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Demonstration of heat-shock protein 70 in the sporozoite stage of malaria parasites

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Abstract Three monoclonal antibodies generated by immunization of mice with *Plasmodium berghei*-infected red blood cells were found to react with the 75-kDa heat-shock protein (HSP70) present in liver stages and erythrocytic forms of the parasites. These antibodies were shown to react with a recombinant protein encoding the carboxyl terminal half of PfHSP70 (aa 365–681). Differently from earlier results, we clearly demonstrated that HSP70 was also expressed in the sporozoite stage, using these monoclonal antibodies in an immunofluorescence and Western immunoblot assay. These monoclonal antibodies react not only with sporozoites of *P. berghei*, the parasites originally used for the immunization, but also with sporozoites of several other rodent and human plasmodial species. Passive transfer of these monoclonal antibodies into naive mice, simultaneously injected with sporozoites, failed to neutralize the infectivity of *P. berghei* sporozoites and to inhibit the development of liver stages of *P. yoelii*.

Introduction

Heat-shock proteins (HSPs) display a high level of evolutionary sequence conservation and are classified into several families based on molecular size. Among these families, HSP70 is one of the most abundant and highly conserved HSPs. It has been found in mammalian cells, yeast, plants, bacteria, and parasites (Lindquist and Craig 1988). Although HSPs are major antigens detected in many infectious diseases, their role in these host-parasite relationships remains to be elucidated.

In *Plasmodium falciparum*, a 75-kDa molecule, PfHSP70, has been identified as a member of the HSP70 family (Bianco et al. 1986; Ardeshir et al. 1987; Yang et al. 1987; Kumar et al. 1988; Mattei et al. 1989). Cloning of the genes encoding HSP70 from *P. chabaudi* (Sheppard et al. 1989) and *P. cynomolgi* (Eckert et al. 1992) revealed a near identity between the HSP70 molecules of the different plasmodial species, displaying as much as 97% sequence homology at the protein level. Several studies have shown that HSP70 from *P. falciparum* and *P. berghei* is expressed in the erythrocytic and liver stages of these parasites (Bianco et al. 1986; Ardeshir et al. 1987; Yang et al. 1987; Mattei et al. 1989; Rénia et al. 1990; Kumar et al. 1993). Failure to detect HSP in sporozoites has led to the suggestion that this protein is not expressed in this parasite stage (Bianco et al. 1986; Kumar et al. 1993).

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. A recent study has provided evidence suggesting that PfHSP70 could be a target of antibody-dependent cell-mediated cytotoxicity (ADCC) and that this molecule, or related epitopes, are expressed on the surface of hepatocytes infected with malarial parasites (Rénia et al. 1990). Other studies have demonstrated that plasmodial HSP70 is recognized by antibodies and T-cells obtained from individuals living in malaria-endemic areas (Kumar et al. 1990; Behr et al. 1992).

To characterize further the antigenic properties of plasmodial HSP70, we generated a series of monoclonal antibodies, which are specific for this plasmodial HSP and do not cross-react with the mammalian counterpart. We investigated the expression of HSP70 in different life-cycle stages of *P. berghei* and the presence of shared antigenic determinants among different plasmodial species. We also investigated the effect that these

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monoclonal antibodies may have on sporozoite infectivity and on the development of exoerythrocytic forms of malaria upon their passive transfer to mice challenged with viable sporozoites.

Materials and methods

Parasites and animals

Sporozoites of *Plasmodium berghei* (NK65 strain) and *P. yoelii* (17XNL) were obtained from the salivary glands of *Anopheles stephensi* mosquitoes 2 weeks after the latter had received an infective blood meal on *P. berghei*-infected hamsters and *P. yoelii*-infected mice, respectively (Vanderberg et al. 1968). Sporozoites of human [*P. falciparum* (NF54 strain) and *P. vivax* (Thai strain)] and simian [*P. knowlesi* (H strain)] malaria were obtained from the salivary glands of different species of infected mosquitoes.

BALB/c mice (4–8 weeks old) were used for immunization and adoptive transfer experiments. These animals were purchased from Jackson Laboratories (Bar Harbor, Me.). *P. berghei*-infected hamsters were used as the source of an infective blood meal for mosquitoes and as the source of infected red blood cells for a Western immunoblot assay. Hamsters (6–7 weeks old) were purchased from Charles River Laboratories (Wilmington, Mass.).

Production of anti-HSP70 monoclonal antibodies

BALB/c mice were immunized every 10 days for five times with an antigen extract obtained from *P. berghei*-infected red blood cells (PRBC). The infected red blood cells were lysed by saponin treatment, extensively washed, and sonicated, and the supernatant was used as antigen. The first immunization was given intraperitoneally, with an antigen extract corresponding to 1×10^7 PRBC emulsified in complete Freund's adjuvant. For the subsequent immunizations, the same antigen dose was emulsified in incomplete Freund's adjuvant. At 3 days after the last immunization, immune spleen cells were collected and fused with the nonsecreting BALB/c plasmacytoma P3U1 (Köhler and Milstein 1975). Three hybridomas secreting IgG1 monoclonal antibodies (mAbs) 2E6, 4C9, and 4E4, respectively, were obtained. To obtain ascites, the hybridoma cells were injected into pristane-treated mice, and the respective antibodies were purified using an Affi-Gel Protein A column (Bio-Rad Laboratories, Richmond, Calif.).

Indirect immunofluorescence assay

The immunofluorescence assays were performed as previously described (Nardin et al. 1982). Briefly, sporozoites from several plasmodial species were placed on multispot glass slides, air-dried, and stored at −20° C. After 1 h of incubation with the different antibodies diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (PBS-BSA), the slides were washed with PBS and incubated with fluorescein isothiocyanate-labeled, affinity-purified goat anti-mouse immunoglobulin (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md). After 1 h, the slides were washed and mounted in PBS containing 50% glycerol and 1% (w/v) *p*-phenylenediamine to reduce bleaching.

Western immunoblot assay

Antigen extracts from *P. berghei* erythrocytic forms were prepared from PRBC obtained from infected hamsters. The infected erythrocytes were lysed by saponin treatment, and after an extensive washing, the parasites were disrupted by sonication and parasite extracts were prepared as described elsewhere (Tsuji et al. 1990). Extracts of normal red blood cells (NRBC) were processed similarly. The extracts of sporozoite-infected and noninfected mosquito salivary glands were prepared by directly sonicating the salivary glands obtained by dissection.

Samples of parasite extracts were placed in sample buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.005% bromophenol blue for 10 min. The extracts, corresponding to 2×10^6 parasites/lane, were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% stacking gel and a 7.5% running gel (Laemmli 1970) and then electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, Calif.). This membrane was treated with PBS containing 1% bovine serum albumin (PBS-BSA) and 10% goat serum for 3 h and then incubated with the purified mAbs (50 μg/ml) for 1 h. After being washed with PBS-BSA containing 0.05% Tween-20, the membrane was further incubated with ^{125}I-labeled goat anti-mouse Ig for 1 h and the membranes were autoradiographed at −70° C using an X-Omat AR film and an image-intensifying screen.

Source and expression of the recombinant HSP70 polypeptides of P. falciparum

The generation of the gene fragment (R44) encoding the carboxyl terminal half of PfHSP70 (aa 365–681) has previously been described (Mattei et al. 1989). This fragment was expressed as a fusion protein with the glutathione S-transferase after the transformation of *Escherichia coli* with a recombinant pGEX vector (Smith and Johnson 1988). The R44 fusion protein was purified by affinity chromatography after the incubation of *E. coli* extracts with glutathione bound to Sepharose 4B beads (Pharmacia LKB, Uppsala, Sweden). The fusion protein bound to the glutathione-Sepharose beads was eluted by competition with soluble glutathione (5 m*M*) following the procedures recommended by the manufacturers. As a control, we purified glutathione S-transferase obtained from *E. coli* transformed with parental, nonrecombinant pGEX vector. The purity of these protein preparations was assessed by SDS-PAGE followed by staining with Coomasie blue as described elsewhere (Smith and Johnson 1988).

Radioimmunoassay

Polyvinylchloride microtiter plates were coated with 20 μg of the recombinant HSP70 of *P. falciparum* (R44)/ml. After 2 h of saturation with PBS-BSA, serial dilutions of ascites containing mAbs 2E6, 4C9, or 4E4 were added to the wells, followed by a 1-h incubation. The wells were thoroughly washed with PBS-BSA, and ^{125}I-labeled, affinity-purified goat anti-mouse immunoglobulin (Ig) was added to each well. After 1 h, the wells were washed and the bound radioactivity was measured.

Passive transfer of mAbs against plasmodial HSP70

BALB/c mice were injected intravenously with a 0.2-ml suspension containing 1×10^3 *P. berghei* sporozoites and 300 μg of the respective mAbs. Their parasitemia was monitored daily by peripheral blood smears obtained form the 3rd to the 12th day after challenge. In addition, the effect of these mAbs on the development of liver stages of *P. yoelii* parasites was assessed by measuring the plasmodial ribosomal RNA (rRNA) in the liver of mice challenged with viable sporozoites as described in detail elsewhere (Arreaza et al. 1991). Briefly, mice were injected intravenously with 3×10^5 sporozoites suspended in PBS containing 300 μg of the respective mAbs. Control mice were injected with sporozoites suspended in PBS without antibodies. After 42 h, the livers of the infected mice were excised, the total RNA was purified, and the amount of plasmodial rRNA was determined in a dot-blot hydridization assay using ^{32}P-labeled plasmodial-specific rRNA probes.

Results and discussion

Immunization with blood stages of Plasmodium berghei induces antibodies that react with the HSP of rodent, simian, and human malarial parasites

Three mAbs specific for plasmodial HSP70 were derived from a BALB/c mouse immunized with multiple doses of *P. berghei* blood-stage extracts. These antibodies, 2E6, 4C9, and 4E4, all of the IgG1 subclass, react with erythrocytic forms of *P. berghei* and *P. falciparum* as determined by an indirect immunofluorescence assay using acetone-fixed parasites. Immunoperoxidase staining of in vitro cultured hepatoma cells, which had been infected with *P. berghei* sporozoites, revealed that mAb 2E6 also reacts specifically with liver-stage schizonts (data not shown). SDS-PAGE analysis of extracts of *P. berghei*-parasitized erythrocytes followed by Western immunoblotting revealed that mAb 2E6 recognizes an antigen with an apparent molecular weight of 75 kDa that is present in parasitized red blood cells (Fig. 1, lane 3). No reactivity was observed with an extract obtained from an equivalent number of non-parasitized red blood cells (Fig. 1, lane 4). Similar results were obtained with antibodies 4C9 and 4E4.

Definitive evidence that the three mAbs (2E6, 4C9, and 4E4) recognize plasmodial HSP70 was obtained in a radioimmunoassay performed with a fusion protein (R44) that contains the amino acid sequence 365–681 of PfHSP70. This HSP70 fragment was obtained from *Escherichia coli* transformed with a recombinant pGEX vector, and it is expressed as a fusion protein with glutathione S-transferase (Smith and Johnson 1988). As shown in Fig. 2, mAbs 2E6, 4C9, and 4E4 reacted in a dose-dependent manner with recombinant HSP, whereas no reactivity could be detected when we used a control antigen obtained from *E. coli* transformed with the nonrecombinant pGEX vector. These antibodies did not react with another recombinant PfHSP70 fusion protein containing the amino acid sequences 490–518 (data not shown). An unrelated IgG1 mAb, 3D11, specific for the circumsporozoite (CS) protein of *P. berghei* also did not react with the R44 fusion protein. Taken together, these results clearly demonstrate that mAbs 2E6, 4C9, and 4E4 recognize plasmodial HSP70 and confirm previous work reporting the expression of HSP70 in liver and erythrocytic stages of malarial parasites.

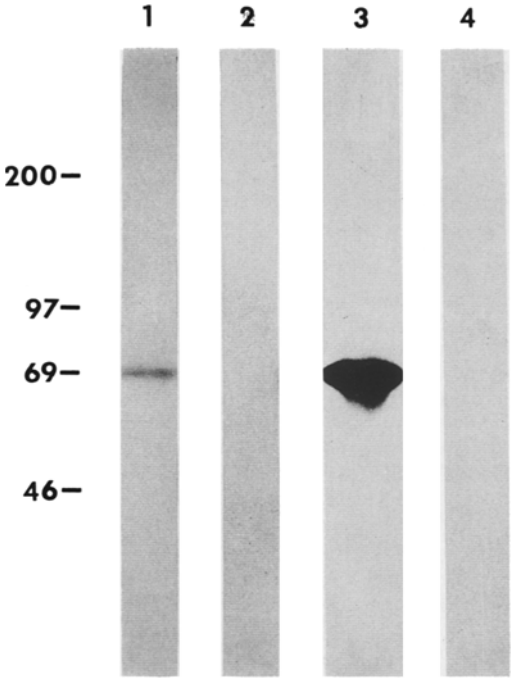

Fig. 1 Western immunoblot assay of *Plasmodium berghei* parasites. *Lanes 1 and 3* were loaded with extracts of 2×10^6 sporozoites and parasitized red blood cells, respectively. *Lane 2* was loaded with an extract of normal salivary glands derived from the same number of noninfected mosquitoes as the infected ones containing 2×10^6 sporozoites (*lane 1*). *Lane 4* was loaded with an extract of normal red blood cells containing an amount of erythrocytes equivalent to the parasitized red blood cells. All lanes were then probed with mAb 2E6. The binding of mAb 2E6 to parasite antigen was assessed by incubation with ^{125}I-labeled goat anti-mouse Ig

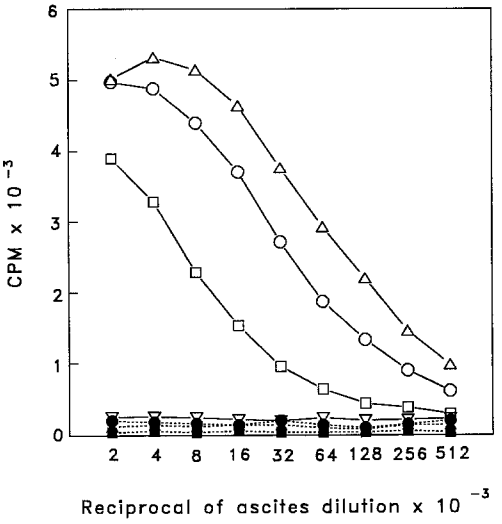

Fig. 2 Binding of antiplasmodial mAbs to recombinant HSP70 of *P. falciparum* (R44)-coated wells. Dilution of ascites of mAbs 2E6 (○), 4C9 (△), 4E4 (□), and 3D11 (▽) were added to wells coated with 20 µg of recombinant PfHSP70 (R44)/ml. As controls, mAbs 2E6 (●), 4C9 (▲), and 4E4 (■) were also added to wells coated with an equivalent protein concentration of extracts obtained from *Escherichia coli* transformed with control pGEX vector. After 1 h, the wells were washed and incubated with ^{125}I-labeled goat anti-mouse Ig

Expression of HSP70 in plasmodial sporozoites

Besides reacting with erythrocytic and liver stages of malarial parasites, the anti-HSP70 mAbs we had obtained also reacted with the sporozoite stage as shown by an indirect immunofluorescence assay using antibody 2E6 incubated with air-dried sporozoites of *P. berghei* (Fig. 3).

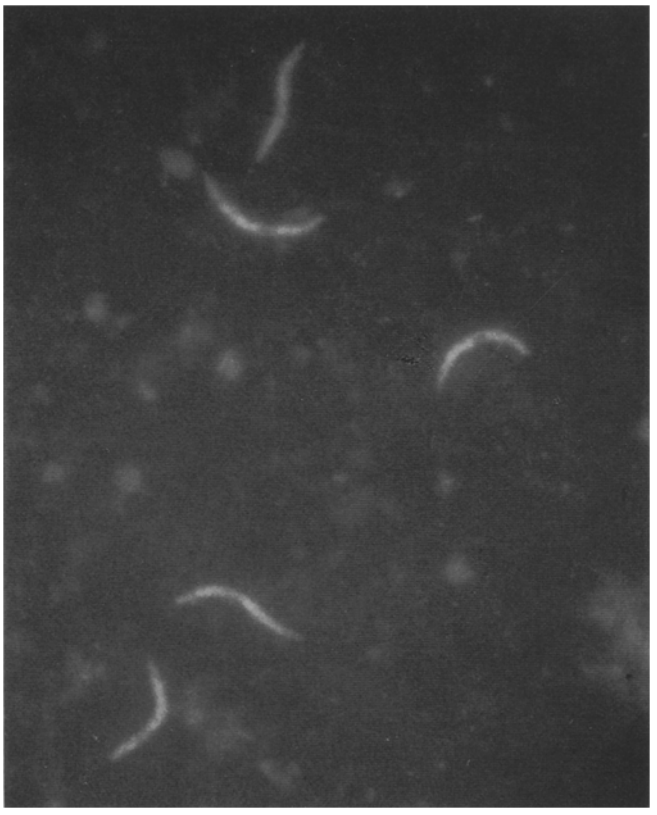

Fig. 3 Indirect immunofluorescence of air-dried sporozoites of *P. berghei* incubated with mAb 2E6. *P. berghei* sporozoites were placed in multispot glass slides, air-dried, and stored at −20° C without further treatment. After sequential incubation with mAb 2E6 and fluorescein isothiocyanate-labeled goat anti-mouse Ig, the preparations were mounted in PBS-glycerol containing *p*-phenylenediamine to reduce bleaching

Furthermore, Western immunoblot assays using mosquito salivary glands infected with sporozoites revealed that these anti-HSP antibodies recognize a 72- to 75-kDa parasite antigen displaying the same molecular weight as the HSP molecule expressed in blood stages (Fig. 1, lane 1). The antigen recognized by these antibodies in the sporozoite extract is parasite-specific, as no reactivity was obtained with an extract obtained from an equivalent number of noninfected mosquito salivary glands (Fig. 1, lane 2). These anti-HSP70 mAbs do not recognize the native CS protein (54/44 kDa) expressed in *P. berghei* sporozoites. Western blot analysis performed with the anti-HSP70 mAbs and also with anti-CS mAbs demonstrated that these antibodies recognize distinct proteins and do not display cross-reactivity (data not shown). Taken together, these results clearly indicate that the HSP is expressed in malarial sporozoites. It is also apparent that as compared with blood stages, sporozoites express significantly lower amounts of this protein. It remains to be determined whether HSP70 is actively synthesized by sporozoites or whether its presence at this stage represents gene expression in earlier sporogonic stages.

Table 1 Immunofluorescence titers of antiplasmodial HSP70 and control monoclonal antibodies incubated with sporozoites of different *Plasmodium* species

Plasmodium species	IFA titer ($\times 10^3$)[a]				
	mAb against HSP70			mAb against CS protein	
	2E6	4C9	4E4	3D11 Pb	2A10 Pf
P. berghei	3.2	3.2	1.6	51.2	Neg[b]
P. yoelii	3.2	3.2	1.6	Neg	Neg
P. falciparum	3.2	3.2	1.6	Neg	25.6
P. vivax	1.6	1.6	0.8	Neg	Neg
P. knowlesi	1.6	1.6	0.8	Neg	Neg

[a] Air-dried sporozoites were incubated with serial dilutions of ascites containing the respective monoclonal antibodies. Antibody binding was revealed using fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin. Neg, Negative
[b] Titer less than 0.2×10^3

These findings differ from the results of previously published studies, which failed to detect expression of HSP70 in *P. berghei* and *P. falciparum* sporozoites (Bianco et al. 1986; Kumar et al. 1993). The negative results obtained using Western blot analysis (Kumar et al. 1993) are most likely attributable to the relatively small number of sporozoites (5×10^4) used by these investigators. In our experiments, we detected HSP70 only when we used an extract of 2×10^6 *P. berghei* sporozoites.

The indirect immunofluorescence assay (Nardin et al. 1982) revealed that these mAbs react with air-dried sporozoites but not with live or glutaraldehyde-fixed parasites, indicating that HSP70, or at least the epitopes of HSP70 recognized by these mAbs, are not expressed on the surface of the sporozoites. This pattern of reactivity contrasts with that observed for anti-CS mAbs, which strongly react with live as well as glutaraldehyde-fixed sporozoites. As shown in Table 1, these mAbs react not only with *P. berghei* sporozoites but also with sporozoites of *P. yoelii*, *P. falciparum*, *P. knowlesi* and *P. vivax*. The immunofluorescence titers obtained by incubating these antibodies with *P. berghei* sporozoites and with sporozoites of various other plasmodial species were similar (Table 1), suggesting that the epitopes recognized by these anti-HSP antibodies are conserved among the different plasmodial species. In contrast, the control mAbs, 3D11 and 2A10, which recognize the repeat domain of the CS protein of *P. berghei* and *P. falciparum*, respectively, reacted only with the sporozoites of the corresponding species. The extensive cross-reactivity observed with the anti-HSP70 mAbs was not unexpected in view of the high degree of conservation of this protein in different plasmodial species (Bianco et al. 1986; Sheppard et al. 1989; Eckert et al. 1992).

Table 2 Lack of effect of anti-HSP70 mAbs on the outcome of a challenge with *P. berghei* sporozoites

mAb	Number of mice infected/ number challenged[a]	Prepatent period (days)
Control	5/5	4.4
2E6	5/5	4.8
4C9	5/5	4.4
4E4	5/5	4.8

[a] All mice were challenged i.v. with 1×10^3 *P. berghei* sporozoites together with 300 μg of the corresponding mAb. Thin blood smears were prepared daily, stained with Giemsa, and examined by light microscopy to detect erythrocytic parasites. All experimental and control mice were dead at 3 weeks after inoculation

Table 3 Lack of effect of anti-HSP70 mAbs on liver-stage development of *P. yoelii*

	mAb	ng of *P. yoelii* rRNA[a] (mean ± SD)
Experiment 1:	Control	15.7 ± 1.2
	2E6	16.7 ± 4.2
	4C9	16.3 ± 3.8
Experiment 2:	Control	28.2 ± 1.5
	4E4	33.0 ± 5.5

[a] All mice were injected i.v. with 3×10^5 sporozoites of *P. yoelii*, and the amount of *P. yoelii* rRNA present in the liver of infected mice was measured at 42 h after the challenge. The mean value ± SD was obtained from three mice

Antiparasite activity of mAbs against HSP70

Previous studies have suggested that anti-HSP70 antibodies might have an antiparasite activity against malarial liver stages by mediating an antibody-dependent cell-mediated cytotoxicity (ADCC) activity (Rénia et al. 1990). To determine whether the mAbs we had obtained could inhibit sporozoite invasion in vivo and/or inhibit the development of liver stages, these mAbs were passively transferred into naive mice, which were simultaneously inoculated with viable *P. berghei* or *P. yoelii* sporozoites. As shown in Table 2, mice injected with *P. berghei* sporozoites together with the respective mAbs (300 μg) as well as mice inoculated only with sporozoites became patent at 4–5 days after sporozoite challenge and died 3 weeks later, indicating the absence of any protective effect of the mAbs.

To determine whether these antibodies could partially inhibit the development of the liver stages, we measured the amount of plasmodial ribosomal RNA (rRNA) in the liver of mice infected with viable *P. yoelii* sporozoites upon the passive transfer of anti-HSP70 mAbs. As shown in Table 3, there was no significant difference between the parasite loads detected in mice injected with mAbs and sporozoites and those found in control mice injected only with sporozoites.

Although these experiments failed to demonstrate that these mAbs affect parasite development, it is nonetheless possible that anti-HSP70 antibodies displaying different epitope specificity and/or belonging to different immunoglobulin isotypes may exert a certain antiparasite activity, as has previously been suggested by other investigators (Rénia et al. 1990).

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