Pre-erythrocytic antigens of *Plasmodium falciparum*: from rags to riches?

Anne Charlotte Grüner[1], Georges Snounou[1], Karima Brahimi[1], Franck Letourneur[2], Laurent Rénia[2] and Pierre Druilhe[1]

[1]Unité de Parasitologie Biomédicale, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex 15, France
[2]Département d'Immunologie et Service Commun de Séquençage, Institut Cochin, INSERM U567, CNRS UHR8104, Université René Descartes, Hôpital Cochin, Paris 75014, France

A growing number of *Plasmodium* genomes have joined the sequencing treadmill, and the genome of *Plasmodium falciparum* has recently been published. Most malaria vaccinologists will soon be confronted by a bewildering array of new potential antigens from the recently completed genome of this parasite. However, for those aiming to target the pre-erythrocytic stages of the hepatic parasite, the wait might be long. In the absence of readily available materials and specific reagents, the selection of pre-erythrocytic antigens from raw sequence data is likely to prove difficult. Here, current knowledge of pre-erythrocytic antigens is updated in the light of recent results, and the post-genomic prospects of completing the antigenic repertoire of these immunologically important and intriguing stages is discussed.

The importance of pre-erythrocytic* malaria antigens lies in the fact that experimentally induced sterilizing protection against malaria in humans has so far been obtained only through immunization with viable radiation-attenuated sporozoites [1–3]. In 1983, the first malaria gene to be sequenced [4] encoded a circumsporozoite (CS) protein found on the surface of the sporozoite, the form that initiates infection in the mammalian host. Attempts to develop pre-erythrocytic vaccines centred mainly on improving formulations based on CS, whereas little effort was made to search actively for other sporozoite surface antigens. It was later demonstrated that induction of protection is associated with the transformation of the irradiated sporozoites into persistent growth-arrested hepatic trophozoites [5–7]. Thus, identification of antigens expressed in liver-stage parasites became an essential step to developing pre-erythrocytic vaccines.

The choice of the metabolically complex hepatocyte as the site for the first obligatory round of schizogony in the mammalian host is a mystery. This stage represents the only time when the parasite is found in a nucleated cell that also expresses major histocompatibility complex (MHC) molecules. The occurrence of hypnozoites in some species argues against a purely multiplicative function. This crucial and complex transition step between the two hosts of the parasite remains to be elucidated.

Lack of sufficient material has impeded research on the biology and immunology of the hepatic stage. Isolation of infected hepatocytes from infected humans is not an option. Only very limited numbers can be obtained from the livers of those rare primates (e.g. chimpanzees) susceptible to *Plasmodium falciparum* and, although *in vitro* cultivation methods have been developed [8], yields remain very low. Consequently, the number of antigens considered to be pre-erythrocytic (16 to date) is limited compared with the number of blood-stage antigens. Six of these represent a subset of antigens discovered through the only systematic analysis of pre-erythrocytic antigens to have been performed [9]. Further antigens uncovered by this approach, together with the prospects of current and future strategies to increase the pre-erythrocytic antigenic repertoire, are outlined below.

The long search for liver-stage antigens

Until recently, the only means to identify hepatic-stage proteins was the immunological screening of parasite nucleic acid expression libraries, but methods to obtain pre-erythrocytic-specific reagents had to be elaborated. Attempts to obtain mouse monoclonal antibodies (mAbs) failed because too few liver-stage parasites were present in immunizing preparations. A large set of human mAbs positive by indirect fluorescence antibody test (IFAT) on sporozoites and liver stages (Fig. 1) were successfully derived from African donors, incidentally providing an early indication of the antigenic complexity of these stages [10]. Unfortunately, these cell lines proved highly unstable and methods to immortalize them were not reliable. It was then reasoned that individuals subjected to sporozoite inoculation while undergoing prophylactic treatment against blood-stage infection would produce sera specific for pre-erythrocytic antigens. The breakthrough came with the identification of a group of missionaries who had remained under daily chloroquine prophylaxis during

Corresponding author: Pierre Druilhe (druilhe@pasteur.fr.).
* In view of the confusion inherent in the term exo-erythrocytic, we decided long ago to group all antigens expressed in the sporozoite and the liver stages under the term 'pre-erythrocytic' antigens.

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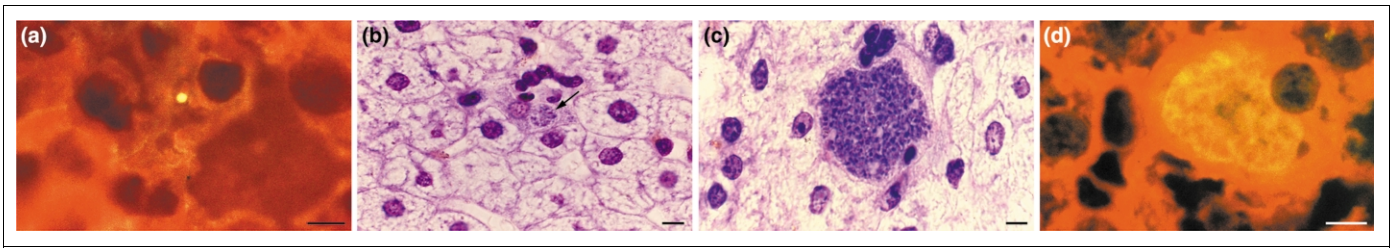

Fig. 1. Hepatic stages of *Plasmodium falciparum* in South American primates. (a) Young hepatic trophozoites, 30 h post-infection (p.i.), can only be detected after immunological staining. (b) Early hepatic schizont, 72 h p.i., containing a few nuclei, as detected in Giemsa-stained preparations. The cytoplasm of the infected hepatocyte is usually condensed (arrow). (c) Mature hepatic schizont, 7 days p.i., showing a multitude of pseudocytomeres and the initial appearance of merozoites. Growth of the parasites led to a fivefold increase in the size of the hepatocyte and compressed its nucleus against the edge of the cell. (d) Epstein–Barr virus (EBV)-transformed African lymphocytes produce monoclonal antibodies that show distinct indirect fluorescence antibody test (IFAT) patterns for sub-mature 5-day-old hepatic *P. falciparum* schizonts. Scale bars = 10 μm.

twenty or more years of residence in hyperendemic areas in Africa [9]. The sera they donated were found to contain high titres of antibodies against the sporozoites and the resulting liver stages to which they were constantly exposed, but showed only minor reactivity against the blood stages, which the chloroquine had suppressed.

The first set of characterized antigens

Using serum collected from the group of missionaries, 120 clones were identified in 1985 by screening 1200 clones pre-selected by hyperimmune sera from a *P. falciparum* genomic expression library. At that time, characterization of all the inserts could not be envisaged. Expression on sporozoites, hepatic-stage parasites, or both, was confirmed by IFAT using antibodies affinity-purified from hyperimmune sera on the different recombinant proteins (K. Brahimi, PhD thesis, Université Paris VII, France, 1997). These antibodies were also tested on sporozoites from two rodent malaria parasite species (K. Brahimi, op. cit.), revealing an interesting predominance of cross-reactivity against *P. yoelii* (32 clones) as compared with *P. berghei* (four clones) [7].

The first antigen of *P. falciparum* to be characterized was liver-stage antigen (LSA) 1 [11], and this remains the only antigen established as being expressed solely at the hepatic stages. This was followed by sporozoite threonine- and asparagine-rich protein (STARP) [12] and sporozoite- and liver-stage antigen (SALSA) [13], both of which are found in liver stages and sporozoites, and were selected because they were consistently found on the surface of sporozoites derived from a series of wild isolates. A further two pre-erythrocytic antigens with similar characteristics were found to be *P. falciparum* erythrocyte membrane protein (PfEMP) 3 [14] and erythrocyte-binding antigen (EBA) 175 [15], previously known as important blood-stage antigens [16–18]. Because the aim of the exercise was to identify parasite antigens that are potentially responsible for the immunity induced by pre-erythrocytic stages, the characterization of a further antigen, LSA3, was based on differential recognition by antibodies from protected and nonprotected human volunteers immunized with irradiated sporozoites [19]. Immunization with LSA3 was shown to protect chimpanzees against a *P. falciparum* sporozoite challenge [19], hence LSA3 is now undergoing Phase I and Phase IIa clinical trials.

As the *P. falciparum* Genome Project neared completion, it became possible to identify all of the genes represented by the 120 clones selected originally. BLAST searches against the *P. falciparum* genome database [20] revealed that 28 *P. falciparum* genes were represented, and some of their characteristics are summarized in Table 1. These genes, and those of the other known pre-erythrocytic antigens, are distributed on all 14 chromosomes of the parasite (Fig. 2). There does not appear to be any clustering of genes, nor is there a biased distribution to the telomeric ends of the chromosomes. Of the genes we identified, introns were predicted in 19, and were confirmed for the five genes that were further investigated [12–15,19], thus demonstrating that they are transcribed during these stages. The genes varied in size from 1–25 kb, and 15 genes were represented by a single clone and 13 by multiple individual clones. For some genes (e.g. *lsa3*), the antibody-selected inserts corresponded to a confined region whereas, for others (e.g. *pfemp3*), these were distributed along the whole coding sequence (Fig. 3).

Proteins by numbers

Over the past 20 years, only nine pre-erythrocytic-stage antigens, excluding CS, have been identified by other approaches (Table 1). The strategy exploiting serum from the missionaries provided 26 new antigens. The reliability of this strategy has been further confirmed by formal demonstrations that 64 of the 112 inserts, representing ten antigens including CS and glutamate-rich protein (GLURP), are indeed expressed at the sporozoite and/or hepatic stages [12–15,19]. Thus, 36 pre-erythrocytic proteins are now known that are antigenic to humans under natural conditions of exposure. However, the expression library used does not represent the complete repertoire of pre-erythrocytic epitopes, not least because the selected subset corresponds to the antibodies elaborated by a single individual. Thus, the antigens obtained in this manner obviously represent only a fraction of the total number of pre-erythrocytic antigens. It is interesting that the abundant merozoite surface proteins (MSP) 1, MSP2 and MSP3, as well as the apical membrane antigen (AMA) 1, were not selected by the missionary serum employed; although some (e.g. MSP3) were represented in the 1200 clones screened (P. Druilhe, unpublished).

The fact that the numerous antigens that have now been characterized are present on the surface of the

Table 1. Characteristics of *Plasmodium falciparum* pre-erythrocytic antigen genes[a]

Gene	Chromosome number	kb	Spz	Ls	Es	Refs
msp1	9	5.1	A	P	P	[26]
pfs16	4	0.4	P[b]	A	P	[27,28]
trap/*Pfssp2*	13	1.7	P	P	P	[29,30]
pfexp1	11	0.9	A	P	P	[31,32]
hsp70-1	8	2.0	A	P	P	[33]
Pfgrp78	9	2.3	A	P	P	[34,35]
mb2	5	2.7	P	P	P	[36]
Pfarp	1	6.6	P	ND	P	[37]
glurp	10	3.8	P	P	P	[38]
cs	3	1.2	P	A	A	[39,40]
lsa1	10	4.8	A	P	A	[11]
starp	7	1.8	P	P	P	[12]
salsa/*msp4*	2	1.0	P	P	P	[13,41]
lsa3	2	4.8	P	P	A	[19]
Pfemp3	2	7.5	P	P	P	[14]
eba175	7	4.9	P	P	P	[15]
ORF 1–20[c]	1–8, 10–14	1.5–26	12P/6nd	11P/8nd	13P/6nd	[d,e]

[a]Abbreviations: A, absence; *cs*, circumsporozoite; *eba*, erythrocyte-binding antigen; Es, erythrocytic stage; IFAT, indirect fluorescence antibody test; *glurp*, glutamate-rich protein; *hsp*, heat shock protein; Ls, liver stage; *lsa*, liver-stage antigen; *msp*, merozoite surface protein; ND, not determined; ORF, open reading frame; P, present; *Pfarp*, *P. falciparum* asparagine-rich protein; *Pfemp*, *P. falciparum* erythrocyte membrane protein; *Pfexp1*, *P. falciparum* exported protein; *Pfgrp*, *P. falciparum* glucose-regulated protein; *Pfssp*, *P. falciparum* sporozoite surface protein; *salsa*, sporozoite- and liver-stage antigen; Spz, sporozoite; *starp*, sporozoite threonine- and asparagine-rich protein; *trap*, thrombospondin-related adhesive protein.
[b]The available data is open to different interpretation.
[c]The 20 ORFs correspond to the inserts present in the selection obtained by screening with the missionaries' serum. Data for Spz, Ls and Es expression is derived from IFAT reactivity using human antibodies affinity-purified against one or more of the recombinant proteins corresponding to the different ORFs.
[d]K. Brahimi, PhD thesis, Université Paris VII, France, 1997.
[e]A.C. Grüner, PhD thesis, Université Paris VII, France, 2001.

sporozoite indicates that this form is potentially as antigenically complex as the extensively investigated blood-stage merozoite. Furthermore, the concept that pre-erythrocytic stages are antigenically silent no longer stands. Indeed, for those antigens studied in detail (LSA1, SALSA, STARP and LSA3), a high prevalence of natural immune responses (antibody, T helper and cytotoxic T lymphocytes) could be detected in individuals living in endemic areas [12–15,19,21,22]. This is remarkable considering that the immune system of an individual in a hyperendemic area is exposed yearly to ~10 million infected erythrocytes for each sporozoite (and the resulting liver-stage parasite) that is inoculated by the mosquito (taking a high estimate of 365 infective bites each consisting of 25 sporozoites versus a low estimate of 0.002% blood-stage parasitaemia over six months).

New tools, same obstacles

Given that 6000 proteins are predicted to be encoded by the *P. falciparum* genome, two problems face the post-genomic parasitologist: (1) how to establish rapidly which of these proteins are present in the pre-erythrocytic stages; and (2) how to identify those that are antigenic? The ultimate step is of course identification of those antigens that can serve as a basis for an efficient vaccine.

A solution to the first problem might well come from recent exciting technical advances, such as mass spectroscopy or laser microdissection. The transcriptome of *P. yoelii* sporozoites has been published recently [23], and the proteome of *P. falciparum* sporozoites is eagerly awaited. However, several million sporozoites were needed in these studies, and the same achievement would be difficult to envisage for hepatic-stage parasites, where access to pure parasite material (which can now be obtained by laser microdissection) is limited to several hundred infected hepatocytes [24,25]. An alternative approach might rely on the identification of stage-specific promoters. However, our studies indicate that this strategy might be less straightforward than originally expected. Antigens unique to the infected hepatocyte appear to be rare (LSA1 is the only one known), whereas many of the antigens are shared by sporozoites and infected hepatocytes, where they are actively synthesized (rather than representing a carry-over following sporozoite invasion, as is the case for CS). Furthermore, a substantial proportion of antigens are expressed throughout the pre- and erythrocytic stages. The fact that immunity is stage specific raises the interesting speculation that antigenic presentation for a given protein might differ depending on the host cell in which it develops.

These post-genomic approaches do not offer a solution to the second problem, namely to indicate which of the many proteins are actually antigenic. The antigens identified so far are structurally diverse, and initial analysis did not reveal a specific feature such as signal or anchor sequences that might have been exploited to identify others. Antigenic screening of all the proteins identified through transcriptome or proteome analysis would require the development of a high-throughput expression system. Although the screening strategy employed nearly 20 years ago remains effective, the spread of drug resistance now makes it difficult to acquire sera similar to those obtained from missionaries. Thus, humans or animals experimentally inoculated with sporozoites might prove to be the only remaining option to obtain stage-specific reagents. In this context, material from protected and nonprotected hosts immunized by irradiated sporozoites offers the added advantage of a differential screen leading to the selection of vaccine candidates [3,19]. In conclusion, the availability of pre-erythrocytic-specific immunological reagents remains

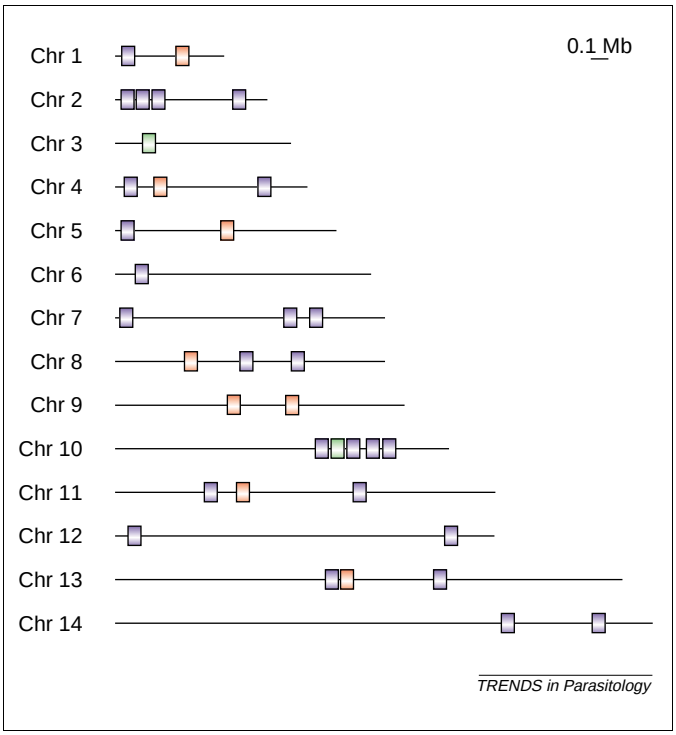

Fig. 2. Distribution and approximate position of the known pre-erythrocytic genes on the 14 chromosomes (Chr) of *Plasmodium falciparum*. Genes corresponding to those found in the selection obtained through screening with serum from a group of missionaries (who had remained under daily chloroquine prophylaxis during twenty or more years of residence in hyperendemic areas in Africa) are represented in purple, those discovered independently are represented in red, and the two genes previously established as expressed in pre-erythrocytic stages and which were also found in the selection are in green. Using serum collected from the group of missionaries, 120 clones had been identified previously by screening 1200 clones pre-selected by hyperimmune sera from a *P. falciparum* genomic expression library. Of these, six clones could not be recovered from the original 120 clones that were obtained in 1986. The inserts from the remaining 114 clones were sequenced and a BLAST search against the latest *P. falciparum* genome database [20] was performed. The insert was found to have been lost in five of the clones. For the remaining 109 clones, 11 were found to have a double insert, thus a total of 120 different sequences were obtained. Four sequences were of human origin, three had no known homologies, and one represented the *P. falciparum* telomere repeat. The 112 *P. falciparum* sequences recognized by the pre-erythrocytic-stage-specific serum represent a total of 28 malaria genes.

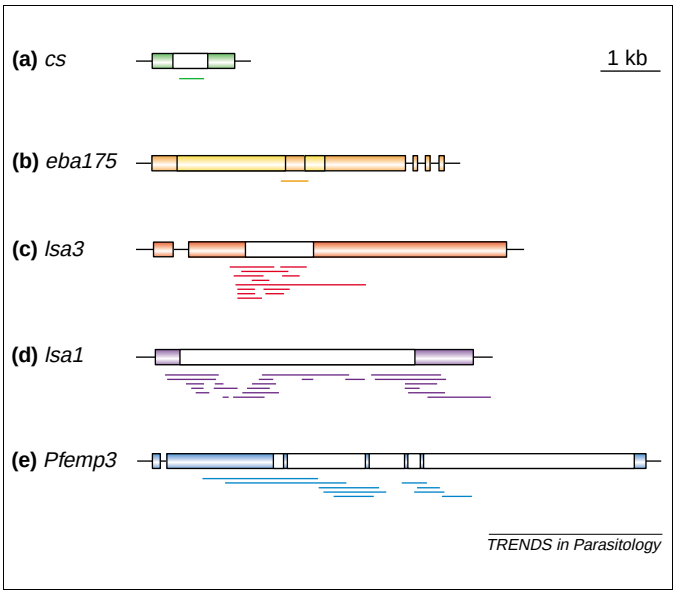

Fig. 3. Distribution of inserts along five representative *Plasmodium falciparum* pre-erythrocytic antigen genes. The inserts correspond to those selected from serum from missionaries who had remained under daily chloroquine prophylaxis during twenty or more years of residence in hyperendemic areas in Africa. Among the selected clones, some genes are represented by a single clone that corresponds to either a repeated [circumsporozoite (*cs*)] or a unique [erythrocyte binding antigen 175 (*eba175*)] portion of the gene. Other genes are represented by numerous inserts that span the whole gene [22 inserts for liver-stage antigen 1 (*lsa1*)], or are represented by clustered and broadly overlapping inserts to one region (17 inserts for *lsa3*) or to more than one region [ten inserts for *P. falciparum* erythrocyte membrane protein 3 (*pfemp3*) of the gene. For a given gene, independent inserts of a similar size and position have not been shown. Repetitive regions in the different genes are represented by white boxes. The *eba175* region II to the 5′-end of the insert and the Camp strain insert to the 3′-end of the insert are represented by boxes of a lighter shade of yellow [15].

an unavoidable requirement for antigen identification. Approaches relying on natural human immune responses offer several substantial advantages.

Despite the major investment in genomics, the liver stage is a glaring gap in the malaria proteome analysis. The lack of concomitant investment in the study of the biology of the liver stages has impeded the identification of critical defence mechanisms. Today, this translates into a scarcity of functional tools to exploit genomic data.

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