A Novel Merozoite Surface Antigen of *Plasmodium falciparum* (MSP-3) Identified by Cellular-Antibody Cooperative Mechanism Antigenicity and Biological Activitiy of Antibodies

Claude Oeuvray, Hasnaa Bouharoun-Tayoun, Heléne Grass-Masse*, Jean Paul lepers**, Laurence Ralamboranto**, André Tartar***, Pierre Druilhe

Laboratoire de Parasitologie Médicale, Institut Pasteur, 28, rue du Docteur Roux, 75015 Paris France *Laboratoire de Chimie des Bio-Molécules, Institut Pasteur de Lille **URA CNRS 1309 1, rue du Pr. Calmette, BP 245 59019 Lile, France ***Unité de Recherche sur le Paludisme, Institut Pasteur de Madagascar BP 1274 Antananarivo, Madagascar

We report the identification of a 48kDa antigen targeted by antibodies which inhibit Plasmodium falciparum in vitro *growth by cooperation with blood monocytes in an ADCI assay correlated to the naturally acquired protection. This protein is located on the surface of the merozoite stage of* P. falciparum, *and is detectable in all isolates tested. Epidemiological studies demonstrated that peptides derived from the amino acid sequence of MSP-3 contain potent B and T-cell epitopes recognized by a majority of individuals living in endemic areas. Moreover human antibodies either purified on the recombinant protein, or on the synthetic peptide MSP-3b, as well as antibodies raised in mice, were all found to promote parasite killing mediated by monocytes.*

Key words: malaria - *Plasmodium falciparum* - merozoite - MSP-3

As a means to select for molecules capable of inducing protective immunity to malaria, we have chosen to rely on those mechanisms mediating premunition, a naturally-occurring type of protection, which is known to be acquired progressively by individuals repeatedly exposed to *Plasmodium falciparum*. We have shown previously that antibodies from protected adults had no major effect upon parasite invasion and growth in red blood cells, but rather acted indirectly by an antibody-dependent cellular inhibition (ADCI) effect mediated by blood monocytes (Druilhe & Bouharoun-Tayoun 1992). The relevance of ADCI to clinical protection was confirmed by passively transferring immunity in humans by means of IgG (Sabchareon et al. 1991) and by performing *in vitro* correlations with the biological material collected during the passive transfer (Bouharoun-Tayoun et al. 1990). Since this indirect effect of monocytes depends on the ability to raise antibodies which are cytophilic to monocytes, the isotype distribution of antimalarial antibodies is critical. It was shown that non-cytophilic IgG2 and / or IgM classes predominated in the various groups of non-protected subjects, whilst cytophilic IgG3 and also IgG1 classes were the main isotypes produced by protected individuals (Bouharoun-Tayoun & Druilhe 1992).

On the basis of the malaria polypeptides recognized by cytophilic/non-cytophilic antibodies and the results of *in vitro* screening with the ADCI assay, we identified a merozoite surface protein, of 48 kDa molecular weight, and characterized its B and T epitopes.

RESULTS

IDENTIFICATION OF THE 48 KDA PROTEIN

One monoclonal antibody of IgM class which we produced previously (Soulier et al. 1982) was the only one found among many studied, able to block the ADCI effect mediated by protected adult IgG. When used to screen a *P. falciparum* DNA library (Guerin-Marchand et al. 1987), this mAb did not label any of the clones tested. Since the mAb 245 was able to show a positive result with native proteins by IFA and to compete with other antibodies in the recognition of native proteins in bio-assays (ADCI), it was decided to screen a DNA library by competition assays. The recombinant proteins from a subset of 100 clones were used in a

IFA competition assay with the mAb. Six clones were found to be able to block more or less completely the reactivity of mAb 245 to parasite proteins.

ADCI assays and analysis of the isotypic distribution of antimalarial antibodies were performed as described previously (Bouharoun-Tayoun et al. 1992).

The human antibodies affinity-purified on the recombinant product of one of the 6 clones, DG210, were found to strongly inhibit *P. falciparum* growth when allowed to cooperate with monocytes in the ADCI assay (Fig. 1).

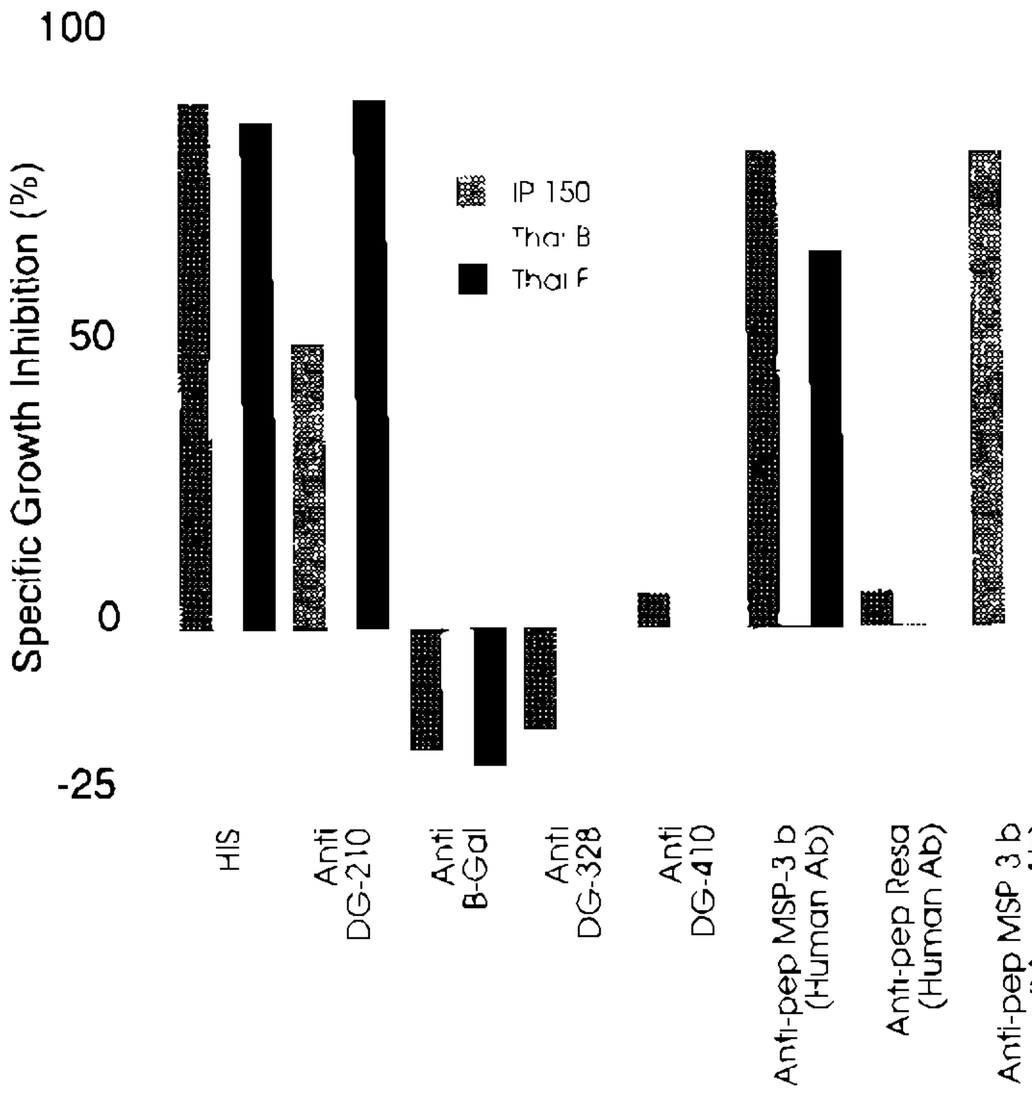

Fig. 1: antibody dependent cellular inhibition assay
Specific growth inhibitory indexes (SGI) were calculated from the results of 96 hours ADCI performed using three different *P. falciparum* strains: one African strain (IP 150: hatched bars) and two Thai strain (Thai B: white bars and Thai F: black bars). HIS: Purified IgG from hyperimmune serum (collected from an African adult), used as positive control. The other antibodies used in these assays were obtained from three differents sources; i) humans antibodies from HIS serum were affinity purified on various recombinant proteins (anti DG 210: antibodies purified on the select clone; anti b(BETA)gal: antibodies purified on control non-recombinant clone from the same DNA library; anti DG328, anti DG414: antibodies purified on control recombinant proteins non related to the clone DG210); ii) human antibodies (from HIS serum) affinity-purified on peptide MSP-3b [Anti-pepMSP-3b (Human Ab.)] or on a RESA peptide [(H-(EENVEHDA)$_2$-(EENV)$_2$-OH)], [Anti-pepRESA (Human Ab.)]; iii) antibodies raised in mice by immunization with the peptide MSP-3b [Anti-pepMSP-3b (mouse Ab.)].

On the other hand, these antibodies recognized, in western blots, a *P. falciparum* protein of 48 kDa. The study of the isotypic distribution of the human antibodies against this antigen revealed that this protein is the target of cytophilic antibodies (IgG1 or IgG3) from protected individuals, and is recognized by non-cytophilic isotypes (IgG2 or/and IgM) in non-protected individuals.

This protein fitted our selection criteria, and was characterized further.

LOCALIZATION OF THE NATIVE PROTEIN IN THE PARASITE

Triton X 114 extraction was performed as described by Bordier (1981). For the phospholipase C treatment we followed the technique of Braun-Breton et al. (1990).

The location of the molecule on the merozoite surface was indicated by the typical grape-like IFA pattern on mature schizonts. Triton X 114 treatment of highly purified *P. falciparum* merozoites was performed and solubilized the 48 kDa protein thus suggesting that it was component of merozoite membranes. Treatment of purified merozoites by *B. cereus* phospholipase C freed the 48 kDa protein, indicating a phosphatidyl-inositol-phosphate anchorage and a surface location. Finally ultrastructural studies using anti DG210 antibodies and gold labelled second antibodies also localized the antigen at the merozoite membrane level. Therefore the antigen was called MSP3: Merozoite Surface Protein 3.

CHARACTERIZATION OF THE DG210 RECOMBINANT CLONE

DG210 belongs to a 1.3 kb gene according to results from Mung bean digestion performed in the conditions described by McCutchans (1984). Results from northern blots indicated that this gene is expressed in blood stages. Sequence analysis of the 192 base pair insert showed that it contained no sequence motif repeated several times in contrast with many other malarial genes in which B-cell epitopes are frequently defined by blocks of repeated polypeptides. No homology was found in DNA and amino-acid sequence with other malarial and non-malarial molecules by screening data banks. The conservation of the gene and of the epitopes defined in the DG210 clone were investigated by PCR, [using the primers: 3': GAAAGGGCAAAAAATGCTTATC;5':ATTTTCCTTAGATATATTTTCC and the following cycle: (94°C, 14'; 50°C, 1'; 72°C, 1')x 40 cycles; (72°C, 5')x 1 cycle] the DNA fragment could be amplified in the

33 isolates tested. The fragment of the gene was present in all these isolates with no evidence of size polymorphism.

EPIDEMIOLOGICAL STUDIES

Three synthetic peptides
MSP-3a:
HERAKNAYQKANQAVLKAKEASSY;
MSP-3b:
AKEASSYDYILGWEFGGGVPEHKKEEN;
MSP-3c:
PEHKKEENMLSHLYVSSKDKENISKENE

were derived from the antigen in order to study the recognition by B and T lymphocytes from malaria exposed individuals. Low levels of antibodies were detected to peptides MSP-3a and MSP-3c. The antibody response to the peptide MSP-3b of 46 individuals living in the hyperendemic area of Donse (Burkina Faso, West Africa) was studied by ELISA in four different age groups. As shown in Fig. 2 the prevalence of responders is high and tend to increase with the time of exposure, starting at 55% in the young children group and reaching a maximal value of 88% in the adults. These results showed that the peptide sequence of MSP-3b defines a major B-cell epitope. The increased prevalence of responses in the 6-10 year age group is surprising. Further investigations will be required to analyze this phenomenon which remains poorly understood.

T cell proliferative responses were analyzed as described previously (Behr et al. 1992), in Ankazobe, a low endemicity village from the highlands of Madagascar (ca 3 infective mosquito bites per year). 13 out of 20 individuals studied showed specific T-cell responses (stimulation index ≥ 2 and Δ cpm ≥ 1000) to at least one of three MSP-3 peptides. Specific lymphoproliferative responses were detected with each of the MSP-3 peptides (Fig. 3), indicating that each of them contain a T-cell antigenic determinant. Out of the twenty individuals tested, 9 showed a positive proliferative index for MSP-3a (45%), 8 for MSP-3b (40%) and 7 for MSP-3c (35%). Healthy controls from the non endemic area of Antananarivo did not respond to any of them. Thus a high prevalence of T-cell specific responses to the molecule identified could be detected, this being signifant particularly when considering the relatively low degree of exposure to malaria of the individuals studied.

BIOLOGICAL ACTIVITY OF ANTIBODIES DIRECTED TO MSP-3

Peptide MSP-3b was used to immunize outbred mice and proved to be immunogenic when injected

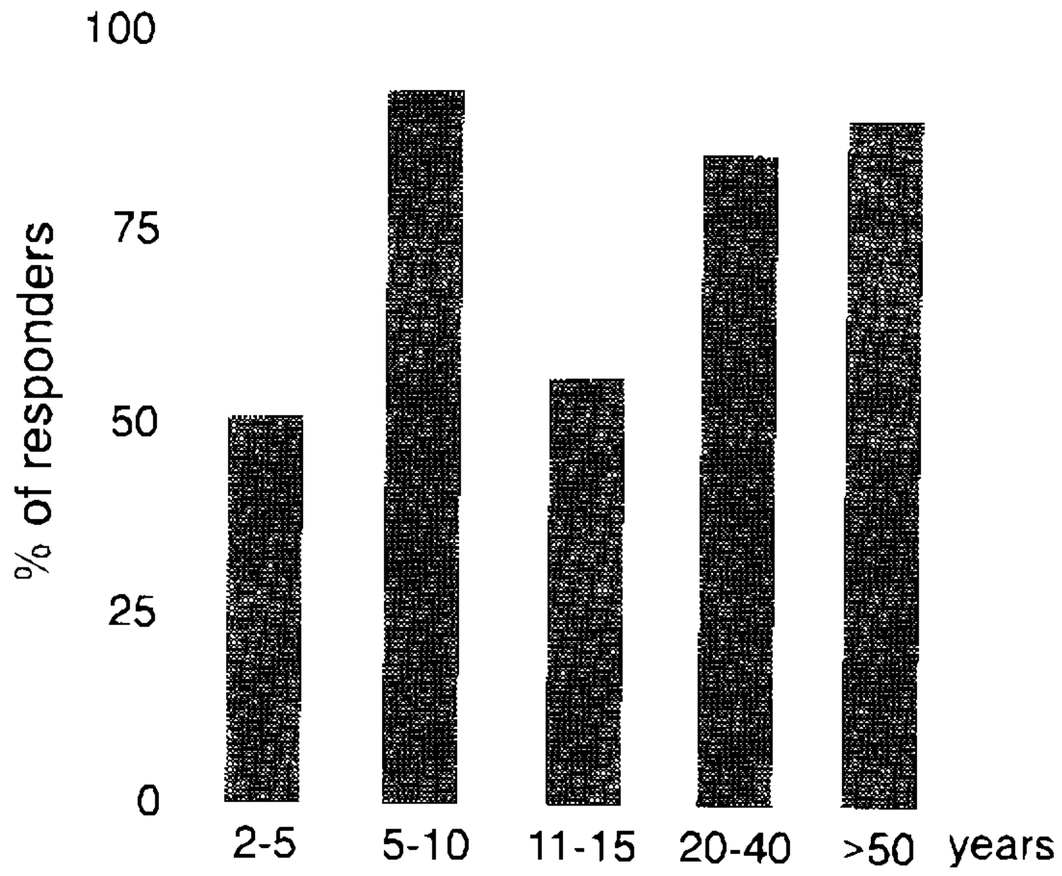

Fig. 2: Prevalence of antibodies to peptide MSP-3b
The prevalence of B-cell responses to the peptide MSP-3b was assessed in an ELISA assay using the sera from 46 individuals living in a malaria hyperendemic area (Donse, Burkina Faso). The prevalence of responders is showed for each age group. Results are expressed as the ratio of the mean OD_{492} of the test sample over the mean OD_{492} + 2 standard deviations of six control sera. A ratio > 2 is scored as a positive reaction.

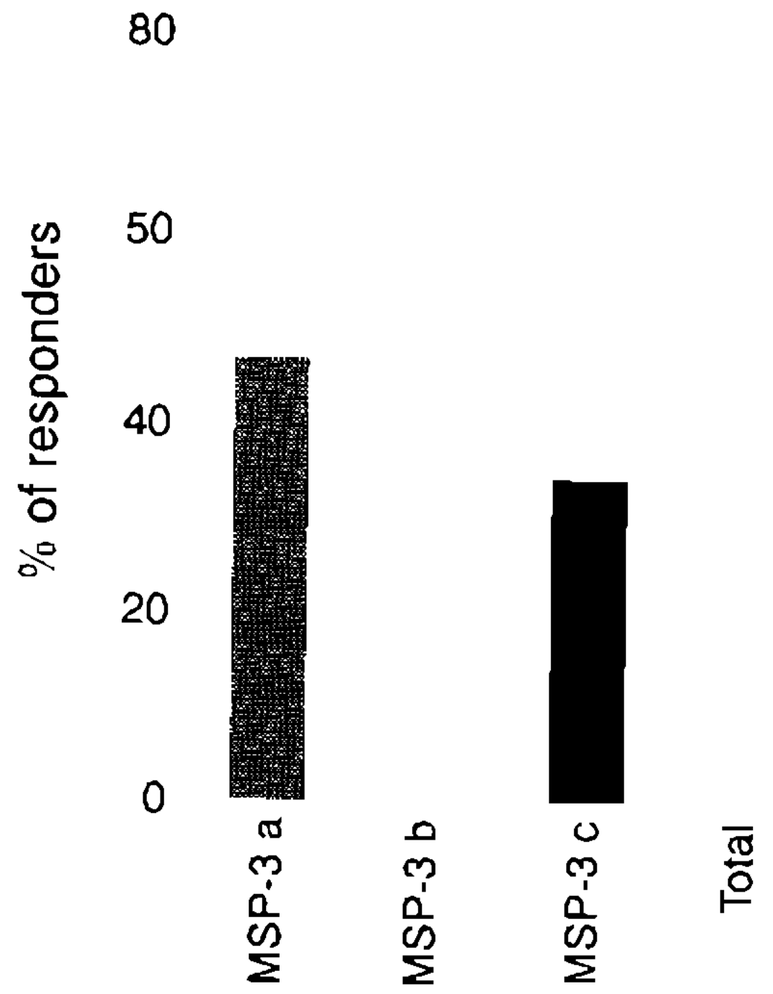

Fig. 3: T-cell responses against MSP-3 peptides
The 3 peptides derived from the sequence of the DG210 clone were tested simultaneously in a T-cell proliferation assay using peripheral blood mononuclear cells from 20 individuals living in Ankazobe (Madagascar). Show here are the percentage of responders to each peptide (MSP-3a; MSP-3b; MSP-3c), as well as the prevalence of individuals showing a positive proliferation index to at least one of them (total).

alone without carrier molecule. Sera from these mice, as well as human antibodies affinity purified on peptide b (Brahimi et al. 1993), were found able to recognize the parasite protein in IFA and western blots.

Human antibodies immuno-purified on peptide MSP-3b, as well as antibodies raised in mice, were studied *in vitro* upon cultured erythrocytic stages. No direct inhibition of parasite growth was found but a strong specific growth inhibition was recorded using these antibodies in the ADCI assay (Fig. 1). Antibodies to RESA peptide, used as control, did not show any ADCI effect (Fig. 1). Taken together our results indicate that human antibodies from protected individuals, immuno-purified on DG210 recombinant protein or on MSP-3b peptide, as well as mice antibodies raised against MSP-3b peptide are able to mediate parasite killing by monocytes.

CONCLUSION

MSP-3 is the first antigen to be screened by means of a mechanism, ADCI, that we found to correlate best to immune protection. The epitopes defined by the DNA fragment identified are target for naturally occurring B and T-cell responses. In view of immunization experiments the availability of synthetic peptide constructs representing B and T cell epitopes of the native protein should provide a way to evaluate at the submolecular level the fine cellular mechanisms that will determine the critical isotypic distribution of the antibody response towards either a blocking or a protective effect.

REFERENCES

Behr C, Sarthou J-L, Rogier C, Trape J.-F, Dat MHQ, Michel J-C, Aribot G, Dieye A, Claverie J-M, Druilhe P, Dubois P 1992. Antibodies and reactive T cells against the malaria heat-shock protein Pf72/Hsp70-1 and derived peptides in individuals continuously exposed to *Plasmodium falciparum*. *J Immunol 149:* 3321-3330.

Bordier, C 1981. Phase separation of integral membranes proteins in Triton X-114 solution. *J Biol Chem 256:* 1604-1607.

Bouharoun-Tayoun H, Attanath P, Sabchaeron A, Chongsuphajaisiddhi T, Druilhe P 1990. Antibodies that protected humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion *in vitro*, but act in cooperation with monocytes. *J Exp Med 172:* 1633-1641.

Bouharoun-Tayoun H, Druilhe P 1992. *P. falciparum* malaria. Evidence for an isotype imbalance which may be responsible for the delayed acquisition of protective immunity. *Infect Immun 60:* 1473-1481.

Brahimi K, Pérignon JL, Bossus M, Gras-Masse H, Druilhe P 1993. Fast immunopurification of small peptides bound to ELISA plates. *J Immunol Meth 162:* 69-75.

Braun-Breton, C, Rosenberry TL, Pereira da Silva LH 1990. Glycolipid anchor of *P. falciparum* surface antigen. *Res Immunol 141:* 743-775.

Druilhe P, Bouharoun-Tayoun H 1992. *Natural Immunities. Res Immunol 142:* 637-643.

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

McCutchan TF, Hansen JL, Dame JB, Mullins JL 1984. Mung Bean nuclease cleaves Plasmodium genomic DNA at sites before and after genes. *Science 225:* 625-627.

Oeuvray C, Bouharoun-Tayoun H, Filgueira M-C, Grass-Masse H, Tartar A, Druilhe P 1993. Characterization of a *Plasmodium falciparum* merozoite surface antigen targeted by defense mechanism developed in immune individuals. *C R Acad Sci Paris 316:* 395-399.

Sabchareon AT, Burnouf D, Ouattara T, Attanath H, Bouharoun-Tayoun P, Chantavanich C, Foucault T, Chongsuphajaisiddhi, Druilhe P 1991. Parasitological and clinical response to immunoglobulin administration in *P. falciparum* malaria. *Am J Trop Med Hyg 45:* 297-308.

Soulier JP, Lee H, Prou O, Raymondjean N, Dubarry M, Druilhe P 1982. Anticorps monoclonaux anti-*Plasmodium falciparum*. *Rev Fanc Trans Imm Hemat 4:* 373-381.