceptibility of males with respect to females, with pregnant females being the least susceptible of all; or corticosteroids which increase the overall susceptibility (Verhave *et al.* 1985).

4.4 Limitations of *in vitro* models

The situation is even more complex under *in vitro* conditions, as the numbers of developing parasites can be exceedingly low as compared to *in vivo* infections, e.g. 10,000 to 100,000 times less (Mazier 1986a; Beaudoin *et al.* 1988*)*. Efficiency of intrahepatocytic development also depends on the source of hepatocytes. Cells derived from the natural *Thamnomys* host are more susceptible than rat or mouse hepatocytes. Parasite strains or species also display different efficiencies in invasion rate, for example *P. berghei* is more infective *in vitro* than *P. yoelii*. In addition *P. berghei* differs strikingly from *P. yoelii* in that Spz of the former can also penetrate and develop in the human HepG2 hepatoma cell line or in human

Hela cells (in which full maturation requires twice as long as in hepatocytes, i.e. 96 versus 42 h; Hollingdale *et al.* 1981; Calvo-Calle *et al.* 1994). Surprisingly, there is an inverse relationship between the two species in their *in vivo* and *in vitro* invasion rates: the invasion rate of *P. yoelii* is high *in vivo* though low *in vitro*, whereas *P. berghei* stands in the opposite situation with the highest invasion rate of all malaria species *in vitro*, both in hepatoma and in hepatocytes, and comparatively low *in vivo* success in mice. Similar and unaccounted differences are also observed between *P. falciparum* and *P. vivax*: both develop completely with similar efficiency in primary human hepatocyte cultures however only *P. vivax* can mature in HepG2 cells (Mazier *et al.* 1984; Smith *et al.* 1984; Hollingdale *et al.* 1985).

Taking into account these differences between models is essential to the interpretation of and particularly the comparison between experiments. For example the tumoral hepatoma cell line, HepG2-A16 (Hollingdale *et al.* 1983) has for practical reasons been much more used to assess the effect of immune effectors (principally to *P. falciparum*) than has the more problematic human hepatocyte. In spite of the logistical complications, several critical differences show that it is imperative to use primary human hepatocytes to obtain reliable data. In practical terms the lack of *P. falciparum* development in HepG2 directly influences the assessment of invasion inhibition studies. In our experience, it has proven extremely difficult to distinguish between a uninuclear, blocked parasite inside a HepG2 cell and a rounded-up Spz that is immediately adjacent though still extracellular (although double-labeling techniques can help in this area (Rénia *et al.* 1988). In contrast, within hepatocytes, the normal development of the parasite leads to the production of liver schizonts that after 3-5 days are readily distinguishable from non-invaded Spz by their large size, intrahepatocytic localization and morphology. Moreover, HepG2 cells clearly differ functionally from host hepatocytes, as evidenced by the former's totally artificial receptivity for *P. berghei* contrasting with its inability to permit intrahepatocytic development of *P. falciparum* (Hollingdale *et al.* 1983). This demonstrates critical differences which may also concern their susceptibility to invasion. Indeed, multiple comparative studies have revealed major discrepancies in the inhibitory effect of Ab to Spz molecules, frequently being near-total for HepG2 while dramatically weaker for hepatocytes (Mazier *et al.* 1986b; Hollingdale *et al.* 1987, 1990a; Moelans *et al.* 1995; Ramirez *et al.* 1995). In one study, Ab found to significantly inhibit *P. falciparum* Spz invasion of HepG2 cells gave the opposite result, i.e. an increase in the numbers of invaded Spz, in human hepatocytes (Hollingdale *et al.* 1990a). Furthermore, data from three human vaccine trials shows a lack of

correlation between levels of inhibition of Spz invasion (ISI) in HepG2 and protection status (Fries *et al.* 1992; Egan *et al.* 1993; Brown *et al.* 1994). In favor of the relevance of the primary hepatocyte assay, a close correlation has been observed, for both *P. falciparum* and *P. yoelii*, between the levels of anti-Spz immunity *in vivo* and the *in vitro* inhibition found with the relevant anti-Spz Ab (Mellouk *et al.* 1986, 1990b; Charoenvit *et al.* 1991). These findings lend weight to the assertion that data obtained using human hepatocytes will more faithfully reflect the true capacity of these Ab to limit Spz invasion *in vivo*.

5. ANTIGENIC REPERTOIRE

Detailed knowledge of the antigenic repertoire of intrahepatocytic parasites is still in its early days. Indeed, for many years it was widely believed that immunity to IrrSpz was mediated by Ab active against the CS protein, thought to be the sole major antigen expressed by Spz (Nardin *et al.* 1982; Nussenzweig and Nussenzweig 1985; Tam and Zavala 1989). This led to a staggering quantity of research on this one protein to the exclusion of any other antigenic proteins that might have been expressed during the Spz or LS. A series of disappointing results with recombinant CS vaccine trials, coupled with increasing recognition of the importance of LS parasites in IrrSpz-induced immunity and the discovery of many new pre-erythrocytic stage Ags, has begun to correct this imbalance. While the field is still challenged by the significant technical complexities and limitations of working with LS parasites, innovative approaches in the past few years from a number of groups have enabled substantial progress to be made. This section serves to illustrate these difficulties and approaches taken to overcome them and also to review our knowledge on the considerable number of *P. falciparum* Ags currently identified as being expressed during the liver stage.

Limited access to LS material has significantly impeded the pace of molecular LS investigations. At the protein level, analysis of human Plasmodia LS Ags has mostly been restricted to direct labeling assays (e.g. IFA) using specific antisera on primate or cultured LS at various stages of development. Indeed, the single reported Western blot analysis of a *P. falciparum* LS antigen (LSA-1) required pooling of 6 months of cumulated infected hepatocyte cultures before sufficient protein was obtained (Fidock *et al.* 1994a). The IFA assays harbor the danger that a positive signal

may not come from the Ag under investigation but from another against which the Ab cross-react. Cross-reactivity is frequent amongst *P. falciparum* Ags, mostly as a result of the abundance of repeat units in these Ags that often share a limited subset of predominant amino acids. Infected hepatocyte IFA assays with Ab directed to distinct epitopes present in different regions of the molecule are thus needed before actual expression during the LS can be inferred with confidence.

5.1 Identification by human antibodies specific to pre-erythrocytic stage antigens

Direct evidence for the existence of non-CS pre-erythrocytic stage Ags came with the demonstration that a considerable proportion of human antisera reacted against the Spz surface though had no reactivity against the immunodominant CS repeats (Galey *et al.* 1990). This confirmed earlier indications of multiple *P. falciparum* Spz surface proteins (Druilhe *et al.* 1986) and was consistent with results indicating the presence of multiple non-CS Spz Ags in *P. yoelii* (Charoenvit *et al.* 1987; Wortman *et al.* 1989). Finally, evidence for the LS-specific expression of novel molecules (Druilhe *et al.* 1984) and the importance of LS parasites in protection (see section 3) prompted their identification.

Given the limitations in accessing LS material, the only way to identify these additional Ags appeared to be by their isolation from a genomic library. This first required the development of specific probes. Attempts to produce monoclonal Ab specific for LS were unsuccessful in mice and while they were produced in human cells they were unsuitable for screening purposes *(*Marchand *et al.* 1990*).* Subsequently, we developed a strategy to identify *P. falciparum* pre-erythrocytic stage Ags on the basis of screening with human stage-restricted sera. Notably, we selected seven individuals who had been living throughout Africa for 15-26 years and had continuously adhered to a daily chloroquine prophylactic regimen (at a time when chloroquine was still effective). Since this drug acts on heme polymerization it has no effect on pre-erythrocytic stages. These individuals, including a priest known as “Père Mauvais” (“PM”), had not suffered from malaria attacks due to blood-stage infection however had clearly been repeatedly exposed to pre-erythrocytic stages, as determined by IFA (their average titers on Spz, LS and ABS were 1/3200, 1/3200 and 1/100 respectively) and Western blots on Spz and ABS (Marchand *et al.* 1990).

Screening of a *P. falciparum* genomic DNA expression library with these sera, in particular the "PM" serum, led to selective identification of multiple Ags expressed by Spz and/or LS (Marchand *et al.* 1990). In total, 120 clones were isolated, of which only one corresponded to the immunodominant repeats of the CS protein.

5.2. Molecular characteristics of the "PM" antigens

Cross-hybridization and cross-reactivity studies on the subset of 120 "PM" clones indicate the presence of twenty or so separate Ags. While many have been partially characterized, the greatest attention has so far been paid to LSA-1, SALSA, STARP and LSA-3 and the following section is therefore restricted to these four Ags.

LSA-1 (Liver Stage Antigen-1) was the first antigen reported to be expressed during the LS (Guérin-Marchand *et al.* 1987) and is still unique in being the only known Ag whose expression is restricted to the intrahepatocytic parasite (Table I). The LSA-1 gene is 5.8-kb in length composed of a single intron with a long block of 17 amino acid repeat units (Zhu and Hollingdale 1991). The LSA-1 sequence is highly conserved in the strains examined to date (Zhu and Hollingdale 1991; Fidock *et al.* 1994a; Aidoo *et al.* 1995; Yang *et al.* 1995) and importantly is generally well conserved in regions containing T helper and CTL epitopes (Aidoo *et al.* 1995; Yang *et al.* 1995) (see below). Identification of a *P. berghei* gene (*P. berghei* LSA-2) that may be the homologue of LSA-1 has been reported (Hollingdale *et al.* 1990b) though the nucleotide sequence and protein characterization has yet to be published. Western blot analysis of infected hepatocytes indicates that the *P. falciparum* LSA-1 has a molecular mass of 200 kDa, while IFAs show that expression of this Ag begins shortly after formation of the LS trophozoite and continues at a high level throughout schizogony (Fidock *et al.* 1994a). This Ag was localized to the parasitophorous vacuole (PV) space by immuno-EM. Late in schizogony when segmentation occurs the plasmalemma appears to break down and LSA-1 can be seen associating with the flocculent material that literally bathes the liver merozoites at the time of hepatocyte rupture and merozoites release. This flocculent material was previously observed in *P. berghei* to be preferentially phagocytosed upon rupture of infected hepatocytes (Meis *et al.* 1987). Interestingly, the α-helical coiled-coil organization and extraordinary length (ca. 220 nm) of the LSA-1 central region is reminiscent of streptococcal M

proteins, whose role is to protect bacteria from complement -mediated phagocytosis. Hypothetically, LSA-1 may play a similar role in actively impairing host phagocytosis or complement-mediated activation upon hepatocyte rupture. One might also postulate that immune interactions at the time of rupture are responsible for the tremendous Ab responses to LSA-1 and other flocculent material-associated Ags (including SALSA and LSA-3) detected in the sera of naturally-exposed individuals (see below).

LSA-3 has been identified as a 200-kDa protein detected on the Spz surface and in internal organelles of Spz as well as in infected hepatocytes, where its localization appears similar to those of LSA-1 and SALSA. The single-copy LSA-3 coding sequence is 5.5-kb with a 5' mini-exon / intron / large exon structure. This gene contains a central repeated region, rich in glutamic acid, and a large non-repeated C-terminal region interrupted by a short repetitive block. The main repeats are notable in their complexity of internal duplications combined with a very high degree of nucleotidic conservation in *P. falciparum*, preserving this region's strikingly regular α-helical structure (P. Daubersies *et al.*, in preparation). This high level of conservation extends to the non-repetitive regions and importantly, is also observed in sequences defining epitopes recognized by inhibitory Ab or human CTLs (Aidoo, submitted for publication; see below). Expression of LSA-3 proved restricted to Spz and LS as assessed by RT-PCR (positive on Spz mRNA), Northern blot (negative on ABS mRNA), Western blot and IFA using Abs to three non cross-reactive epitopes. A homologous protein sharing several B and T helper epitopes with *P. falciparum* has been identified in *P. yoelii* (BenMohamed *et al.*, 1997, unpublished data). Ab specific to LSA-3 detect a 205 kDa protein in *P. yoelii* Spz extracts and can totally block *P. yoelii* Spz invasion of rodent hepatocytes (K. Brahimi, unpublished data), leading to a current drive to isolate the corresponding gene from this rodent species. Interest in this antigen is supported by protection data. For instance, immunization of mice by *P. falciparum* LSA-3 was found to induce full protection in mice against a *P. yoelii* Spz challenge (see Figure 2). (K. Brahimi *et al.*, manuscript in preparation).

The STARP antigen was initially chosen for investigation based on IFAs indicating its consistent expression on the surface of Spz from a geographically wide range of endemic regions (Fidock *et*

al. 1994b). Immuno-EM studies on Spz and LS using Ab directed to either repetitive or non-repetitive regions confirmed the location of this protein on the Spz surface and indicated active synthesis during the intrahepatocytic stage. Sporozoite expression was verified using Northern analysis and RT-PCR on Spz RNA, taking advantage of the size difference between STARP cDNA and genomic DNA resulting from the splicing of the 5' intron during mRNA synthesis. STARP was also found to be expressed by cultured ABS parasites at the early ring stage following merozoite invasion. The function of this 78-kDa protein in the human host remains entirely unknown. One possible clue to its function in the mosquito vector comes from the finding that STARP-specific Ab recognize a determinant on the surface of uninfected salivary glands of *Anopheles gambiae* (C. Guérin-Marchand and P. Sinnis, independent unpublished observations). One speculation is that STARP may be performing molecular mimicry enabling the Spz to evade the mosquito vector's recognition/defense mechanisms; an idea that can now be tested using gene transfection and knockout techniques (Wu *et al.* 1995; Crabb *et al.* 1997; Fidock and Wellems, 1997b). The STARP gene harbors a complex array of internal repetitive regions which are consistently conserved in *P. falciparum* and this gene has also been sequenced from *P. reichenowi*, phylogenetically very closely related to *P. falciparum* and which parasitizes chimpanzees. Cross-hybridization studies further indicate the presence of a STARP homologue in rodent malaria parasites though sequence information has not yet been obtained (Fidock *et al.* 1994c).

SALSA (Bottius *et al.* 1996a) was selected on the basis of its strong reactivity with antisera that recognized neither CS nor LSA-1 yet were highly reactive with pre-erythrocytic stage parasites. IFA and immuno-EM localize SALSA to the Spz surface and to the flocculent material in the PV of the developing LS parasite. Spz expression was confirmed by definitive results from RT-PCR studies and by labeling of Spz blots with two non cross-reactive populations of SALSA-specific Ab which consistently detected a 70-kDa protein. Western blotting also detected a 41-kDa protein in ABS protein extracts. This was thought to possibly indicate a cross-reactive protein as sera of transfusion malaria patients having high levels of Ab to ABS parasites were uniformly nonreactive to the SALSA Ag. Independent work however has demonstrated ABS expression of a 40-Kda protein recognized by Ab raised to the MSP-4 gene, a single-copy locus with almost perfect homology to the SALSA fragment previously reported (Marshall *et al.* 1997). MSP-4 was

furthermore proposed, on the basis of Triton X-114 partitioning and IFA and preliminary immuno-EM patterns, to be expressed on the surface of erythrocytic stage merozoites. The MSP-4 protein has a putative signal sequence, appears to be GPI-anchored to the ABS merozoite surface and contains an EGF-like domain that may be important for protein-protein interactions. While the cause of the discrepancy in the protein sizes detected in Spz and ABS remains to be solved (likely reflecting either cross-reactions or stage-specific alternative splicing), all the evidence to date points to MSP-4 and SALSA being expressed by the same gene.

Partial sequence data obtained from 8 additional Ags of the initial 120 in the "PM" subset recently determined that six were previously unidentified pre-erythrocytic stage Ags. This provides further evidence that profound modifications in gene expression are associated with the intra-hepatic location of the parasite (A.-C. Grüner and P. Druilhe, unpublished data).

5.3. Antigens initially identified in other stages or in rodent *Plasmodia*

The major emphasis on malaria vaccine research has generated detailed analysis of a multitude of Plasmodium Ags, leading in a number of cases to evidence of the pre-erythrocytic stage expression of *P. falciparum* Ags initially identified in other stages or in other *Plasmodium* parasites. As previously mentioned, the paucity of infected hepatocyte material available to investigators has restricted most molecular studies to LS IFAs, with the inherent danger that the Abs tested may recognize cross-reactive proteins and not reflect true intrahepatocytic expression. This danger must always be addressed prior to proposing the LS expression of an Ag however has been clearly recognized in some studies, with measures taken to reduce the chance of cross-reactivity. In consideration of chapter length constraints, this section will not discuss LS-expressed Ags identified only in rodent Plasmodia. In particular, this chapter will not deal with the CS protein as studies indicate that this Spz surface Ag, while carried into the infected hepatocyte during invasion, is not actively synthesized by the transformed LS parasite (Danforth *et al.* 1978; Suhrbier *et al.* 1988; Atkinson *et al.* 1989; Millet *et al.* 1991a; our unpublished observations).

TRAP/PfSSP2 was first identified as a result of its homology to region II of the CS protein and was thought to be expressed by ABS parasites (Robson *et al.* 1988). This protein was subsequently

recognized as being the *P. falciparum* homologue of the *P. yoelii* SSP2 protein, expressed by Spz and found in Spz micronemes and occasionally on the surface (Hedstrom *et al.* 1990; Cowan *et al.* 1992; Rogers *et al.* 1992a, 1992b). This was an important finding as rPySSP2 protein had been found to confer 100% protection against Spz challenge when used in conjunction with rCS protein as an experimental vaccine in mice (Khusmith *et al.* 1991). Furthermore, a $CD8^+$ CTL clone recognizing PySSP2 conferred protection upon passive transfer (Khusmith *et al.* 1994). Expression of the 90-kDa TRAP/PfSSP2 protein has been detected in early to mid stage LS parasites, while the 140-kDa PySSP2 protein is detected throughout LS development of *P. yoelii*-infected hepatocytes (Aikawa *et al.* 1990). TRAP/SSP2 orthologues have now been characterized for 7 species of *Plasmodium* (Robson *et al.* 1988, 1990, 1997; Rogers *et al.* 1992b; Sijwali *et al*. 1997; Templeton and Kaslow 1997) and share an integrin-like magnesium-binding A-domain, a thrombospondin-like sulfatide-binding domain (similar to the CS protein region II), an acidic repeat region, a transmembrane domain and a carboxyl acidic cytoplasmic domain. Recently, generation and analysis of TRAP-knockout *P. berghei* parasites uncovered a key role for TRAP/SSP2 in Spz motility and infectivity for both salivary glands and hepatocytes (Sultan *et al.* 1997).

PfEXP-1 was identified many years ago in blood stages (Hope *et al.* 1984, 1985; Coppel *et al.* 1985; Kara *et al.* 1988; Simmons *et al.* 1987). The PyHEP17 Ag was identified in *P. yoelii* with the aid of a mAb (NYLS3) raised to *P. yoelii*-infected mouse hepatocytes (Charoenvit *et al.* 1995). This mAb is to date the only Ab described as being active against *P. yoelii*-infected hepatocytes *in vitro*. Upon passive transfer it delayed onset and reduced the density of *P. yoelii* blood stage parasitemia after Spz or blood stage challenge (Charoenvit *et al.* 1995). Cloning of PyHEP17 and studies on its antigenicity and expression profile provided convincing evidence that this is the homologue of the *P. falciparum* protein PfEXP-1 (Doolan *et al.* 1996a). Both proteins appear to be expressed a few hours after Spz invasion, with expression continuing throughout LS development and ABS stages (Sanchez *et al.* 1994; Charoenvit *et al.* 1995). Interest in incorporating PfEXP-1 into a pre-erythrocytic stage vaccine has been strengthened by the finding that naked DNA vaccines incorporating PyHep17 alone or in combination with the CS gene could induce protection in mice (Doolan *et al.* 1996b).

The *P. falciparum* heat-shock proteins Pfhsp70, Pfgrp and PfHSP60 are also thought to be expressed during LS development (Rénia *et al.* 1990; Kumar *et al.* 1993; Das *et al.* 1997) and Ab data has been reported suggesting that the hsp70 protein in *P. yoelii* and *P. falciparum* may be present on the surface of the infected hepatocyte (Rénia *et al.* 1990).

Intrahepatocytic expression has also been suggested for a number of ABS Ags. One innovative approach took advantage of a genetic cross (Walliker *et al.* 1987). Evidence for MSP-1 expression was provided by IFA analysis of LS obtained in chimpanzees infected with either the serotypically-distinct parental clones or with parasites resulting from meiotic recombination (Szarfman *et al.* 1988b). IFA studies on the same set of hepatic biopsies also provided indications for LS expression of several other ABS Ags, including PfEMP2, SERA, ABRA and RAP-1 proteins (Szarfman *et al.* 1988a). In these cases however only a single Ab was tested, thereby failing to rule out the possibility that the positive signal may have come from separate cross-reactive Ags. Liver stage expression of GLURP has also been evoked, however this study suffers from the same caveat of possible cross-reactivity with true LS Ags (Borre *et al.* 1991). Continued investigation of which ABS Ag are expressed during the LS will help shed light on the extent of similarity between LS and ABS merozoites, in particular the extent to which the same molecules might be playing a role in RBC invasion by merozoites produced by these two stages. At a more general level, it can be predicted that many ABS genes will also be expressed by later-stage LS, particularly housekeeping genes. From a vaccine viewpoint however, co-expression of a given Ag in both LS and ABS parasites may diminish its potential for development as a pre-erythrocytic stage vaccine candidate since it is known that protective immunity induced against pre-erythrocytic stages does not protect against ABS challenge and vice versa. This may indicate that Ags expressed in both stages are not involved in protection.

Clearly, the preceding list is only the tip of the iceberg, as exemplified by the fact that only a single Ag has been identified whose expression is unique to the LS (LSA-1). Nevertheless, the situation is clearly far better than a decade ago when only one protein was known. It can be anticipated that research and understanding of the antigenic repertoire and molecular biology of Plasmodium LS will rapidly accelerate as a direct result of the improved recognition of the importance of LS in both

the *induction* and *targeting* of IrrSpz-induced immunity. Innovative advances in model and *in vitro* and systems and improved molecular tools of analysis are nevertheless essential before this field can progress at the pace it deserves. In particular, technical innovations enabling routine analyses of LS parasite RNA and cDNA, such as *in situ* amplification of RNA, will be an important adjunct to Ab data for unequivocal molecular demonstration of true LS expression of a particular antigen. One way may be to employ sensitive protocols that enable generation of cDNA libraries from minor quantities of messenger RNA. Such techniques have recently been successfully applied to generate a cDNA library enriched for Spz-specific sequences (D. Fidock, T. Nguyen and A. James, unpublished data) and it is likely that similar approaches can be adopted to develop LS-specific cDNA libraries.

5.4 Antigenicity and immunogenicity

Many studies in this area have focused on the CS and TRAP proteins, for which the reader is referred to extensive reviews presented elsewhere (Nardin and Nussenzweig 1993; Hoffman *et al.* 1996); chapter 33 this book). In this section we will focus on studies concerning the "PM" Ags subset previously described.

A problem frequently encountered in malaria vaccine research has been the poor immunogenicity frequently seen with many *P. falciparum* Ags. Present data argues that LS Ags constitute a noticeable exception: it is unlikely to be a coincidence that sterile protection can be induced by a few blocked liver trophozoites and that most of the above molecules demonstrate very strong antigenicity and immunogenicity. Given the striking differences between LS and ABS Ags in terms of the quantities exposed to the human immune system, the results are indeed striking. With a liver schizogony lasting 5.5 days and an average of about half a dozen LS forms following Spz infection, contrasting with blood infections of up to 10^8 - 10^{10} ABS parasites circulating all year round for the first 20 years of life, the average amount of each molecule / day / immune system can be estimated to be in the order of 10^{10} - 10^{12} for ABS versus LS Ags.

Measurements of total Ab responses to the Spz surface and to LS schizonts by IFA have shown a relationship between the level of malaria transmission and the intensity of Ab responses to Spz surface molecules (though not the CS) (Druilhe *et al.* 1986 and unpublished data) and have underlined the strong

antigenicity of LS: in an area of very low transmission anti-LS Ab titers were nearly 100 times higher than Ab titers directed at the Spz surface (Figure 3). Subsequent ELISA studies with synthetic peptides derived from the four Ags LSA-1, LSA-3, SALSA and STARP, showed a high prevalence of B-cell responses in areas of low, moderate and high transmission by mosquitoes (Fidock *et al.* 1994c, 1997a; Bottius *et al.* 1996a). This was particularly true for LSA-3-derived peptides (Figure 4). *In vitro* those human Abs exert strong, although frequently sub-total, inhibition of invasion (see section 6). When combined with the transmission-dependent levels of the Spz surface-specific Ab, these data suggest that these Ab might regulate LS load as opposed to their exerting a total invasion blocking effect.

T-helper cell studies similarly showed an unusually high prevalence of responders measured as lymphoproliferation or IFN-γ production when compared to ABS Ags studied in parallel (Fidock *et al.* 1994a; Bottius *et al.* 1996a; C. Roussilhon, unpublished data; P. Brasseur, unpublished data). It is noteworthy that T-helper responses also differed from B-cell responses in that the prevalence of the former was more tightly correlated with the level of transmission, with the lowest prevalence observed in low transmission areas (including the Madagascar highlands) and extremely high prevalences (87-92 % to individual peptides) in the very high transmission area of Djoumouna (P. Brasseur, unpublished data). These latter results suggest a particularly low genetic restriction of T-cell epitopes in these molecules. For the LSA-1 molecule, strong lymphoproliferative and IFN-γ responses, derived primarily from $CD8^+$ cells, were also detected in PBMC from a Papua New Guinean endemic population (Connelly *et al.* 1997). Interestingly, the combination of proliferative and IL-10 responses to the LSA-1 C-terminal region has been found to be predictive of resistance to reinfection in chronically exposed Kenyan individuals, while IFN-γ or TNF-α responses were not associated with protection from malaria (P. Duffy, personal communication).

A number of epitopes targeted by human cytotoxic T lymphocytes *in vitro* have also been defined for these four Ags (Hill *et al.* 1992; Aidoo *et al.* 1995, submitted for publication; Lalvani *et al.* 1996; Doolan *et al.* 1997; L. BenMohammed, unpublished data). Interestingly, the prevalence of CTL response can in some cases be high however the level of cytolosis is generally low (Aidoo *et al.* 1995). Further studies have since confirmed that CTL against LS Ags are present at very low levels in peripheral blood mononuclear cells (PBMC) of naturally-exposed individuals (Plebanski *et al.* 1997). This raises the promising possibility that raising the frequency of *P. falciparum* pre-erythrocytic stage-specific CTL in

human PBMC may be sufficient to confer, or increase, effective protection against LS infection (Aidoo *et al.* 1995).

In agreement with the good antigenicity data from individuals harboring very small numbers of LS schizonts, immunogenicity studies have revealed that the same molecules appear to not share the problems of frequent polymorphism in immunologically interesting regions, high genetic restriction, and limited immunogenicity found with several other *P. falciparum* Ags, particularly from ABS. These immunogenicity studies were carried out in mice, *Aotus* monkeys and chimpanzees using recombinant proteins, synthetic peptides and naked DNA vectors, with or without different adjuvants. In each species, these various formulations were found to elicit responses from all the major effectors of the immune system. This is demonstrated, for instance, by the induction of B, Th and CTL responses in animals of these three species immunized by peptides administered in the absence of adjuvant (BenMohamed *et al.* 1997, submitted for publication; B. Perlaza *et al.* in press).

The reasons for the surprisingly high immunogenicity of the four Ags discussed above are not fully understood. One possible explanation stems from the general assumption that among malarial Ags evolution has selected the least antigenic molecules. Liver stage antigens would have escaped the rule, because due to their low numbers they are semi-concealed from the immune system. Another explanation is that this is not a general feature of LS Ags and that the favorable immunological properties of these four molecules results from their selection based on the immune responses induced in humans under natural conditions of exposure (as opposed to selection being based on the immune response in mice). It is worthwhile to recall how this selection occurred:

- immunodominant and conserved B-cell epitopes were deliberately selected. Notably, among the 120 pre-erythrocytic stage clones analyzed (see section 5.2), we performed an initial selection based on: i) epitope conservation among several isolates - made by screening affinity purified Ab against Spz from twelve parasite isolates; ii) consistent recognition of recombinant proteins by 15 African hyper-immune sera; and iii) evaluation of the duration of immune responses in "post-immune" individuals who had been without antigenic boost for the past 8-10 years. This was based on the reasoning that conserved Ags inducing consistent and long-lasting immune responses would contain strong T-helper epitopes in addition to dominant and non-polymorphic B-cell epitopes. Results obtained in non-human primates,

combined with the high B- and T-cell antigenicity observed in the field, validate this empirical approach since of twelve peptides chosen for synthesis, 11 and 12 defined universal B- and T-cell epitopes.

6. MECHANISMS OF DEFENSE AGAINST PRE-ERYTHROCYTIC STAGES

The analysis of events taking place in the liver and of available data about immunity to pre-erythrocytic stages reveals many puzzling contradictions. On the one hand, as many as half of the twenty or fewer Spz deposited in the skin manage to make their way past all the immune cells and invade and develop within an hepatocyte in naive animals. In total contradiction is the fact that many of the mammalian defense effectors have been reported as being able to partially or totally block this part of the cycle (Figure 5). On this basis, the sum of innate (non-adaptive) immunity and of adaptive responses to malarial Ags should be able to block all pre-erythrocytic stage parasites. Moreover, pre-erythrocytic stage Ags appear to be extremely antigenic and immunogenic. We believe that two major issues underlie these apparent contradictions: i) though not yet widely recognized, there are already several indications that individuals living in endemic areas can develop immunity capable of preventing the majority of, though not all, pre-erythrocytic stage infections (see section 7); and ii) the powerful defense mechanisms described in experimental rodent hosts may not actually take place in natural host/parasite combinations, e.g. *P. falciparum* in humans (see below). Another issue here involves the quantitative analysis of the efficacy of particular immune effectors, which for example has led to a reevaluation of IFN-γ whose effect appears much more modest today as compared to several of the initial independent reports (reviewed in Suhrbier 1991).

This field has been dominated by IrrSpz induced immunity. Indeed, studies have concentrated almost exclusively on an analysis of the basis of immunity induced by injection of the irradiated forms of the Spz stage, to the exclusion of other mechanisms that may be essential in defense against natural pre-erythrocytic stage infections. Nevertheless, the particularly strong level of resistance induced by IrrSpz immunization has set the gold standard for the field. Whether the same can be reproduced by subunit vaccines, instead of a live attenuated parasite, has still to be demonstrated. The only clear conclusion from the human and (mostly) rodent vaccine studies is that when some degree of protection was achieved, it was marginal, as vaccinated subjects resisted only borderline infective live Spz doses, in contrast with the resistance to very large challenge doses in IrrSpz recipients.

6.1 The intrahepatocytic parasite: a very susceptible stage

During a malaria infection, the pre-erythrocytic stages offer the widest range of effectors of the immune system, particularly because the hepatocyte is the sole host cell of the parasite able to express MHC Class I and Class II molecules. While the induction of an Ab response to Spz, easily detectable by the circumsporozoite "precipitation" (a tail-like precipitate of the outer membrane; Vanderberg *et al.* 1969), was thought for many years to be of premier importance (Nussenzweig *et al.* 1972a), subsequent studies highlighted the important role of pure T cell-mediated mechanisms (Chen *et al.* 1977; Spitalny *et al.* 1977; Verhave *et al.* 1978). In the past decade there has been a dramatic shift towards focusing on the infected hepatocyte (reviewed by Nardin and Nussenzweig 1993; Hoffman *et al.* 1996). The extensive and almost sole use of rodent models has indeed helped to unveil the value of LS as targets of defense and to describe a large number of mechanisms able to inhibit or destroy intrahepatocytic parasite development. Indeed, this stage has been found susceptible to: i) Ab acting either alone or in conjunction with non-parenchymal cells (presumably Kupffer or NK cells) to produce Ab-mediated cell cytotoxicity (ADCC) (Rénia *et al.* 1990); ii) $CD8^+$ (Romero *et al.* 1989; Rodrigues *et al.* 1991), $CD4^+$ (Tsuji *et al.* 1990; Rénia *et al.* 1991, 1993a) or γδ T cells (Tsuji *et al.* 1994, 1996) and iii): a large number of cytokines and other mediators (see section 6.4). The strong, frequently total effect of single effectors and the difficulty in producing protection *in vivo* by means that induce similar effectors must cast doubts about the relevance of the models employed in those studies.

6.2. The irradiated sporozoite model

Consistent with results in rodent models, intravenous injection of IrrSpz has been shown to confer sterile protection in naive humans, though only borderline immunity was obtained in monkeys (reviewed in Cochrane and Nussenzweig 1989). However it is seldom stressed that in humans the number of doses needed is particularly high and that acquisition of sterile immunity is not as consistent as in rodents. These studies have revealed that induction of a protective response is highly dependant on the host/parasite combination, the dose of irradiation, the number of Spz injected and the number of immunizations (Weiss *et al.* 1989; Rodrigues *et al.* 1993). As an example, one injection of as few as 1000 *P. berghei* IrrSpz induced protection in A/J mice (Weiss *et al.* 1989), and protection was always increased when going from a single to three injections of either *P. yoelii* in BALB/c mice or *P. berghei* in A/J mice (Jaffe *et al.* 1990).

In fact, protection appears to be inversely correlated with the susceptibility of the host to the Spz infection. This is clearly a matter of concern for vaccine development. For example, C57BL/6 mice are exquisitely sensitive to the development of *P. berghei* and are the most difficult to protect, requiring 3 immunizations by 30,000 IrrSpz (Jaffe *et al.* 1990). On the other hand BALB/c are quite refractory to *P. berghei* infection and are the easiest to protect. In the natural *Thamnomys* host, sterile protection could not be induced even using the high doses effective in C57BL/6 (Chatterjee *et al*., personal communication). In humans, protection to *P. falciparum* was inconsistently achieved even after 12-14 exposures to IrrSpz in vaccination protocols lasting nearly an entire year, and 8-10 immunizations proved ineffective (Herrington *et al.* 1991; Edelman *et al.* 1993). Intramuscular introduction of IrrSpz (which does not result in a liver infection) was also ineffective in humans (Bray 1976), recalling the importance of liver trophozoites.

Of particular note are recent reports showing that liver trophozoites resulting from IrrSpz are both persistent and essential for induction of protection (Londono *et al.* 1991; Scheller and Azad 1995a; Scheller *et al.* 1995b; refer to section 3.1). Recent studies have confirmed that infected hepatocytes produced *in vivo* or *in vitro* are immunogenic and can induce Ab to both Spz and LS parasites (Millet *et al.* 1991b; Scheller and Azad 1995a). Protection against an infectious Spz challenge has been achieved following immunization of mice and rats with liver trophozoite-infected hepatocytes resulting from injection of irradiated or normal *P. yoelii* or *P. berghei* Spz (Rénia *et al.* 1994; Scheller and Azad 1995a). These findings reinforce the crucial role of LS parasites in the *induction* of the so-called anti-Spz immunity.

6.3 Antibodies

Immunizations with IrrSpz induce Ab which react specifically with the surface of the Spz (Vanderberg *et al.* 1969). These Ab produced in mice have many clear, and sometimes powerful, biological effects. Paradoxically however these can be totally opposite in their outcome, e.g. leading to protection or in contrast to facilitation of the infection. Abs can enhance Spz clearance *in vivo* (Nussenzweig *et al.* 1972a), facilitate their phagocytosis by macrophages (Danforth *et al.* 1980; Seguin *et al.* 1989) and reduce Spz penetration into hepatocytes *in vitro* (Hollingdale *et al.* 1984; Mellouk *et al.* 1990b). Abs to the CS protein in particular have been extensively studied (Hollingdale *et al.* 1982; Mazier *et al.* 1986b;

Charoenvit *et al.* 1991) and have been reported to have significant blocking activity, particularly in HepG2 cells. In contrast, inhibition of invasion into hepatocytes was always found to be sub-total **(**Mazier *et al.* 1986; Mellouk *et al.* 1986, 1990). These Ab were also found to inhibit intrahepatocytic development after invasion (Mazier *et al.* 1986b; Nudelman *et al.* 1989). Recently human and mouse Ab to other Spz-surface associated Ag such as SALSA, STARP and LSA-3 were found to display strong inhibitory activity *in vitro,* those directed to LSA-3 having the most profound effect (Fidock et al. 1997a; K. Brahimi *et al*., unpublished data}. Human Abs to LSA-3 were the only ones found able to totally inhibit *P .yoelii* invasion in mouse hepatocytes (K. Brahimi *et al*., unpublished data).

In contrast, anti-Spz Abs have also been reported to (sometimes strongly) enhance Spz penetration and subsequent development in rodent or in human hepatocytes *in vitro* (Nudelman *et al.* 1989; Mellouk *et al.* 1990b; Etlinger *et al.* 1991). This paradoxical effect was reported both at low and high Ab concentrations and was more pronounced in *Thamnomys* hepatocytes than in rat or mouse hepatocytes or hepatoma cells (Nudelman *et al.* 1989; Hollingdale *et al.* 1990a). This reinforces the idea that experimentation in unnatural hosts may lead to false conclusions and urges for the sole use of only the most relevant assays, i.e. those employing homologous hepatocytes, to assess the protective capacity of Ab to defined Ags (see section 4.4).

Passive Ab transfers *in vivo* have also yielded contrasting results. When mixed *in vitro* with Spz prior to *in vivo* injection, Ab to the CS protein were shown to neutralize Spz infectivity in mice as well as *Saimiri* and chimpanzees (Yoshida *et al.* 1980; Gysin *et al.* 1984). In passive transfer experiments, only monoclonal but not polyclonal Ab to the CS protein were effective in conferring sterile protection (Potocnjak *et al.* 1980; Mellouk *et al.* 1990b; Charoenvit *et al.* 1991). Vaccine trials have also demonstrated the lack of a clear relationship between levels of anti-CS Ab and protection.

6.4 T cells

It has long been known that mice depleted of B cells by treatment with anti-μ chain Ab and immunized with IrrSpz can resist Spz challenge in the absence of detectable anti-Spz Ab (Chen *et al.* 1977). Studies have since shown that depending on the host/parasite combination being tested, $CD8^+$ and $CD4^+$ cells, acting alone or in concert, can be involved in mediating protection conferred by IrrSpz (Schofield *et al.*

1987a; Weiss *et al.* 1988, 1989; Weiss 1990a; Rodrigues *et al.* 1993) (Table II). These T cells have been shown to inhibit LS both *in vivo* and *in vitro*. Use of $CD8^+$ clones or lines has also enabled identification of T cell epitopes in parasite proteins of either rodent (Romero *et al.* 1989; Weiss *et al.* 1990b, 1992; Rodrigues *et al.* 1991, 1992) or human origin (Malik *et al.* 1991; Hill *et al.* 1992; Sedegah *et al.* 1992; Doolan *et al.* 1993; Blum-Tirouvanziam *et al.* 1995; Lalvani *et al.* 1996; BenMohamed *et al.* 1997; Plebanski *et al.* 1997). In a Gambian population, the HLA class I antigen HLA-B53 was associated with resistance to severe malaria and its further study led to the identification of a conserved HLA-B53-dependent CTL epitope in the LSA-1 Ag (Hill *et al.* 1991, 1992). However a similar association could not be confirmed in Kenya (Yates *et al.* 1994). Low-level $CD8^+$ T cells recognizing *P. falciparum* Ags have been identified in a proportion of naturally-exposed or IrrSpz-immunized individuals, though without a clear relationship with protection (Doolan *et al.* 1991, 1993; Malik *et al.* 1991; Hill *et al.* 1992; Sedegah *et al.* 1992; Aidoo *et al.* 1995; Wizel *et al.* 1995a, 1995b). In one study in Kenyan individuals, T cell response to a $CD8^+$ T cell epitope in the CS protein showed a statistically positive association with resistance to reinfection (Hoffman *et al.* 1989b). These data supported, though failed to firmly establish the role of $CD8^+$ cells in protection so that today their role in humans is still a source of debate.

Less clear yet is the role of CD4+ T cells. In some rodent model infections $CD4^+$ cells clearly are important in IrrSpz-induced protection; however it is unclear whether they directly eliminate the parasite (Rodrigues *et al.* 1993; Weiss *et al.* 1993). Protection conferred by immunization with infected hepatocytes can be mediated by both CD4+ and CD8+ T cells, in the absence of detectable Ab to either Spz, LS or ABS (Rénia *et al.* 1994). Adoptive transfer of CD4+ T cell clones and induction of CD4+ T cells by peptide immunization have further shown the ability of these cells to confer sterile protection against Spz challenge (Rénia *et al.* 1993a; Wang *et al.* 1996). In contrast the role of cytolytic CD4+ T cells is still elusive in human malaria. Cytotoxic $CD4^+$ T cell clones have been obtained from an IrrSpz-immunized volunteer (Moreno *et al.* 1991) and it has been proposed that these cells might directly eliminate LS parasites through an IFN-γ dependent pathway (Zevering *et al.* 1994).

The capacity of rodent CD4+ and CD8+ T cells to inhibit LS parasites clearly demonstrates that rodent hepatocytes can present Ags in association with MHC class I and class II molecules. Rodent hepatocytes normally express low levels of MHC class I molecules and expression of MHC class II molecules can

easily be induced by IFN-α and IFN-γ (Franco *et al.* 1988). However, human hepatocytes differ from rodents in that Class I expression is undetectable (without induction). It is still unknown whether malaria parasites, like viruses, can influence MHC Ag expression in human hepatocytes. Furthermore, hepatocytes are heterogeneous in their phenotype and conceivably some parasites may hide in hepatocytes expressing no, or very low level MHC Ag and thus be protected from T cell attack.

6.4.1 Direct cytotoxicity

Based on the known activity of CTL against hepatotropic viruses or bacteria, it has been inferred that CTL should be able to directly lyse *Plasmodium*-infected hepatocytes. This assumption has been reinforced by the identification of malarial CD8+ or $CD4^+$ T cell epitopes in *in vitro* systems employing peptide-pulsed or -transfected P815 mouse mastotocytoma cells or mouse B lymphoma A20 cells (Romero *et al.* 1989; Tsuji *et al.* 1990; Weiss *et al.* 1990b), as well as EBV-transformed human peripheral blood cells (PBL) either peptide-pulsed (Moreno *et al.* 1991; Hill *et al.* 1992) or infected with recombinant vaccinia virus (Aidoo *et al.* 1997). However, clear evidence in favor of or against direct cytolytic action towards infected hepatocytes has not been obtained, due in large part to the inherent difficulty of using infected hepatocytes in *in vivo* systems or in *in vitro* ^{51}Cr release assays. Using C57BL/6 mice deficient for the perforin gene or presenting a mutation in Fas and Fas ligand molecules (involved in T cell cytotoxicity), Renggli *et al.* (1997) were unable to demonstrate a role for these proteins in IrrSpz protection. *In vitro* experiments, using infected hepatocytes as target cells, demonstrated that the activity of both CD4+ and CD8+ T cells against infected hepatocytes could not be blocked either by the use of anti-lymphokine Ab (mainly anti-IL-6, and anti-IFN-γ), culture supernatants, or cyclosporin A (an immunosuppressive agent highly specific for T lymphocytes and which prevents the activity of IFN-γ, TNF-α and IL-1) (Rénia *et al.* 1991, 1993a). Recently, through the use of a hepatitis B model in which transgenic mice express the hepatitis B virus genome, it has been shown that CD8+ T cells can act by inhibiting viral mRNA transcription through a TNF-α and IFN-γ dependent mechanism, i.e. without lysing the host cell (Guidotti *et al.* 1996; Chisari 1997) Similar types of *in vivo* effect against Plasmodium LS development deserve to be investigated in cell lines displaying antimalarial *in vitro* cytolytic activity.

6.4.2 Cytokine mediated parasite elimination

For over 20 years it has been known that cytokine inducers (eg. viruses, PolyIC, BCG and killed *Corynebacterium parvum)* can totally sterilize a Spz challenge (Nussenzweig 1967b; Jahiel *et al.* 1970; Smrkovski and Strickland 1978; Verhave *et al.* 1980). More recently, cytokine-specific Ab have enabled investigation of whether T lymphocytes or other cells can eliminate the LS parasite through lymphokine release. A number of studies have demonstrated that the use of anti-IFN-γ Ab can reverse IrrSpz-induced protection and this cytokine has also been implicated in protective T cell-mediated parasite elimination (Schofield *et al.* 1987b; Hoffman *et al.* 1989a; Seguin *et al.* 1994). However, results have been conflicting depending on the host/parasite combination and the T cell clone under investigation (Hoffman *et al.* 1989a; Weiss *et al.* 1992; Rénia *et al.* 1993a). These findings underline the need to exercise caution when studying T cell clones since they can differ in their fine specificity of Ag recognition and their effector mechanisms.

In direct assays, several lymphokines have been clearly shown to inhibit LS development. *In vivo* and *in vitro* studies have demonstrated that IFN-γ, IL-1 and IL-6 can directly inhibit, while TNF-α and IL-12 indirectly inhibit hepatocytic development of malaria parasites (Ferreira *et al.* 1986; Maheshwari *et al.* 1986; Mellouk *et al.* 1987; Schofield *et al.* 1987a, 1987b; Vergara *et al.* 1987; Puri *et al.* 1988; Pied *et al.* 1990, 1992; Nussler *et al.* 1991a, 1991b; Sedegah *et al.* 1994; Vreden *et al.* 1992; Hoffman *et al.* 1997). These lymphokines could conceivably be produced either by activated T cells, or by nonparenchymal cells (such as Kupffer cells, endothelial cells, NK cells, epithelial cells and Itoh cells) present in close proximity to hepatocytes and potentially capable of participating in the inhibition of LS development (Rénia *et al.* 1990; Nussler *et al.* 1991a; Pied *et al.* 1992). In these assays, IFN-γ had no direct effect on LS parasite development (Vigario *et al.*, manuscript submitted).

With the advent of mice deficient for specific lymphokine genes, it has been possible to directly address the role of these lymphokines (summarized in Table III). Despite the valuable information that can be obtained through the use of these mice, care should be taken to uncover possible mechanisms of compensation. Moreover, the relevance of these data to human malaria is compromised by the major differences observed between model host/parasite combinations, which potentially could result in some lymphokines being essential in one system though largely irrelevant in another.

Many studies have indicated that lymphokines inhibit LS parasites through a L-arginine dependent pathway and through induction of reactive oxygen intermediates (Pied *et al.* 1990; Mellouk *et al.* 1991; Nussler *et al.* 1991b, 1993; Mellouk *et al.* 1994). Of particular note is nitric oxide (NO), produced by nitric oxide synthase (iNOS) and capable of being generated by hepatocytes and Kupffer cells (Billiar *et al.* 1989; Curran *et al.* 1989). Studies in *P. berghei* IrrSpz-immunized rats have shown that protection can correlate with the restricted expression of mRNA iNOS in hepatocytes (Klotz *et al.* 1995). In a study performed in mice immunized with *P. berghei* IrrSpz, depletion of CD8+ or neutralization of IFN-γ at the time of challenge prevented iNOS expression in the liver and abrogated protection in immunized animals (Seguin *et al.* 1994). Moreover, it has been proposed that NO plays a role in promoting influx of CD8+ T cells to the site of *P. berghei* infected hepatocytes (Scheller *et al.* 1997). However, recent experiments using iNOS deficient mice failed to reveal a discernible impact on IFN-γ mediated protection against *Plasmodium yoelii* LS parasites (M. Tsuji and F. Zavala; cited in Nathan 1997). Potentially, other reactive products such as reactive oxygen intermediates may be involved, as has been demonstrated for IL-6 activity (Pied *et al.* 1990).

The pro-inflammatory cytokines IL-1, IL-6 and TNF-α are also known to act as potent inducers of hepatocyte synthesis acute phase proteins (APP) (Andus *et al.* 1991). C-reactive protein, hemopexin, α1-anti-trypsin and α2-macroglobulin have all been shown to inhibit Spz penetration and subsequent intrahepatocytic development *in vitro* (Pied *et al.* 1989, 1995; Nussler *et al.* 1991c; Rénia *et al.* 1993b; Vreden *et al.* 1995). Induction of these APP has been shown to render rats resistant *to P. yoelii* or *P. berghei* Spz infection. A high basal level of APP has been proposed as a factor contributing to innate resistance to Spz infection and LS development in rats (Nussler *et al.* 1991c; Vreden *et al.* 1995).

6.5 Protective responses elicited by co-infections

Given the very large range of non Ag-specific mediators that can affect the liver schizogony, one might expect that infection by other microorganisms would affect the success of the pre-erythrocytic stages. Human beings are far from being axenic organisms. For instance, individuals in malaria endemic areas frequently harbor viral infections, which are known to induce substantial levels of several immune mediators such as IFN-γ. Theoretically therefore a response to such co-infections might deeply affect or even sterilize a Spz inoculum, even in non-immune individuals, or supplement specific, acquired

mechanisms in semi-immune individuals. BCG has been shown in rodent malaria to inhibit LS development and suppress immunity conferred by IrrSpz immunization (Smrkovski and Strickland 1978; Smrkovski 1981). In rodents, malaria blood stages are also known to induce lymphokines such as IFN-γ, which could render hepatocytes refractory to Spz infection (Nussler *et al.* 1993). This is reminiscent of the phenomenon of concomitant protection in schistosomiasis infection (Mitchell 1990).

6.6 Conclusion

It is today evident that the LS can be the target of a very wide range of both innate and Ag-specific immune mechanisms. Nonetheless, our knowledge of these mechanisms is derived from experiments performed in a wide range of host/parasite combinations that include a large number of parasite and rodent species. It would be foolhardy to propose a single scheme that attempts to assemble and prioritize immune defense mechanisms based on current knowledge. The use of these models has nevertheless had the advantage of quickly testing whether one or several mechanisms may participate in defense against LS. Their relevance to the human situation however will technically be difficult to assess. In view of the considerable emphasis put on MHC class I- and class II- dependent cellular mechanisms, it is important to recall that rodent and humans may well differ with respect to the expression of those molecules.

It is likely that the adaptation of the parasite to its natural host will translate in immunological terms by a more restricted range of less effective defense mechanisms than those seen in unnatural hosts. The difficulty in inducing protective immunity in humans by immunization with IrrSpz is testament to this idea. Whether immune effectors against human plasmodia will be simply less numerous, or differ from one individual to the other depending on the human genetic background (as is seen in mice), remains to be investigated.

7. NATURALLY ACQUIRED IMMUNITY TO PRE-ERYTHROCYTIC STAGES. -

Irradiated Spz are thought to induce very strong, sterile immunity, whereas repeated exposure to non-irradiated Spz in endemic areas is generally considered as inducing no protection whatsoever. These assumptions deserve to be re-examined. The first is based mostly on the results from single

challenges in immunized volunteers, despite the fact that a) not all volunteers were protected and b) when a second challenge was made, only some of the previously protected individuals resisted a second time. The second assumption, that no pre-erythrocytic stage immunity develops in naturally exposed individuals, is based solely on the observation of a high prevalence of blood stage infection in individuals of all ages in endemic areas. This is taken as data from which to conclude that no individual has reached a refractory state.

Because under natural conditions of exposure it is much more difficult to assess protection to pre-erythrocytic stages (since one cannot examine the LS load), the question of naturally acquired immunity has not been directly addressed. Nevertheless, available data suggests that the level of natural pre-erythrocytic stage immunity has been significantly underestimated. Indeed, there are several indications that strong immunity can develop that is protective against the majority of individual challenges by live non-attenuated Spz (see below).

As previously mentioned (sections 5 and 6), non-irradiated Spz can be just as efficient as irradiated ones in inducing sterile immunity in rodents. Furthermore, analyses of Ab present in the sera of naturally exposed individuals demonstrate that both total IgG (Mellouk *et al.* 1990b) and Spz Ag-specific Ab can strongly inhibit Spz invasion. These analyses also show that these Ab increase as a direct function of Spz inoculation rates. Naturally-acquired immunity to just LS parasites is more difficult to assess. Nevertheless, among the many diverse mechanisms (including $CD4^+$, $CD8^+$ T cells and many cytokines) found to partially or totally block LS development in rodents, most if not all have also been described in individuals living under natural conditions of exposure. These mechanisms may well affect LS development in humans as much as they are believed to do so in rodents.

Existence of naturally-acquired pre-erythrocytic stage immunity is strongly argued for by data from the immense Garki project conducted in Tanzania. Notably, it proved impossible to establish a model of transmission that corresponded to field observations without introducing a factor representing a strong density-dependent pre-erythrocytic filter, or "brake" (Molineaux and Gramiccia 1980; L. Molineaux, personal communication). This work also demonstrated that

residual insecticide spraying, which dramatically reduced the inoculation rate, resulted in only minor or no modification of the incidence of malaria attacks. This suggests the modification of an adaptive human response, e.g. of an immunological type, itself dependent on Spz density.

Because the LS load resulting from natural Spz challenges is inaccessible, we have developed an indirect means to assess natural resistance to pre-erythrocytic stages (Ndieye *et al.*, 1997 and other unpublished studies). In areas where transmission is regularly monitored by entomological methods, radical cure of pre-existing parasitemia by blood schizontocides and further monitoring for new blood stage infections in the following weeks can provide indications of the occurrence of a successful liver cycle. In one such study in Senegal, 10 % of a 147 person cohort fully resisted natural challenges for more than 14 weeks, corresponding to an estimated 41 consecutive challenges by infective mosquito bites. In another very high transmission area in the Congo, full resistance extended to up to an estimated 150 consecutive challenges in 30 % of the followed-up individuals (Brasseur et al, unpublished data). Similar studies conducted in Irian Jaya and Kenya have also recorded similar data (Jones *et al.* 1994; Beadle *et al.* 1995). This would indicate the existence of full sterile resistance to a staggering number of consecutive challenges in the above individuals, a situation that volunteers immunized by means of IrrSpz have never faced. We note that none of these IrrSpz-immunized protected volunteers were ever subjected to conditions of prolonged natural challenge comparable to the individuals in field studies, where there may occur as many as three challenges per day. Thus, anti pre-erythrocytic stage immunity acquired under natural conditions might be even more efficacious than that induced by IrrSpz. Nevertheless, the observation that highly-exposed individuals constantly harbor blood stage infections (Bottius *et al.* 1996b) conversely indicates that sterile resistance may extend to many but clearly not all natural challenges initiated by live Spz infection.

8. CONCLUDING REMARKS.

Despite the small the number of researchers involved in this difficult field, considerable advances have been made over the last 10 years. These concern an improved understanding of the respective roles of LS and Spz, improvement in our knowledge of the antigenic repertoire of pre-erythrocytic stages and an

inventory of the many distinct mechanisms of defense taking place against these two stages. In addition, these have led to improved recognition of the dangers in extrapolating rodent and *in vitro* data to the human situation and consequently a more modest attitude in this respect, the development of new tools to analyze events taking place within the liver, and finally the more recent understanding that LS may not be only a preferential target for defense but also the critical inducer of IrrSpz immunity, the most effective defense known against malaria infection. Thus, sufficient evidence has now accumulated to indicate that despite the technical difficulties, the study of the LS Ag repertoire and of the immune mechanisms elicited against this stage will play a critical role in the development of the "anti-Spz" vaccine.

However in this, as in other fields of malaria vaccine development, needed progress is significantly impeded by the absence of i) rodent host/parasite combinations clearly relevant to immunity in man; and ii) an immune response clearly correlated with the protective status, which could be used as a surrogate marker to guide vaccine development. Such a marker was earlier believed to be Ab directed to the Spz surface, then was supplanted by the diametrically opposed $CD8^+$ CTL response to infected hepatocytes, and evidence is accumulating that both options may not be correct or at least not universal. The identification of a relevant marker would be critical at the levels of vaccine molecule selection, the choice of an Ag delivery system (in order to induce the effector arms identified as critical) and the monitoring of pre-clinical studies in animals and clinical studies in humans. Other limitations remain the very difficult handling and limited access offered by the present tools to these obscure and long-forgotten stages. A major requirement is clearly the development of better tools. Since results gathered in rodents can be misleading, more attention has to be given to developing suitable models for *P. falciparum*. The strong indications obtained recently in favor of the critical role of LS in the strongest protection achievable against malaria at any stage should stimulate a renewed interest in making the effort to develop the necessary tools.

9. REFERENCES

Aggarwal, A., S. Kumar, R. Jaffe, D. Hone, M. Gross, and J. Sadoff. 1990. Oral Salmonella: malaria circumsporozoite recombinants induce specific CD8+ cytotoxic T cells. *J. Exp. Med.* **172**: 1083-90.

Aidoo M., A. Lalvani, S. C. Gilbert, H. C. Whittle, P. Daubersies, P. Druilhe, and A. V. S. Hill. Submitted for publication. CTL epitopes for HLA-B53 and other HLA types in the malaria vaccine candidate liver-stage antigen-3.

Aidoo, M., A. Lalvani, H. C. Whittle, A. V. Hill, and K. J. Robson. 1997. Recombinant vaccinia viruses for the characterization of Plasmodium falciparum-specific cytotoxic T lymphocytes: recognition of processed antigen despite limited re-stimulation efficacy. *Int. Immunol.* **9**: 731-7.

Aidoo, M., A. Lalvani, C. E. M. Allsopp, M. Plebanski, S. Meisner, P. Krausa, M. Browring, S. Morris-Jones, S. McAdam, F. M. Gotch, D. A. Fidock, M. Takiguchi, K. J. H. Robson, B. M. Greenwood, P. Druilhe, H. C. Whittle, and A. V. S. Hill. 1995. Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria. *Lancet.* **345**: 1003-7.

Aikawa, M., C. T. Atkinson, L. M. Beaudoin, M. Sedegah, Y. Charoenvit, and R. L. Beaudoin. 1990. Localization of CS and non-CS antigens in the sporogonic stages of *Plasmodium yoelii*. *Bull. World. Health. Org.* **68, Suppl.**: 165-171.

Alger, N. E., and J. Harant. 1976. *Plasmodium berghei*: heat-treated sporozoite vaccination of mice. *Exp. Parasitol.* **40**: 261-8.

Andus, T., J. Bauer, and W. Gerok, W. 1991. Effects of cytokines on the liver. *Hepatology* **13:** 364-375.

Arreaza, G., V. Corredor, and F. Zavala. 1991. *Plasmodium yoelii*: quantification of the exoerythrocytic stages based on the use of ribosomal RNA probes. *Exp. Parasitol.* **72**: 103-5.

Atkinson, C.T., M. Aikawa, S. B. Aley, and M. R. Hollingdale. 1989. Expression of *Plasmodium berghei* circumsporozoite antigen on the surface of exoerythrocytic schizonts and merozoites. *Am. J. Trop. Med. Hyg.* **41:** 9-17

Bafort, J. 1971. The biology of rodent malaria with particular reference to *Plasmodium vinckei vinckei* Rodhain. *Ann. Soc. belge Méd. Trop.* **51:** 1-204.

Beadle, C., P. D. McElroy, C. N. Oster, J. C. Beier, A. J. Oloo, F. K. Onyango, D. K. Chumo, J. D. Bales, J. A. Sherwood, and S. L. Hoffman. 1995. Impact of transmission intensity and age on *Plasmodium falciparum* density and associated fever: implications for malaria vaccine trial design. *J. Infect. Dis.* **172**: 1047-54.

Beaudoin, R. L., C. P. Strome, F. Mitchell, and T. A. Tubergen. 1977. *Plasmodium berghei*: immunization of mice against the ANKA strain using the unaltered sporozoite as an antigen. *Exp. Parasitol.* **42**: 1-5.

Beaudoin, R.L., D. Mazier, and M. R. Hollingdale. 1988. *In vitro* cultivation of the exoerythrocytic stages of malaria parasites, p. 321-330. *In* W. H. Wernsdorfer, and I. McGregor (eds.), *Malaria: principles and practice of malariology*. Churchill Livingstone, Edinburgh London and New York.

BenMohamed, L., H. Gras-Masse, K. Brahimi, W. Eling, J. Langermans, A. Thomas, and P. Druilhe. Submitted for publication. High immunogenicity in chimpanzees of eleven peptides derived from four malaria pre-erythrocytic stage molecules.

BenMohamed, L., H. Gras-Masse, A. Tartar, P. Daubersies, K. Brahimi, M. Bossus, A. Thomas, and P. Druilhe. 1997. Lipopeptide immunization without adjuvant induces potent and long- lasting B, T helper, and cytotoxic T lymphocyte responses against a malaria liver stage antigen in mice and chimpanzees. *Eur. J. Immunol.* **27**: 1242-53.

Bianco, A. E., J. M. Favarolo, T. R. Burkot, J. G. Culvenor, P. E. Crewther, G. V. Brown, R. F. Anders, R. L. Coppel, and D. J. Kemp. 1986. A repetitive antigen of *Plasmodium falciparum* that is homologous to heat shock protein 70 of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.* **83**: 8713-8717.

Billiar, T. R., R. D. Curran, D. J. Stuehr, M. A. West, B. G. Bentz, and R. L. Simmons. 1989. An L-arginine-dependent mechanism mediates Kupffer cell inhibition of hepatocyte protein synthesis *in vitro*. *J. Exp. Med.* **169**: 1467-72.

Blum-Tirouvanziam, U., C. Servis, A. Habluetzel, D. Valmori, Y. Men, F. Esposito, L. Del Nero, N. Holmes, N. Fasel, and G. Corradin. 1995. Localization of HLA-A2.1-restricted T cell epitopes in the circumsporozoite protein of *Plasmodium falciparum*. *J. Immunol.* **154**: 3922-31.

Borre, M. B., M. Dziegiel, B. Hogh, E. Petersen, K. Rieneck, E. Riley, J. F. Meis, M. Aikawa, K. Nakamura, M. Harada, A. Wind, P. H. Jakobsen, J. Cowland, S. Jepsen, N. H. Axelsen, and J. Vuust. 1991. Primary structure and localization of a conserved immunogenic *Plasmodium falciparum* glutamate rich protein (GLURP) expressed in both the preerythrocytic and erythrocytic stages of the verterbrate life cycle. *Mol. Biochem. Parasitol.* **49**: 119-132.

Bottius, E., L. BenMohamed, K. Brahimi, H. Gras, J. P. Lepers, L. Raharimalala, M. Aikawa, J. Meis, B. Slierendregt, A. Tartar, A. Thomas, and P. Druilhe. 1996a. A novel *Plasmodium falciparum* sporozoite and liver stage antigen (SALSA) defines major B, T helper, and CTL epitopes. *J. Immunol.* **156**: 2874-2884.

Bottius, E., A. Guanzirolli, J. F. Trape, C. Rogier, L. Konate, and P. Druilhe. 1996b. Malaria: even more chronic in nature than previously thought; evidence for subpatent parasitaemia detectable by the polymerase chain reaction. *Trans. R. Soc. Trop. Med. Hyg.* **90**: 15-9.

Boulard, Y., P. Druilhe, F. Miltgen, I. Landau, and J. Rinjard. 1982. Ultrastructure du schizonte hépatique de *Plasmodium falciparum* chez le singe *Cebus appella*. *Protistologica* **18:** 253-258.

Bray, R. S. 1976. The use of irradiated sporozoites in the fields. *Trans.R.Soc.Trop.Med.Hyg.* **70**: 258-262.

Bray, R. S. 1962. Studies on malaria chimpanzee; VI. *Laverania falciparum*. Am. J. Trop. Med. Hyg. **7:** 20-24.

Briones, M. R., M. Tsuji, and V. Nussenzweig. 1996. The large difference in infectivity for mice of *Plasmodium berghei* and *Plasmodium yoelii* sporozoites cannot be correlated with their ability to enter into hepatocytes. *Mol. Biochem. Parasitol.* **77**: 7-17.

Brown, A. E., P. Singharaj, H. K. Webster, J. Pipithkul, D. M. Gordon, J. W. Boslego, K. Krinchai, P. Su-archawaratana, C. Wongsrichanalai, W. R. Ballou, B. Permpanich, K. C. Kain, M. R. Hollingdale, J. Wittes, J. U. Que, M. Gross, S. J. Cryz, and J. C. Sadoff. 1994. Safety, immunogenicity and limited efficacy study of a recombinant *Plasmodium falciparum* circumsporozoite vaccine in Thai soldiers. *Vaccine.* **12**: 102-8.

Bucher, N. L. R., G. S. Robinson and S. R. Farmer. 1990. Effects of extracellular matrix on hepatocyte growth and gene expression: implications for hepatic regeneration and the repair of liver injury. *Seminars in Liver Disease* **10:** 11-19.

Calvo-Calle, J.M., A. Moreno, W. M. C. Eling, and E. H. Nardin1994. *In vitro* development of infectious liver stages of *P.yoelii* and *P.berghei* malaria in human cell lines. *Exp. Parasitol.* **79:** 362-373.

Charoenvit, Y., S. Mellouk, M. Sedegah, T. Toyoshima, M. F. Leef, P. De la Vega, R. L. Beaudoin, M. Aikawa, V. Fallarme, and S. L. Hoffman. 1995. *Plasmodium yoelii*: 17-kDa hepatic and erythrocytic stage protein is the target of an inhibitory monoclonal antibody. *Exp. Parasitol.* **80**: 419-29.

Charoenvit, Y., S. Mellouk, C. Cole, R. Bechara, M. F. Leef, M. Sedegah, L. F. Yuan, F. A. Robey, R. L. Beaudoin, and S. L. Hoffman. 1991. Monoclonal, but not polyclonal, antibodies protect against *Plasmodium yoelii* sporozoites. *J. Immunol.* **146**: 1020-5.

Charoenvit, Y., M. F. Leef, L. F. Yuan, M. Sedegah, and R. L. Beaudoin. 1987. Characterization of *Plasmodium yoelii* monoclonal antibodies directed against stage-specific sporozoite antigens. *Infect Immun.* **55**: 604-8.

Chatterjee, S., G. Francois, P. Druilhe, G. Timperman, and M. Wery. 1996. Immunity to *Plasmodium berghei* exoerythrocytic forms derived from irradiated sporozoites. *Parasitol. Res.* **82**: 297-303.

Chen, D. H., R. E. Tigelaar, and F. I. Weinbaum. 1977. Immunity to sporozoite-induced malaria infection in mice. I.The effect of immunization of T and B cell-deficient mice. *J. Immunol.* **118**: 1322-1327.

Chisari, F. V. 1997. Cytotoxic T cells and viral hepatitis. *J. Clin. Invest.* **99**: 1472-7.

Clyde, D. F., V. C. McCarthy, R. M. Miller, and W. E. Woodward. 1975. Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. *Am. J. Trop. Med. Hyg.* **24**: 397-401.

Cochrane, A. H., R. S. Nussenzweig, and E. H. Nardin. 1980. Immunization against sporozoites, p. 163-202. *In* J. P. Kreier (ed.), *Malaria*. Academic Press, New York.

Cogswell, F. B. 1992. The hypnozoite and relapse in primate malaria. *Clin. Microbiol. Reviews.* **5**: 26-35.

Cogswell, F. B., W. E. Collins, W. A. Krotoski, and R. C. Lowrie. 1991. Hypnozoites of *Plasmodium simiovale*. *Am. J. Trop. Med. Hyg.* **45**: 211-213.

Collins, W. E., J. C. Skinner, P. G. Contacos, and M. J. Geoffrey (eds.). 1981. Observations on relapse patterns and delayed prepatent periods in *Plasmodium simiovale*. Parasitological Topics, Society of Protozoologists, Special Publications.

Collins, W. E., P. G. Contacos, P. S. Stanfill, and B. B. Richardson. 1973. Studies on human malaria in *Aotus* monkeys. I. Sporozoite transmission of *Plasmodium vivax* from El Salvador. *J. Parasitol.* **59**: 606-8.

Connelly, M., C. L. King, K. Bucci, S. Walters, B. Genton, M. P. Alpers, M. Hollingdale, and J. W. Kazura. 1997. T-cell immunity to peptide epitopes of liver-stage antigen 1 in an area of Papua New Guinea in which malaria is holoendemic. *Infect. Immun.* **65**: 5082-7.

Coppel, R. L., J. M. Favaloro, P. E. Crewther, T. R. Burkot, A. E. Bianco, H. D. Stahl, D. J. Kemp, R. F. Anders, and G. V. Brown. 1985. A blood stage antigen of *Plasmodium falciparum* shares determinants with the sporozoite coat protein. *Proc. Natl. Acad. Sci. USA.* **82**: 5121-5125.

Cowan, G., S. Krishna, A. Crisanti, and K. Robson. 1992. Expression of thrombospondin-related anonymous protein in *Plasmodium falciparum* sporozoites. *Lancet.* **339**: 1412.

Crabb, B. S., B. M. Cooke, J. C. Reeder, R. F. Waller, S. R. Caruana, K. M. Davern, M. E. Wickham, G. V. Brown, R. L. Coppel, and A. F. Cowman. 1997. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. *Cell.* **89**: 287-296.

Curran, R. D., T. R. Billiar, D. J. Stuehr, K. Hofmann, and R. L. Simmons. 1989. Hepatocytes produce nitrogen oxides from L-arginine in response to inflammatory products of Kupffer cells. *J. Exp. Med.* **170**: 1769-74.

Danforth, H. D., M. Aikawa, A. H. Cochrane, and R. S. Nussenzweig. 1980. Sporozoites of mammalian malaria: attachment to, interiorization and fate within macrophages. *J. Protozool.* **27**: 193-202.

Danforth, H. D., A. U. Orjih, and R. S. Nussenzweig. 1978. Immunofluorescent staining of exoerythrocytic schizonts of *Plasmodium berghei* in fixed liver tissue with stage-specific immune serum. *J. Parasitol.* **64:** 1123-1125.

Das, A., C. Syin, H. Fujioka, H. Zheng, N. Goldman, M. Aikawa, and N. Kumar. 1997. Molecular characteriztion and ultrastructural localization of *Plasmodium falciparum* Hsp60. *Mol.Biochem.Parasitol.* **88**: 95-104.

Dieye, A., C. Rogier, J. –F. Trape, J. –L. Sarthou, and P. Druilhe. 1997. HLA-Class I associated resistance to severe malaria: a parasitological re-assessment. *Parasitol. Today* 13: 48-49.

Doby, J.M. and R. Barker. 1976. Essais d'obtention des formes pré-érythrocytaires de *Plasmodium vivax* en cultures de cellules hépatiques humaines inoculées par sporozoïtes. *Comp. Rend. Acad. Sci. Biol.* **170:** 661-665.

Doolan, D. L., R. C. Hedstrom, M. J. Gardner, M. Sedegah, H. Wang, R. A. Gramzinski, M. Margalith, P. Hobart, and S. L. Hoffman. 1998. DNA vaccination as an approach to malaria control: current status and strategies. *Curr Top Microbiol Immunol.* **226**: 37-56.

Doolan, D. L., S. L. Hoffman, S. Southwood, P. A. Wentworth, J. Sidney, R. W. Chesnut, E. Keogh, E. Appella, T. B. Nutman, A. A. Lal, D. M. Gordon, A. Oloo, and A. Sette. 1997. Degenerate cytotoxic T cell epitopes from *P. falciparum* restricted by multiple HLA-A and HLA-B supertype alleles. *Immunity.* **7**: 97-112.

Doolan, D. L., R. C. Hedstrom, W. O. Rogers, Y. Charoenvit, M. Rogers, P. de la Vega, and S. L. Hoffman. 1996a. Identification and characterization of the protective hepatocyte erythrocyte protein 17 kDa gene of Plasmodium yoelii, homolog of *Plasmodium falciparum* exported protein 1. *J Biol Chem.* **271**: 17861-8.

Doolan, D. L., M. Sedegah, R. C. Hedstrom, P. Hobart, Y. Charoenvit, and S. L. Hoffman. 1996b. Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8+ cell-, interferon gamma-, and nitric oxide-dependent immunity. *J Exp Med.* **183**: 1739-46.

Doolan, D. L., C. Khamboonruang, H. P. Beck, R. A. Houghten, and M. F. Good. 1993. Cytotoxic lymphocyte T cell low-responsiveness to the *Plasmodium falciparum* circumsporozoite protein in naturally exposed endemic populations: analysis of human CTL response to most known variants. *Int. Immunol.* **5**: 37-46.

Doolan, D. L., R. A. Houghten, and M. F. Good. 1991. Location of human cytotoxic T cell epitopes within a polymorphic domain of the *Plasmodium falciparum* circumsporozoite protein. *Int. Immunol.* **3**: 511-516.

Draper, C. C., J. Dorrel, P. G. Shute, and J. M. Bafort. 1971. Exoerythrocytic schizogony of *Plasmodium vivax* in *Aotus* monkeys. *Trans. R. Soc. Trop. Med. Hyg.* **65:** 417-418.

Druilhe, P., O. Pradier, J. P. Marc, F. Miltgen, D. Mazier, and D. Parent. 1986. Levels of antibodies to *Plasmodium falciparum* sporozoite surface antigens reflect malaria transmission rates and are persistent in the absence of reinfections. *Infect. Immun.* **53**: 393-397.

Druilhe, P., R. M. Puebla, F. Miltgen, L. Perrin, and M. Gentilini. 1984. Species- and stage-specific antigens in exoerythrocytic stages of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **33**: 336-341.

Druilhe, P., and C. Marchand. 1989. From sporozoite to liver stages: the saga of the irradiated sporozoite vaccine, p. 39-48. *In* K. McAdam (ed.), *New strategies in parasitology*, Churchill Livingstone, Edinburgh.

Edelman, R., S. L. Hoffman, J. R. Davis, M. Beier, M. B. Sztein, G. Losonsky, D. A. Herrington, H. A. Eddy, M. R. Hollingdale, D. M. Gordon, and D. F. Clyde. 1993. Long-term persistence of sterile immunity in a volunteer immunized with X-irradiated *Plasmodium falciparum* sporozoites. *J. Infect. Dis.* **168**: 1066-1070.

Egan, J. E., S. L. Hoffman, J. D. Haynes, J. C. Sadoff, I. Schneider, G. E. Grau, M. R. Hollingdale, W. R. Ballou, and D. M. Gordon. 1993. Humoral immune responses in volunteers immunized with irradiated *Plasmodium falciparum* sporozoites. *Am. J. Trop. Med. Hyg.* **49**: 166-73.

Egan, J. E., J. L. Weber, W .R. Ballou, M. R. Hollingdale, W. R. Majarian, D. M. Gordon,W. L. Maloy, S. L. Hoffman, R. A. Wirtz, I. Schneider, G. R. Woollett, J. F. Young, and W. T. Hockmeyer. 1987. Efficacy of murine malaria sporozoite vaccines : implications for human vaccine development. *Science* **236:** 453-456.

Etlinger, H. M., L. Rénia, H. Matile, M. Manneberg, D. Mazier, A. Trzeciak, and D. Gillessen. 1991. Antibody response to a synthetic peptide-based malaria vaccine candidate : influence of sequence variants of the peptide. *Eur. J. Immunol.* **21**: 1505-1511.

Ferreira, A., V. Enea, T. Morimoto, and V. Nussenzweig. 1986. Infectivity of *Plasmodium berghei* sporozoites measured with a DNA probe. *Mol. Biochem. Parasitol.* **19**: 103-9.

Ferreira, A., L. Schofield, V. Enea, H. Schellekens, P. Van Der Meide, W. E. Collins, R. S. Nussenzweig, and V. Nussenzweig. 1986. Inhibition of development of exoerythrocytic forms of malaria parasites by gamma interferon. *Science.* **232**: 881-884.

Fidock, D. A., V. Pasquetto, H. Gras, E. Badell, W. Eling, W. R. Ballou, J. Belghiti, A. Tartar, and P. Druilhe. 1997a. *Plasmodium falciparum* sporozoite invasion is inhibited by naturally-acquired or experimentally-induced polyclonal antibodies to the STARP antigen. *Eur. J. Immunol.* **27**: 2502-2513.

Fidock, D. A., and T. E. Wellems. 1997b. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proc. Natl. Acad. Sci. USA*. **94**: 10931-10936.

Fidock, D. A., H. Gras-Masse, J. P. Lepers, K. Brahimi, L. Benmohamed, S. Mellouk, C. Guerin-Marchand, A. Londono, L. Raharimalala, J. F. Meis, G. Langsley, C. Roussilhon, A. Tartar, and P. Druilhe. 1994a. *Plasmodium falciparum* liver stage antigen-1 is well conserved and contains potent B and T cell determinants. *J. Immunol.* **153**: 190-204.

Fidock, D. A., E. Bottius, K. Brahimi, I. I. M. Moelans, M. Aikawa, R. N. H. Konings, U. Certa, P. Olafsson, T. Kaidoh, A. Asavanich, C. Guerin-Marchand, and P. Druilhe. 1994b. Cloning and characterization of a novel *Plasmodium falciparum* sporozoite surface antigen, STARP. *Mol. Biochem. Parasitol.* **64**: 219-232.

Fidock, D. A., S. Sallenave-Sales, J. A. Sherwood, G. S. Gachihi, M. F. Ferreira-da-Cruz, A. W. Thomas, and P. Druilhe. 1994c. Conservation of the *Plasmodium falciparum* sporozoite surface protein gene, STARP, in field isolates and distinct species of *Plasmodium*. *Mol. Biochem. Parasitol.* **67**: 255-267.

Franco, A., V. Barnaba, P. Natali, C. Balsano, A. Musca, and F. Balsano. 1988. Expression of Class I and Class II Major Histocompatibility Complex antigens on human hepatocytes. *Hepatology.* **8**: 449-454.

Francois, G., M. Van Looveren, and G. Timperman. 1997. Evaluation of alpha-difluoromethylornithine (DFMO) as a tool to induce protective immunity against *Plasmodium berghei* malaria. *Ann. Trop. Med. Parasitol.* **91**: 103-6.

Franke, E. D., G. Corradin, and S. L. Hoffman. 1997. Induction of protective CTL responses against the *Plasmodium yoelii* circumsporozoite protein by immunization with peptides. *J. Immunol.* **159**: 3424-3433.

Fries, L. F., D. M. Gordon, I. Schneider, J. C. Beier, G. W. Long, M. Gross, J. U. Que, S. J. Cryz, and J. C. Sadoff. 1992. Safety, immunogenicity, and efficacy of a *Plasmodium falciparum* vaccine comprising a circumsporozoite protein repeat region peptide conjugated to Pseudomonas aeruginosa toxin A. *Infect. Immun.* **60**: 1834-9.

Galey, B., P. Druilhe, I. Ploton, C. Desgranges, A. Asavanich, T. Harinasuta, C. Marchand, K. Brahimi, Y. Charoenvit, C. Paul, M. Gross, and R. L. Beaudoin. 1990. Evidence for diversity of *Plasmodium falciparum* sporozoite surface antigens derived from analysis of antibodies elicited in humans. *Infect. Immun.* **58**: 2995-3001.

Garnham, P.C .C., R. G. Bird, J. R. Baker R. Killick-Kendrick. 1969. Electron microscope studies on the motile stages of malaria parasites. VII. The fine structure of the merozoites of exoerythrocytic schizonts of *Plasmodium berghei yoelii*. *Trans. R. Soc. Med. Hyg.* **63:** 328-339.

Garnham, P. C. 1966. Malaria parasites and other haemosporidia. Oxford, UK, Blackwell Scientific Publications.

Garnham, P. C. C. 1947. Exoerythrocytic schizogony in *Plasmodium kochi* Laveran. A preliminary note. *Trans. R. Soc. Trop. Med. Hyg.* **40**: 719-722.

Golenser, J., J. Heeren, J. P. Verhave, H. J. Kaay, and J. H. Meuwissen. 1977. Crossreactivity with sporozoites, exoerythrocytic forms and blood schizonts of *Plasmodium berghei* in indirect fluorescent antibody tests with sera of rats immunized with sporozoites or infected blood. *Clin. Exp. Immunol.* **29**: 43-51.

Guérin-Marchand, C., P. Druilhe, B. Galey, A. Londono, J. Patarapotikul, R. Beaudoin, C. Dubeaux, A. Tartar, O. Mercereau-Puijalon, and G. Langsley. 1987. A liver stage-specific antigen of *Plasmodium falciparum* characterized by gene cloning. *Nature.* **329**: 164-167.

Guidotti, L. G., T. Ishikawa, M. V. Hobbs, B. Matzke, R. Schreiber, and F. V. Chisari. 1996. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity.* **4**: 25-36.

Gysin, J., J. Barnwell, D. H. Schlesinger, V. Nussenzweig, and R. S. Nussenzweig. 1984. Neutralization of the infectivity of sporozoites of *Plasmodium knowlesi* by antibodies to a synthetic peptide. *J. Exp. Med.* **160**: 935-40.

Hedstrom, R. C., J. R. Campbell, M. L. Leef, Y. Charoenvit, M. Carter, M. Sedegah, R. L. Beaudoin, and S. L. Hoffman. 1990. A malaria sporozoite surface antigen distinct from the circumsporozoite protein. *Bull World Health Organ.* **68 Suppl**: 152-7.

Herrington, D., J. Davis, E. Nardin, M. Beier, J. Cortese, H. Eddy, G. Losonsky, M. Hollingdale, M. Stzein, M. Levine, R. S. Nussenzweig, D. Clyde, and R. Edelman. 1991. Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected vaccinees. *Am. J. Trop. Med. Hyg.* **45**: 539-547.

Hill, A. V. S., J. Elvin, A. C. Willis, M. Aidoo, C. E. M. Allsopp, F. M. Gotch, X. M. Gao, M. Takiguchi, B. M. Greenwood, A. R. M. Townsend, A. J. McMichael, and H. C. Whittle. 1992. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. *Nature.* **360**: 434-439.

Hill, A. V. S., C. E. M. Allsopp, D. Kwiatkowski, N. M. Anstey, P. Twumasi, P. A. Rowe, S. Bennett, D. Brewster, A. J. McMichael, and B. M. Greenwood. 1991. Common West African HLA antigens are associated with protection from severe malaria. *Nature.* **352**: 595-600.

Hoffman, S. L., J. M. Crutcher, S. K. Puri, A. A. Ansari, F. Villinger, E. D. Franke, P. P. Singh, F. Finkelman, M. K. Gately, G. P. Dutta, and M. Sedegah. 1997. Sterile protection of monkeys against malaria after administration of interleukin-12. *Nat. Med.* **3**: 80-83.

Hoffman, S. L., E. D. Franke, M. R. Hollingdale, and P. Druilhe. 1996. Attacking the infected hepatocyte, 35-76. *In* S. L. Hoffman (ed.), *Malaria vaccine development: a multi-immune response approach*. ASM Press, Washington D.C.

Hoffman, S. L., V. Nussenzweig, J. C. Sadoff, and R. S. Nussenzweig. 1991. Progress towards malaria preerythrocytic vacines. *Science.* **252**: 520-521.

Hoffman, S. L., D. Isenbarger, G. W. Long, M. Sedegah, A. Szarfman, L. Waters, M. R. Hollingdale, P. H. van der Meide, D. S. Finbloom, and W. R. Ballou. 1989a. Sporozoite vaccine induces genetically restricted T cell elimination of malaria from hepatocytes. *Science.* **244**: 1078-81.

Hoffman, S.L., C .N. Oster, C. Mason, J. C. Beier, J. A. Sherwood, W. R. Ballou, M. Mugambi, and J. D. Chulay. 1989b. Human lymphocyte proliferative response to a sporozoite T cell epitope correlates with resistance to falciparum malaria. *J. Immunol.* **142:** 1299-1303.

Holder, A. A., M. J. Lockyer, K. G. Odink, J. S. Sandhu, V. Riveros-Moreno, S. C. Nicholls, Y. Hillman, L. S. Davey, M. L. Tizard, R. T. Schwarz, and et al. 1985. Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites. *Nature.* **317**: 270-3.

Hollingdale, M. R., A. Appiah, P. Leland, V. E. do Rosario, D. Mazier, S. Pied, D. A. Herrington, J. D. Chulay, W. R. Ballou, T. Derks, S. H. Yap, R. L. Beaudoin and J. P. Verhave 1990a. Activity of human volunteer sera to candidate *Plasmodium falciparum* circumsporozoite protein vaccines in the inhibition of sporozoite invasion assay of human hepatoma cells and hepatocytes. *Trans. R. Soc. Trop. Med. Hyg.* **84**: 325-329.

Hollingdale, M. R., M. Aikawa, G. Chen, J. F. G. Meis, K. Sakhuja, B. Sina, and J. Zhu. 1990b. Pre-erythrocytic stage malaria parasites: non-circumsporozoite protein antigens. *Bull. OMS.* **68 (Suppl.)**: 178-180.

Hollingdale, M. R. 1988. Biology and immunology of sporozoite invasion of liver cells and exoerythrocytic development of malaria parasites. *Prog Allergy*. **41**: 15-48.

Hollingdale, M. R., W. R. Ballou, S. B. Aley, J. F. Young, S. Pancake, L. H. Miller, and W. T. Hockmeyer. 1987. *Plasmodium falciparum*: elicitation by peptides and recombinant circumsporozoite proteins of circulating mouse antibodies inhibiting sporozoite invasion of hepatoma cells. *Exp. Parasitol.* **63**: 345-51.

Hollingdale, M. R., P. Leland, and A. L. Schwartz. 1985. *In vitro* culture of two populations (diviving and nondividing) of exoerythrocytic parasites of *Plasmodium vivax*. *Am J Trop Med Hyg*. **34**: 216-222.

Hollingdale, M. R., E. H. Nardin, S. Tharavanij, A. L. Schwartz, and R. S. Nussenzweig. 1984. Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells; an *in vitro* assay of protective antibodies. *J. Immunol.* **132**: 909-913.

Hollingdale, M. R., P. Leland, and A. L. Schwartz. 1983. *In vitro* cultivation of the exoerythrocytic stage of Plasmodium berghei in a hepatoma cell line. *Am J Trop Med Hyg.* **32**: 682-4.

Hollingdale, M. R., F. Zavala, R. S. Nussenzweig, and V. Nussenzweig. 1982. Antibodies to the protective antigen of *Plasmodium berghei*. *J. Immunol.* **128**: 1929-1930.

Hollingdale, M. R., J. L. Leef, M. McCullough, and R. L. Beaudoin. 1981. *In vitro* cultivation of the exoerythrocytic stage of *Plasmodium berghei* from sporozoites. *Science.* **213**: 1021-2.

Hope, I. A., M. Mackay, J. E. Hyde, M. Goman, and J. Scaife. 1985. The gene for an exported antigen of the malaria parasite *Plasmodium falciparum* cloned and expressed in *Escherichia coli*. *Nucleic Acids Res.* **13**: 369-379.

Hope, I. A., R. Hall, D. L. Simmons, J. E. Hyde, and J. G. Scaife. 1984. Evidence for immunological cross-reaction between sporozoites and blood stages of a human malaria parasite. *Nature.* **308**: 191-4.

Hulier, E., P. Petour, G. Snounou, M. P. Nivez, F. Miltgen, D. Mazier, and L. Rénia. 1996. A method for the quantitative assessment of malaria parasite development in organs of the mammalian host. *Mol. Biochem. Parasitol.* **77**: 127-35.

Jaffe, R. I., G. H. Lowell, and D. M. Gordon. 1990. Differences in susceptibility among mouse strains to infection with *Plasmodium berghei* (ANKA clone) sporozoites and its relationship to protection by gamma-irradiated sporozoites. *Am. J. Trop. Med. Hyg.* **42**: 309-13.

Jahiel, R. I., J. Vilcek, and R. S. Nussenzweig. 1970. Exogenous interferon protects mice against *Plasmodium berghei* malaria. *Nature.* **227**: 1350-1.

Jakstys, B. P., N. E. Alger, J. A. Harant, and P. Silverman. 1974. Ultrastructural analysis of Plasmodium berghei sporozoite antigens prepared by freeze thawing and heat inactivation. *J Protozool.* **21**: 344-8.

Jones, T. R., J. K. Baird, M. J. Bangs, B. A. Annis, Purnomo, H. Basri, S. Gunawan, S. Harjosuwarno, P. D. McElroy, and S. L. Hoffman. 1994. Malaria vaccine study site in Irian Jaya, Indonesia: *Plasmodium falciparum* incidence measurements and epidemiologic considerations in sample size estimation. *Am. J. Trop. Med. Hyg.* **50**: 210-8.

Kara, U., D. Stenzel, L. Ingram, G. Bushell, J. A. Lopez, and C. Kidson. 1988. Inhibitory monoclonal antibody agaisnt a (Myristylated) small-molecular weight antigen from *Plasmodium falciparum* asociated with the parasitophorous vacuole membrane. *Infect. Immun.* **56**: 903-909.

Kaufmann, S. H. 1993. Immunity to intracellular bacteria. *Annu. Rev. Immunol.* **11**: 129-63.

Khan, Z. M., C. Ng, and J. P. Vanderberg. 1992. Early hepatic stages of *Plasmodium berghei*: release of circumsporozoite protein and host cellular inflammatory response. *Infect. Immun.* **60:** 264-270.

Khan, Z. M. and J. P.Vanderberg. 1991a. Role of host cellular response in differential susceptibility of nonimmunized BALB/c mice to *Plasmodium berghei* and *yoelii* sporozoites. *Infect. Immun*. **59:** 2529-2534.

Khan, Z. M. and J. P. Vanderberg. 1991b. Eosinophil-rich, granulomatous inflammatory response to *Plasmodium berghei* hepatic schizonts in nonimmunized rats is age-related. *Am. J. Trop. Med. Hyg.* **45:** 190-201.

Khusmith, S., M. Sedegah, and S. L. Hoffman. 1994. Complete protection against *Plasmodium yoelii* by adoptive transfer of a CD8+ cytotoxic T-cell clone recognizing sporozoite surface protein 2. *Infect. Immun.* **62**: 2979-83.

Khusmith, S., Y. Charoenvit, S. Kumar, M. Sedegah, R. L. Beaudoin, and S. L. Hoffman. 1991. Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein. *Science.* **252**: 715-718.

Klotz, F. W., L. F. Scheller, M. C. Seguin, N. Kumar, M. A. Marletta, S. J. Green, and A. F. Azad. 1995. Co-localization of inducible-nitric oxide synthase and *Plasmodium berghei* in hepatocytes from rats immunized with irradiated sporozoites. *J. Immunol.* **154**: 3391-5.

Kramer, L. D., and J. P. Vanderberg. 1975. Intramuscular immunization of mice with irradiated *Plasmodium berghei* sporozoites. Enhancement of protection with albumin. *Am. J. Trop. Med. Hyg.* **24**: 913-6.

Krotoski, W. A., W. E. Collins, R. S. Bray, P. C. C. Garnham, F. B. Cogswell, R. W. Gwadz, R. Killick-Kendrick, R. H. Wolf, R. E. Sinden, L. C. Koontz, and P. S. Stanfill. 1982a. Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium vivax* infection. *Am. J. Trop. Med. Hyg.* **31**: 1291-1293.

Krotoski, W. A., R. S. Bray, P. C. C. Garnham, R. W. Gwadz, R. Killick-Kendrick, C. C. Draper, G. A. T. Targett, D. M. Krotoski, M. W. Guy, L. C. Koontz, and F. B. Cogswell. 1982b. Observations on early and late post-sporozoite tissue stages in primate malaria. II. The hypnozoite of *Plasmodium cynomolgi bastianellii* from 3 to 105 days after infection, and detection of 36- to 40-hour pre-erythrocytic forms. *Am. J. Trop. Med. Hyg.* **31**: 211-225.

Krotoski, W. A., D. M. Krotoski, P. C. C. Garnham, R. S. Bray, R. Killick-Kendrick, C. C. Draper, G. A. T. Targett, and M. W. Guy. 1980. Relapses in primate malaria: discovery of two populations of exoerythrocytic stages. Preliminary note. *Br. Med. J.* **1**: 153-154.

Kumar, N., H. Nagasawa, J. B. Sacci, B. Sina, M. Aikawa, C. Atkinson, P. Uparanukraw, L. B. Kubiak, A. F. Azad, and M. R. Hollingdale. 1993. Expression of members of the heat-shock protein 70 family in the exoerythrocytic stages of *Plasmodium berghei* and *Plasmodium falciparum*. *Parasitol. Res.* **79**: 109-113.

Kumar, N., C. A. Syin, R. Carter, I. Quakyi, and L. H. Miller. 1988. Plasmodium falciparum gene encoding a protein similar to the 78-kDa rat glucose-regulated stress protein. *Proc. Natl. Acad. Sci. U S A.* **85**: 6277-81.

Lalvani, A., N. Hurt, M. Aidoo, P. Kibatala, M. Tanner, and A. V. Hill. 1996. Cytotoxic T lymphocytes to *Plasmodium falciparum* epitopes in an area of intense and perennial transmission in Tanzania. *Eur. J. Immunol.* **26**: 773-9.

Lambiotte, M., I. Landau, N. Thierry, and F. Miltgen. 1981. [Development of schizonts in cultured hepatocytes of adult rats after *in vitro* infection with *Plasmodium yoelii* sporoazoites]. *C. R. Seances Acad. Sci. III.* **293**: 431-3.

Li, J., J. D. Zhu, A. Appiah, T. F. McCutchan, G. W. Long, W. K. Milhous, and M. R. Hollingdale. 1991. Plasmodium berghei: quantitation of *in vitro* effects of antimalarial drugs on exoerythrocytic development by a ribosomal RNA probe. *Exp Parasitol.* **72**: 450-8.

Link, H. T., K. White, and U. Krzych. 1993. *Plasmodium berghei*-specific T cells respond to non-processed sporozoites presented by B cells. *Eur. J. Immunol.* **23**: 2263-2269.

Londono, J. A., M. Sedegah, Y. Charoenvit, R. L. Beaudoin, and P. Druilhe. 1991. Antigenic analysis of *Plasmodium yoelii* liver stages by fluorescence antibody assays. *Trop. Med. Parasitol.* **42**: 381-5.

Maheshwari, R. K., C. W. Czarniecki, G. P. Dutta, S. K. Puri, B. N. Dhawan, and R. M. Friedman. 1986. Recombinant human gamma interferon inhibits simian malaria. *Infect. Immun.* **53**: 628-630.

Malik, A., J. E. Egan, R. A. Houghten, J. C. Sadoff, and S. L. Hoffman. 1991. Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. *Proc. Natl. Acad. Sci. USA.* **88**: 3300-3304.

Marchand, C., P. Druilhe, A. Tartar, A. Thomas, and P. Druilhe. 1990. How to select *Plasmodium falciparum* pre-erythrocytic antigens in an expression library without defined probe. *Bull. WHO.* **68**: 158-164.

Marshall, V. M., A. Silva, M. Foley, S. Cranmer, L. Wang, D. J. McColl, D. J. Kemp, and R. L. Coppel. 1997. A second merozoite surface protein (MSP-4) of *Plasmodium falciparum* that contains an epidermal growth factor-like domain. *Infect. Immun.* **65**: 4460-7.

Mattei, D., L. S. Ozaki, and L. P. de Silva. 1988. A *Plasmodium falciparum* gene encoding a heat shock-like antigen related to the rat 78 Kd glucose-regulated protein. *Nucleic Acids Res.* **16**: 5204.

Mazier, D., W. E. Collins, S. Mellouk, P. M. Procell, N. Berbiguier, G. H. Campbell, F. Miltgen, R. Bertolotti, P. Langlois, and M. Gentilini. 1987. *Plasmodium ovale*: *in vitro* development of hepatic stages. *Exp. Parasitol.* **64**: 393-400.

Mazier, D. 1986a. Use of cultured hepatocytes in parasitology, p. 378-397. *In* A. Guillouzo, and C. Guguen-Guillouzo (eds.), *Research in isolated and cultured hepatocytes*. J. Libtey Eurotext Ltd./Inserm, Paris.

Mazier, D., S. Mellouk, R. L. Beaudoin, B. Texier, P. Druilhe, W. Hockmeyer, P. Trosper, Y. Paul, Y. Charoenvit, J. Young, F. Miltgen, L. Chedid, J. P. Chigot, B. Galley, O. Brandicourt, and M. Gentilini. 1986b. Effect of antibodies to recombinant and synthetic peptides on *Plasmodium falciparum* sporozoites *in vitro*. *Science.* **231**: 156-159.

Mazier, D., R. L. Beaudoin, S. Mellouk, P. Druilhe, B. Texier, J. Trosper, F. Miltgen, I. Landau, C. Paul, O. Brandicourt, C. Guguen-Guillouzo, P. Langlois, and M. Gentilini. 1985. Complete development of hepatic stages of *Plasmodium falciparum in vitro*. *Science.* **227**: 440-442.

Mazier, D., I. Landau, P. Druilhe, F. Miltgen, C. Guguen-Guillouzo, D. Baccam, J. Baxter, J. P. Chigot, and M. Gentilini. 1984. Cultivation of the liver forms of *Plasmodium vivax* in human hepatocytes. *Nature.* **307**: 367-9.

Mazier, D., I. Landau, F. Miltgen, P. Druilhe, M. Lambiotte, D. Baccam, and M. Gentilini. 1982. [*In vitro* infestation of adult *Thamnomys* hepatocytes with *Plasmodium yoelii* sporozoites; schizogony and release of infecting merozoites]. *C. R. Seances Acad. Sci. III.* **294**: 963-5.

Meis, J. F. G. M., T. Ponnudurai, B. Mons, A. van Belkum, P. M. C. A. van Eerd, P. Druilhe, and H. Schellekens. 1990. *Plasmodium falciparum*: studies on mature exoerythrocytic forms in the liver of the chimpanzee, *Pan troglodytes*. *Exp. Parasitol.* **70**: 1-11.

Meis, J. F., and J. P. Verhave. 1988. Exoerythrocytic development of malarial parasites. *Adv. Parasitol.* **27**: 1-61.

Meis, J. F. G. M., P. H. K. Jap, M. R. Hollingdale, and J.-P. Verhave. 1987. Cellular response against exoerythrocytic forms of *Plasmodium berghei* in rats. *Am. J. Trop. Med. Hyg.* **37**: 506-510.

Meis, J. F. G. M., J.-P. Verhave, P. H. K. Jap, and J. H. E. T. Meuwissen. 1985. Fine structure of exoerythrocytic merozoite formation of *P. berghei* in rat liver. *Am. J. Trop. Med. Hyg.* **37**: 506-510.

Meis, J. F., J. P. Verhave, P. H. Jap, and J. H. Meuwissen. 1983. An ultrastructural study on the role of Kupffer cells in the process of infection by *Plasmodium berghei* sporozoites in rats. *Parasitology.* **86**: 231-42.

Mellouk, S., S. L. Hoffman, Z. Z. Liu, P. de la Vega, T. R. Billiar, and A. K. Nussler. 1994. Nitric oxide-mediated antiplasmodial activity in human and murine hepatocytes induced by gamma interferon and the parasite itself: enhancement by exogenous tetrahydrobiopterin. *Infect. Immun.* **62**: 4043-6.

Mellouk, S., S. J. Green, C. A. Nacy, and S. L. Hoffman. 1991. IFN-γ inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. *J. Immunol.* **146**: 3971-3976.

Mellouk, S., F. Lunel, M. Sedegah, R. L. Beaudoin, and P. Druilhe. 1990a. Protection against malaria induced by irradiated sporozoites. *Lancet.* **335**: 721.

Mellouk, S., N. Berbiguier, P. Druilhe, M. Sedegah, B. Galey, L. Yuan, M. Leef, Y. Charoenvit, C. Paul, S. Hoffman, and R. Beaudoin. 1990b. Evaluation of an *in vitro* assay aimed at measuring protective antibodies against sporozoites. *Bull. WHO.* **68 (Suppl.)**: 52-59.

Mellouk, S., R. K. Maheshwari, A. Rhodes-Feuillette, R. L. Beaudoin, N. Berbiguier, H. Matile, F. Miltgen, I. Landau, S. Pied, J. P. Chigot, R. M. Friedman, and D. Mazier. 1987. Inhibitory activity of interferons and interleukin 1 on the development of *Plasmodium falciparum* in human hepatocyte cultures. *J. Immunol.* **139**: 4192-4195.

Mellouk, S., D. Mazier, P. Druilhe, N. Berbiguier, and M. Danis. 1986. *In vitro* and *in vivo* results suggesting that anti-sporozoite antibodies do not totally block *Plasmodium falciparum* sporozoite infectivity. *N. Engl. J. Med.* **315**: 20889-5055.

Meuwissen, J. H., J. Golenser, and J. P. Verhave. 1978. Development of effective antisporozoite immunity by natural bites of *Plasmodium berghei*-infected mosquitoes in rats under prophylactic treatment with various drug regimens. *Isr. J. Med. Sci.* **14**: 601-5.

Millet, P., C. T. Atkinson, M. Aikawa, M. R. Hollingdale, and W. E. Collins. 1991a. Strain specificity in the liver-stage development of *Plasmodium falciparum* in primary cultures of new world monkey hepatocytes. *Am. J. Trop. Med. Hyg.* 45: 236-242.

Millet, P., W. E. Collins, C. T. Atkinson, G. H. Campbell, J. R. Broderson, B. G. Brown, V. Filipski, M. Aikawa, and P. Ngyen-Dinh. 1991b. *Plasmodium cynomolgi*: Immunization of a rhesus monkey with exoerythrocytic stages cultured in autologous hepatocytes. *Exp. Parasitol. 72*: 91-98.

Millet, P., W. E. Collins, T. L. Fisk, and P. Nguyen-Dinh. 1988a. *In vitro* cultivation of exoerythrocytic stages of the human malaria parasite Plasmodium malariae. *Am J Trop Med Hyg.* **38**: 470-3.

Millet, P., T. L. Fisk, W. E. Collins, J. R. Broderson, and P. Nguyen-Dinh. 1988b. Cultivation of exoerythrocytic stages of Plasmodium cynomolgi, *P. knowlesi*, P. coatneyi, and P. inui in Macaca mulatta hepatocytes. *Am J Trop Med Hyg*. **39**: 529-34.

Mitchell, G.F. 1990. A note on concomitant immunity in host/parasite relationships: a successfully transplanted concept from tumor immunology. *Advances in Cancer Research* **54:** 319-333.

Moelans, II, J. Cohen, M. Marchand, C. Molitor, P. de Wilde, J. F. van Pelt, M. R. Hollingdale, W. F. Roeffen, W. M. Eling, C. T. Atkinson, M.Aikawa, J. G. Schoenmakers, and R. N. H. Konings. 1995. Induction of Plasmodium falciparum sporozoite-neutralizing antibodies upon vaccination with recombinant Pfs16 vaccinia virus and/or recombinant Pfs16 protein produced in yeast. *Mol Biochem Parasitol.* **72**: 179-92.

Molineaux, L., and G. Gramiccia. 1980. The Garki project: Research on the epidemiology and control of malaria in the Sudan savanna of West Africa. *Wolrd Health Organization, Geneva.*

Moreno, A., P. Clavijo, R. Edelman, J. Davis, M. Sztein, D. Herrington, and E. H. Nardin. 1991. Cytotoxic CD4+ T cells from a sporozoite-immunized volunteer recognize the *Plasmodium falciparum* CS protein. *Int. Immunol.* **3**: 997-1003.

Müller, H. M., I. Reckmann, M. R. Hollingdale, H. Bujard, K. J. Robson, and A. Crisanti. 1993. Thrombospondin related anonymous protein (TRAP) of *Plasmodium falciparum* binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. *Embo J.* **12**: 2881-2889.

Mulligan, H. W., P. F. Russell, and B. N. Mohan. 1941. Active immunization of fowls against *Plasmodium gallinaceum* by injections of killed homologous sporozoites. *J. Malar. Inst. India.* **4**: 25-34.

Nardin, E. H., and R. S. Nussenzweig. 1993. T cell responses to pre-erythrocytic stages of malaria. *Ann. Rev. Immunol.* **11**: 687-727.

Nardin, E. H., V. Nussenzweig, R. S. Nussenzweig, W. E. Collins, K. T. Harinasuta, P. Tapchaisri, and Y. Chomcharn. 1982. Circumsporozoite proteins of human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. *J. Exp. Med.* **156**: 20-30.

Nathan, C. 1997. Inducible nitric oxide synthase: what difference does it make? *J. Clin. Invest.* **100**: 2417-23.

Nudelman, S., L. Rénia, Y. Charoenvit, L. Yuan, F. Miltgen, R. L. Beaudoin, and D. Mazier. 1989. Dual action of anti-sporozoite antibodies *in vitro*. *J. Immunol.* **143**: 996-1000.

Nussenzweig, R. S., J. Vanderberg, G. L. Spitalny, C. I. Rivera, C. Orton, and H. Most. 1972a. Sporozoite-induced immunity in mammalian malaria. A review. *Am. J. Trop. Med. Hyg.* **21**: 722-8.

Nussenzweig, R. S., J. Vanderberg, H. Most, and C. Orton. 1969a. Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. III. Specificity of the protection. *Nature.* **222**: 488-489.

Nussenzweig, R. S., J. Vanderberg, and H. Most. 1969b. Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. IV. Dose response, specificity and humoral immunity. *Milit. Med.* **134**: 1176-1182.

Nussenzweig, R. S., J. Vanderberg, H. Most, and C. Orton. 1967a. Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. *Nature.* **216**: 160.

Nussenzweig, R.S. 1967b. Increased non specific resistance to malaria produced by administration of killed *Corynebacterium parvum*. Exp. Parasitol. **21:** 224-231.

Nussenzweig, R.S., R. Herman,J. P. Vanderberg, M. Yoelii, and H. Most. 1966. Studies on sporozoite induced infections of rodent malaria. III. The course of sporozoite induced *Plasmodium berghei* in different hosts. *Am. J. Trop. Med. Hyg*. **15:**. 684-689.

Nussenzweig, V., and R. S. Nussenzweig. 1989. Rationale for the development of an engineered sporozoite malaria vaccine. *Adv. Immunol.* **45**: 283-334.

Nussenzweig, V., and R. S. Nussenzweig. 1985. Circumsporozoite proteins of malaria parasites. *Cell.* **42**: 401-403.

Nussler, A. K., L. Rénia, V. Pasquetto, F. Miltgen, H. Matile, and D. Mazier. 1993. *In vivo* induction of the nitric oxide pathway in hepatocytes after injection with irradiated malaria sporozoites, malaria blood parasites or adjuvants. *Eur. J. Immunol.* **23**: 882-887.

Nussler, A., S. Pied, J. Goma, L. Rénia, F. Miltgen, M. Gentilini, G. E. Grau, and D. Mazier. 1991a. TNF inhibits malaria hepatic stages *in vitro* via IL-6 liver synthesis. *Int. Immunol.* **3**: 317-321.

Nussler, A., J. C. Drapier, L. Rénia, S. Pied, F. Miltgen, M. Gentilini, and D. Mazier. 1991b. L-arginine-dependent destruction of intrahepatocytic malaria parasites in response to tumor necrosis factor and/or interleukin 6 stimulation. *Eur J Immunol.* **21**: 227-30.

Nussler, A. S. Pied, M. Pontet, F. Miltgen, L. Rénia, M. Gentilini, and D. Mazier. 1991c. Inflammatory status and preerythrocytic stages of malaria: role of the C-reactive protein. *Exp Parasitol.* **72**: 1-7.

Nussler, A., J. Y. Folzou, F. Miltgen, and D. Mazier. 1989. Effect of irradiation on *Plasmodium* sporozoites depends on the species of hepatocytes infected. *Trop. Med. Parasitol.* **40**: 468-469.

Orjih, A. U., A. H. Cochrane, and R. S. Nussenzweig. 1982. Comparative studies on the immunogenicity of infective and attenuated sporozoites of *Plasmodium berghei*. *Trans. R. Soc. Trop. Med. Hyg.* **76**: 57-61.

Pape, K.A., E. R. Kearney, A. Khoruts, A. Mondino, R. Merica, Z. -M. Chen, E. Ingulli, J. White, J. G. Johnson, and M. K. Jenkins. 1997. Use of adaptative transfer of T-cell antigen-receptor-transgenic T cells for the study of T-cell activation *in vivo*. *Immunol. Rev.* **156:** 67-78.

Perlaza, B. L., M. Arévalo-Herrera, K. Brahimi, G. Quintero, J. C. Palomino, H. Gras-Masse, A. Tartar, P. Druilhe, and S. Herrera. Submitted for Publication. Immunogenicity of four *Plasmodium falciparum* pre-erythrocytic Antigens in *Aotus lemurinus* monkeys.

Pied, S., M. D. Tabone, G. Chatellier, M. Marussig, C. Jardel, F. Nosten, and D. Mazier. 1995. Non specific resistance against malaria pre-erythrocytic stages: involvement of acute phase proteins. *Parasite.* **2**: 263-8.

Pied, S., A. Civas, F. Berliot-Picard, L. Rénia, F. Miltgen, M. Gentilini, J. Doly, and D. Mazier. 1992. IL-6 induced by IL-1 inhibits malaria pre-erythrocytic stages but its secretion is down-regulated by the parasite. *J. Immunol.* **148**: 197-201.

Pied, S., L. Rénia, A. Nussler, F. Miltgen, and D. Mazier. 1990. Inhibitory activity of IL-6 on malaria hepatic stages. *Parasite Immunol.* **13**: 211-217.

Pied, S., A. Nussler, M. Pontent, F. Miltgen, H. Matile, P. H. Lambert, and D. Mazier. 1989. C-reactive protein protects against preerythrocytic stages of malaria. *Infect. Immun.* **57**: 278-82.

Pirson, P. 1982. Culture of the exoerythrocytic liver stages of *Plasmodium berghe*i sporozoites in rat hepatocytes. *Trans. R. Soc. Trop. Med. Hyg.* **76:** 422-6764.

Plebanski, M., M. Aidoo, H. C. Whittle, and A. V. Hill. 1997. Precursor frequency analysis of cytotoxic T lymphocytes to pre-erythrocytic antigens of *Plasmodium falciparum* in West Africa. *J. Immunol.* **158**: 2849-55.

Ponnudurai, T., A. H. W. Lensen, G. J. A. van Gemert, M. G. Bolmer, and J. H. E. Meuwissen. 1991. Feeding behaviour and sporozoite ejection by infected *Anopheles stephensi*. *Trans. R. Soc. Trop. Med. Hyg*. **85**: 175-180.

Potocnjak, P., N. Yoshida, R. S. Nussenzweig, and V. Nussenzweig. 1980. Monovalent fragment (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. *J. Exp. Med.* **151**: 1504-1513.

Puri, S. K., R. K. Maheshwari, G. P. Dutta, R. M. Friedman, and M. M. Dhar. 1988. Human interferon-gamma protects rhesus monkeys against sporozoite- induced *Plasmodium cynomolgi* malaria infection. *J. Interferon Res.* **8**: 201-206.

Quistorff, B. 1990. Metabolic heterogeneity of liver parenchymal cells. *Essays in Biochemistry*. **25**: 83-136.

Rajan, T. V. 1997. Why does *Plasmodium* have a pre-erythrocytic cycloe. *Parasitol. Today.* **13**: 284-286.

Ramirez, A. D., E. M. Rocha, and A. U. Krettli. 1995. Antisporozoite antibodies with protective and nonprotective activities: *in vitro* and *in vivo* correlations using *Plasmodium gallinaceum*, an avian model. *J. Eukaryot. Microbiol.* **42**: 705-8.

Ramsey, J. M., M. R. Hollingdale, and R. L. Beaudoin. 1982. Infection of tissue-stage cells with ^{60}CO gamma-irradiated malaria sporozoites, p. 19-25. *In* M. Ristic (ed.), *Nuclear techniques in the study of parasitic infections*. International Atomic Energy, Vienna.

Reed, R. C., V. Louis-Wileman, E. V. Cosmai, S. Fang, D. L. Jue, R. M. Wohlhueter, R. L. Hunter, and A. A. Lal. 1997. Multiple antigen constructs (MACs): induction of sterile immunity. *Vaccine.* **15**: 482-488.

Reed, R. C., V. Louis-Wileman, R. L. Wells, A. F. Verheul, R. L. Hunter, and A. A. Lal. 1996. Re-investigation of the circumsporozoite protein-based induction of sterile immunity against *Plasmodium berghei* infection. *Vaccine.* **14**: 828-36.

Renggli, J., M. Hahne, H. Matile, B. Betschart, J. Tschopp, and G. Corradin. 1997. Elimination of *P. berghei* liver stages is independent of Fas (CD95/Apo- I) or perforin-mediated cytotoxicity. *Parasite Immunol.* **19**: 145-148.

Rénia, L., M. M. Rodrigues, and V. Nussenzweig. 1994. Intrasplenic immunization with infected hepatocytes: a mouse model for studying protective immunity against malaria pre-erythrocytic stage. *Immunology.* **82**: 164-8.

Rénia, L., D. Grillot, M. Marussig, G. P. Corradin, F. Miltgen, P.-H. Lambert, D. Mazier, and G. Del Giudice. 1993a. Effector functions of circumsporozoite peptide-primed CD4+ T cell clones against *Plasmodium yoelii* liver stages. *J. Immunol.* **150**: 1471-1478.

Rénia, L., D. Xia, D. Samols, and V. Nussenzweig. 1993b. Transgenic mice expressing C-reactive protein are susceptible to infection with *Plasmodium yoelii* sporozoites. *Infect. Immun.* **61**: 348-9.

Rénia, L., M. Salone-Marussig, D. Grillot, G. Corradin, F. Miltgen, G. Del Giudice, and D. Mazier. 1991. *In vitro* activity of CD4+ and CD8+ T lymphocytes from mice immunized with a malaria synthetic peptide. *Proc. Natl. Acad. Sci. USA.* **88**: 7963-7967.

Rénia, L., D. Mattei, J. Goma, S. Pied, P. Dubois, F. Miltgen, A. Nüssler, H. Matile, F. Menégaux, M. Gentilini, and D. Mazier. 1990. A malaria heat shock like protein epitope expressed on the infected hepatocyte surface is the target of antibody-dependent cell-mediated cytotoxic mechanisms by non-parenchymal liver cells. *Eur. J. Immunol.* **20**: 1445-1449.

Rénia L., F. Miltgen, Y. Charoenvit, T. Ponnudurai, J. P. Verhave, W. E. Collins, and D. Mazier. 1988. Malaria sporozoite penetration: a new approach by double staining. *J. Immunol. Meth.* **112:** 201-205.

Rieckmann, K. H., R. L. Beaudoin, J. S. Cassells, and K. W. Sell. 1979. Use of attenuated sporozoites in the immunization of human volunteers against falciparum malaria. *Bull WHO.* **57 (suppl.)**: 261-265.

Robson, K. J., S. Naitza, G. Barker, R. E. Sinden, and A. Crisanti. 1997. Cloning and expression of the thrombospondin related adhesive protein gene of *Plasmodium berghei*. *Mol. Biochem. Parasitol.* **84**: 1-12.

Robson, K. J. H., J. R. S. Hall, L. C. Davies, A. Crisanti, A. V. S. Hill, and T. E. Wellems. 1990. Polymorphism of the TRAP gene of *Plasmodium falciparum*. *Proc. R. Soc. Lond. B.* **242**: 205-216.

Robson, K. J. H., J. R. S. Hall, M. W. Jennings, T. J. R. Harris, K. Marsh, C. I. Newbold, V. E. Tate, and D. J. Weatherall. 1988. A highly-conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. *Nature*. **335**: 79-82.

Rodrigues, M., R. S. Nussenzweig, and F. Zavala. 1993. The relative contribution of antibodies, CD4+ and CD8+ T cells to sporozoite-induced protection against malaria. *Immunology.* **80**: 1-5.

Rodrigues, M., R. S. Nussenzweig, P. Romero, and F. Zavala. 1992. The *in vivo* cytotoxic activity of CD8+ T cell clones correlates with their levels of expression of adhesion molecules. *J. Exp. Med.* **175**: 895-905.

Rodrigues, M. M., A. S. Cordey, G. Arreaza, G. Corradin, P. Romero, J. L. Maryanski, R. S. Nussenzweig, and F. Zavala. 1991. CD8+ cytolytic T cell clones derived against the *Plasmodium yoelii* circumsporozoite protein protect against malaria. *Int. Immunol.* **3**: 579-585.

Rogers, W. O., A. Malik, S. Mellouk, K. Nakamura, M. D. Rogers, A. Szarfman, D. M. Gordon, A. K. Nussler, M. Aikawa, and S. L. Hoffman. 1992a. Characterization of *Plasmodium falciparum* sporozoite surface protein 2. *Proc. Natl. Acad. Sci. USA*. **89**: 9176-9180.

Rogers, W. O., M. D. Rogers, R. C. Hedstrom, and S. L. Hoffman. 1992b. Characterization of the gene encoding sporozoite surface protein 2, a protective *Plamodium yoelii* sporozoite antigen. *Mol. Biochem. Parasitol.* **53**: 45-52.

Romero, P. J., J. L. Maryanski, G. P. Corradin, R. S. Nussenzweig, V. Nussenzweig, and F. Zavala. 1989. Cloned cytotoxic T cells recognize an epitope on the circumsporozoite protein and protect against malaria. *Nature.* **341**: 323-325.

Rosenberg, R., R. A. Wirtz, I. Schneider, and R. Burge. 1990. An estimation of the number of malaria sporozoites ejected by a feeding mosquito. *Trans. R. Soc. Trop. Med. Hyg.* **84**: 209-212.

Rossan, R. N., and D. C. Baerg. 1975. Demonstration of exoerythrocytic stages of *Plasmodium vivax* in *Saimiri sciureus*. *Trans. R. Soc. Trop. Med. Hyg.* **69**: 471-2.

Rouvière, H. 1981. Lymphatiques du foie, p. 351-358. *In* H. Rouviére (ed.), *Anatomie des lymphatiques de l'homme*. Masson (Publisher), Paris.

Sanchez, G. I., W. O. Rogers, S. Mellouk, and S. L. Hoffman. 1994. *Plasmodium falciparum*: exported protein-1, a blood stage antigen, is expressed in liver stage parasites. *Exp. Parasitol.* **79**: 59-62.

Scheller, L. F., S. J. Green, and A. F. Azad. 1997. Inhibition of nitric oxide interrupts the accumulation of CD8+ T cells surrounding *Plasmodium berghei*-infected hepatocytes. *Infect. Immun.* **65**: 3882-3888.

Scheller, L. F., and A. F. Azad. 1995a. Maintenance of protective immunity against malaria by persistent hepatic parasites derived from irradiated sporozoites. *Proc Natl Acad Sci U S A.* **92**: 4066-8.

Scheller, L. F., K. C. Stump, and A. F. Azad. 1995b. *Plasmodium berghei*: Production and quantitation of hepatic stages derived from irradiated sporozoites in rats and mice. *J. Parasitol.* **81**: 58-62.

Scheller, L. F., R. A. Wirtz, and A. F. Azad. 1994. Susceptibility of different strains of mice to hepatic infection with *Plasmodium berghei*. *Infect. Immun.* **62**: 4844-7.

Schofield, L., J. Villaquiran, A. Ferreira, H. Schellekens, R. S. Nussenzweig, and V. Nussenzweig. 1987a. Gamma interferon, CD8+T cells and antibodies required for immunity to malaria sporozoites. *Nature.* **330**: 664-666.

Schofield, L., A. Ferreira, R. Altszuler, V. Nussenzweig, and R. S. Nussenzweig. 1987b. Interferon-gamma inhibits the intrahepatocytic development of malaria parasites *in vitro*. *J. Immunol.* **139**: 2020-2025.

Sedegah, M., F. Finkelman, and S. L. Hoffman. 1994. Interleukin 12 induction of interferon gamma-dependent protection against malaria. *Proc. Natl. Acad. Sci. USA*. **91**: 10700-2.

Sedegah, M., B. K. Sim, C. Mason, T. Nutman, A. Malik, C. Roberts, A. Johnson, J. Ochola, D. Koech, B. Were, and S. L. Hoffman. 1992. Naturally acquired CD8+ cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. *J. Immunol.* **149**: 966-971.

Seed, T. M., M. Aikawa, C. Sterling, and J. Rabbege. 1974. Surface properties of extracellular malaria parasites: morphological and cytochemical study. *Infect Immun.* **9**: 750-61.

Seguin, M. C., F. W. Klotz, I. Schneider, J. P. Weir, M. Goodbary, M. Slayter, J. J. Raney, J. U. Aniagolu, and S. J. Green. 1994. Induction of nitric oxide synthase protects against malaria in mice exposed to irradiated *Plasmodium berghei* infected mosquitoes: involvement of interferon-gamma and CD8+ T cells. *J. Exp. Med.* **180**: 353-358.

Seguin, M. C., W. R. Ballou, and C. A. Nacy. 1989. Interactions of *Plasmodium berghei* sporozoites and murine Kuppfer cells *in vitro*. *J. Immunol.* **143**: 1716-1722.

Shin, S.C., J. P. Vanderberg, and J. A. Terzakis. 1982. Direct infection of hepatocytes by sporozoites of *Plasmodium berghei*. *J. Protozool.* **29:** 448-454.

Shortt, H. E., N. H. Fairley, G. Covell, P. G. Shute, and P. C. C. Garnham. 1951. The pre-erythrocytic stage of *Plasmodium falciparum*. *Trans. Roy. Soc. Trop. Med. Hyg.* **44**: 405-419.

Shortt, H. E., and P. C. C. Garnham. 1948a. Pre-erythrocytic stage in mammalian malaria parasites. *Nature.* **161**: 126.

Shortt, H. E., P. C. C. Garnham, G. Covell, and P. G. Shute. 1948b. The pre-erythrocytic stage of human malaria, *Plasmodium vivax*. *Br. Med. J.* **1**: 1225-1228.

Shortt, H. E., and P. C. C. Garnham. 1948c. The pre-erythrocytic development of *Plasmodium cynomolgi* and *Plasmodium vivax*. *Trans. Roy. Soc. Trop. Med. Hyg.* **41**: 785-795.

Sidjanski, S., and J. P. Vanderberg. 1997. Delayed migration of *Plasmodium* sporozoites from the mosquito bite to the blood. *Am. J. Trop. Med. Hyg.* **57**: 426-429.

Siegler, C. I., P. Leland, and M. R. Hollingdale. 1984. In infectivity of irradiated *Plasmodium berhei* sporozoites to cultured hepatoma cells. *Am. J. Trop. Med. Hyg.* **33:** 544-547.

Sijwali, P. S., P. Malhotra, S. K. Puri, and V. S. Chauhan. 1997. Cloning and sequence analysis of the thrombospondin-related adhesive protein (TRAP) gene of *Plasmodium cynomolgi bastianelli*. *Mol. Biochem. Parasitol.* **90**: 371-376.

Simmons, D., G. Woolett, M. Bergin-Cartwright, K. Day, and J. Scaife. 1987. A malaria antigen exported into a new compartment within the host erythrocyte. *EMBO J.* **6**: 485-491.

Sinden, R.E. and J. Smith. 1980. Culture of liver stages (exoerythrocytic schizonts) of rodent malaria parasites from sporozoites *in vitro*. *Trans. R. Soc. Trop. Med. Hyg.* **74:** 134-136.

Sinnis, P., and V. Nussenzweig. 1996. Preventing sporozoite invasion of hepatocytes, 15-34. *In* S. L. Hoffman (ed.), *Malaria vaccine development: a multi-immune response approach* , ASM Press, Washington, D.C.

Smith, J. E., J. F. Meis, T. Ponnudurai, J. P. Verhave, and H. J. Moshage. 1984. *In-vitro* culture of exoerythrocytic form of *Plasmodium falciparum* in adult human hepatocytes. *Lancet.* **2**: 757-8.

Smrkovski, L. L. 1981. Effect of route of *Mycobacterium bovis* BCG administration on induction of suppression of sporozoite immunity in rodent malaria. *Infect.Immun.* **31**: 408-412.

Smrkovski, L. L., and G. T. Strickland. 1978. Rodent malaria: BCG-induced protection and immunosuppression. *J. Immunol.* **121**: 1257-1261.

Spitalny, G. L., J. P. Verhave, J. H. E. T. Meuwissen, and R. S. Nussenzweig. 1977. *Plasmodium berghei*: T-cell dependence of sporozoite-induced immunity. *Exp.Parasitol.* **42**: 73-81.

Spitalny, G. L., and R. S. Nussenzweig. 1972. Effect of various routes of immunization and methods of parasite attenuation on the development of protection against sporozoite-induced rodent malaria. *Proc. Helminthol. Soc. (Washington, DC).* **39**: 506-514.

Stewart, M. J., and J. P. Vanderberg. 1992. Electron microscopic analysis of circumsporozoite protein trail formation by gliding malaria sporozoites. *J. Protozool.* **39**: 663-671.

Strome, C.P., P. L. De Santis, and R. L. Beaudoin. 1979. The cultivation of the exoerythrocytic stages of *Plasmodium berghei* from sporozoites. *In vitro* **15:** 531-536.

Suhrbier, A. 1991. Immunity to the liver stage of malaria. *Parasitol. Today.* **7**: 160-163.

Suhrbier, A., L. A. Winger, E. Castellano, and R. E. Sinden. 1990. Survival and antigenic profile of irradiated malarial sporozoites in infected liver cells. *Infect. Immun.* **58**: 2834-2839.

Suhrbier, A., A. Hamilton, J. Nicholas, and R. E. Sinden. 1988. The fate of the circumsporozoite proteins during the exoerythrocytic development of *P. berghei*. *Eur. J. Cell. Biol. 46*, 25-35.

Sultan, A. A., V. Thathy, U. Frevert, K. J. Robson, A. Crisanti, V. Nussenzweig, R. S. Nussenzweig, and R. Ménard. 1997. TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. *Cell.* **90**: 511-522.

Szarfman, A., J. A. Lyon, D. Walliker, I. Quakyi, R. J. Howard, S. Sun, W. R. Ballou, K. Esser, W. T. London, R. A. Wirtz, and R. Carter. 1988a. Mature liver stages of cloned *Plasmodium falciparum* share epitopes with proteins from sporozoites and asexual blood stages. *Parasite Immunol.* **10**: 339-351.

Szarfman, A., D. Walliker, J. S. McBride, J. A. Lyon, I. A. Quakyi, and R. Carter. 1988b. Allelic forms of gp195, a major blood stage antigen of *Plasmodium falciparum*, are expressed in liver stages. *J. Exp. Med.* **167**: 231-236.

Tam, J. P., P. Clavijo, Y. Lu, V. Nussenzweig, R. S. Nussenzweig, and F. Zavala. 1990. Incorporation of T and B epitopes of the circumsporozoite protein in a chemically defined synthetic vaccine against malaria. *J. Exp. Med.* **171**: 299-306.

Tam, J. P., and F. Zavala. 1989. Multiple antigen peptide. A novel approach to increase detection sensitivity of synthetic peptides in solid-phase immunoassays. *J. Immunol. Methods.* **124**: 53-61.

Templeton, T. J., and D. C. Kaslow. 1997. Cloning and cross-species comparison of the thrombospondin-related anonymous protein (TRAP) gene from *Plasmodium knowlesi*, *Plasmodium vivax* and *Plasmodium gallinaceum*. *Mol. Biochem. Parasitol.* **84**: 13-24.

Tsuji, M., C. L. Eyster, R. L. O'Brien, W. K. Born, M. Bapna, M. Reichel, R. S. Nussenzweig, and F. Zavala. 1996. Phenotypic and functional properties of murine gamma delta T cell clones derived from malaria immunized, alpha beta T cell-deficient mice. *Int. Immunol.* **8**: 359-366.

Tsuji, M., Y. Miyahira, R. S. Nussenzweig, M. Aguet, M. Reichel, and F. Zavala. 1995. Development of antimalaria immunity in mice lacking IFN-gamma receptor. *J. Immunol.* **154**: 5338-44.

Tsuji, M., P. Mombaerts, L. Lefrancois, R. S. Nussenzweig, F. Zavala, and S. Tonegawa. 1994. gamma delta T-Cells contribute to immunity against the liver stages of malaria in alpha beta T-cell-deficient mice. *Proc. Natl. Acad. Sci. USA.* **91**: 345-349.

Tsuji, M., P. J. Romero, R. S. Nussenzweig, and F. Zavala. 1990. CD4+ cytolytic T cell clone confers protection against murine malaria. *J. Exp. Med.* **172**: 1353-1358.

van Pelt, J. F., H. J. Moshage, E. Depla, J. P. Verhave, and S. H. Yap. 1997. Identification of human liver carboxylesterase as one of the proteins involved in Plasmodium falciparum malaria sporozoite invasion in primary cultures of human hepatocytes. *J. Hepatol.* **27**: 688-98.

Vandenberghe, P., J. Delabie, M. de Boer, C. L. De Wolf-Peeters, and J. L. Ceuppens. 1992. *In situ* expression of B7/BB1 on antigen-presenting cells and activated B cells: an histochemical study. *Int. Immunol. **5:*** 317-321.

Vanderberg, J.P., Z. M. Khan, and M. J. Stewart. 1993. Induction of hepatic inflammatory response by *Plasmodium berghei s*porozoites protects BALB/c mice against challenge with *Plasmodium yoelii* sporozoites. *J. Parasitol*. **79:** 763-767.

Vanderberg, J. P., S. Chew, and M. J. Stewart. 1990. *Plasmodium* sporozoite interactions with macrophages *in vitro*: a videomicroscopic analysis. *J. Protozool.* **37**: 528-536.

Vanderberg, J. P., R. S. Nussenzweig, and H. Most. 1969. Protective immunity produced by the injection of X-irradiated. *Military.Medecine.* **134**: 1183-1190.

Vergara, U., A. Ferreira, H. Schellekens, and V. Nussenzweig. 1987. Mechanisms of escape of exoerythrocytic forms (EEF) of malaria parasites from the inhibitory effects of interferon. *J. Immunol.* **138**: 4447-4449.

Verhave, J. P., and J. F. Meis. 1984. The biology of tissue forms and other asexual stages in mammalian plasmodia. *Experientia.* **40**: 1317-29.

Verhave, J.P., J. F. Meis, T. M. De Boo, and J. H. Meuwissen. 1985. The delivery of exoerythrocytic parasites of *Plasmodium berghei*: a hormone controlled process. *Ann. Soc. belge Méd. Trop.* 65: 35-44.

Verhave, J.P., J. H. E. P. Meuwissen, and J. Golenser. 1980. The dual role of macrophages in the sporozoite-induced malaria infection. A hypothesis. *Int J Nucl. Med. Biol.* **7:** 149-156.

Verhave, J. P., G. T. Strickland, H. A. Jaffe, and A. Aftab. 1978. Studies on the transfer of protective immunity with lymphoid cells from mice immune to malaria sporozoites. *J.Immunol.* **121**: 1031-1033.

Verhave, J.P. 1975. Immunization with sporozoites. Thesis. Catholic University of Nijmegen.

Vinetz, J. M., J. Li, T. F. McCutchan, and D. C. Kaslow. 1998. Plasmodium malariae infection in an asymptomatic 74-year-old Greek woman with splenomegaly. *N. Engl. J. Med.* **338**: 367-71.

Vreden, S. G., M. F. Van den Broek, M. C. Oettinger, W. Boers, N. Van-Rooijen, J. H. Meuwissen, and R. W. Sauerwein. 1995. Susceptibility to *Plasmodium berghei* infection in rats is modulated by the acute phase response. *Parasite Immunol.* **17**: 445-50.

Vreden, S. G. 1994. The role of Kupffer cells in the clearance of malaria sporozoites. *Parasitol. Today.* **10**: 304-308.

Vreden, S. G. S., M. F. van den Broek, M. C. Oettinger, J. P. Verhave, J. H. Meuwissen, and R. W. Sauerwein. 1992. Cytokines inhibit the development of liver schizonts of the malaria parasite *Plasmodium berghei in vivo*. *Eur. J. Immunol.* **22**: 2271-2275.

Walliker, D., I. A. Quakyi, T. E. Wellems, T. F. McCutchan, A. Szarfman, W. T. London, L. M. Corcoran, T. R. Burkot, and R. Carter. 1987. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science.* **236**: 1661-1666.

Wang, R., Y. Charoenvit, G. Corradin, P. De La Vega, E. D. Franke, and S. L. Hoffman. 1996. Protection against malaria by *Plasmodium yoelii* sporozoite surface protein 2 linear peptide induction of CD4+ T cell- and IFN-gamma-dependent elimination of infected hepatocytes. *J. Immunol.* **157**: 4061-7.

Wang, R., Y. Charoenvit, G. Corradin, R. Porrozzi, R. L. Hunter, G. Glenn, C. R. Alving, P. Church, and S. L. Hoffman. 1995. Induction of protective polyclonal antibodies by immunization with *Plasmodium yoelii* circumsporozoite protein multiple antigen peptide vaccine. *J. Immunol.* **155**: 1637.

Waters, A. P., D. G. Higgins, and T. F. MCutchan. 1993. The phylogeny of malaria: a useful study. *Parasitol. Today.* **9**: 246-250.

Waters, A. P., D. G. Higgins, and T. F. McCutchan. 1991. Plasmodium falciparum appears to have arisen as a result of lateral transfer between avian and human hosts. *Proc Natl Acad Sci U S A*. **88**: 3140-4.

Weiss, W. R., M. Sedegah, J. A. Berzofsky, and S. L. Hoffman. 1993. The role of CD4+ T cells in immunity to malaria sporozoites. *J. Immunol.* **151**: 2690-2698.

Weiss, W. R., J. A. Berzofsky, R. A. Houghten, M. Sedegah, M. Hollingdale, and S. L. Hoffman. 1992. A T cell clone directed at the circumsporozoite protein which protects mice against both *Plasmodium yoelii* and *Plasmodium berghei*. *J. Immunol.* **149**: 2103-2109.

Weiss, W. R. 1990a. Host/parasite interactions and immunity to irradiated sporozoites. *Immunol. Lett.* **25**: 39-42.

Weiss, W. R., S. Mellouk, R. A. Houghten, M. Sedegah, S. Kumar, M. F. Good, J. A. Berzofsky, L. H. Miller, and S. L. Hoffman. 1990b. Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. *J. Exp. Med.* **171**: 763-773.

Weiss, W. R., M. F. Good, M. R. Hollingdale, L. H. Miller, and J. A. Berzovsky. 1989. Genetic control of immunity to *Plasmodium yoelii* sporozoites. *J. Immunol.* **143**: 4263-4266.

Weiss, W. R., M. Sedegah, R. L. Beaudoin, L. H. Miller, and M. F. Good. 1988. CD8+ T cells (cytotoxic/supressor) are required for protection on mice immunized with irradiated sporozoites. *Proc. Natl. Acad. Sci. USA*. **85**: 573-576.

Wéry, M. 1968. Studies on the sporogony of rodent malaria parasites. *Ann. Soc. belge Méd. Trop.* **48:** 1-137.

White, K. L., H. L. Snyder, and U. Krzych. 1996. MHC class I-dependent presentation of exoerythrocytic antigens to CD8+ T lymphocytes is required for protective immunity against *Plasmodium berghei*. *J. Immunol.* **156**: 3374-81.

Wisse, E., R. B. De Zanger, K. Charles, P. Van der Smissen, and R. S. Mc Cuskey. 1985. The liver sieve: considerations concerning the structure and function of endothelium fenestrae, the sinusoidal wall and the space of Disse. *Hepatology* **5:** 683-692.

Wizel, B., R. Houghten, P. Church, J. A. Tine, D. E. Lanar, D. M. Gordon, W. R. Ballou, A. Sette, and S. L. Hoffman. 1995a. HLA-A2-restricted cytotoxic T lymphocyte responses to multiple *Plasmodium falciparum* sporozoite surface protein 2 epitopes in sporozoite-immunized volunteers. *J. Immunol.* **155**: 766-75.

Wizel, B., R. A. Houghten, K. C. Parker, J. E. Coligan, P. Church, D. M. Gordon, W. R. Ballou, and S. L. Hoffman. 1995b. Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the *Plasmodium falciparum* sporozoite surface protein 2. *J. Exp. Med.* **182**: 1435-45.

Wortman, A., P. Rogers, Y. Charoenvit, A. McDermott, M. Leef, M. Sedegah, and R. L. Beaudoin. 1989. Cloning of Plasmodium yoelii genes expressing three different sporozoite-specific antigens. *Microb Pathog.* **6**: 227-31.

Wu, Y., C. D. Sifri, H. H. Lei, X. Z. Su, and T. E. Wellems. 1995. Transfection of *Plasmodium falciparum* within human red blood cells. *Proc. Natl. Acad. Sci. USA.* **92**: 973-977.

Yang, C., Y. P. Shi, V. Udhayakumar, M. P. Alpers, M. M. Povoa, W. A. Hawley, W. E. Collins, and A. A. Lal. 1995. Sequence variations in the non-repetitive regions of the liver stage-specific antigen-1 (LSA-1) of Plasmodium falciparum from field isolates. *Mol. Biochem. Parasitol.* **71**: 291-4.

Yates S. N. R., R. Snow, C. E. M. Allsopp, C. R. J. Newton, D. Palmer, N. Peschu, K. Marsh, C. I. Newbold and A. V. S. Hill. 1994. HLA associated differential susceptibility to severe malaria in children from Kilifi, Kenya. 6th Malaria meeting of The British Society of Parasitology, September 19-21, Liverpool, England. Page 19.

Yoshida, N., R. S. Nussenzweig, P. Potocnjak, V. Nussenzweig, and M. Aikawa. 1980. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasites. *Science.* **207**: 71-73.

Zavala, F., J. P. Tam, P. J. Barr, P. J. Romero, V. Ley, R. S. Nussenzweig, and V. Nussenzweig. 1987. Synthetic peptide vaccine confers protection against murine malaria. *J. Exp. Med.* **166**: 1591-1596.

Zevering, Y., C. Khamboonruang, and M. F. Good. 1994. Natural amino acid polymorphisms of the circumsporozoite protein of *Plasmodium falciparum* abrogate specific human CD4+ T cell responsiveness. *Eur. J. Immunol.* **24**: 1418-1425.

Zhu, J., and M. Hollingdale. 1991. Structure of *Plasmodium falciparum* liver stage antigen-1. *Mol. Biochem. Parasitol.* **48**: 223-226.

10. FIGURE LEGENDS

Figure 1. Three-day (left) and seven-day old (right) *P. falciparum* liver schizonts obtained respectively in *Aotus trivirgatus* and in *Saimiri sciureus* following inoculation of 10^6 *P. falciparum* Spz (produced by feeding *A. stephensi* mosquitoes on membrane feeeders containing patient gametocytes; P. Druilhe, unpublished material). Note the denser cytoplasm of the hepatocyte hosting the young LS, which is about the size of the hepatocyte nucleus (3-5 μm). The liver cycle is slightly slower in unnatural hosts: the large (80-100 μm) 7-day schizont obtained in South American monkeys is almost fully mature, i.e. about the same stage as would be obtained on day 6 in chimpanzees and about day 5.5 in humans. Staining is by Giemsa-colophonium.

Figure 2. Investigation of cellular events in the liver. C3H mice were immunized with a recombinant LSA-3 protein and challenged by intravenous inoculation of 10^6 *P. yoelii* Spz. A large number of schizonts were seen in control mice receiving adjuvant alone (top). None were detected upon examination of serial sections in rLSA-3 immunized animals (center), even though a similar number of cell granuloma were seen in these animals (enlarged image on bottom). Data from K. Brahimi, P. daubersies and P. Druilhe.

Figure 3. Comparative assessment of antibody response to sporozoite surface and liver stage proteins in an area of very low malaria transmission (Podor, Northern Senegal, averaging 1-5 infective bites per individual per year). Sera were individually tested from 55 individuals covering all age groups. Antibody titers were determined to the sporozoite surface using a "wet" sporozoite IFA assay (Druilhe *et al.*, 1986), and to liver stage parasites by IFAs on 5 μm liver sections from a *P. falciparum* infected Cebus monkey (Druilhe *et al.*, 1984). The figure shows the $\log_{10}$ geometric mean of titers measured in each age group. On average, titers to liver stage antigens were 150 times higher than those measured against the sporozoite surface (K. Brahimi and P. Druilhe, unpublished data).

F**igure 4.** Prevalence of antibody responses to synthetic peptides derived from LSA-3, LSA-1, SALSA and CS among a 210 individual cohort from Dielmo, Senegal (P. Druilhe, unpublished data). Positive responses were defined as an OD_{492} test serum value ≥ the mean + 3 SD of a group of control naïve human sera assayed in parallel (Fidock *et al.*, 1994a).

Figure 5. Schematic representation of immune effectors experimentally demonstrated to be active against pre-erythrocytic stage parasites. Ab: antibodies; ADCC: antibody-dependent cellular cytotoxicity; APP: acute phase proteins; CTL: cytotoxic T lymphocytes; Mϕ: macrophages; NOI: nitric oxide intermediates; ROI: reactive oxygen intermediates.

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