Merozoite Surface Protein-3: A Malaria Protein Inducing Antibodies That Promote *Plasmodium falciparum* Killing by Cooperation With Blood Monocytes

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We have previously found that the acquired protection against malaria implicates a mechanism of defense that relies on the cooperation between cytophilic antibodies and monocytes. Accordingly, an assay of antibody-dependent cellular inhibition (ADCI) of parasite growth was used as a means of selecting for molecules capable of inducing protective immunity to malaria. This allowed us to identify in the sera of clinically protected subjects an antibody specificity that promotes parasite killing mediated by monocytes. This antibody is directed to a novel merozoite surface protein (MSP-3) of a molecular mass of 48 kD. Purified IgG from protected subjects are effective in ADCI and those directed against MSP-3 are predominantly cytophilic. In contrast, in nonprotected individuals, whose antibodies are not effective in ADCI, anti-MSP-3 antibodies are mostly noncytophilic. A region in MSP-3 targetted by antibodies effective in the ADCI assay was identified and its sequence was determined; it contains an epitope not defined by a repetitive structure and does not appear to be polymorphic. Antibodies raised in mice against a peptide containing this epitope, as well as human antibodies immunopurified on this peptide, elicit a strong inhibition of *Plasmodium falciparum* growth in ADCI assay, whereas control antibodies, directed to peptides from other molecules, do not. The correlation between isotypes of antibodies produced against the 48-kD epitopes, clinical protection, and the ability of specific anti-MSP-3 antibodies to block the parasite schizogony in the ADCI assay suggests that this molecule is involved in eliciting protective mechanisms.

PREMUNITION IS a naturally occurring type of protection known to be progressively acquired by individuals repeatedly exposed to *Plasmodium falciparum*.[1-3] It is a nonsterilizing type of immunity found to be mediated mainly by IgG[3,4] that results in maintaining parasite loads to low, subpatent levels. We have previously shown that antibodies from individuals who have reached a state of premunition (malaria protected) had no major direct effect on parasite invasion and growth in red blood cells, but rather acted indirectly by an antibody-dependent cellular inhibition (ADCI) effect mediated by blood monocytes.[5,6] The relevance of ADCI to clinical protection was further supported when experiments of passive transfer of immunity in humans by means of IgG were repeated[4] and when in vitro studies could be performed using the biologic material collected during the in vivo passive transfer; this clearly showed that clinically effective IgG acted by cooperating with blood monocytes.[7] Because this effect of monocytes depends on antibodies that are cytophilic to those cells, the isotype distribution can be expected to be critical. Indeed, we further observed that noncytophilic classes (IgG2 and/or IgM) predominated among the antimalarial antibodies developed by the various groups of nonprotected subjects, whereas cytophilic subclasses (IgG1 and mainly IgG3) were the most abundant isotypes produced by individuals who had reached a state of protection.[8]

These unique features of antimalarial antibodies were exploited in an attempt to identify the antigen(s) targetted by the subset of antibodies responsible for mediating protection in humans. We have relied on both the differential recognition of antigens by various isotypes and the screening of well-defined antibody specificities in the ADCI assay to identify a 48-kD molecule and, at the submolecular level, a polypeptide defining an epitope targetted by antibodies mediating parasite killing.

MATERIALS AND METHODS

Parasite Culture and ADCI Assay

The following parasite strains were used: NF54, Palo Alto (Uganda), two of the Thai isolates studied previously,[7] as well as 10 fresh African isolates collected in primary attack cases in European travellers.

P falciparum blood stage parasites were cultured in RPMI + 10% human serum as described.[9] Synchronization of the parasite asexual blood stage cycle was achieved by several sorbitol treatments[10] and flotations on plasmagel.[11]

Merozoites were purified using negatively charged Versapor filters (Gelman Sciences, Ann Arbor, MI) as described by Mrema et al.[12]

The ADCI assays were performed as previously described.[7] Briefly, adherent mononuclear cells from healthy blood donors[13] were distributed in a 96-well plate (Nunclon; Nunc, Copenhagen, Denmark) and cocultured with *P falciparum* synchronized schizonts-infected red blood cells (RBC) added at a ratio of 200 RBC per monocyte. Purified IgG were used at a final concentration of 2 mg/mL, which corresponds to about 10% of their initial concentration in the donor's serum. Affinity-purified antibodies were added at a final concentration of 5 μg/mL (10% vol/vol). The parasitemia after 96 hours of culture was estimated in thin smears from each well by microscopic examination of more than 10,000 erythrocytes. Control wells consisted of (a) parasites alone; (b) parasites and control IgG (NIgG from healthy blood donors); (c) parasites and monocytes; (d) parasites and antibodies without monocytes; and (e) parasites, control IgG, and monocytes.

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To take into account the possible inhibiting or facilitating effect induced by IgG or cells alone, a specific growth inhibition index (SGI) taking into account controls b, d, and e was calculated as SGI = 1 − ([percentage of parasitemia with monocyte and test IgG/percentage of parasitemia with test IgG]/[percentage of parasitemia with monocyte and NIgG/percentage of parasitemia with NIgG]) × 100, and used to express results for ADCI.

Competition ADCI assays were performed as described previously[8] with IgGs from hyperimmune individuals together with IgG from first attack patients (PA) or with the mouse monoclonal antibody (MoAb) 2-45.

Antigen Preparations

Blood stage antigens. *P falciparum* synchronized blood stage parasites (either rings, schizonts, or merozoites) were washed in RPMI and the antigens were directly extracted by boiling in Laemmli sample buffer for 5 minutes. In some experiments, purified merozoites were divided into three equal batches at each purification round. One part was kept as total extract. The second part was submitted to Triton X-114 extraction (Sigma, St Louis, MO).[14] Both aqueous and detergent phases were kept at −20°C. The third part was incubated with 5 U/mL *Bacillus cereus* phospholipase C (Boehringer, Manheim, Germany).[15] The resulting supernatant and pellet phases were frozen. The various fractions made from approximately 10^8 merozoites each were extracted with Laemmli sample buffer. All antigen preparations were run on 7.5% or 10% sodium dodecyl sulfate (SDS) acrylamide gel and transferred onto nitrocellulose filters (BA 85; Schleicher & Schuell Inc, Dassel, Germany).

Recombinant antigens. *P falciparum* genomic DNA (clone T9/96) was purified from saponin-lysed cultures using standard techniques.[16] After recloning of DG-210 into the pGEX-2a expression vector,[17] gluthatione-S-transferase fusion proteins (GST) were purified by affinity chromatography on gluthatione-agarose (Sigma), as described.[17]

Synthetic antigens. Three overlapping synthetic peptides (MSP-3a, HERAKNAYQKANQAVLKAKEASSY; MSP-3b, AKEASSYDYILGWEFGGGVPEHKKEEN; and MSP-3c, PEHKKEENMLSHLYVSSKDKENISKENE) derived from the amino acid sequence encoded by DG-210 were synthetized by the method of Merrifield with acetyl and carboxamide N and C termini, respectively. They were purified to greater than 95% by high performance liquid chromatography (HPLC) and characterized by amino acid analysis and by mass spectrometry. The RESA peptide [H-(EENVEHDA)$_2$-(EENV)$_2$-OH] was purchased from Bachem (Bubendorf, Switzerland).

Antibodies

Human antibodies. The pool of human IgG (PIAG) previously found to confer passive protection upon transfer[4] had been purified from 180 pooled sera of protected hyperimmune African subjects from the Ivory Coast as described previously.[7] Eight individual human sera were also used: a first group of three hyperimmune sera (HIS) from the Ivory Coast (same group of donors as for PIAG preparation), the second from five European travellers recovering from a *P falciparum* PA (or first attack). These PA cases are subsequently referred to as sera from nonprotected subjects because they are known to be susceptible to further malarial infections, whereas sera from African adults are referred to as sera from protected subjects because they had reached a state of premunition, ie, of clinical protection to malaria, as further confirmed by an experiment of passive transfer of IgG.[4] For all individual sera, the IgG were prepared on a GF05-trisacryl column (IBF, Villeneuve-la-Garenne, France) followed by a diethylaminoethyl (DEAE)-trisacryl column (IBF, Villeneuve-la-Garenne, France) and extensively dialyzed against phosphate-buffered saline (PBS), pH 7.4.

Defined antibodies were prepared by immunopurification from human hyperimmune serum on the recombinant proteins DG-210, DG-328, and DG-414 or on synthetic peptides MSP-3a, MSP-3b, MSP-3c, or RESA as described.[18] The final concentration was 50 μg/mL. Results of immunopurification were assessed for specificity and purity by Western blots of recombinant and native parasite proteins extracts and by enzyme-linked immunosorbent assay (ELISA) in the case of synthetic peptides.[18]

Mouse antibodies. Anti–DG-210-GST and antipeptide MSP-3b antibodies were raised in outbred mice by the injection of 50 μg of either purified recombinant protein or peptide MSP-3b on day 0 in complete Freund's adjuvant, and on days 21, 42, and 63 with incomplete Freund's adjuvant. Sera were collected on day 80.

A mouse MoAb of IgM class, MoAb 2-45, was one that we produced previously.[19]

Rabbit sera. Sera from rabbits immunized with the merozoite surface antigen-1 (MSA-1) or with MSA-2 were kindly supplied by Dr Jana McBride (University of Edinburgh, Edinburgh, UK).

Antibody Assays

The immunoblotting assays were performed as previously described[7] with human sera, mouse sera, or rabbit sera (diluted 1/100) or undiluted affinity-purified human antibodies and shown using alkaline-phosphatase–conjugated goat antibodies (diluted 1/7,500; Promega, Madison, WI).

Isotype-specific Western blots. Isotype-specific Western blots were performed using mouse MoAbs directed to human IgGs, as described.[8] NL16 (anti-IgG1), ZG4 (anti-IgG3), and GB7B (anti-IgG4) were purchased from Merck (Nogent-sur-Marne, France). HP 6002 (anti-IgG2) and MB11 (anti-IgM) were purchased from Sigma.

Immunofluorescence Assay (IFA)

IFAs were performed on air-dried, acetone-fixed, thin smears of *P falciparum* asexual blood stages as described.[20]

For IFA competition assays, fusion proteins from 100 DNA clones and from 10 control clones (concentrated to approximatively similar amounts as evaluated on Western blots shown by anti–β-galactosidase antibodies) were incubated for 2 hours with the MoAb 2-45 diluted 1/250. The competition between each soluble recombinant protein and native proteins in the parasite was assessed by running these samples in IFA.

Immunoelectron Microscopy

Immunoelectron microscopy was performed using extracellular merozoites of *P falciparum,* prepared as described,[21] embedded in LR White resin (Polyscience, Inc, Warrington, PA). Sections were blocked in PBS, 1% bovine serum albumin, and 0.01% Tween 20, and incubated in anti–MSP-3 mouse serum or control sera (preimmunization samples and sera raised against control peptides belonging to antigens expressed during the liver stage) shown with goat antimouse IgG conjugated to 15-nm gold particles. The sections were fixed with 2% glutaraldehyde, stained with 2% uranyl acetate and lead citrate, and examined by a JEOL 100CX electron microscope (JEOL, Japan).

DNA Studies

DNA sequence. The 192-bp *P falciparum* DG-210 fragment was excised by digestion with *Eco*RI, cloned in both orientations into the *Eco*RI site of the single-stranded phage m13mp8, and sequenced by the chain termination method.[22]

Polymerase chain reaction (PCR) analysis. The DNA from each

isolate was extracted by a classical phenol-chloroform procedure. We produced one set of oligonucleotides primers from the DG-210 DNA (oligo 1, GAAAGGGCAAAAAATGCTTATC; oligo 2, ATTTTCCTTAGATATATTTTCC). They were used to amplify, by PCR, DNA from various laboratory and field *P falciparum* isolates in a Hybaid thermal cycle (Hook and Tucker Instruments Ltd, UK) using the following cycle schedule: 94°C for 14 minutes, 50°C for 1 minute, and 72°C for 1 minute for 40 cycles and 72°C for 5 minutes for one cycle.

Northern blot and Mung Bean digestion analysis. The DG-210 insert amplified by PCR was size fractionated on a 4% low melting NuSieve GTG agarose gel (FMC, Rockland, ME) and radiolabeled (Megaprime; Amersham, Les Ullis, France). This probe was hybridized under high stringency conditions (2× SSC, 65°C) with total *P falciparum* RNA extracted from NF 54 cultured parasites as described in Bonnefoy et al[23] or with total *P falciparum* DNA that had been digested by the Mung Bean nuclease as previously described by McCutchan et al[24] in 45% formamide, transferred to Hybond N according to Amersham protocol. A circumsporozoite gene probe radiolabeled in the same way, which showed a 1.3-kb fragment, was used as control of the output of the digestion by Mung Bean nuclease.

RESULTS

Identification of a 48-kD Protein

The identification of a 48-kD blood stage protein as a target of protective antibodies relied on two independent studies that finally proved to be convergent, ie, (1) the isotypic distribution of antimalarial antibodies in protected and nonprotected subjects, and (2) the characterization of the MoAb 2-45. The two approaches differed in their principle and the second one may appear somewhat complex and indirect. However, both relied on the results from ADCI assays as the final and critical readout.

Firstly, when studying the isotype distribution of antimalarial antibodies from individuals with various states of immunity to malaria, it was found that many of the exposed though nonprotected subjects had, as previously described,[8] an imbalance in favor of noncytophilic Ig classes such as IgG2 or IgM (Fig 1). In some of these individuals, the majority of IgG2 or IgM antibodies specific for malarial antigens was found directed only towards a single apparent polypeptide, frequently a 48-kD polypeptide, often appearing as a closely spaced doublet (Fig 1, First Attack) or a polypeptide in the 90- to 100-kD range,[8] thus drawing attention to these molecules. In contrast, the same 48-kD protein doublet was better recognized by the cytophilic subclasses, IgG1 and IgG3, in sera from individuals who had acquired a state of clinical resistance to the disease (African adults; Fig 1, PIAG).

The sera mentioned above having a disproportionately high amount of IgG2 directed principally to the 48-kD doublet were tested in competition ADCI assays. The IgG purified from these sera partially reversed the ADCI effect obtained with IgG from protected Africans[7] (Fig 2c, PIAG + First Attack). This led us to speculate (1) that noncytophilic IgG2 directed to the 48-kD doublet can compete with cytophilic IgG1 and IgG3 directed to this polypeptide and (2) that the latter antibodies play a major role in the ADCI effect.

Secondly, an MoAb of IgM class, MoAb 2-45, which we produced previously,[19] was the only one found among over

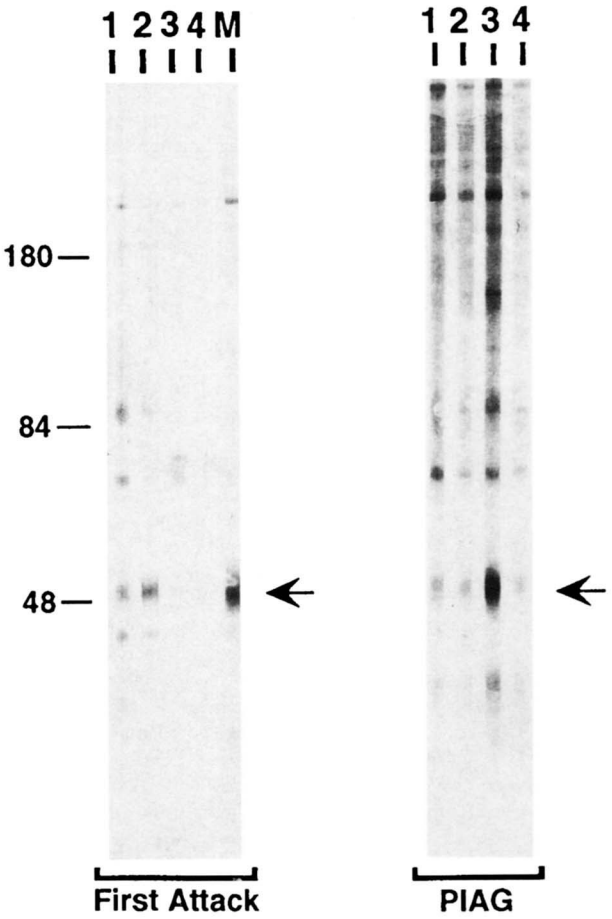

Fig 1. IgG subclass distribution (parasite extract). A Western blot of *P falciparum* late schizont antigens extract (strain NF 54) was incubated with a 1/100 dilution of PIAG, a pool of IgG from 180 hyperimmune African adults,[4] or with First Attack, a serum from a European recovering from a primary attack, and shown by MoAb specific to human IgG1, IgG2, IgG3, IgG4, and IgM. The arrows indicate the MSP-3 polypeptide.

a large series studied (among which several were of IgM class and serve as controls) that was able to reverse the ADCI effect mediated by protected adult IgG (Fig 2B and C, PIAG + MoAb 2-45). We thus suspected that this MoAb may recognize a major target of protective antibodies. This MoAb was positive at high titer by IFA on asexual blood stage parasites. Unfortunately, in Western blots it reacted very weakly to numerous polypeptides, so that it was impossible to identify its target. It did not recognize any of the 1,200 genomic expression clones from a nonamplified λgt11 DNA library.[25] We surmised that MoAb 2-45 could not recognize its target bound to nitrocellulose. We thus decided to screen recombinant clones by means of an assay using soluble recombinant proteins, based on the idea that the epitope in solution may readily bind to the specific antibody paratope. A subset of 100 DNA clones expressing malarial proteins consistently recognized by sera from eight protected African adults was isolated, and the corresponding recombinant protein lysates were used in IFA competition assays

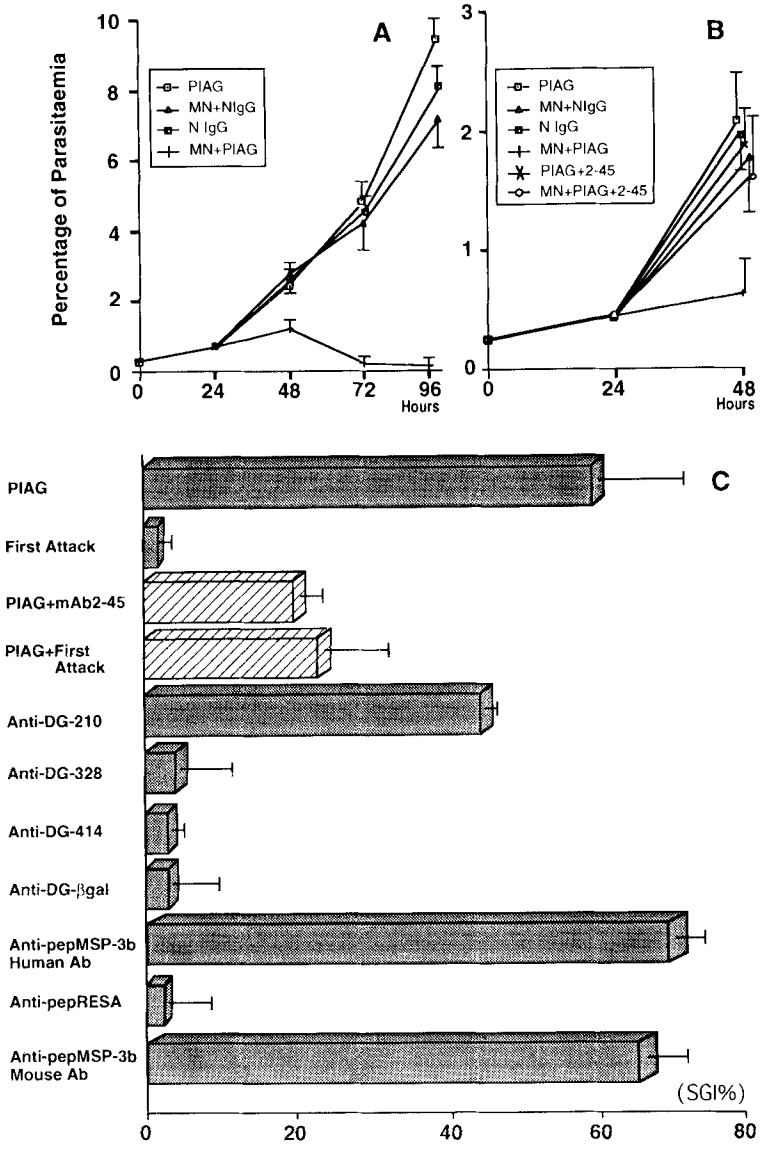

Fig 2. ADCI (A) and (B) show the data obtained in individual ADCI experiments that are used to calculate the SGI. Most results are summarized in (C), in which the means ± SD of several experiments are shown. In (A) is shown the course of parasitemia in a typical ADCI assay performed over 4 days. The inhibitory effect of a pool of PIAG or of NIgG was assessed on a 96-hour *P falciparum* culture in the presence or absence of normal human monocytes (MN). In (B) is shown an ADCI assay of only 48 hours in which a competition between the PIAG and the MoAb 2-45 was tested. In (C) are shown the SGI indexes calculated from results of 96-hour ADCI assays performed using PIAG alone (positive control); First Attack (primary attack; shown is the mean SGI obtained in ADCI assays performed with IgG from 5 primary attack cases), PIAG+mAb2-45 (an ADCI competition assay between PIAG and MoAb 2-45), PIAG + First Attack (a competition between PIAG and IgG from 1 first attack case*), Anti-DG-210 (a direct ADCI assay using human antibodies affinity-purified on the recombinant protein DG-210), DG-328 and DG-414 (control antibodies purified on various recombinant proteins, nonrelated to DG-210; which correspond to 80/110-kD and 56/110-kD proteins, respectively, on Western blot), pepMSP-3b (human antibodies; human antibodies affinity-purified on the synthetic peptide MSP-3b), RESA (human antibodies purified on an RESA-peptide), and pepMSP-3b (mouse antibodies; antibodies raised in mice by immunization against MSP-3b). (■) Direct ADCI assays; (▨) ADCI competition assays. *Competition assays performed with several PA cases having an imbalance, either to most malarial polypeptides or to the 48-kD protein doublet, gave a reversal of ADCI of the same order of magnitude; only one result is shown. Similar results showing an inhibition of growth with anti-MSP-3 antibodies were obtained upon two strains from Thailand, ThB, and ThC[7] and are not shown.

with the MoAb 2-45. One of them totally abolished the binding of the MoAb to *P falciparum* blood stages in IFA. The corresponding clone, called DG-210, thus apparently encoded for the target of MoAb 2-45. Antibodies from African immune serum immunopurified on this clone recognized a doublet at 48 kD in Western blots, which appeared to be exactly the same as that involved in the isotype imbalance in nonprotected subjects described above (Fig 3A, lane 2); it differs from MSA-2, a merozoite surface antigen[26,27] that has a higher relative molecular weight (Mr) (Fig 3A, lane 4).

Antibodies to a Recombinant Polypeptide, Part of the 48-kD Protein, Mediate Parasite Killing

The identified DG-210 recombinant protein was studied according to the same two criteria as those used for selection, namely ADCI and isotype distribution. Anti–DG-210 antibodies purified from IgG of protected subjects strongly inhibited *P falciparum* growth when allowed to cooperate with monocytes (Fig 2C, anti–DG-210), whereas these antibodies had no direct effect on merozoite invasion and intraerythrocytic growth (ie, without monocytes; data not shown). These immunopurified antibodies recognized only the 48-kD protein in parasite extracts (data not shown). Control antibodies prepared in the same condition from several control λgt 11 recombinant proteins, and antibodies affinity purified on a RESA peptide, were not inhibitory either in direct inhibition and ADCI assays (Fig 2C, anti–DG-328, 414, βGal, and anti-RESA). Results were consistent in separate experiments on three different isolates (NF54: Fig 2, and two Thai *P falciparum* isolates; data not shown).

The identification of DG-210 as part of the gene coding for the 48-kD parasite protein doublet was also supported by studying the isotype distribution of antibodies directed to the recombinant protein from clone DG-210. IgG2 or IgM were found to be the most abundant isotypes in anti–DG-

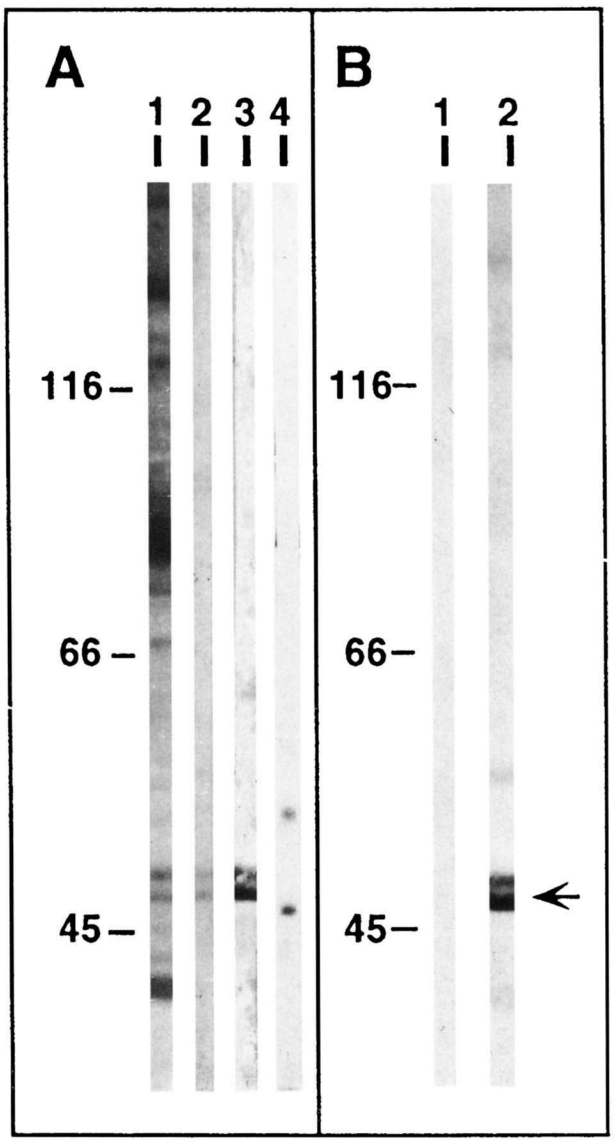

Fig 3. (A) Identification of the MSP-3 protein. Western blot of *P falciparum* blood stage schizont antigens (strain NF 54), assayed using total hyperimmune African serum (PIAG) diluted 1/100 (lane 1); human antibodies affinity-purified on peptide MSP-3b (lane 2); mouse anti–DG-210-GST serum, which in ELISA assays reacts only with peptide MSP-3b (1/100; lane 3); or rabbit anti–MSA-2 serum, which labels the 54-kD protein and its lower Mr processed fragments (1/25; lane 4). (B) Stage specificity of MSP-3. Western blot of *P falciparum* ring stage (strain NF 54; lane 1) and late schizont antigens (lane 2) labeled with human antibodies affinity purified on MSP-3b as above.

210 antibodies from primary attack cases, whereas IgG3 and IgG1 were more abundant in protected African sera (Fig 4). Thus, the epitopes contained in the identified recombinant protein lead the characteristics of the native protein we were looking for, ie, they had induced in vivo antibodies that were predominantly of noncytophilic isotypes in nonprotected subjects, although they were, in contrast, mainly cytophilic in protected individuals and able to promote an ADCI inhibitory effect.

The 48-kD Molecule Is Located on the Merozoite Surface

Previous experiments performed using the ADCI assay had shown that the critical antigens responsible for triggering antibody-armed monocytes are expressed on the surface of merozoites, rather than on the surface of infected RBCs (Bouharoun-Tayoun and Druilhe, manuscript submitted). The stage specificity and precise location of the 48-kD antigen were investigated by several means. IFA was negative with *P falciparum* sporozoites and liver stages. Fully mature asexual blood stage schizonts gave the typical grape-like location of fluorescence around individual merozoites, which is obtained using antibodies directed to other merozoite surface molecules such as MSA-1 and MSA-2 (Fig 5A); in contrast, rings, trophozoites, and submature schizonts were negative. Western blots confirmed these findings by showing that the protein was present in merozoites and in late schizont extracts but not in rings and trophozoites (Fig 3B, lanes 1 and 2 and Fig 6, lane 1). Triton X-114 solubilization of

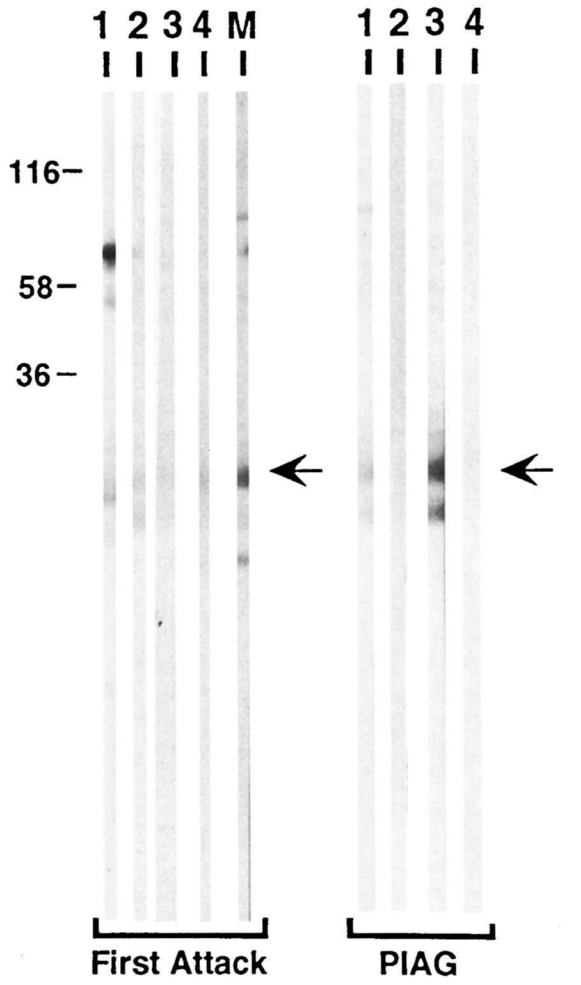

Fig 4. IgG subclass distribution (recombinant protein). A Western blot of purified DG-210-GST recombinant protein was shown as described in the legend of Fig 1. The arrows indicate the recombinant DG-210-GST (the lower Mr bands are degradation products resulting from purification of the recombinant protein).

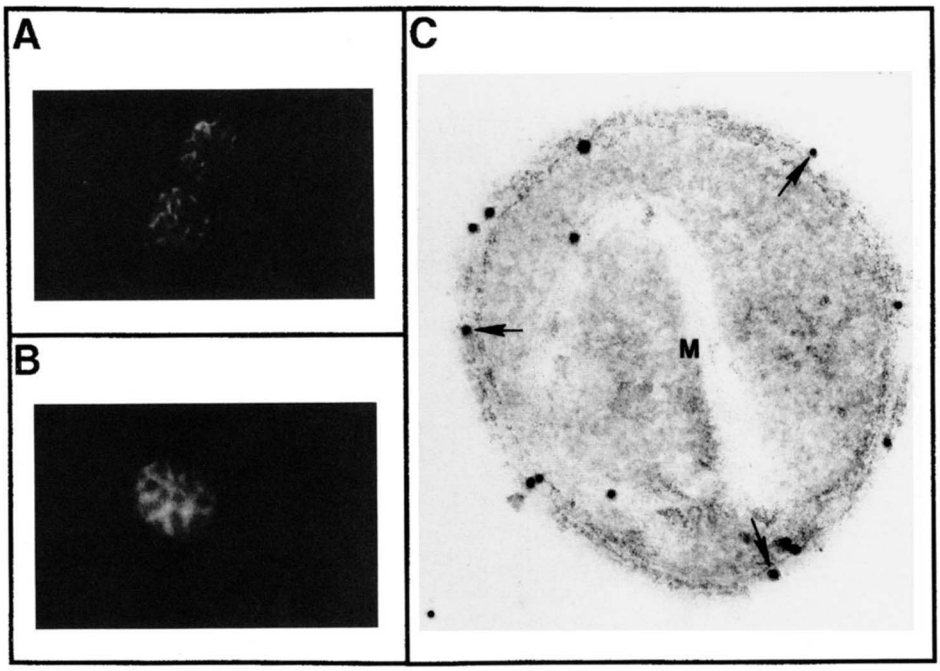

Fig 5. Localization of DG-210/MSP-3 by IFA (A and B) and immunogold labeling (C). Typical grape-like immunofluorescence pattern on *P falciparum* mature asexual blood stages using (A) anti–DG-210-GST mouse serum diluted 1/100 or (B) MoAb 2-45 diluted 1/250. The pattern was the same when tested on several cultured strains, including NF54, Palo Alto, as well as on 10 fresh isolates grown up to late schizont stage in a 35-hour culture. (C) Immunoelectron microscopic localization (Fig 6C) of MSP-3 on *P falciparum* merozoite (M) shown by DG-210-GST mouse serum and antimouse antibodies that were immunogold labeled (original magnification × 65,000).

purified *P falciparum* merozoites extracted approximately 50% of the 48-kD doublet (Fig 6, lane 4), ie, about the same amount as for MSA-1 and MSA-2 (Fig 6, lanes 5 and 6). This data suggested that the 48-kD protein doublet was a component of merozoite membranes. Incidentally, both the polypeptides from the doublet were released by Triton extraction, arguing against the hypothesis that the higher Mr protein could be the cytoplasmic precursor for the lower Mr surface protein and suggesting that the two proteins differ by limited posttranslational modifications. Among surface proteins from various organisms, several have been found to be anchored by a phosphatidyl-inositol-phosphate moiety in cell membranes (eg, surface proteins in trypanosomes, the malarial merozoite proteins, MSA-1 and MSA-2,[26,27] Fc receptors, surface IgE, etc).[28-30] This is likely the case also for the 48-kD antigen that was freed after treatment with *Bacillus cereus* phospholipase C, to the same extent as was MSA-1, whereas MSA-2 was only partially freed (Fig 6, lanes 10 through 15), probably reflecting differences in the lipid content of these anchorage sequences.[28-30]

The location of the antigen was studied at the ultrastructural level by immunogold labeling. The labeling was localized around extracellular merozoites, apparently associated with the outer membrane (Fig 5). Labeling of the surface was much more pronounced than that seen in internal structures. Results from electron microscopy studies confirm the expression of DG-210 in merozoites and are in support of its surface location. Therefore, we named the antigen MSP-3 (merozoite surface protein-3).

Characterization of the Epitopes Targetted by Protective Antibodies

DG-210 is part of a 1.4-kb gene according to the results from Mung Bean digestion performed in the conditions described by McCutchan et al[24] (Fig 7C, lane 1) that showed the full circumsporozoite gene size in the same experiment (Fig 7C, lane 2). DG-210 is expressed in blood stages as shown by results from Northern blot analysis that showed a 1.3-kb transcript (Fig 7C). Both sizes are in agreement with the size of the protein. Only a single clone corresponding to this gene could be found among the T9.96 clones studied because none of the 1,200 antigen-producing clones cross-hybridized nor cross-reacted immunologically with DG-210 (data not shown). DNA studies were restricted to the sequence analysis of the DG-210 clone because this clone contained the critical epitopes of interest. This sequence contains no repetitive motif, in contrast with many other malarial antigens whose dominant B-cell epitopes are frequently defined by repeated blocks of polypeptides (Fig 7A). No homology exceeding 35% in DNA and amino acid sequence with other malarial genes and nonmalarial molecules was found by screening the National Biomedical Research Foundation (NBRF) and Los Alamos data banks.

The conservation of the gene fragment and of the epitopes defined by the DG-210 clone was investigated by several means. Using the oligonucleotides shown in Fig 7A, the DNA fragment could be amplified by PCR in 2 African cultured strains, in 4 Thai isolates, and in 29 African isolates from a Senegalese village (Fig 7B), yielding a single amplified product the identity of which was ascertained by further hybridization (data not shown). The gene was present in all with no evidence of size polymorphism. Similarly, at the protein level, screening of Western blots prepared from six African and Thai isolates with DG-210 affinity-purified antibodies showed the protein doublet in all, with no variation in molecular weight from isolate to isolate. Ten additional Congolese isolates screened by IFA were all positive and all parasites seen in each isolate were labeled by the antibodies.

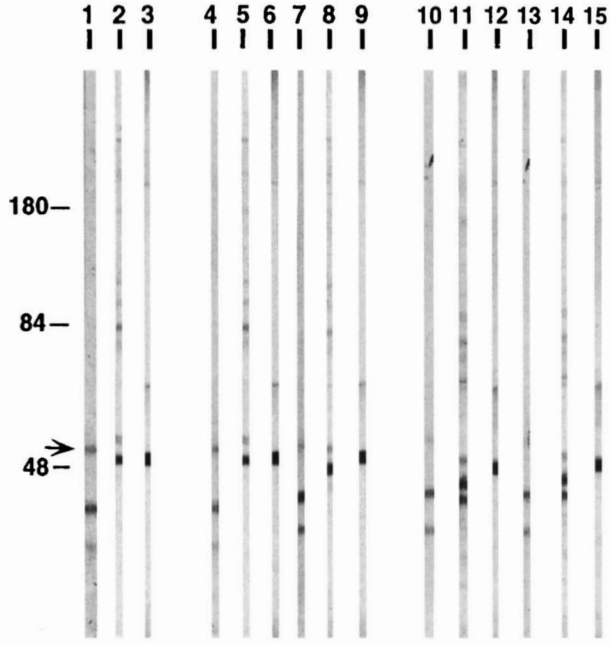

Fig 6. Merozoite membrane location of MSP-3. Shown is the reactivity in a Western blot of a serum anti–DG-210-GST raised in mice (lanes 1, 4, 7, 10, and 13) or a mixture of rabbit sera raised against MSA-1 (lanes 2, 5, 8, 11, and 14) and MSA-2 (lanes 3, 6, 9, 12, and 15) with antigens of the following: lanes 1 through 3, a merozoite preparation extracted in Laemmli sample buffer; lanes 4 through 9, merozoites treated with Triton X-114 (lanes 4, 5, and 6: detergent phase; lanes 7, 8, and 9: aqueous phase); or lanes 10 through 15, merozoites digested by PIPLC (lanes 10, 11, and 12: PIPLC unsoluble phase; lanes 13, 14, and 15: PIPLC soluble phase). Note that, in merozoite extracts, the 48-kD fragment still appears as a doublet, but that two degradation products at 41 and 38 kD are generated in addition. MSA-2 is visible at 56 kD and for MSA-1 the main processed fragments at 83, 43, and 33 kD are detected. The three merozoite proteins are solubilized to approximately the same extent by Triton X-114 treatment. After PIPLC treatment, approximately the same proportion of the 48-kD molecule and its processed fragments are freed as for MSA-1, whereas MSA-2 is only partially freed.

Thus, we could not find any indication of antigenic polymorphism in the region of the molecule bearing the B-cell epitopes targetted by protective antibodies, and no indication of size polymorphism of the protein. These results are in agreement with results from ADCI competition assays in which noncytophilic antibodies from primary attack (first attack) cases competed efficiently with cytophilic ones from protected African adults (Fig 2C). Because first attack cases had raised antibodies in response to a single isolate only briefly harbored before being eradicated by drug treatment, and pooled sera were used from African adults having encountered a large number of highly polymorphic isolates, the efficient competition observed suggests that the target epitopes of protective antibodies are contained in nonpolymorphic regions.

Biologic Effect of Antibodies Directed to Peptide MSP-3b

Three synthetic peptides (MSP-3a, MSP-3b, and MSP-3c, shown in Fig 7A) were derived from the antigen. Antibodies directed to peptide MSP-3b, but not to MSP-3a and MSP-3c, were detected by ELISA in 8 of 10 hyperimmune sera studied (data not shown). Peptide MSP-3b was used to immunize outbred mice and proved to be immunogenic when injected alone without carrier molecule. Both the sera from these mice as well as human antibodies immunopurified on peptide MSP-3b were found to be able to recognize the parasite protein in IFA and Western blots (Fig 3A, lanes 2 and 3), demonstrating that the peptide mimicked properly the epitopes defined by the native protein. Incidently, this confirms that each part of the doublet contains the epitope defined by peptide MSP-3b. Finally, human antibodies immunopurified on peptide MSP-3b, as well as antibodies raised in mice, were studied in vitro on cultured erythrocytic stages. No direct inhibition of parasite growth was found, whereas in the ADCI assay, a strong specific growth inhibition was recorded using those antibodies (Fig 2C, anti–pepMSP-3b, human and mouse antibodies) that recognize only the 48-kD doublet protein (Fig 3A, lanes 2 and 3), whereas antibodies to control peptides did not. This extends the initial observation made with the DG-210 recombinant protein by showing that antibodies corresponding to an epitope(s) defined by the synthetic peptide can mediate parasite killing by monocytes. Antibodies induced in mice by the small sequence found to contain the critical epitope(s) reproduced in vitro the effect obtained with total IgG from protected subjects.

DISCUSSION

We have previously found that sera of subjects clinically immune to malaria contain protective antibodies able to promote the in vitro inhibition of parasite growth by monocytes. In this study, we report the identification in these sera of an antibody specificity with potent in vitro ADCI effect and the characterization of its target epitope part of a novel 48-kD merozoite component.

The particular attention that we have given to the isotype distribution of various antimalarial antibodies was the result of our previous finding that the ADCI mechanism was a critical component of protective responses to asexual blood stages of malaria. Our attention was focused on a 48-kD molecule because of the distinct balance of isotypes found in nonprotected versus protected subjects. The validity of this approach and the interest of this molecule was in turn supported by the strong inhibitory effect obtained in ADCI assays using antibodies specific to the identified antigen. Previously, merozoite surface components have been the object of extensive studies because it was expected that their surface location made them proper targets for defense mechanisms. In the present study, although we knew that the merozoite stage bears the antigen(s) able to trigger ADCI (Bouharoun-Tayoun and Druilhe, manuscript submitted), the approach used was directed at any molecule that could be the object of an isotype imbalance. It is noteworthy that this finally led to the identification of a novel antigen that also appears to be a component of the merozoite surface.

Evidence to support our assertion that the DG-210 clone corresponds to the 48-kD protein doublet comes from the following. Human antibodies purified either on the peptide

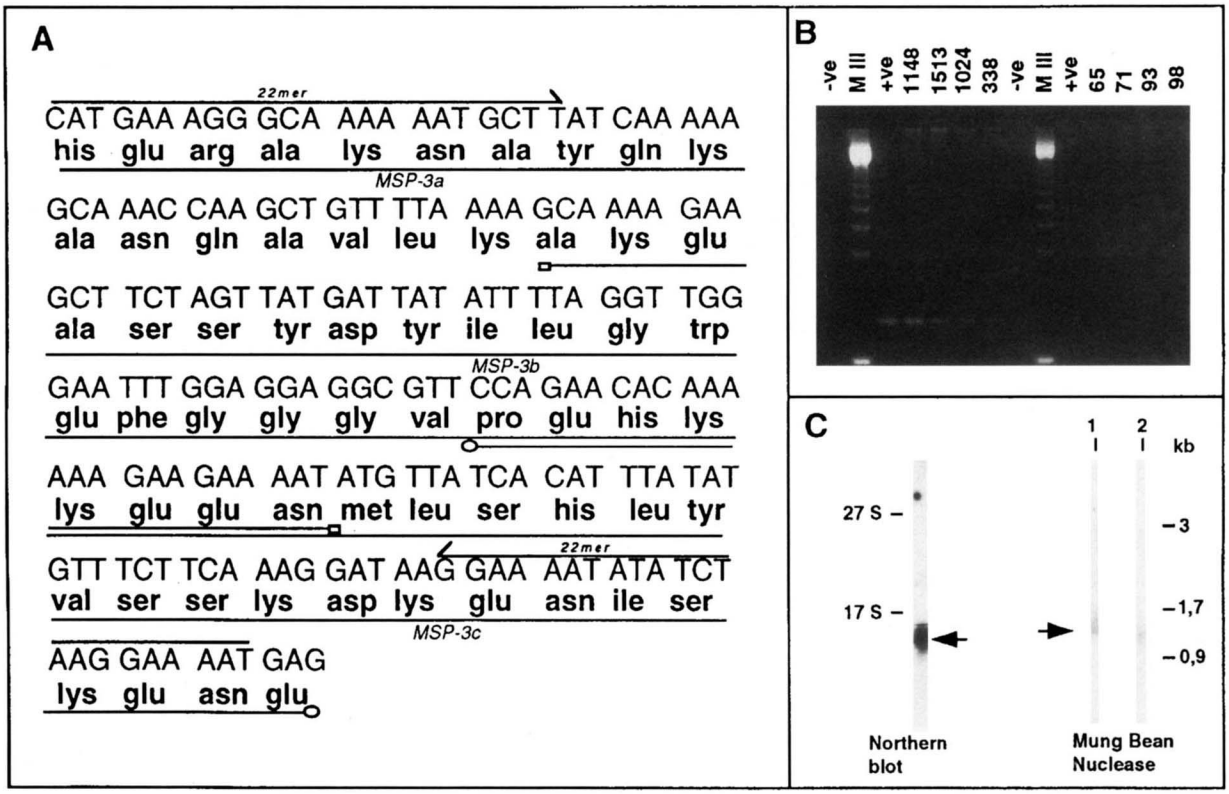

Fig 7. (A) DNA and amino acid sequence. Shown are the three overlapping synthetic peptides (MSP-3a [■ — ■]; MSP-3b [□ — □]; and MSP-3c [○ — ○]) derived from the amino acid sequence and the set of oligonucleotide primers used in PCR (→). (B) PCR analysis. Typical PCR amplification obtained with various field isolates using the two primers described above. (Samples no. 71, 93, and 98 are negative controls.) (C) Northern blot and Mung Bean digestion analysis. Shown is total *P falciparum* RNA from late schizont stage (Northern blot) and total *P falciparum* DNA digested by the Mung Bean nuclease, both labeled with a DG-210 probe (lane 1). Mung Bean nuclease digestion products were probed with a labeled circumsporozoite gene probe as control (lane 2).

or on the recombinant protein, as well as monospecific sera raised in mice, recognize a doublet protein in which both molecular weights are identical to those shown by the discriminating IgG2 (and no other protein is seen). A similar isotype distribution was found against the native parasite protein and recombinant DG-210 material in the same individuals. Finally, Northern blot analysis and Mung Bean data are both in agreement with the mature polypeptide size of the 48-kD doublet.

Over the past 2 decades, several approaches have been taken in an attempt to identify malarial antigens that could be used to vaccinate exposed populations.[31] This research has led to the characterization of a wide range of parasite molecules, many of which were found to be polymorphic from one isolate to the next.[32,33] In contrast, the amount of biologic information on interactions between plasmodia and humans that has been gathered is relatively limited because of the highly restricted host specificity of the parasite. In our opinion, a major limitation to the development of a malaria vaccine has been the lack of a reliable assay that accurately reflects the specific immunity developed by humans. Such an assay would permit direct screening of molecules that are targetted by protective immune responses.

MSP-3 is the first antigen to be screened by means of this mechanism, ADCI, that we have found to closely correlate to immune protection.[7] The epitopes defined by the DNA recombinant protein, particularly those defined by the 27 amino acid MSP-3b peptide, are a target for naturally occurring antibodies. Specific antibodies extracted from the sera of protected subjects are effective in inducing parasite killing mediated by monocytes. These results obviously do not rule out the possible involvement of other molecules able to induce protective antibodies, as well as of other effector mechanisms. However, the correlation between isotype distribution of antibodies directed to the 48-kD protein, clinical protection, and ability of specific anti–MSP-3b antibodies to block the parasite schizogony in the ADCI assay suggests that this molecule could be involved in eliciting protective mechanisms.

It is encouraging for the future of MSP-3 and of a malaria vaccine that (1) in contrast with several malarial proteins there is no indication for major strain polymorphism in the epitopes, nor in the protein studied; and (2) antibodies to protective epitopes in MSP-3 can be induced in mice and also can be readily produced by humans in response to a single encounter with the parasite in natural conditions of exposure to the disease, suggesting that the epitopes targetted by protective antibodies are in fact readily immunogenic

when presented by the parasite, although the antibodies induced after the first attacks are of an ineffective isotype and have a blocking effect in vitro. Thus, there are indications that target antigens of protective mechanisms may be less polymorphic and more immunogenic than is generally presumed.[32,33]

In view of immunization experiments, the availability of synthetic peptide constructs representing B- and T-cell epitopes of the native protein should provide a means 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.

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REFERENCES

1. Sergent ED, Parrot L: L'Immunité, la Prémunition et la Résistance Innée. Arch Inst Pasteur Alger 13:279, 1935

2. Druilhe P, Bouharoun-Tayoun H: Natural immunities. Res Immunol 142:637, 1992

3. Cohen S, McGregor IA, Carrington S: Gamma globulin and acquired immunity to human malaria. Nature 192:733, 1935

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

5. Khusmith S, Druilhe P: Cooperation between antibodies and monocytes that inhibit in-vitro proliferation of *P. falciparum.* Infect Immun 41:219, 1983

6. Lunel F, Druilhe P: Effector cells involved in non specific and antibody-dependent mechanisms directed against *P. falciparum* blood stages in vitro. Infect Immun 57:2043, 1989

7. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphajaisiddhi T, Druilhe P: Antibody which protect man against *P. falciparum* blood stages do not inhibit parasite growth in-vitro but act in cooperation with monocytes. J Exp Med 172:1633, 1990

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

9. Trager W, Jensen JB: Human malaria parasites in continuous culture. Science 193:673, 1976

10. Lambros C, Vanderberg JP: Synchronization of *P. falciparum* erythrocytic stages in culture. J Parasitol 65:418, 1979

11. Reese RT, Langreth SG, Trager W: Isolation of stages of the human parasite *Plasmodium falciparum* from culture and from animal blood. Bull World Health Organ 57:53, 1979

12. Mrema JEK, Langreth SG, Jost RC, Rieckmann KH, Heidrich HG: *P. falciparum:* Isolation and purification of spontaneously released merozoites by membrane sieves. Exp Parasitol 54:285, 1982

13. Boyum A: Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest 21:77, 1968 (suppl 97)

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

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

16. Manniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual (ed 2). Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1989

17. Smith DB, Johnson KS: Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with gluthatione S-transferase. Gene 67:31, 1988

18. Brahimi K, Pérignon J-L, Bossus M, Gras H, Tartar A, Druilhe P: Fast immunopurification of small amounts of specific antibodies on peptides bound to ELISA plates. J Immunol Methods 162:69, 1993

19. Soulier JP, Lee H, Prou O, Raymondjean N, Dubarry M, Druilhe P: Anticorps monoclonaux anti-*Plasmodium falciparum.* Rev Fr Transfus Immunohematol 4:373, 1982

20. Druilhe P, Khusmith S: Epidemiological correlation between levels of antibodies promoting *P. falciparum* merozoites phagocytosis and malaria immune status. Infect Immun 55:888, 1987

21. Aikawa M, Atkinson CD: Immunoelectron microscopy of parasites. Adv Parasitol 29:151, 1990

22. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463, 1977

23. Bonnefoy S, Guillotte M, Langsley G, Mercereau-Puijalon O: *P. falciparum:* Characterization of gene R45 encoding a trophozoite antigen containing a central block of 6 amino acid repeats. Exp Parasitol 74:441, 1992

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

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

26. Epping RJ, Goldstone SD, Ingram LT, Upcroft JA, Ramasamy R, Cooper JA, Bushell GR, Geysen HM: An epitope recognised by inhibitory monoclonal antibodies that react with a 51 kd merozoite surface antigen in *P. falciparum.* Mol Biochem Parasitol 28:1, 1988

27. Clark JT, Donachie S, Anand R, Wilson OF, Heidrich HG, McBride J: 46-53 kd glycoprotein from the surface of *P. falciparum* merozoites. Mol Biochem Parasitol 32:15, 1989

28. Schofield L, Hackett F: Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. J Exp Med 177:145, 1993

29. Ferguson MAJ, Williams AF: Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. Annu Rev Biochem 57:285, 1988

30. Roberts WL, Myher JJ, Kuksis A, Low MG, Rosenberry TL: Lipid analasys of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. J Biol Chem 263:18766, 1988

31. Romero P: Malaria vaccines. Curr Opin Immunol 4:432, 1992

32. Day KP, Marsh K: Naturally acquired immunity to *Plasmodium falciparum.* Parasitol Today 7:A68, 1991

33. Mercereau-Puijalon O, Fandeur T, Guillotte M, Bonnefoy S: Parasite features impeding malaria immunity: Antigenic diversity, antigenic variation and poor immunogenicity. Res Immunol 142:611, 1991