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Cellular and antibody responses to the *Plasmodium falciparum* heat shock protein Pf72/HSP70 during and after acute malaria in individuals from an endemic area of Brazil

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Abstract

Proliferative and antibody responses to three synthetic peptides corresponding to Pf72/HSP70 were followed-up in acute malaria patients from an endemic area of Brazil. In vitro lymphocyte responsiveness to all peptides was relatively low and short-lived and there was a considerable variation in the frequency and magnitude of the individual lymphoproliferative response to the peptides at different periods after the onset of infection. Although 96% of the patients had IgG antibodies to crude *Plasmodium falciparum* asexual blood stage antigens, specific IgG antibody responses to the peptides varied from 12.5 to 40% according to the tested peptides. No significant difference was observed in the proliferative or antibody responses to the peptides between individuals that remained parasitemic after treatment and those that recovered from malaria infection. The different frequencies of proliferative responses in peripheral blood T cells on different occasions after the onset of their infection

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show that, in order to be informative, evaluation of the in vitro cellular immune response to peptides requires longitudinal studies in which each individual is tested repeatedly.

Keywords: Malaria; *P. falciparum*; Heat shock protein

1. Introduction

Heat-shock proteins (Hsps) are the most abundant proteins in living organisms and display a high level of evolutionary sequence conservation. They are classified into several families based on their molecular size and among these families, HSP70 has been found in mammalian cells, yeast, plants, bacteria and parasites (Lindquist and Craig, 1988). In *Plasmodium falciparum*, a m.w. 72 kDa molecule, has been identified as a member of the HSP70 and is extremely well conserved between different plasmodial species (Sheppard et al., 1989; Eckert et al., 1992).

Several studies have shown that HSP70 is expressed in the erythrocytic, liver and sporozoite stages of *P. falciparum* and *Plasmodium berghei* (Bianco et al., 1986; Ardeshir et al., 1987; Yang et al., 1987; Kumar et al., 1993; Tsuji et al., 1994). Immunization in monkeys suggests that certain epitopes in this molecule may induce protection (Dubois et al., 1984) and recent studies have demonstrated that *P. falciparum* Pf72/HSP70 is recognized by antibodies and T-cells obtained from individuals living in endemic malaria areas (Kumar et al., 1990; Behr et al., 1992; Alexandre et al., 1997). In addition, studies conducted in a holoendemic area in Senegal showed that antibodies against native Pf72/HSP70 protein are positively correlated with age and parasite exposure (Behr et al., 1992).

The immunogenicity of this molecule together with its high degree of conservation among different plasmodial species makes the characterization of the immune responses induced by HSP70 of particular interest. As the malaria vaccines presently under consideration are subunit vaccines based on isolated parasite antigen fragments, it is of utmost importance to identify immunologically active epitopes in conserved antigens. In the present work we studied the antibody response and the specific lymphocyte activation to Pf72/HSP70 peptides in malaria patients from endemic areas of the Brazilian Amazon region during and after the acute infection.

2. Material and methods

2.1. Study area and patients

Blood was collected from 32 falciparum malaria patients, 22 males and ten females ranging in age from 11 to 62 years (mean = 28) living in a rural malaria-endemic village in Porto Velho municipality (Rondonia) for less than 4 years. Most of

the individuals (62%) reported their last malaria attack between 1 and 2 months ago and 54% of the patients mentioned more than four episodes of malaria in the past.

Patients were contacted with the cooperation of the local Malaria Control Program Agency (Fundação Nacional de Sade) and after obtaining written consent from the patients or from their parents and taking an oral history, 20 ml of blood were collected before treatment on the day of diagnosis and on two occasions after the onset of their illness. On the day of diagnosis (Day 0) 32 patients participated in the study, 15 days later (Day 15) 25 out of 32 and 30 days later (Day 30) only 13 out of 32 were still being followed up. All patients had mild symptoms and none developed severe malaria. The mean time elapsed between the beginning of malaria symptoms and diagnosis (Day 0) was 4.2 ± 2.3 days and all individuals received an 8-day curative treatment of quinine plus tetracycline (conventional treatment of the Ministry of Health for *P. falciparum*) after blood collection. Parasitemia was evaluated by examination of 500 leukocytes in Giemsa stained thick blood smears. The negative controls used in the proliferative response assay were peripheral blood mononuclear cells (PBMC) from six members of our team and from 19 individuals without a malaria history living in downtown Porto Velho (area considered free of transmission) for a short period of time. Both groups of sera were tested in parallel by ELISA for the presence of antibodies to *P. falciparum* soluble antigen in order to check the usefulness of Porto Velho individuals as negative controls for the lymphoproliferative assays. All of them were found to be negative.

2.2. Synthetic peptides

All three peptides—P5: KLQPAEIETCMKTIT; P6: KNQLAGKDEYEA-KQKEAE and P7: SKIYQDAAGAAGGMPG—were obtained from the Laboratory of Organic Chemistry, Pasteur Institute, Paris, or from Neosystem, Strasbourg, France. All of them were more than 90% pure as evaluated by analytical reverse-phase HPLC. The choice and localization of the peptides in the Pf72/HSP70 sequence has been published elsewhere (Behr et al., 1992).

2.3. Cell proliferation

Plasma was obtained from whole blood after centrifugation and the same volume of RPMI 1640 medium (Sigma) was added. PBMC were purified by density gradient centrifugation (Ficoll–Hypaque) and cell viability was confirmed by Trypan blue staining. The isolated PBMC were incubated on 96 well plates with different stimuli at 2×10^5 cells per well in 200 μl complete RPMI medium supplemented with 3.7 g/l sodium bicarbonate (Grupo Quimica), 2 mM glutamine (Sigma), 10 mM HEPES (Sigma), 100 U/ml penicillin and streptomycin (Sigma), and 10% heat-inactivated human serum AB. The peptides were added at a final concentration of 10 μg/ml and tested in triplicate. Plates were incubated at 37°C in 5% CO_2 for 6 days and 1 μCi of ^{3}H-thymidine was added to each well for the last 18 h of incubation. Cells were harvested onto a glass fiber filter and cellular incorporation of ^{3}H-thymidine was measured by liquid scintillation counting.

Stimulation indices (SI) were calculated from geometric means of triplicates of stimulated wells divided by the geometric means of unstimulated wells. The proliferative response was considered positive when SI were greater than or equal to 2.5 and the difference between the geometric means of stimulated and unstimulated cultures was higher than 1000 cpm.

2.4. Detection of antibodies to Pf72/HSP70 synthetic peptides

Maxisorp plates (Nunc) were coated with 100 μl per well of peptides P5, P6 or P7 at a concentration of 5 μg/ml in PBS and incubated for 3 h at 37°C and overnight at 4°C. The wells were washed three times with PBS-0.1% Tween 20 (T20) and saturated with 200 μl of PBS-0.1%T20-1% BSA for 1 h at 37°C in a humid chamber. After three washes with PBS-0.1%T20, plasmas were diluted 1/100 in PBS-0.1%T20-1% BSA and 100 μl were placed in wells for 1 h at 37°C. The wells were washed three times and 100 μl of peroxidase-labeled goat anti-human IgG or IgM (Zymed) diluted 1/1000 in PBS-0.05%T20 containing 0.1% of BSA were added for 1 h at 37°C. Bound antibodies were detected with 100 μl of the substrate *O*-phenylenediamine (OPD, 10 mg; Sigma) diluted in 25 ml of citrate/phosphate buffer (pH 5.0) containing 10 μl of H_2O_2. The reaction was stopped by the addition of 50 μl of 2 N HCl and the absorbance at 492 nm was recorded on a TiterTeck Multiskan Reader (model II MCC/340 MKII). Samples giving values greater than the mean optical densities (OD) $\pm$ 2 SD of values from 20 malaria-unexposed controls were considered positive (cut off values: P5 = 0.107, 0.195; P6 = 0.132, 0.223 and P7 = 0.125, 0.22 for IgM and IgG, respectively).

2.5. Detection of antibodies to P. falciparum soluble antigens

Parasite antigens were extracted from a *P. falciparum* isolate from Periquitos (Rondonia) maintained in non-synchronous in vitro culture according to the technique of Trager and Jensen (1976). Red blood cells with 6% of parasitemia were washed three times in 20 volumes of PBS, pH 7.2, and the pellet was treated with three volumes of lysis buffer (0.1% saponin in distilled water). After shaking for 15 min at room temperature, 20 volumes of PBS were added and the mixture was centrifuged at 240 × *g* for 20 min at 4°C. Three additional washes were performed and 1 ml of carbonate/bicarbonate buffer, pH 9.6, containing 1 mM of PMSF (phenylmethylsulfonyl fluoride, Sigma) was added to the brown pellet containing the parasite. The antigen extract was sonicated three times (5 min each time) in a Branson CU-6 Ultrasonic Cleaner and centrifuged at 5200 × *g* for 15 min at 4°C. Microplates (Maxisorp) were coated overnight at 4°C with 100 μl of *P. falciparum* antigen (5 μg/ml) diluted in 0.06 M carbonate/bicarbonate buffer, pH 9.6. The plates were washed three times with PBS-0.05%-T20 and blocked with 0.06 M carbonate/bicarbonate buffer, pH 9.6, PBS-T20 containing 5% of skimmed milk (Molico–Nestlé) overnight at 4°C. Plates were washed three times with PBS-0.05%T20, and 100 μl plasma samples diluted 1/100 in PBS-0.05%T20 containing 5% skimmed milk were added and incubated in a humid chamber for 1 h at 37°C.

After three washes a 100-μl of peroxidase-labeled goat anti-human IgG conjugate diluted 1/1000 in PBS-0.05%T20 containing 0.1% of skimmed milk were added. After 1 h incubation at 37°C the plates were washed three times and the reaction was developed using the same protocol for the ELISA described above.

3. Results

3.1. Lymphocyte proliferation

Results in Fig. 1 show that there was a considerable variation in the proportion of responders to the Pf72/HSP70 derived peptides in at least one of the three collections (9.4% for P5, 41% for P6 and 19% for P7). Only two individuals had a proliferative response to all three peptides in a given collection and none presented a persistent response in at least two collections. All tested individuals had a proliferative response to phytohemagglutinin (PHA) in all collections (mean Δ cpm 50589 ± 181183). PBMC from negative controls did not respond to any of the peptides (mean Δ cpm 120 ± 60). Background values obtained with unstimulated PBMC from patients were not different from those recorded for negative controls.

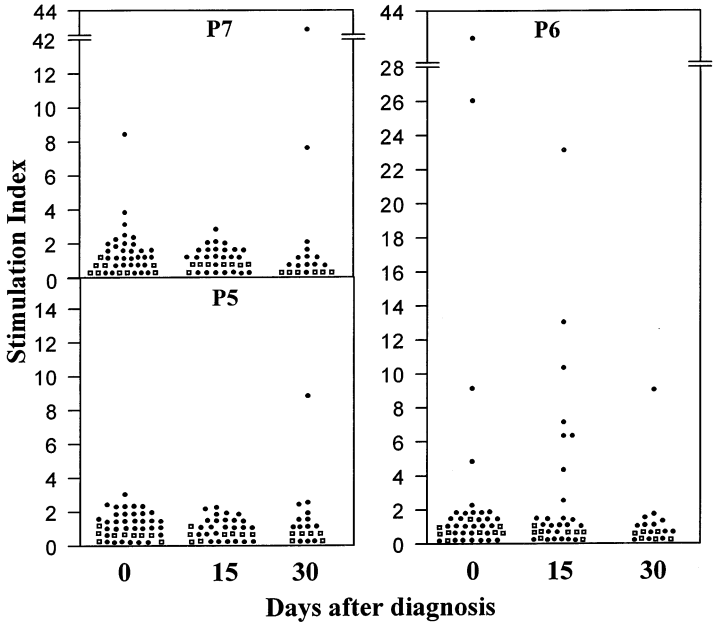

Fig. 1. Lymphoproliferative responses against *P. falciparum* Pf72-HSP70 peptides of patients on different occasions after malaria diagnosis. Each point represents the stimulation index (SI) for one individual (● patients, □ controls). The SI values correspond to the means cpm of triplicates of stimulated wells divided by cpm of unstimulated wells. A proliferative response was considered positive when the SI was ≥ 2.5.

On day 0 all 32 patients were infected with *P. falciparum* and mean parasite density was 7830-parasites/μl (range 487–28555). The frequency of responders was relatively low (3.1% for P5, 12.5% for P6 and 9.4% for P7) and only three of the individuals had SI above 5. No correlation was observed between the SI values and parasitemia levels (P5: $r = 0.12$; P6: $r = 0.28$; and P7: $r = 0.18$). On day 15 the proportion of responders was 32% for P6, 4% for P7 and no response was recorded for P5. At this time when all individuals were supposed to be cured from the malaria infection, ten of the 25 (40%) patients studied were still infected (mean parasitemia—270 parasites/μl of blood). As far as proliferative responses to P6 are concerned, the response tended to be more frequent in parasitemic individuals (40%) than in non-parasitemic subjects (25%) although this result was not statistically significant ($P > 0.05$). Similarly to day 0, no correlation was observed between the SI values and parasitemia levels (P5: $r = -0.18$, P6: $r = 0.11$ and P7: $r = -0.15$). Moreover no difference was recorded between the two groups in mean SI values plus SD (9.1 ± 3.0 and 9.2 ± 9.4). On day 30, only 13 individuals were tested and the percentage of samples presenting a positive response was 15% for P5 and P7, and 8% for P6. Among the ten individuals who were parasitized on day 15, only seven were available for follow up on day 30 and four of them continued to be parasitemic (mean parasitemia 1714 ± 957/μl). Similarly, the six individuals who were negative on day 15 continued to be negative. With respect to the lymphoproliferative response of four individuals who continued to be parasitemic, one responded to all three peptides and one to P7, and among the three non-parasitemic individuals one responded to P5. The treatment failure reported in our study has already been observed in the region by Banic et al. (1998) and could be related to poor compliance to treatment, since supervision of patients was not possible neither in our nor in Banic's study. However, resistance to quinine plus tetracycline has already been reported in Brazil (Alecrim, MG, Instituto de Medicina Tropical de Manaus, Brazil—personal communication—Boulos et al., 1997) and cannot be ruled out at least in some of our patients.

3.2. Antibodies to soluble P. falciparum antigen and peptides

At the time of diagnosis (Day 0) 96% of the individuals had IgG antibodies to soluble *P. falciparum* antigens and the remaining 4% continued to be non-responders on days 15 and 30. In contrast, the responses to peptides P5, P6, and P7 varied according to the tested peptide and to the day of blood collection (Fig. 2). The cumulative frequency for IgM antibodies in patients followed-up with three blood collections ($n = 13$) was very high for P6 and P7 (Fig. 2a) and increased with time after the day of diagnosis. Conversely, IgG antibodies were less frequent and only few individuals became positive after day 15 (Fig. 2b). When the population was considered as a whole ($n = 32$) the frequency of IgM antibodies to P6 and P7 was lower but followed the same pattern (Fig. 2c). The highest frequency of IgG antibody response was recorded for P6 (44%) and few individuals became positive after day 0 (Fig. 2d). No correlation was observed between the levels of antibodies to the peptides and parasitemia levels (P5: $r = -0.07$, P6: $r = 0.24$, P7: $r = 0.05$)

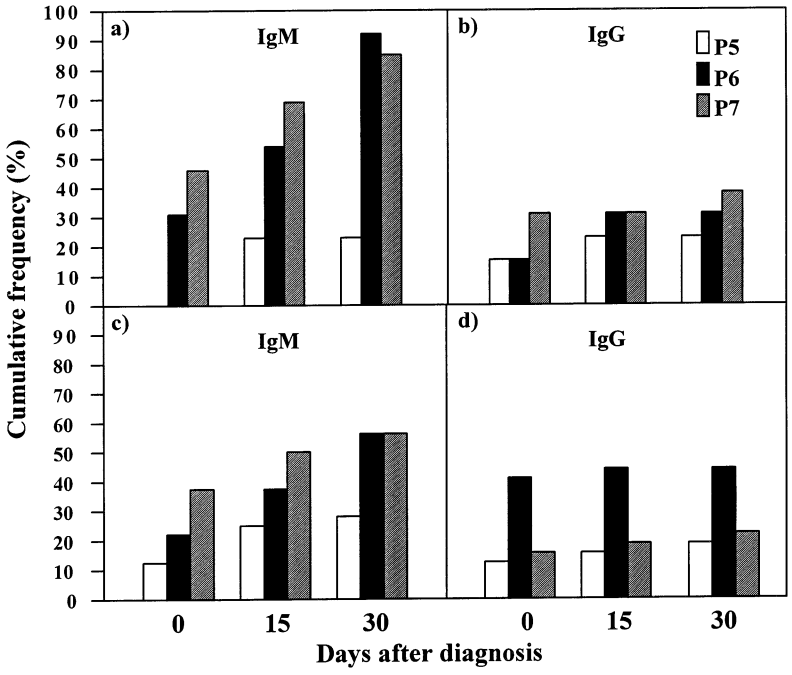

Fig. 2. Cumulative frequency of antibody response to *P. falciparum* Pf72-HSP70 peptides. a and b: represent the cumulative frequency of positive individuals that were followed up over 3 collections ($n = 13$). c and d: represent the cumulative frequency of all tested individuals at each time point.

and no difference was also observed between the frequency of antibodies and the time of residence of migrants in the endemic area or the number of past attacks of malaria ($P > 0.05$). Similarly, no difference was observed between parasitemic and non-parasitemic individuals on days 15 and 30 ($P > 0.05$).

4. Discussion

In this study we evaluated the naturally acquired immune response to Pf72/HSP70 derived peptides during and after acute infection. The specific T cell proliferation was low and short lived and peptide P6 was more frequently recognized. In an African population P6 was also the most recognized, suggesting that this peptide is more immunogenic than P5 and P7 even in populations from different malaria endemic areas with different levels of exposure (Behr et al., 1992). Independently of the Pf72/HSP70 derived peptide used, we found 25% of responders. This frequency does not seem to be in disagreement with the proliferative responses ranging from 6 to 51%, previously reported for other malaria synthetic peptides (Behr et al., 1992; Kabilan et al., 1994; Al-Yaman et al., 1997; Kulane et al., 1997; Banic et al., 1998; Jacobson et al., 1998).

It has been previously suggested (Wyler and Brown, 1977), that the antigen specific in vitro proliferative response is not abrogated during infection; although this may occur in patients with severe, possibly fatal, cerebral malaria (Brasseur et al., 1983). In this context it could be tempting to conclude that data reported here are in agreement with this idea for two main reasons: (i) no difference was observed in the frequency of responders and in the SI values between parasitemic and non-parasitemic individuals or for those with high or low parasitemia; and (ii) at day 15, when all individuals were supposed to be cured and the highest frequency for P6 was recorded, no difference was observed in the proliferative response between parasitemic and non-parasitemic individuals. In addition, similar results for other malaria antigens, (MSP1, MSP2 and Pf155/RESA (Behr et al., 1992; Riley et al., 1993; Kulane et al., 1997)) and even a higher prevalence of proliferative response to one (MSP2) peptide in parasitemic as compared to non-parasitemic individuals (Al-Yaman et al., 1997) have already been reported. In the present work, the mitogen responses were also not suppressed during acute uncomplicated malaria (Day 0), since there were no significant differences in the response to PHA between patients with acute malaria and healthy controls or between parasitemic and non-parasitemic individuals on days 15 and 30. This supports the concept that generalized severe depression of lymphoproliferative responses is not a feature of uncomplicated malaria (Theander et al., 1986). However, in view of the frequency of T cell reactivity recorded to the peptides and the limited number of donors presented here the idea that acute malaria impairs the in vitro specific proliferative response cannot be discarded. In addition one must consider that other factors such as genetic background of donors, reallocation of cells outside the peripheral compartment, low sensitivity of the assays and even an effect of previous chronic malaria exposure could participate to explain the figures recorded here.

In respect to humoral immune responses the antibody frequency to P6 and P7 was relatively high for IgM and the number of responders increased with time after the day of diagnosis but IgG antibodies were very low. Naturally acquired IgG antibodies to the Pf72/HSP70 recombinant protein as well as to peptides of the rhoptry-associated proteins-1 (RAP-1) and RAP-2 reported in other areas of the same region were also relatively low, 26.5 and 17%, respectively (Alexandre et al., 1997; Jacobson et al., 1998). Therefore, since a higher frequency of antibodies to the Pf72/HSP70 recombinant protein was reported in individuals living in the same region for more than 8 years (Alexandre et al., 1997), the low frequency of IgG to Pf72/HSP70 synthetic peptides observed by our group could suggest that these antibodies are directly related to cumulative exposure to malaria.

In the malaria exposed population studied in an endemic area of Brazil, humoral and T cell responses to Pf72/HSP70 are induced, showing the immunogenicity of this Hsp in its natural form. In as much as no consistent tendency of mean level or prevalence of positive responses to vary with the course of infection was recorded, the different frequencies of proliferative responses in peripheral blood T cells on different occasions indicate that, individual responses can vary in an unpredictable manner after the onset of the illness. Since the total number of responders can be underestimated if a single cross-sectional study is done we conclude that in order to

be informative, evaluations of the in vitro cellular immune response to peptides require longitudinal studies in which each individual is tested repeatedly.

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