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ANTIBODIES AND REACTIVE T CELLS AGAINST THE MALARIA HEAT-SHOCK PROTEIN Pf72/Hsp70-1 AND DERIVED PEPTIDES IN INDIVIDUALS CONTINUOUSLY EXPOSED TO *Plasmodium falciparum*[1]

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Pf72/Hsp70-1, a heat-shock protein of m.w. 72 kDa from *Plasmodium falciparum* is one of the Ag of interest to be included in a polyvalent vaccine against malaria. It is one of the major immunogens present in a fraction of purified blood stage parasites that elicited protection against experimental infection of *Saimiri* monkeys with blood stages of *P. falciparum*. It is present at all blood stages and one of its B cell epitopes is also detected on the surface of the infected hepatocyte. Moreover, Pf72 appears to be well conserved among different isolates of *P. falciparum*. We have examined the immune response against Pf72/Hsp70-1 in individuals from different age groups living in a holoendemic area (West Africa). The immune response against the native Ag (purified from schizonts and called Pf/Hsp70) was analyzed both at the humoral level by ELISA and at the cellular level by assessing in vitro proliferation and IFN-γ production of PBMC. Of the individuals studied 52% had a statistically significant level of anti-Pf/Hsp70 antibodies as compared with unexposed individuals. These positive individuals showed a heterogeneous distribution because significant levels of antibodies were found in 70% of the adults but in only 26% of the children. The presence of Pf/Hsp70-specific reactive T cells in the blood was detected in 32% of the individuals. The total anti-Pf/Hsp70 antibody level (IgG + IgM) appeared strongly age related and correlated positively with parasite exposure, whereas the T cell response failed to correlate either with the antibody level or with age. Moreover, PBMC of donors responded to the Pf/Hsp70 in a dissociated way, namely, by either T cell proliferation or IFN-γ production. Ten synthetic peptides based on sequences found in the C-terminal part of Pf72/Hsp70-1 were further tested as potential T cell epitopes. The proliferative response of PBMC from individuals continuously exposed to the parasite showed that three peptides more frequently trigger significant T cell proliferation (in 21% to 27% of the individuals) and three others less frequently (10%). None of these peptides allowed detection of reactive T cells in PBMC of Europeans with no previous exposure to malaria. Some of the stimulating peptides are highly similar to human heat-shock Hsc and Hsp70 with large stretches of identical amino acids. The presence of such reactive T cells at a detectable level in the peripheral blood from individuals naturally exposed to Pf72/Hsp70-1 raises the question of possible cross-reactivities among Hsp70 from *P. falciparum*, other microorganisms, and man, and stresses the necessity of further studies before including such peptide sequences from Pf72/Hsp70-1 in a polyvalent vaccine against malaria.

In holoendemic areas where *Plasmodium falciparum* transmission is continuous, naturally acquired immunity against *P. falciparum* malaria protects humans against severe clinical attacks but does not eliminate the persistence of low levels of parasitemia. In humans, all the effectors of this naturally acquired immunity are still not known. However, antibodies have been shown to be one of the major effectors in protection against the blood stages of the infection (1–3). Thus the components of a multivalent vaccine have to contain B and T cell epitopes able to induce an appropriate immune response in all individuals living in endemic areas.

Pf72/Hsp70-1 is one of the candidates for a multivalent vaccine against malaria. A semi-defined vaccine containing a parasite protein fraction of 75-kDa mean m.w. including this molecule was able to elicit protection of *Saimiri sciureus* monkeys against the asexual blood stage of *P. falciparum* (4). The antibody reactivity in the immunized animals revealed a strong recognition of the Pf72/Hsp70-1 Ag. It is also one of the proteins recognized by sera from protected *S. sciureus* monkeys whose serum is able to transfer protection when injected into naive

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monkeys challenged with *P. falciparum*-infected RBC (3). The Hsp70-1 gene coding for the Pf72 Ag was cloned using anti-75-kDa fraction antibodies as screening tools (5), and proved to be identical to that of a 75-kDa Ag previously cloned using antisera from immune monkeys (6). Sequence analysis revealed that Pf72/Hsp70-1 is a member of the highly conserved family of 70-kDa Hsp (7–9). The Pf72/Hsp70-1 is extremely well conserved among *P. falciparum* isolates (10). It is one of the major proteins expressed in the cytoplasm of all blood stages of the parasite (8, 11) and possibly present on the merozoite surface (8). Furthermore, this molecule could be also implicated in immunity against the hepatic stages: an epitope recognized by an anti-Pf72 mAb is exposed at the surface of hepatocytes infected by *P. falciparum*, and this mAb is able to elicit an antibody-dependent cellular cytotoxicity against infected hepatocytes (12).

Extensive immunologic studies have already been performed in different animal models, allowing the definition of several B cell epitopes on Pf72/Hsp70-1 (13–15). In humans, this molecule seems to be a good immunogen in natural conditions of immunization because sera from individuals living in malaria-endemic areas or having suffered multiple malaria infections strongly react with this Ag (16–18). However, little is known concerning the humoral and the T cell responses against this Ag in relation to the acquisition of protection in humans naturally exposed to the parasite.

The determination of peptide sequences recognized by human T cells is required for vaccine candidate analysis in order to identify a minimal subset of epitopes recognized by a genetically diverse population. This is especially true for the Pf72/Hsp70-1 molecule, which exhibits high sequence similarities with the homolog host proteins, the human cognate Hsc70 (19), and inducible Hsp70 (20). Indeed, inclusion of such homologous peptide sequences in a vaccine could be responsible for undesirable immune cross-reactive responses. Thus, we decided to investigate the humoral and T cell immune responses against the native molecule Pf72/Hsp70-1, purified from *P. falciparum* schizonts, in humans naturally exposed to the parasite, and we examined their eventual relationship to the acquisition of protection.

For this purpose, we have exploited a prospective longitudinal study performed in Dielmo, a village in Senegal (West Africa) located in a holoendemic area, with a closed extensive survey of entomologic, clinical, and parasitologic parameters. This epidemiologic study aims to examine various immunologic parameters in relation to the acquisition of protection by individuals subjected to the typical conditions of *P. falciparum* continuous transmission. Thus, in 84 healthy representative individuals out of the 250 inhabitants of Dielmo, we monitored by ELISA the antibody response (IgG and IgM) against the Pf72/Hsp70-1. The presence of specific reactive T cells in the peripheral blood was measured by assessing proliferation and IFN-γ secretion after in vitro stimulation.

In this report, we analyze the relation between these immunologic parameters and the epidemiologic parameters such as exposure to the parasite or susceptibility to subsequent clinical attacks. In order to locate T cell epitopes within Pf72/Hsp70-1, we assessed the presence of reactive T cells against 10 synthetic peptides from the C-terminal part of the protein that shows the least sequence similarity with the homolog host protein. Six peptides were found to trigger a significant cell proliferative response of PBMC after in vitro stimulation in 10 to 27% of the tested individuals.

MATERIALS AND METHODS

Study Area and Population

The population of the village of Dielmo (Senegal, West-Africa) has been followed since June 1990 in a prospective longitudinal study. This study concerns epidemiologic and immunologic criteria of *P. falciparum* malaria as already described (21). Located in South Senegal, near the frontier of The Gambia, Dielmo was selected on the basis of a high level of malaria prevalence and little uncontrolled use of antimalarial drugs. The 250 inhabitants agreed to participate in the study, which follows all of the rules edicted by the Perfectionnement Council joining the Pasteur Institute of Dakar and the Senegal authorities.

Malaria transmission is perennial with moderate seasonal variations. The village is bordered by a river with permanent anophelian breeding places. The entomologic data were collected 1 wk a month all the year around—night-bite collection on human bait (12 men/night) monthly, and indoor pyrethrum collections (20 rooms)—showing a mean of 115 infective bites/person/yr. A permanent medical team lived in the village. Each household was visited daily to rapidly identify sick persons and collect clinical data. In addition, each individual was followed for blood thick smear collection twice a week during the rainy season (i.e., from June to September) and once a month during the dry season (from October). No chemoprophylaxis was given to the inhabitants and the absence of chemoprophylaxis was controlled by systematic measure of antimalarial drugs in the urine (Saker-Salomons modified test) (22) once a month. Malaria attacks, determined by the association of parasite/leucocyte ratio superior to 2 and body temperature above 38.5°C (23) were immediately treated with quinine-quinidine-cinchonine (Quinimax, 25 mg/kg daily for 3 days). The plasmodial indices measured at the end of the dry season and at the end of the rainy season as well as the average monthly incidence rates of clinical attacks in the different age groups are shown in Table I (21).

Blood Samples

Collection of blood samples in the field. At t_0 in June 1990 (just before the beginning of the rainy season, i.e., the period of high transmission), venous samples were taken from every individual except children less than 2 yr old for PBMC preparation, plasma collection, and malaria parasite analysis. Eighty-four individuals without any symptoms of malaria attack or other infectious diseases were selected for this study, in order to have two groups of around 40 donors representative of age groups 2 to 15 yr old and adults over 20. Teenagers between 15 and 20 were voluntarily underrepresented because of known age-related problems such as extensive individual variations in in vitro cellular responses. Twenty milliliters of venous blood were collected between 8 a.m. and 10 a.m. (to avoid nyctohemeral variations), in two siliconized dry evacuated tubes to which 0.1 ml of heparin (2500 IU/ml, Liquemine, Roche) was added. The tubes were then brought to the Pasteur Institute in Dakar by car in less than 5 h, in isotherm boxes to maintain the temperature between 20°C and 30°C. Blood (5 ml) was also collected in EDTA-containing tubes for hematologic studies.

Collection of the plasma and PBMC preparation. The plasma from heparinized tubes was harvested after 5 min of centrifugation at 800 × *g*, aliquoted, and stored at −80°C until use. After removal

TABLE I

Epidemiologic characteristics of the village (data collected during 1 yr of survey)[a]

	Age Groups						
	<1	1–4	5–9	10–14	15–19	20–39	>40
Plasmodial indices (%)							
End of the dry season	55	88	84	84	57	35	23
End of the rainy season	39	92	90	89	79	61	57
Monthly incidence rate of clinical attacks (%)	25	35	14	2	3	1	<1

[a] Plasmodial indices and incidence rates of clinical attacks are calculated as described in Reference 21.

of the plasma, the initial blood volume was reconstituted with RPMI 1640 containing 3.7 g/liter of sodium bicarbonate and 10 mM HEPES. PBMC were prepared on Ficoll gradients using the classical protocol (Histopaque 1077, Sigma Chemicals, St Louis, MO). Viable cells were counted by trypan blue exclusion and the cell concentration was adjusted to 2×10^6 cells/ml in complete culture medium.

Flow cytometric analysis. Fluorescein- or phycoerythrin-conjugated mAb, anti-CD3, anti-CD2, anti-CD19, anti-CD4, and anti-CD8, were purchased from Coulter Immunology (Hialeah, FL), and anti-TCR-$\gamma\delta$ from T Cell Sciences (Cambridge, MA). They were used according to manufacturer's instructions for total blood and the cell populations analyzed on an Epics Profile flow cytometer (Coulter).

Stimuli Used for PBMC in Vitro Stimulation Experiments

Mitogen and Ag control. Leukoagglutinin (Sigma) and PPD[3] (Statens Serum Institute, Copenhagen, Denmark) were used at a final concentration of 10 μg/ml to check the quality of the culture.

The PFSL was prepared from an in vitro continuous culture (24) in O^+ human erythrocytes of a knob-positive variant of the original FUP-1 isolate (4). Schizont enrichment providing more than 90% mature schizont-infected erythrocytes was performed by Plasmagel (Laboratoire Roger Bellon, Neuilly sur Seine, France) sedimentation (25). Mock cultures of normal RBC from the same batch were run in parallel and treated as above. Schizont preparations and corresponding nonparasitized RBC were washed three times in RPMI 1640 (Flow Laboratories, McLean, VA). The pellets were resuspended in 4 volumes of distilled water, aliquoted, and stored at −80°C until use. The lysates were further diluted 1/800 to 1/1000 in complete culture medium for cellular response studies.

Pf/Hsp70: Purification of the native Ag. Members of the Hsp70 family, such as the *Escherichia coli* DNA K, bind ATP (26). The native AgPf72/Hsp70-1 was thus purified from total PFSL (prepared as described above) on an ATP-affinity column using a modification of described methods (27). Briefly, soluble components were extracted from parasite-infected cells using hypotonic buffer (10 mM Tris-acetate, pH 7.5; 10 mM NaCl; 0.1 mM EDTA) containing the protease inhibitors (1 mM) PMSF/TLCK. The particulate and membranous pellet containing most of the Pf71/Hsp70-2, another heat-shock molecule of the Hsp70 family (5, 28), was discarded by centrifugation at 10,000 × *g*, 4°C. The 0.22-μ filtered soluble extract was applied to a DEAE-cellulose column (DE52, Whatman, Clifton, NJ) in the presence of β-mercaptoethanol (15 mM). Elution was performed by a linear salt gradient from 50 to 500 mM NaCl in the B buffer (Tris acetate, 20 mM, pH 7.6, 0.1 mM EDTA, and 15 mM β-mercaptoethanol). The Pf72/Hsp70-1-containing fractions were supplemented with $MgCl_2$ (3 mM) and then applied to an ATP agarose column (Sigma). The bound protein was specifically eluted using a 3-mM ATP solution in the $MgCl_2$-supplemented Tris-acetate B buffer. The protein content was estimated by the method of Bradford (29), and the purity evaluated by SDS-PAGE (30).

Synthetic peptides. The 10 Pf72-derived synthetic peptides were obtained from either the Laboratory of Organic Chemistry, Pasteur Institute, Paris, France, or from Neosystem, Strasbourg, France. All were more than 90% pure as evaluated by analytic reverse-phase HPLC:

Peptide 1, NTTIPAKKSQIFTTYAD; peptide 2, VEKSTGKQNHITITND; peptide 3, LSQDEIDRMVNDAEKY; peptide 4, YGVKSSLEDQKIKEKL; peptide 5, KLQPAEIETCMKTIT; peptide 6, KNQLAGKDEYEAKQKEAE; peptide 7, SKIYQDAAGAAGGMPG; peptide 8, GNAPAGSGPTVEEVD; peptide 9, GGMPGGMPGGMPY; peptide 10, YGVKSSLEDQKIKEKLQPAEIETCG. The (EENVHDA)2-(EENV)2 peptide containing both B- and T-epitopes from the Pf155/RESA Ag was purchased from Bachem, Switzerland. All synthetic peptides were diluted in RPMI 1640 in a 2 mg/ml stock solution and stored at −80°C. Preliminary experiments allowed the determination of the optimal concentration of 10 μg/ml for all peptides used in the T cell proliferation assay. Thus, the peptide stock solutions were diluted in complete medium at 10 μg/ml and sterile filtered for use in the assays. The localization on the Pf72/Hsp70-1 sequence of peptides 1 to 10 is shown in Figure 1.

Detection of Parasite-Specific T Cells

Culture conditions. PBMC were incubated with the different stimuli at 2×10^5 cells/well in round bottomed, 96-well plates (Costar, Cambridge, MA) in 0.2 ml of complete culture medium: RPMI 1640 supplemented with 3.7 g/liter sodium bicarbonate, 2 mM glutamine, 1% Na-pyruvate, 1% nonessential aminoacids, 10 mM HEPES, 100 U/ml penicillin and streptomycin (Flow Laboratories), and 10% heat-inactivated human serum AB (Jacques Boy, France). The different stimuli were added at appropriate concentrations in quadruplicate wells, and the plates incubated for 6 days at 37°C in 5% CO_2.

Proliferation assays and expression of results. To assess the proliferative responses of cells to the various stimuli, 1 μCi/well of [^{3}H]TdR was added and incorporation evaluated on a LKB Betaplate apparatus after 14 to 16 h. S.I. were calculated from geometric means of quadruplicates as follows: S.I. was defined as the ratio of the geometric mean cpm of quadruplicate test cultures to the geometric mean cpm of quadruplicate control cultures. A positive proliferative response was attributed to samples for which the S.I. was at least >2 and the cpm difference between geometric means of test cultures and control cultures was more than 1000 cpm. The maximum proliferation was obtained at day 6 for the different parasite Ag as PFSL, Pf/Hsp70, and the Pf72/Hsp70-1-derived peptides (assays being initiated on day 0). The T cell response was assessed by checking [^{3}H]TdR incorporation at day 6.

IFN-γ assay and expression of results. The IFN-γ concentration in pooled supernatants from the quadruplicate wells was assessed by the capture ELISA test performed in duplicate. Briefly, wells were coated with 50 μl of anti-IFN-γ mAb RU 40.2 at 5 μg/ml in PBS and vacant attachment sites on the plate were blocked with PBS-BSA (3%). Fifty microliters of undiluted supernatant were added for 16 to 18 h at room temperature, and the wells were then washed three times for 10 min with PBS-Tween 0.1%. A second anti-IFN-γ mAb, RU 308, coupled to peroxidase, diluted in RPMI 1640 containing 50 mM HEPES, was added in the wells at 0.8 μg/ml and left for 3 h at room temperature. The wells were again washed three times and the activity was revealed after a 15-min incubation at room temperature with 100 μl of reaction substrate (*o*-phenylenediamine, 1 mg/ml and H_2O_2 30%, 1 μl/ml in citrate buffer). The reaction was stopped by adding 50 μl/well of 3 N HCl and the OD read at 450 nm on a Dynatech reader. The IFN-γ content of supernatants was calculated from standard curves performed on culture medium containing the known amount of IFN-γ included on each plate. Readings were compared with the international human National Institutes of Health IFN-γ standard Gg23-901-530. This test is able to detect 0.25 IU/ml. Thus, we consider as positive samples for which the quantity of IFN-γ secreted in the presence of the Ag was more than 1 IU/ml. We never found detectable levels of IFN-γ in control wells (PBMC in the absence of Ag). Preliminary studies showed that the optimal IFN-γ production after stimulation with PFSL was obtained around days 3 or 4, but no detectable decrease in concentration was observed at day 6. Thus, culture supernatants (100 μl/well) were harvested in the evening of day 5, frozen at minus 80°C until use, and replaced by 100 μl of fresh medium containing the [^{3}H]TdR for assessing proliferation. Anti-human IFN-γ mAb were kindly given by Mrs. Cousin, Roussel Uclaf, France.

Antibody Detection

Antibodies against P. falciparum. Free parasites were prepared from in vitro cultures of the Banjul strain (gift of Dr. Brian Greenwood) and Ag was extracted as described (31). Nunclon ELISA plates were coated with this antigenic preparation at 5 μg/ml in carbonate buffer, pH 9.6, at 4°C. Preliminary data using a range of extract concentration indicated that 5 μg/ml gave the highest signal to noise ratio. Sera diluted in PBS were incubated for 1 h at 37°C. After three washes in PBS-Tween 1%, the plates were incubated 2 h at 37°C in the presence of goat anti-human IgG + IgM antibodies conjugated to peroxidase (Institut Pasteur Production, France). The peroxidase activity was revealed using *o*-phenylenediamine, 1 mg/ml and H_2O_2 30%, 1 μl/ml, in 0.1 M citrate buffer, pH 5.3); OD was measured at 450 nm.

Antibodies against Pf/Hsp70. Luxlon ELISA plates were coated with 250 ng/well of the native purified Pf/Hsp70 in PBS overnight at 4°C. After saturation (PBS-0.1% Tween 5%-milk for 1 h at 37°C), the wells were washed in PBS 0.1% Tween and incubated 1 h at 37°C with dilutions of test and control plasmas. The wells were washed and either peroxidase-conjugated goat anti-human IgG + IgM (Biosys, Compiegne, France), or goat anti-human IgG, or goat anti-human IgM antibodies (KPL, Gaithersburg, MD) were added for 1 h at 37°C. After incubation the wells were washed and revealed with the 3,3′,5,5′-tetramethylbenzidine substrate (KPL), used according to the suppliers instructions. The reaction was stopped with H_3PO_4 1 M and O.D. read at 450 nm. All the plasma were tested in duplicate at the dilution of 1/100. For comparison of data collected on different ELISA plates, we included in each plate a standard curve corresponding to a positive plasma tested at different dilutions. In order to quantify the level of antibody anti-Hsp70 we ascribed a value of 100 arbitrary units to the reference plasma diluted to 1/100. The level

[3] Abbreviations used in this paper: PPD, purified protein derivative; TLCK, tosyl-L-lysine-chloro-methyl-ketone; PFSL, *Plasmodium falciparum* schizont lysate; a.u., arbitrary units; RESA, ring erythrocyte surface Ag; S.I., stimulation index.

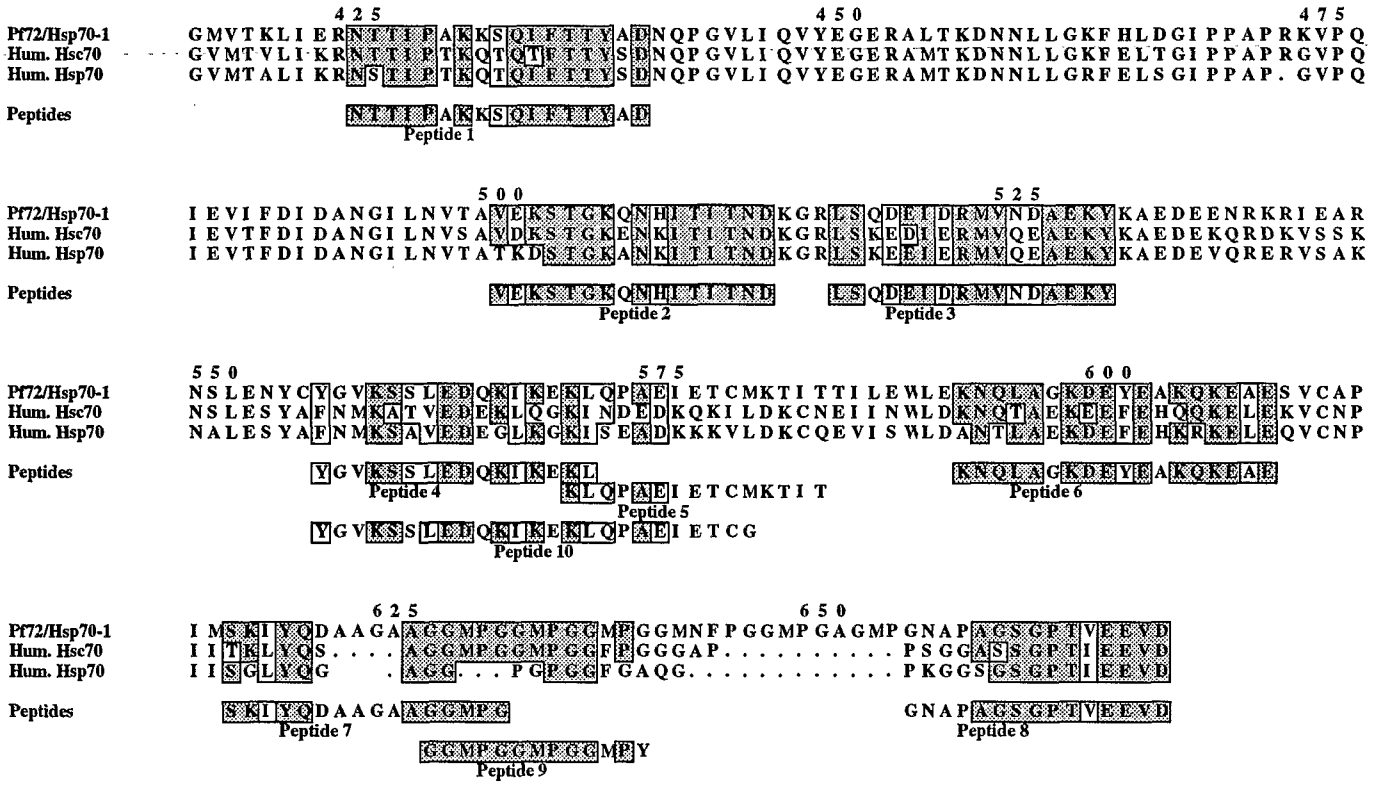

Figure 1. Pf72/Hsp70-1-derived synthetic peptides sequences. homologies between *P. falciparum* Pf72/Hsp70-1 (9) and either human Hsc70 (19) or Hsp70 (20) are highlighted in *shadowed boxes* for identical or *open boxes* for conserved residues.

of antibody anti-Hsp70 of all plasma tested was expressed in arbitrary units compared to the reference plasma.

RESULTS

Prerequisite Criteria to Included Blood Donors

To significantly compare T cell responses to any Ag in the group and to correlate them with an eventual particular immunologic status, it is important to first monitor, as precisely as possible, the hematologic parameters and physiologic status of the immune system of the donors at the time of the study. For that purpose, all the blood samples were analyzed the day of the bleeding for hemoglobin content, for blood cell numbers and differential leukocyte numbers. In addition, the presence of HbS was detected by cellulose acetate and citrate agar electrophoresis and confirmed by the falciformation test (32). Out of the 84 selected individuals, all donors presenting signs of anemia, major hematologic pertubations, or blood cell disorders compared with known average values for Senegal populations were excluded from this study. Were also excluded three individuals displaying the presence of the filaria, *Dipetalonema perstans*. No infection with either *Schistosoma* or other major parasites except intestinal parasites was observed. Thick blood films prepared when the blood was drawn were stained with Giemsa and later examined for the presence of the plasmodial species.

In order to further characterize lymphocyte subpopulations and investigate the possible variation in the measure of the in vitro T cell response, PBMC subpopulations were characterized in approximately half of the samples by FACS analysis. We studied retrospectively the number and the repartition among B and T lymphocyte subsets using anti-CD19, anti-CD2, anti-CD4, and anti-CD8 antibodies. Although 10 individuals out of the 39 tested had a CD4/CD8 ratio slightly different from the normal values (2.3 ± 0.5) in the Senegalese population, we did not observe statistically significant modifications in the T cell response measured by proliferation or IFN-γ secretion against the different Ag and/or mitogens we tested.

Seventy-seven plasmas of the 79 finally selected malaria-exposed donors were tested for the presence of anti-*P. falciparum* antibodies. All the plasma contained antibodies that recognized *P. falciparum* asexual blood stages as detected by ELISA with a titer equal to or above 1/200. Nonexposed donors had undetectable or very low levels of antibodies (titer less than 1/20).

Purification of the Native Hsp70 from P. falciparum-Infected Erythrocytes (Pf/Hsp70)

The Pf/Hsp70 preparation purified by affinity chromatography on ATP-agarose column showed by Coomassie blue staining on SDS-PAGE analysis only one major single band of 72 kDa m.w., which is specifically recognized on Western blots by Pf72/Hsp70-1-specific mAb (Fig. 2). The preparation was therefore assessed to be at least 90% pure. However, we cannot exclude the possibility that the preparation could contain other members of the Hsp70 family, harboring identical m.w. and ATP-binding properties. Indeed, one of the known members of this heat-shock 70 family, Pf71/Hsp70-2 Ag (28), is also called P75 Ag, and is homologous to the rat glucose-regulated protein GRP 78 (33) and could also bind to the ATP-agarose column but has biologic properties slightly different from PF72, such as its membranous localization in the parasite (11). Thus, to avoid the contamination by Pf71/Hsp70-2 as much as possible, we prepared a soluble extract of schizonts without detergent. No 71-kDa band

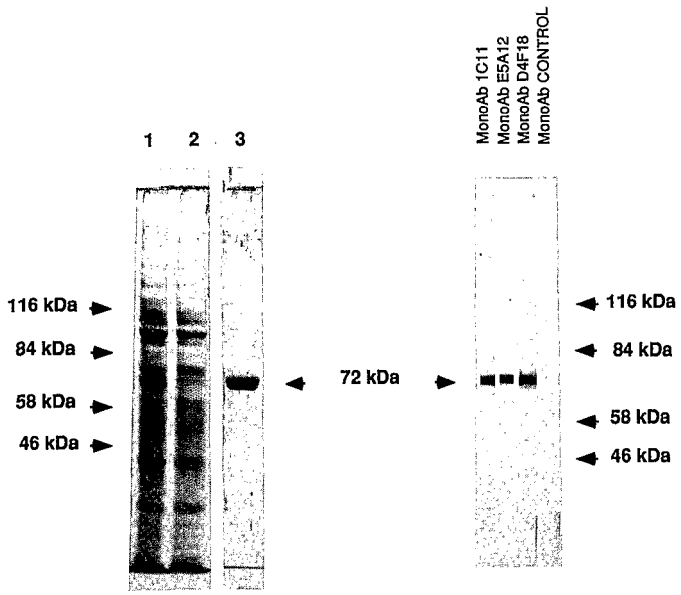

Figure 2. Purification of Pf/Hsp70 from *P. falciparum* schizonts. *A*, Coomassie blue staining of: *1*, parasite lysate before ATP-agarose column; *2*, after ATP-agarose column; *3*, Pf/Hsp70 specifically eluted with ATP. *B*, Western blot analysis of the purified Pf/Hsp70 with specific mAb (13).

was seen in the preparation. A third member of the family has also been described, but is characterized so far only at the DNA level (34), with no data available on its ATP-binding capacity and subcellular localization.

ATP-agarose chromatography performed on equivalent extracts from cultured noninfected RBC did not provide any detectable Hsp70-like proteins, ensuring the absence of contaminating human components in our *P. falciparum*-derived preparation (data not shown).

Presence of Anti-Pf/Hsp70 IgM and IgG Antibodies is Positively Correlated with Parasite Exposure

The level of circulating antibodies specific for Pf/Hsp70 was measured by ELISA for the 77 tested individuals. The results are expressed in a.u. as described in *Materials and Methods*. Plasma from 20 Europeans who had never been exposed to malaria was also tested in the same way. The distribution of the population expressed as a function of the antibody level corresponded to a normal distribution with a mean of 11 ± 6 a.u. in the assay for total IgG + M. Thus, we decided to fix the cut-off for a positive value in anti-Pf/Hsp70 antibodies to 26 U (mean ± 2.5 SD). Similar values were obtained in IgG- and IgM-specific ELISA tests.

Using this criterion, 50.6% of the population (i.e., 39/77 individuals) is positive for anti-Pf/Hsp70 IgG + IgM antibodies (Tables II and Fig. 3). Only 26% of the children under 16 were positive whereas 70% of the adults over 20 have significant levels of anti-Pf/Hsp70 antibodies (Tables II and Fig. 3). Similar results were obtained for anti-Pf/Hsp70 IgG antibodies—54% of the 74 individuals analyzed were positive, whereas only 30.5% were positive for IgM antibodies (Table II).

We observed that the average IgG + IgM antibody level increases with age in the population and becomes significantly positive (>26 a.u) in the 11- to 15-yr-old age group. A similar increase was observed for IgG antibody level, a delay was, however, observed for a significant level of IgM antibodies (Fig. 4*B*). In the same population, the number of malaria attacks during the ensuing year decreased rapidly with age, suggesting the acquisition of clinical protection before 10 years of age (Fig. 4*A*). This contrasts with the slow decrease with age of the average of parasitemia on the day on which subjects were bled. Low frequencies of high parasitemias (F2 and F3) were observed in the 11- to 15- and 16- to 20-yr-old groups in which significant levels of anti-Pf/Hsp70 antibodies appeared (Fig. 4*C*). Thus, our data suggest an inverse relationship between the Pf/Hsp70 level of antibody and the control of circulating *P. falciparum* parasites. However, no direct correlation between anti-Pf/Hsp70 antibodies (Fig. 4*B*) and the frequency of subsequent malaria attacks can be drawn at the individual level. The parasitemia at the time of the bleeding was also not correlated with the individual level of anti-Hsp70 antibodies.

TABLE II

Proportion of positive responders to Pf/Hsp70-1 in relation to malaria attacks in children and adults

Positive Responses to Pf/Hsp 70	All Ages	Under 16 Yr of Age	Over 20 Yr of Age
Antibodies			
IgG + IgM	39/77 (50.6%)	9/34 (26.5%)	30/43 (69.8%)
IgG	40/74 (54%)	12/33 (36.4%)	28/41 (63.6%)
IgM	23/75 (30.7%)	5/33 (15.1%)	18/42 (42.9%)
Proliferation	10/68 (14.7%)	5/31 (16.1%)	5/37 (13.5%)
IFN-γ production	15/63 (23.8%)	5/26 (19.2%)	10/37 (27.0%)
Malaria attacks	21/79 (26.6%)	17/35 (48.6%)	4/44 (9.1%)

Pf/Hsp70-Reactive T Cells Respond Either by Proliferation or by IFN-γ Secretion

The presence of Pf/Hsp70-reactive T cells in the blood of the 79 selected individuals was tested by both proliferation and IFN-γ secretion. Positive proliferative responses and IFN-γ secretion were assessed by comparison with control cultures without Ag as described in *Material and Methods*. For all individuals, PBMC gave a high proliferative T cell response and produced large amounts of IFN-γ in response to the stimulation by the control mitogen leukoagglutinin and Ag PPD, ensuring the lack of any nonspecific massive disorders in the capacity of T cells to proliferate or to produce IFN-γ (data not shown). In response to stimulation by the total parasite lysate PFSL, 57 of 77 individuals tested gave positive PBMC proliferative responses with a high S.I. (calculated using noninfected RBC as control stimulus) ranging from 2 to 242.8 (mean 30.28 ± SD 43.7) whereas PBMC from only for 36 individuals produced IFN-γ. These 36 individuals also showed a proliferative response (data not shown).

Positive proliferative responses against the purified Pf/Hsp70 were obtained in 10 individuals from the 68 effectively analyzed, i.e., 14.7% of the samples. S.I. of the positive responses were on the average weak (mean 5.02 ± SD 3.49), ranging from 2 to 13, compared with those obtained with PPD (mean 71.96 ± SD 84.47), ranging from 2.55 to 452.8. In response to purified Pf/Hsp70, the PBMC of 16 individuals of the 63 tested produced IFN-γ (25.3%), however in only three samples were both the proliferative response and IFN-γ production associated (Fig. 3).

Dissociated cellular responses, i.e., without any corre-

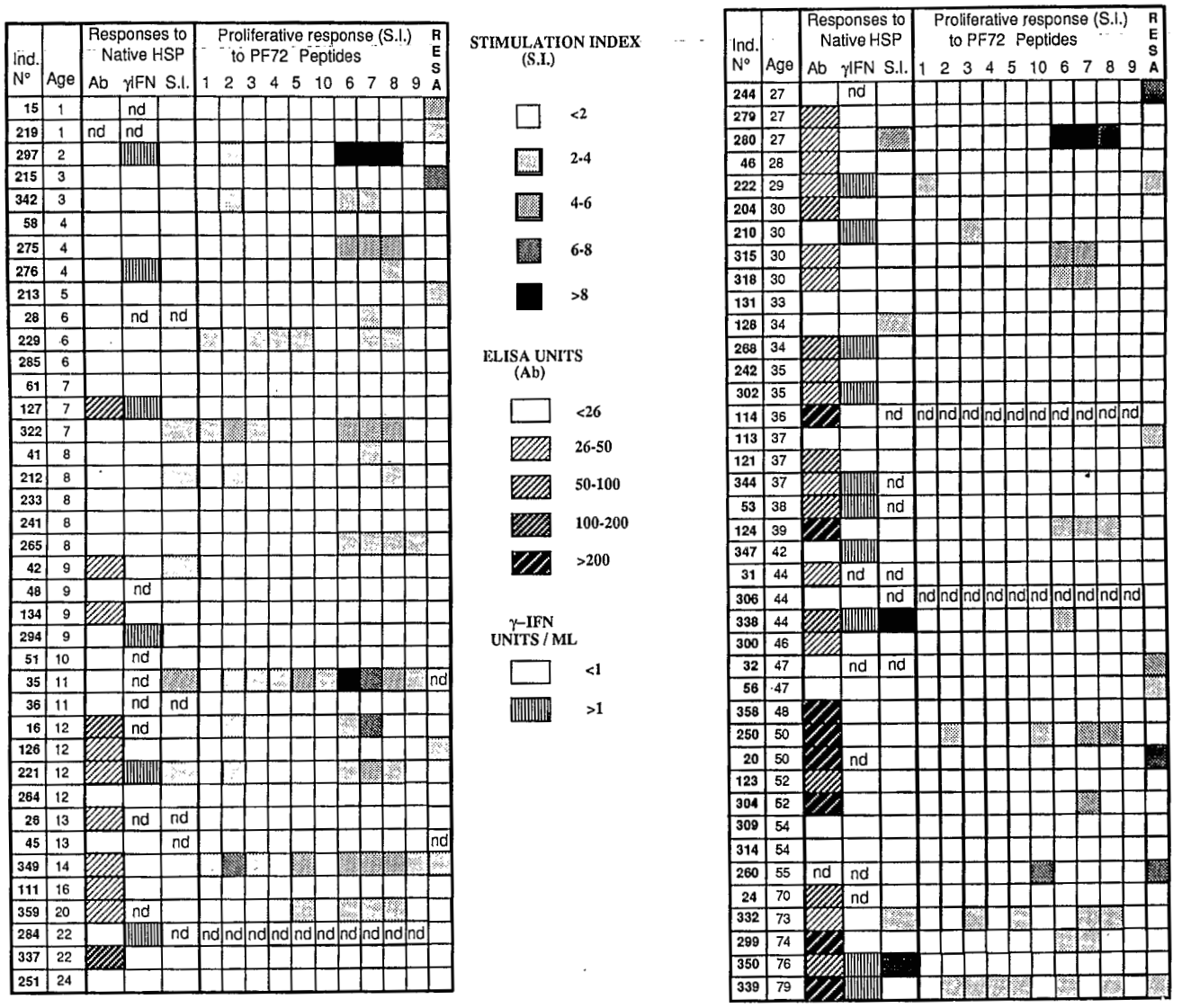

Figure 3. Individual responses against the native Pf/Hsp70 and the different peptides. S.I., IFN-γ, and anti-Pf/Hsp70 antibody units are calculated as described in *Materials and Methods*.

lation between proliferative response and IFN-γ secretion were also observed when using the Pf155/RESA peptide as a comparative element; indeed, although the PBMC of only 14 individuals proliferate, those of 6 others only produce IFN-γ with 2 being double responders (data not shown).

Absence of Correlation between Antibody (IgM and IgG) Response and T Cell Response against Pf/Hsp70

No statistically significant correlations between humoral and cellular responses to the Pf/Hsp70 were obtained. Indeed proliferative responders and IFN-γ producers were equally represented in the antibody-positive or antibody-negative populations.

Table II presents a synoptic summary of the positive responders to Pf/Hsp70 in antibodies, T cell proliferation, or IFN-γ secretion in relation to malaria attacks in children and adults.

Hsp70-Derived Peptides, Even Those Exhibiting Homology to Self, Are Able to Induce T Cell Proliferation

Choice of the peptides. The amino acid sequence of Pf72/Hsp70-1 Ag was analyzed by computer programs designed to predict T cell immunogenicity on the basis of three different criteria. We selected the peptides for the presence of rare amino acid patterns (35). Some of these peptides also corresponded to regions of α-amphipathicity (36) and/or specific sequence motifs (37) (Table III). The search for the presence of putative epitopes was confined to the C-terminal part of the Ag because it is least similar to the host Hsp70 and Hsc70 (20, 38). The 10 selected peptides and their similarity with the human Hsc70 and Hsp70 are shown in Figure 1.

Analysis of the T cell response to the different peptides. All samples screened for the presence of Pf72/Hsp70-1 peptide-reactive T cells were also screened for the presence of T cells reactive to the synthetic peptide from the Pf155/RESA molecule (EENVHDA)2-(EENV)2. This was done in order to first compare our analysis with other studies in terms of proportion of responders. Indeed this peptide was shown to be recognized by a good proportion of individuals exposed to *P. falciparum* in different endemic areas but not recognized by T cells from nonexposed individuals (39, 40), and second, to have a negative reference to test the association between responses to different peptides of the Pf72/Hsp70-1 and

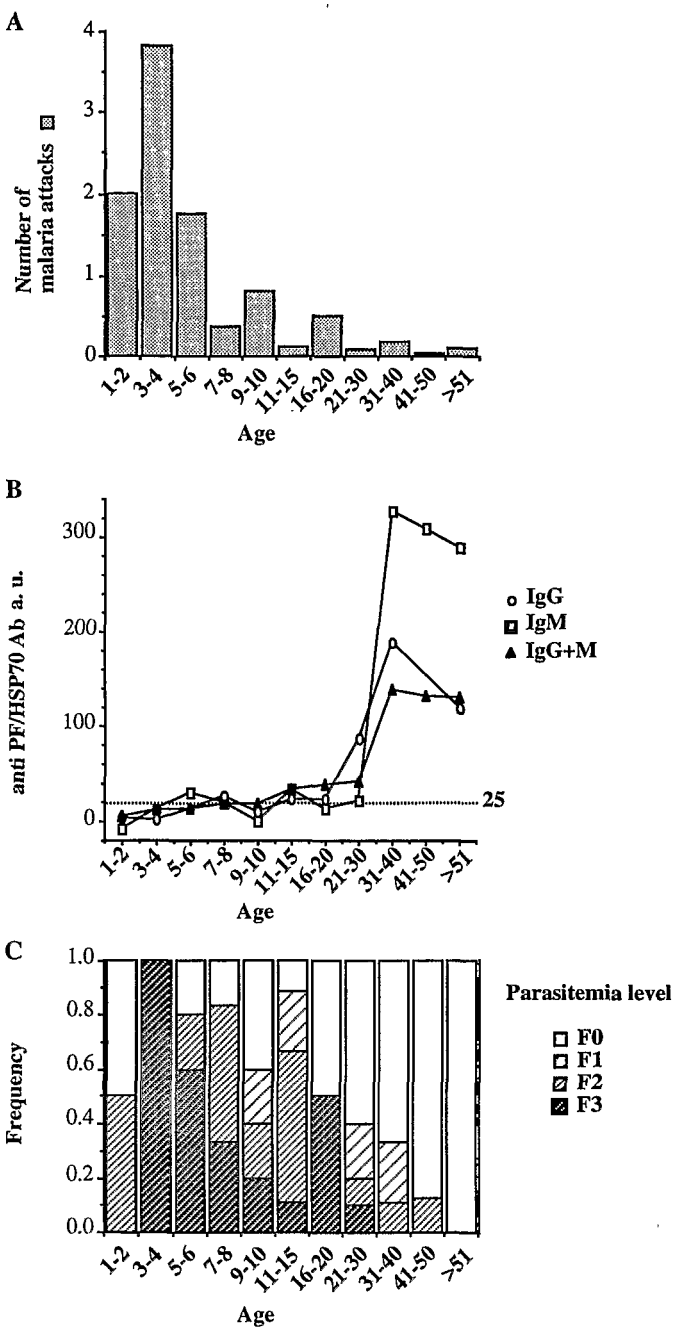

Figure 4. Level of anti-Pf/Hsp70 antibodies (IgG + IgM), number of malaria attacks, and parasitemia in the different age groups. *A*, average number of malaria attacks per individual during the 11 mo after blood sampling at t_0. *B*, Average level of anti-Pf/Hsp70 antibodies in different age groups as assessed by ELISA test. (▲, total IgG + IgM; O, IgG; □, IgM.) *C*, frequency in each group of levels of parasitemia in the blood samples collected at t_0 expressed as follows: F0, 0 to 5; F1, 5 to 50; F2, 50 to 500; and F3, 500 to 5000 parasites/μl of blood. Number of individuals in each age group: 1 to 2 yr old, 2; 3 to 4, 6; 5 to 6, 5; 7 to 8, 7; 9 to 10, 5; 11 to 15, 9; 16 to 20, 2; 21 to 30, 11; 31 to 40, 9; 41 to 50, 8; >51, 9.

the response to the native Pf/Hsp70 molecule. Twenty Caucasians never exposed to malaria did not give any proliferative response to any of these 10 peptides, or to the Pf155/RESA-derived peptide (EENVHDA)2-(EENV)2 which was used under the same conditions. Individual proliferative responses to the 10 Pf72-derived peptides and the Pf155/RESA-derived peptide (EENVHDA)2-(EENV)2 are shown in Figure 3 in comparison with the response to the purified parasite Pf/Hsp70.

Screening of PBMC from individuals from Dielmo showed that although three peptides, p6, p7, and p8, were able to induce proliferation with a relatively high frequency, in 17(22%), 21(27%), and 16(20%) samples, respectively, peptides p2, p3, and p5 less frequently induced proliferation in 10 (13%), 7 (9%), and 6 (8%) samples (Table III). The Pf155/RESA-derived peptide induced proliferation in 14/76 (18%), a frequency closely similar to the frequency we obtained with the p6, p7, p8 peptide samples. This frequency is somewhat lower but comparable with already published results in other endemic areas (39–41). The presence of T cells reactive to at least two of the three peptides, p6, p7, and p8, was detected in 18 individuals.

TABLE III

Pf72/Hsp70-1-derived peptides: T cell epitope predictions according to different models and frequence of responders

Group	Peptide No.	Claverie Model	Berzofsky Model	Rothbard Model	% of Individuals with Responsive T Cells
1	9	–	–	+	4
	1	+	–	–	5
	4	–	–	–	4
2	5	+	+	–	8
	3	+	+	+	9
	2	+	+	±	13
3	8	–	+	–	20
	6	+	–	–	22
	7	–	+	+	27

When checked for a correlation between proliferative responses to the peptides and to the native protein, between one-third and one-fourth of the individuals responding to peptides 6, 7, or 8 responded to native Pf/Hsp70, whereas none of the Pf155/RESA responders responded to the native Pf/Hsp70. In other words, more than half of the individuals (7/10) giving a proliferative response to Pf/Hsp70 gave also a positive proliferative response to one or more of the three peptides (p6, p7, and p8), whereas no correlation was observed with the proliferative response to the Pf155/RESA peptide.

No peptide was able to induce a significant level of IFN-γ production. In only two individuals, we detected IFN-γ in the supernatants of PBMC stimulated with either of the 10 peptides (data not shown). By comparison, PBMC of 9/63 individuals produced more than 1 U/ml when stimulated by the Pf155/RESA peptide (data not shown), a proportion lower but still close to the published results on cellular responses to this Pf155/RESA peptide (40). It should be stressed that in our hands, even if the percentage of positive responses to this Pf155/RESA peptide was repeatedly lower than those published, in only two individuals of the 14/77 whose PBMC proliferated was IFN-γ also detectable. This confirms the already published data and is closely comparable with the results we obtained for the native Pf/Hsp70 Ag where proliferative responses and IFN-γ production were dissociated (40).

DISCUSSION

As the protective immune response against blood stage infection seems to be mainly mediated by antibodies (1–3), vaccine components have to contain B and T cell epitopes able to induce an appropriate immune response in all individuals. Epidemiologic studies in endemic areas are required to collect information about antibody and T cell-immune responses against defined parasite mole-

cules during natural sensitization against malaria.

To perform this study we decided not to use a recombinant protein of the Pf72/Hsp70-1 but the native Ag purified from the parasite, even if our preparation could contain more than one member of the Hsp70 family. Indeed, the sera of individuals can recognize conformational epitopes present on the native form but absent in the recombinant fusion protein because of differences in folding and glycosylation patterns. At the T cell level, preliminary experiments performed in Senegal using a βgal-fusion protein showed that the same levels of proliferation of PBMC were frequently obtained both with the rAg and with the control carrier alone. Furthermore, the majority of specific T cell clones we produced recognized epitopes located in the carrier part or at the junction between the carrier part and the insert part of the fusion molecule (C. Behr et al., unpublished observations).

Heat-shock proteins such as Pf72/Hsp70-1 belong to a peculiar category of Ag. Indeed, such stress proteins have been highly conserved during evolution and members of this family are found in all cells from prokaryotes to humans. They retain more than 50% of amino acid sequence identity between the pathogen and their human counterparts with, in some cases, large identical stretches of amino acids. Nevertheless, Hsp-reactive T cells and antibodies have been found dominant during and after many different infections with either parasitic pathogens or bacterial pathogens (for reviews see References 18 and 42). Furthermore, strong immune responses against stress proteins have been also found to be associated with autoimmune diseases such as SLE in which high levels of antibodies against the self-Hsp70 and Hsp90 are found in the sera of patients (43). Also, in the different rat models of arthritis or in human arthritis, the pathology seems related to the cellular and humoral immune response to Hsp70 and Hsp65 (44, 45). Paradoxically, last Ag seems to be able, in certain situations, to protect or prevent against arthritis (46–48). Because of these different possible aspects, the human immune response to parasite heat-shock proteins such as the Pf72/Hsp70-1 is very complex to analyze, especially in endemic areas where individuals are in close contact with other unrelated microorganisms. Thus, in a first step, we decided to simply check for the presence of antibodies or T cells able to react with the native Pf72/Hsp70-1 or some derived synthetic peptides, independently of which microorganisms may have induced such a response.

Out of the 77 plasma we tested, we found that more than half contained antibodies reactive with Pf/Hsp70. We observed a strong age-related increase in the total level of (IgM + IgG) and the IgG antibodies reactive against the native Pf/Hsp70 protein; this increase is correlated with the decrease in parasitemia. This is in contrast to results of Kumar et al. (17), probably because we tested larger samples and had the possibility to test age groups under 10 years of age. However, as revealed by individual analysis, an increased level of total antibodies appears to be linked to parasite exposure and not to the acquisition of clinical protection.

In the population under study, we detected the presence of specific Pf/Hsp70-1-reactive T cells in the peripheral blood of approximately one-third of the population. Whereas the presence of specific antibodies is clearly age related, the presence of specific reactive T cells shows an age-independent distribution. Such differences between the humoral and T cell responses with regard to age have already been described for all the malarial Ag studied so far (39, 49). These results may indicate that the T cell response as it is measured (namely proliferation and IFN-γ secretion) probably reflects much more the transient recirculation of short lived, recently primed, T cells than the persistence in the peripheral blood of long lived memory T cells.

For almost all individuals whose PBMC responded to the Pf/Hsp70, these PBMC responded in a dissociated way either by proliferation or IFN-γ secretion. This was also described for the Pf155/RESA, the PfCS (circumsporozoite) protein, and the 48/45-kDa gametocyte Ag in different endemic areas (49, 50–52). In this regard, it would also be interesting to check IL-4 secretion, but at the time of the study a sufficiently sensitive test was not available. Cytokine production in the absence of T cell proliferation might suggest that the Pf72/Hsp70-1 could activate different subsets of T cells through different epitopes. This dissociated response could also simply be the consequence of a preactivation of the cells present in the different PBMC samples. Indeed, already committed and differentiated T cells have already been shown to behave differently from specific cell precursors after in vitro stimulation. Thus it must be stressed that the evaluation of the presence of Ag-specific reactive T cells in the peripheral blood of donors must be done using multiple assays of the T cell responses such as proliferation, secretion of certain lymphokines, and surface expression of activation markers.

The precise identification of T cell epitopes on Pf72/Hsp70-1 is very important. It would be critical to include sequences in a multivalent vaccine that are able to induce appropriate immune responses in genetically diverse populations and to eliminate those that would cause possible pathologic events caused by self-cross-reactive epitopes. When using the 10 selected peptides to detect Pf72/Hsp70-1-specific T cells, no response of PBMC from nonexposed Caucasian individuals was found either by proliferation or IFN-γ secretion. Analysis of the response of PBMC from 79 malaria-exposed individuals focused our attention on six peptides. Three peptides (p6, p7, and p8) were frequently recognized and three others (p2, p3, and p5) less frequently. The proliferative response obtained in the presence of the peptides was not consistent with predictions made by any of the models. The peptide p6, which presents a relatively high level of homology in the amino acid sequence with both its human counterparts (11/18 identical residues), was recognized by T cells from naturally exposed individuals (17/76). It is interesting to note that it belongs to the same protein region as peptides 4 and 5 (and then peptide 10), which was previously analyzed by Richmann et al. (15) and described as being immunogenic in rabbits as a noncoupled peptide. The presence of human T cell epitopes specific for the *P. falciparum* Pf72/Hsp70-1, in peptide 8, is possibly of interest. Located at the C terminus part of the molecule, it contains a 10-amino acid stretch that is almost totally homologous to both human sequences (9 identical and 1 conservative difference). Thus, it is of prime importance to know if this stretch can by itself constitute a T cell epitope possibly common to the proteins from the host. Analysis of homologies between human and parasitic

heat-shock 70 points to another peptide, the p3 peptide. This peptide presents a very high degree of similarity with the human Hsc70 and Hsp70. Out of the 16 amino acids of p3, 11 are identical to the Hsc70 and 4 conservatively changed (D for E, Q for N). In the case of the Hsp, 10 identical amino acids are noted together with five conservative changes. Despite this similarity, p3 is recognized by a relatively large number of individuals (8/72).

All these results could suggest that during natural infections cross-reactivities between the human and parasitic Hsp70 could occur at the T cell level, as has been suspected (53–55). Indeed, immunologic cross-reactions have been already described at the antibody level in mice (14), and in humans antibodies cross-reacting with the human Hsc70 were described after several natural infections (18). Thus, the next step will be to analyze possible cross-reactivity between these epitopes and corresponding sequences from human heat-shock proteins and from Hsp70 of other unrelated microorganisms. Such a comparative analysis is currently being investigated in the same cohort of individuals.

We very rarely found IFN-γ secretion in the supernatants of PBMC of donors of Dielmo, stimulated by one of the peptides selected from the Pf72/Hsp70, whereas the Pf155/RESA-peptide, under the same conditions, induced IFN-γ in a significant number of individuals as already described (40, 41). Our results suggest that the subset of T cells that is stimulated in vitro in the presence of the native Pf/Hsp70 or in the presence of the selected peptides are not the same. In humans, among different distinguishable T cell subsets, $CD45RA^+$ T cells, usually considered as "naive" T cells are able to produce no or very low levels of IFN-γ or IL-4, whereas $CD45RO^+$ T cells do produce IL-2, IFN-γ, and IL-4 after in vitro stimulation (56). One of the possible explanations for the fact that T cells stimulated by the native Pf/Hsp70 molecule can respond by IFN-γ secretion whereas they are unable to respond upon the stimulation of the tested peptides derived from the Pf72 would be that the native protein, compared with these peptides, would activate preferentially cells already primed in vivo. We also cannot exclude that the peptides lack critical epitopes found in the native protein. Another explanation could be that the native Ag could activate preferentially T cells with a Th1 profile of cytokine secretion whereas the peptides will activate T cells with a Th2 profile independent of the degree of previous in vivo priming. The responders to Pf72 peptides responded more frequently to the native Ag than to the Pf155/RESA peptide, but not all the responders to the peptides did respond to the native Ag. This phenomenon can be attributed to either differences in the ability of APC to process and present peptides and native Ag to the T cells or to the possible presence in the native protein of T cell epitopes that could be suppressors of the proliferative response (57).

The present study clearly shows that in an endemic area of continuous transmission of malaria, humoral and T cell responses to the Pf72/Hsp70-1 are induced. However, it is impossible to ascribe a significant correlation between naturally acquired immunity to the parasite and the presence of Pf72/Hsp70-1-reactive T cells detected by proliferation and IFN-γ secretion. The present data were collected in the context of a longitudinal study; therefore, the evolution of both humoral and cellular responses (antibody isotypes, T lymphocytes subsets, and lymphokine secretion) and their possible correlations with the acquisition of malaria immunity can be analyzed both against the Pf72/Hsp70-1 and the derived peptides.

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