How to select *Plasmodium falciparum* pre-erythrocytic antigens in an expression library without defined probe

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The restricted access to Plasmodium falciparum *liver stages has greatly limited the analysis of the antigenic content of that stage. Due to the lack of material to perform immunochemical studies, of access to mRNA, and of monoclonal probes, we decided to screen a genomic library with stage-restricted human antibodies. This strategy led to the identification of a large number of DNA fragments encoding both sporozoite specific as well as liver-stage specific epitopes. Following the initial characterization of one liver-stage antigen, further screening was performed by using additional selective human antibodies. These were defined as having a high degree of reactivity with native antigens on either of the two stages while being negative with the already known molecules of the two stages. From this second screening and the study of cross-reactions, several subsets of DNA clones expressing antigens present on the surface of sporozoites, or in liver stages, or in both, could be identified. In exposed individuals a high prevalence of antibodies to several of these antigens was found.*

Introduction

Our interest in the study of liver-stage (LS) antigens derives firstly from initial IFA studies using *P. falciparum* LS which revealed the presence of unique stage-specific antigens to which a high prevalence of antibodies was found in endemic areas (*1*), and secondly from the conclusions we made several years ago that γ-irradiated-sporozoite-induced protection may depend more on antigens appearing when the parasites enter the hepatocyte, than on antigens present on the sporozoite.

However, the liver phase of development of human malaria has attracted little interest in the past particularly from an immunological point of view. In this context of nearly absent immunological research for 40 years most of the tools required for characterizing liver-stage antigens were not available.

Sometimes in research constraints may happen to have unexpected advantages. In the case of LS the lack of access to parasites of that stage, of tools, and of probes for LS-specific antigens has obliged us to use what may appear as a complicated approach, which in turn now seems to us to be more fruitful than the more classical ones.

Methods and results

Methods enabling production of *P. falciparum* and *P. vivax* liver schizonts could be developed. *In vivo* in chimpanzees but also in other monkeys not susceptible to blood forms, such as *Cebides*, LS can develop and become fully mature (*1*). *In vitro* the progress in the cultivation of human hepatocytes could be applied successfully to the cultivation of malaria LS which in most cases remain submature (*2*). However, with both groups of methods the yield remains very low and no satisfactory concentration technique could be developed. Thus, despite important progress, the production of parasitic material of human malaria species did not allow the performance of immunochemical techniques aimed at analysing antigens and did not allow the extraction of messenger RNA. In addition, using the available *in vivo* and *in vitro* material the immunization of Balb C mice was attempted but proved unsuccessful. The lack of a detectable immune response to LS antigens prevented us from attempting to produce mouse monoclonal antibodies (MAb).

In order to circumvent these difficulties and also to raise probes to those epitopes recognized by the human immune system we attempted to produce human monoclonal antibodies. Two clones specific to the LS antigens were obtained by fusion of human peripheral lymphocytes with myeloma lines, but were lost (Goutner & Druilhe, unpublished results). Much better results were obtained by Epstein-Barr virus transformation of lymphocytes from 28 immune subjects (*3*). Cloning of these lymphoblastoid lines and screening by IFA on infected liver sections allowed identification of several human MAb specific to LS. These human MAb, however, turned out to be very unstable and were thought to be inappropriate for DNA screening purposes.

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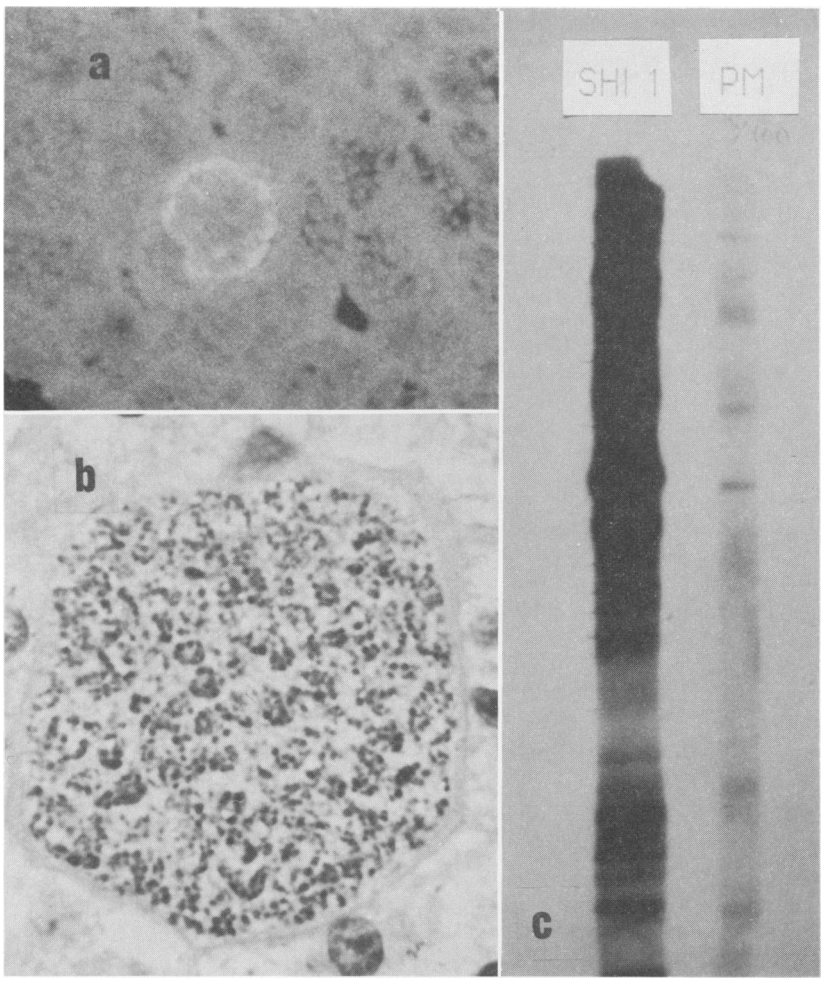
In the absence of a monospecific probe, we decided to search for stage-specific antisera and to screen a genomic DNA library by such polyclonal antibodies. "Restricted specificity" stage-specific sera were searched for among several individuals who stayed for many years in endemic areas with uninterrupted chloroquine prophylaxis which prevents blood-stage infections.

Three sera from missionaries who lived for 24–28 years in highly endemic areas while under continuous chloroquine prophylaxis reacted at very high titres with pre-erythrocytic stages but, as shown in Fig. 1, were almost negative with blood-stage antigens as compared with hyperimmune sera, which were positive in all stages.

Among all antigens producing DNA clones which were found positive with a pool of hyperimmune sera containing antibodies to the three stages (sporozoite, liver stage, and blood stage), the liver schizont encoding clones were expected to be those that were positive with the missionaries' serum but negative with transfusion malaria sera and antisporozoite monoclonal antibodies. This procedure also enabled selection of sporozoite antigens differing from the circumsporozoite antigen (CS).

Fig. 1. **Pattern of reactivity of the selective missionaries' serum.** By IFA (*a*) on sections of infected liver and (*c*) on blood-stage extracts following SDS-PAGE and Western blotting (right lane, PM), as compared with hyperimmune African serum with same titre on liver stages (left lane, SHI1); (*b*) Giemsa-stained liver schizont.



More than 85% of 1200 genomic DNA clones were in this way excluded. One hundred and twenty clones thought to correspond mainly to pre-erythrocytic-stage antigens were selected by the missionaries' sera (Fig. 2a).

Thus, it is because of the initial difficulties encountered that we were led to use a totally different screening procedure which turned out to be more global and, we believe, more fruitful since it allowed us to select at once several gene fragments encoding several of the pre-erythrocytic antigens. This methodology had one major advantage compared to screening by mouse monoclonals: to select preferentially those epitopes which are recognized by the human immune system. In addition, the epitopes selected were likely to be among the ones well conserved within several parasite isolates since the library was made from one cloned parasite from Asia and was screened with African sera.

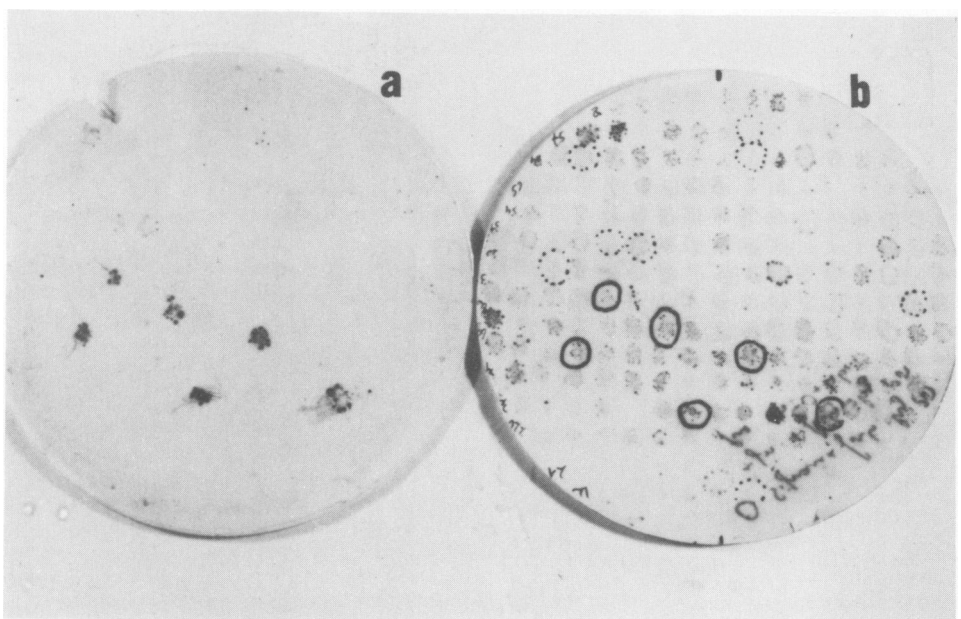
In the subset of 120 clones we made first a very common error of choice: to select clones giving a high immunological signal which apparently biased the choice towards repetitive structures.

The first liver-stage antigen we selected, called LSA, was made of 51 base-pair repeats, encoding a highly conserved seventeen aminoacid repeat polypeptide which was predicted to form an α helix (*4*).

Human antibodies affinity purified on the recombinant protein, as well as sera from mice, rabbits and chimpanzees immunized with either LSA synthetic or recombinant protein, reacted with *P. falciparum* liver schizonts giving the typical stage-specific labelling of the periphery of the schizont (*1*). The same antibodies were negative with *P. falciparum* sporozoites and blood stages as well as with all stages of heterologous malaria species.

Among several synthetic peptides, those made of one or more of the basic seventeen amino acid structure were antigenically reactive in direct recognition as in antigen inhibition assays. Several overlapping peptides of 8 and 12 amino acids, as well as one shifted seventeen aminoacid peptide overlapping two repeats, did not contain the epitope present in the repeated structure (Londono et al., in preparation). Circular dichroism studies confirmed the predicted α helical structure in the antigenically reactive peptides and showed that those that were non-reactive were not organized, thus suggesting that the antigenicity of the LSA molecule was defined by the secondary structure of the molecule.

Fig. 2. **Selection of DNA recombinant phages by the missionaries' serum (*a*) compared to African adult serum (*b*).** Nitrocellulose filters soaked with IPTG (10-1M) were applied on a petri dish spotted with 100 different colonies, reacted with each serum (1/100 in TBS-milk), then with anti-human peroxidase labelled antibodies, and revealed with DAB substrate.



The comparison of results obtained by immunofluorescence on native liver schizont proteins, by ELISA with LSA synthetic peptides, or Western blot with LSA fusion protein, showed that all subjects from all endemic areas reacted with the native proteins. In contrast the prevalence of antibodies to the LSA repeated peptide and the LSA recombinant protein was about 95% in individuals from forest and savanna areas and 80% in a very low endemic area at the northern part of the Sahel. In other words these studies, although they revealed a high prevalence of antibodies to LSA, also indicated that important additional epitopes or additional LS molecules were lacking.

At that stage it may have appeared appropriate to first of all complete investigations on the structure of the LSA gene, i.e., to study the whole sequence of the gene. However we had no indication that the lacking epitopes we were interested in were contained within that gene. Since our interest was primarily in the immune response to various LS epitopes we decided to complete our molecular study of LS antigens by making a further epitope selection in the subset of pre-erythrocytic clones, irrespective of the location of these epitopes in the genome.

Table 1: **Choice of selective sera**[a]

Sera	IFA-LS	ELISA-307 LSA_1 (ratio)
1	800	3.3
2	400	0.5
3	3200	7.1
4	3200	0.9
5	6400	1.5
6	6400	16.6
7	12800	25.0
8	1600	0.8
9	25600	3.3
10	6400	12.0

Sera	IFA-SPZ	ELISA-CS (ratio)
1	<100	0.7
2	400	1.8
3	6400	11.8
4	6400	1.1
5	1600	5.8
6	800	5.6
7	12800	0.6
8	6400	8.6
9	<100	1.9
10	25600	0.9

[a] Results were obtained by IFA either on wet sporozoites of NF54 strain of *P. falciparum* (IFA-SPZ), or on liver stages in sections of infected liver (IFA-LS), and by ELISA using as antigens either R32tet32 (ELISA-CS) or LSA1 peptide made of 2.5 repeats (results of IFA expressed as reciprocal titres; results of ELISA expressed as a ratio of the OD given by the test serum compared to the mean ± 2 SD of 6 control negative sera).

This complementary screening was based on principles similar to those used for LSA, i.e., on differential screening by human antibodies. For this purpose additional human selective sera able to detect both non-CS sporozoite surface antigens and complementary liver-stage antigens were necessary.

In sera from endemic areas, we selected those sera negative with the LSA1 molecule but having high titres on native LS antigens and similarly sera negative with the CS protein but having high titres of antibodies directed to sporozoite surface antigens. (Table 1).

The pattern of reactivity given by these sera with recombinant proteins allowed us to make a first classification in three categories: a first group of clones which were all negative with all five sera; a second group which were all positive with at least 4 of these 5 sera; and a third group with highly dissociated results.

Since we were mostly interested in conserved antigens yielding consistent immune responses, we thereafter evaluated the reactivity of each fusion protein with a complementary series of 8 hyperimmune sera and selected the clones that were more consistently reactive with those sera.

We then evaluated the stage specificity of antigens by reacting affinity-purified antibodies on sporozoites, liver forms and blood forms of *P. falciparum* and of heterologous species. About 47 positive clones were identified up to now. Some were positive only on liver stage, some only on sporozoites, and some on both.

Surprisingly the first group of clones, negative with the five selective sera, correspond only to liver-stage specific clones and in fact all belong to the large LSA1 family. The second group, positive with selective sera, correspond (nearly all of them) to antigens shared between liver and sporozoite stages, such as the SALSA (sporozoite and liver-stage antigen) (Marchand et al., in preparation). In the third group we found mostly if not only sporozoite surface-specific antigens.

In order to detect redundant epitopes we thereafter studied the immunological cross-reactions between fusion proteins by reacting the antibodies, affinity-purified on the product from one clone, with the recombinant product of each of the remaining clones. For example, antibodies corresponding to two different clones reveal exactly the same pattern of clones. This procedure finally led us to construct a table of cross-reactivities (part of the results are shown in Fig. 3), i.e., to classify clones in relatively clear-cut immunological families.

—The large LSA1 family represents nearly 20% of the clones. However, apparently some of the larger

Fig. 3. **Classification based on immunological cross-reactivities.** Human antibodies were affinity purified on the recombinant product from each clone and reacted with recombinant proteins of all the remaining clones as described in Fig. 2. The intensity of the reaction was rated 0 to + + +. For clarity all the negative results are not printed (vertical axis, recombinant proteins tested; horizontal axis, antibodies to each recombinant protein).

inserts encode additional epitopes differing from those contained in the seventeen-amino acid repeats.

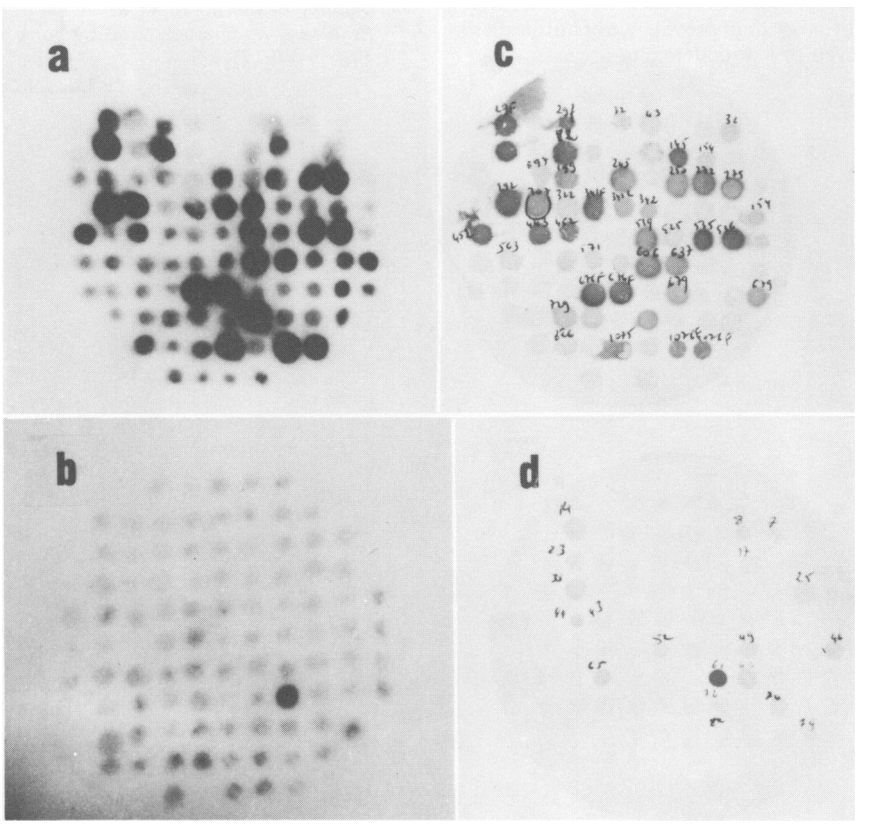
—The SALSA is poorly included in any of those families, but there is a second large family having similar features with encoding antigens present on both sporozoite surface and liver stage. The peripheral labelling of the liver schizont obtained with antibodies corresponding to those clones is similar to what has been defined as being LS specific.

—The three remaining smaller groups shown in Fig. 3 correspond to sporozoite-specific antigens. Only one, corresponding to the CS-repeated tetrapeptide, was identified and another, corresponding to the non-repetitive part of the CS.

We are aware that such a classification based on epitope cross-reactivities has great limitations. It was made on the assumption that the genomic library being made of small-sized DNA fragments, each small DNA fragment would express only one or a limited number of epitopes. Obviously this will prove wrong in a percentage of cases and also cross-reactions can be seen between distinct molecules. This is why we are now investigating the similarities and differences between these clones at the DNA level.

Fig. 4 compares the results obtained with LSA1 and SALSA at the DNA level and the immunological level. Each clone is located in the same position in the two assays. It is surprising that, among the 22 immunologically cross-reactive clones of the LSA1 family, all except two also cross-hybridize and that no additional clone was detected in this way. Similarly, the SALSA, which is recognized only by antibodies purified on itself, also hydridize with no other clone

Fig. 4. **Comparison of cross-immunological (*c*, *d*) and cross-hybridization studies (*a*, *b*) with SALSA (*b* and *d*) and LSA (*a* and *c*) DNA inserts.** Cross-immunological studies were performed as described in Fig. 3. Cross-hybridizations were made using P^{32}-labelled DNA inserts of either SALSA or LSA clone on nitrocellulose membranes containing 100 bacteriophage plaques. In the two techniques the phages are distributed in the same order on the filter.



than itself. Such a close correlation between DNA structure and antigenic features of the corresponding protein was unexpected and would probably not occur when studying other molecules.

Conclusions

(1) The results outlined above confirm our earlier work indicating firstly that several antigens were accessible on the sporozoite surface, and secondly that antigens specific to LS or to both LS and sporozoites exist.

(2) The initial absence of access to parasites of the liver stage and to a specific probe for the antigens of that stage led us to use an alternative strategy of screening based on selection by human antibodies which is exactly opposite to the mouse MAb selective process used for many *P. falciparum* antigens.

(3) This screening procedure opened an access to a rather large range of molecules both from liver stage and sporozoite stage in which a single copy of the CS protein was found.

(4) Our main selective criterion has been and will remain the prevalence of immune responses among malaria exposed individuals. In this respect the importance of some of the identified molecules is supported by the very high prevalence of antibodies in populations, even of low-endemicity areas.

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