

Blocked hepatic-stage parasites and decreased susceptibility to *Plasmodium berghei* infections in BALB/c mice

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(Received 29 April 1998; revised 21 May 1998; accepted 21 May 1998)

SUMMARY

The BALB/c strain of mice is comparatively more resistant to sporozoite infections of *Plasmodium berghei* than the C57BL6 strain. Infection with live sporozoites results in the formation of small hepatic forms in the BALB/c liver that persist for as long as 6 days. Upon infection with small numbers of sporozoites, some of the parasites are destroyed in the liver whereas the rest persist as blocked forms. When larger numbers of sporozoites are injected the same process occurs but, in addition, a fraction of the liver-stage parasites complete full development and give rise to blood forms. Although blocked liver forms persist until day 6 post-infection they actually develop to only 24 h of maturity. The nature of these persistent forms is similar to those obtained from irradiated sporozoite immunization. There is a stronger cell proliferation to liver-stage antigens by spleen lymphocytes of irradiated sporozoite-immunized BALB/c mice in comparison to that of immunized C57BL6 mice suggesting that a stronger priming to liver-stage antigens, probably due to the presence of blocked hepatic forms in the liver for a longer period of time (as compared to C57BL6), occurs in the BALB/c mice. This could be a reason for the long-lasting protective memory observed in BALB/c mice.

Key words: *Plasmodium berghei*, BALB/c mice, sporozoites, immunity.

INTRODUCTION

Studies on the susceptibility of mouse strains to *P. berghei* sporozoite infections indicate that the BALB/c strain is resistant to infection in contrast to the C57BL6 strain which is relatively susceptible (Jaffe *et al.* 1990), although the mechanisms involved in these observed differences are not fully understood.

Of significant interest are the inherent differences in the ability of gamma-irradiated sporozoites to induce protective immunity in these mice strains. BALB/c mice are relatively easy to protect, requiring fewer numbers and doses of irradiated sporozoites to induce protection against experimental sporozoite challenge. Moreover, this level of protection is long-lived (Winger & Sinden, 1992). In contrast, C57BL6 mice require multiple doses of larger numbers of irradiated sporozoites to induce complete protection which is short lived. The requirement for different immunization schedules for different strains of mice strongly suggests differences in the parasite behaviour inside host hepatocytes.

Another aspect which emerges is that the observed protective memory in BALB/c mice may be a function of long-lived memory cells that are maintained by constant antigenic activation, probably

with hepatic stage antigens. The aim of this study was to determine whether (1) the relative resistance to sporozoite infection in the BALB/c mice is due to prolonged parasite persistence following live sporozoite infection and (2) whether the relative difference in the duration of protective memory after irradiated sporozoite immunization between BALB/c and C57BL6 mice is reflected by the difference in immune responses generated to hepatic-stage antigens.

MATERIALS AND METHODS

Parasite

The *Plasmodium berghei* ANKA strain (Vincke, Bafort & Scheepers, 1966) was used in all the experiments. *P. berghei* ANKA sporozoites were dissected from the salivary glands of *Anopheles stephensi* mosquitoes 21 days after an infective bloodmeal. The ANKA strain is maintained by cyclical transmission in mice and *Anopheles* mosquitoes, in our insectarium.

Maintenance of the vector and sporozoite production

Breeding of *A. stephensi* and development of the sporogonic stages of *P. berghei* ANKA was performed as mentioned before (Chatterjee *et al.* 1996).

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Irradiation and processing of sporozoites

The dose to infected mosquitoes was delivered by ^{60}Co irradiation by means of a teletherapy machine (Theratron-780, courtesy of Mr Schaecken, Middelheim Hospital, Antwerp, Belgium). The method of irradiation has been previously described (Chatterjee *et al.* 1996).

Animals and immunization protocol

Female 6-week-old C57BL6 and BALB/c mice (IFFA Credo, Brussels) were used for inoculation with normal or irradiated sporozoites. Groups of 6 mice were infected i.v. with 500 000 live *P. berghei* sporozoites. In another experiment, groups of C57BL6 mice were immunized 3 times every 2 weeks by i.v. injections of 30 000 irradiated sporozoites. An irradiation dose of 12 krad was selected for the experiment. A separate group of BALB/c mice was immunized with single doses of 10 000 irradiated sporozoites. At 2 weeks after the last immunization, mice were challenged with live sporozoites (20 sporozoites for the C57BL6 mice and 1000 sporozoites for the BALB/c mice). From day 4 post-challenge, thin blood films were made from the tail blood, fixed with methanol, and Giemsa-stained. The numbers of parasites/10 000 erythrocytes were counted and the mean parasitaemia calculated. Protected mice were rechallenged with similar numbers of sporozoites 1 month after the first challenge.

Liver dissection and detection of liver stages

Forty-eight hours after the inoculation with 500 000 live sporozoites intravenously, mice were sacrificed and their livers removed. In separate groups of mice similarly treated with sporozoites, livers were removed at 2, 6, 14 and 22 days post-infection, fixed in Carnoy's solution, embedded in paraffin, and serial paraffin sections (4 μm) cut. The presence of *P. berghei* hepatic stages in liver sections was determined using the Giemsa collophonium staining technique as described before (Wéry, 1968).

Evaluation of number of liver forms

The number of liver stages in the whole liver was calculated by multiplying the mean number of the liver forms in each slide by the volume of the mouse liver ($\cong 1.3 \text{ cm}^3$). The resultant value was divided by the volume of the liver section (mean liver stages \times volume of the liver section/volume of the mouse liver/volume of the section).

Generation of antibodies to liver forms in infected BALB/c mice

Groups of BALB/c mice were inoculated with 150, 300, 500, 1000 and 10 000 live sporozoites and given

chloroquine treatment (25 mg chloroquine/kg) on days 2, 3 and 4. On day 7 all the mice were bled for sera and sera from mice infected with the same dose of sporozoites were pooled and stored at -20°C until required for the immunofluorescent antibody test (IFAT) (Chatterjee *et al.* 1996).

Hep-G2 cells

The *in vitro* development of *P. berghei* sporozoites into hepatic stages was carried out in Hep-G2 monolayers as previously described (Chatterjee *et al.* 1996). Slides were incubated at 37°C and 5% CO_2 for various periods from 24 to 72 h. The cells were then fixed with cold methanol and the parasites counted using 50 μl of optimally diluted test serum/chamber, collected from BALB/c mice infected with different doses of sporozoites. Slides were incubated for 30 min at 37°C . Chamber slides were rinsed with phosphate-buffered saline (PBS), and then incubated either with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulin G, or FITC-labelled goat anti-mouse immunoglobulin M (Diagnostics Pasteur, Paris) diluted 1/100 in Evans blue solution for 30 min at 37°C , and then finally rinsed again with PBS. Slides were mounted with a glass cover-slip with glycerol, and parasites observed under a fluorescent microscope.

Proliferative responses to liver-stage antigens by irradiated sporozoite primed lymphocytes of C57BL6 and BALB/c mice

Spleens were dissected out from groups of C57BL6 and BALB/c mice immunized with irradiated sporozoites at 2 weeks after the last immunization. Spleen cells were extracted into RPMI 1640 medium (Gibco, Grand Island, NY). As a control, spleen cells were also isolated from unprimed spleens of naive animals. Spleen cells were washed twice in the same medium and adjusted to a concentration of 2×10^6 cells/ml in complete RPMI medium containing 2 mM L-glutamine, 25 mM HEPES, 5% heat-inactivated FCS and 5×10^{-5} M mercaptoethanol. Then 200 μl of the cell suspension were deposited in 96-well microtitre plates (Falcon) together with dilutions of *P. falciparum* CS protein and LSA-1 protein-based peptides. The peptides used were: a 32-mer based on the CS protein region II based sequence 331–362; a linear peptide and a multiple antigen peptide (MAP) based on the LSA-1 protein sequence 1742–1760; and a linear peptide representing a hybrid of the LSA-1 repeat sequence: EQQSDLEQERLAKEKLEQEQSDLEQERRAKEKLQ. All peptides were provided by Dr V. S. Chauhan, ICGB, New Delhi. Test wells were assayed in triplicate. Cells were incubated with peptide for 2 days at 37°C in a 5% CO_2 incubator. Eighteen hours before harvest, 0.5 μCi of [^3H]TdR (Amersham, Sydney, Australia) was added to each

well. At the end of this period wells were harvested and the uptake of ^3H estimated by liquid scintillation counting using a LKB beta plate system (Pharmacia, Finland).

RESULTS

Number of liver-stage parasites developing from live sporozoites at 50 h post-infection in C57BL6 and BALB/c strains of mice

At 50 h post-sporozoite infection (with 500 000 sporozoites), the average number of mature liver stage parasites/section in the BALB/c mice was calculated to be 0.048, the estimated total number of liver forms/BALB/c liver was 156 and the percentage of normal development of sporozoites was calculated to be 0.03%. In contrast, the average number of mature liver-stage parasites/section in the C57BL6 liver was calculated to be 4.53, the estimated total number of liver forms/infected liver was 14 722 and the percentage parasite development was calculated to be 2.94%. Thus the C57BL6 strain of mice was of the order of about 100 times more susceptible to hepatic-stage infection as compared to the BALB/c strain.

Persistence of liver-stage parasites in BALB/c mice hepatocytes

BALB/c mice inoculated with 500 000 live sporozoites were sacrificed on days 2, 6, 14 and 22. The numbers of liver-stage parasites at each time-point were counted in Giemsa-stained sections. In 38 sections screened on day 2, 4 hepatic-stage parasites were found. On day 6, 8 hepatic stage parasites were counted in 43 sections. In 30 and 24 sections screened on days 14 and 22 respectively, no parasites could be identified. Thus liver-stage parasites were observed until day 6 post-infection in BALB/c mice. In liver sections the hepatic schizonts on day 2 varied in size between 22.75 and 28.00 μm . In contrast, the hepatic schizonts found in day 6 sections were smaller, measuring only 8.75 μm .

Generation of antibodies to blocked liver forms in BALB/c mice

Serum antibodies generated in BALB/c mice infected with different doses of sporozoites were tested for reactivity to liver stage parasites developing in Hep G2 cells at different times of maturation varying from 24–72 h. Sera from mice that received a single infection were screened for reactivity to liver-stage parasites using anti-mouse IgM conjugated to FITC, as the second antibody in IFAT. Sera from mice that had received 3 infections were screened for reactivity to liver-stage parasites using anti-mouse IgG conjugated to FITC. A

monoclonal antibody 3D11, specific for the CS protein repeat sequences of *P. berghei*, was used as a positive control while normal mouse serum was used as negative control.

Sera from mice that received a single sporozoite inoculation showed the following pattern of reactivity: infection with 150 and 300 sporozoites generated IgM antibodies up to 24-h-old liver stages but not to 48-h-old liver stages. Sera from mice infected with 500, 1000 and 10000 sporozoites showed IgM reactivity to liver forms at all stages of maturation.

Sera from mice that received 3 inoculations showed the following pattern of reactivity: infection with 150 and 300 sporozoites did not generate IgG antibodies to liver forms at 24 nor at 48 h of maturity. Sera from mice infected with 500 and 1000 sporozoites showed IgG reactivity to liver forms at 24 h of maturity but not at 48 h, while sera from mice infected with 10000 sporozoites showed IgG reactivity to liver forms at all stages of maturation. The control antibody 3D11 showed reactivity to liver stages at all hours of maturity.

In all experiments normal mouse serum showed no reactivity to hepatic-stage parasites. Blood smears throughout were negative because of chloroquine treatment.

The protective status generated upon immunization with irradiated sporozoites in C57BL6 and BALB/c strains of mice

Once host protection following immunization with 12 krad irradiated sporozoites was observed, repeated sporozoite challenges were carried out in the immune C57BL6 and BALB/c mice to determine their respective protective memories. Challenge was carried out with the minimum infective dose relevant to the mouse strain; 20 sporozoites for the C57BL6 mice and 1000 sporozoites for the BALB/c strain. The C57BL6 mice already began to lose protective immunity by 2 months post-immunization. The second live sporozoite challenge resulted in blood-stage parasitaemia in 2 of the 8 immunized animals. In contrast the BALB/c mice maintained complete protective status after 3 live sporozoite challenges with 1000 sporozoites, at 3 months post-immunization.

Proliferative responses to liver-stage antigens by irradiated sporozoite primed lymphocytes of C57BL6 and BALB/c mice

Spleen cells obtained from groups of immune C57BL6 mice (that had received 3 immunizations with 30000 irradiated sporozoites) and immune BALB/c mice (that had received 1 immunization with 10000 irradiated sporozoites) were stimulated with dilutions of mitogen, concanavalin A (Con A)

Table 1. Stimulation index values obtained by triggering irradiated sporozoite-primed spleen cells of C57BL6 and BALB/c mice with CS and LSA-1-based peptides

Triggering antigen	Stimulation Index	
	C57BL6	BALB/c
Con A (1 µg/well)	5.2	12.1
LPS (1 µg/well)	11.9	10.6
CS region II-based peptide (2 µg/well)	2.2	4
LSA-1-based linear peptide (2 µg/well)	1.5	3.2
LSA-1-based MAP (1 µg/well)	1.4	3.6
LSA-1-based repeat peptide (1 µg/well)	1.2	2.8

and lipopolysaccharide (LPS) or specific antigen (CS protein and LSA-1 based peptides). As a control, spleen cells were also isolated from naive animals and stimulated in the similar way. All test wells were assayed in triplicate. The uptake of ³H estimated by liquid scintillation counting was a measure of the antigen-specific proliferation by the spleen lymphocytes that had previously been primed *in vivo* with irradiated sporozoites. Results are depicted as stimulation index (cpm obtained upon antigen stimulation of primed spleen cells/cpm obtained upon homologous antigen stimulation of naive spleen cells), and shown in Table 1.

DISCUSSION

Blocked liver forms in infected BALB/c and resistance to sporozoite infections

In the BALB/c liver inflammatory responses are generated as soon as 24 h post-infection (Khan & Vanderberg, 1991), and this has been suggested to be responsible for the almost 100 times increased susceptibility of C57BL6 mice to sporozoite infections as compared to the BALB/c mice. We have not observed any inflammatory responses in infected C57BL6 liver sections after sporozoite infection. Thus even if the sporozoite invasion rate is the same between the 2 mice strains, once inside the hepatocytes a major part of the young developing liver forms may be destroyed in the BALB/c liver due to a non-specific inflammatory response, a phenomenon that does not occur in the C57BL6 liver. This is believed to be the reason why a larger number of sporozoites are required to initiate a blood infection in the BALB/c strain of mice. Our results, however, lead to a different conclusion. We observed persistent liver forms in the BALB/c liver even at 6 days post-infection. This is unusual since under normal situations the hepatic schizogony of *P. berghei* lasts about 54 h. The persistence of small, blocked,

hepatic-stage parasites for extended periods of time may be a reason for making this mouse strain so resistant to sporozoite infections. Upon infection with suboptimal numbers of sporozoites, the parasites enter the hepatocytes, where some of the hepatic stages are destroyed by inflammatory responses. The rest develop further but are blocked, probably by the same inflammatory response, and therefore persist in the liver (in our observations for as long as 6 days). This observation mimics the persistent, blocked hepatic forms developing from irradiated sporozoites in the liver, that is now believed to induce protective immunity (Scheller & Azad, 1995).

The blocked liver forms are developed to about 24 h of maturity

To identify the developmental stage of the blocked hepatic forms in BALB/c liver sections, serum antibodies (IgM raised after 1 infection and IgG raised after 3 immunizations) were tested for reactivity to hepatic stages at different hours of maturation in Hep G2 cells *in vitro*. Infection with a low sporozoite load (150 and 300) resulted in IgM antibody generation to young 24-h-old forms only. Similarly repeated infections with 500 or 1000 sporozoites generated IgG with reactivity to 24-h-old hepatic forms. Upon infection with a lower number of sporozoites, partial development occurs to give rise to young hepatic forms in the BALB/c liver that may not develop beyond 24-h-old forms. This is partly due to the fact that the sporozoite load was small, and the effect of the non-specific inflammatory responses. However, upon infection with a large number of sporozoites (10000) development of all the stages of the parasite occurs, generating antibodies to the different stages. Some forms may have perished, a few others possibly developed further but were blocked, whereas the rest completed development to maturity and gave rise to blood-stage infections. The inflammatory response maintained by the blocked forms may be insufficient or too specific to confer protection to blood stages.

Immune response to liver-stage antigens may be responsible for the long-term protective memory in irradiated sporozoite-immunized BALB/c mice

To further test the hypothesis that blocked hepatic stages are responsible for inducing resistance to sporozoite infections, we immunized groups of C57BL6 and BALB/c mice with the optimal schedule of irradiated sporozoites in order to obtain protection upon challenge. We observed a short-lived protective memory in the C57BL6 mice as compared to the BALB/c mice. It is possible that in the C57BL6 mice the blocked hepatic forms arising from the irradiated sporozoites do not persist as long

as the forms developing from irradiated sporozoites in the BALB/c mice. The possibility that blocked hepatic forms are responsible for expressing protective epitopes that maintain host protection was checked by studying the cellular proliferative responses generated to sporozoite and liver-stage antigens by spleen lymphocytes of irradiated sporozoite-primed C57BL6 and BALB/c animals. A significantly stronger response was observed to LSA-1-based peptides (to linear non-repeat and repeat sequences and non-repeat peptide based MAP) by BALB/c lymphocytes. In comparison the response generated by the C57BL6 spleen lymphocytes to LSA-1-based peptides was insignificant. Proliferative responses to the region II-based conserved sequences of the CS protein was significantly higher in the BALB/c lymphocytes as compared to the C57BL6.

Taken together it appears that a strong priming of memory T cells to sporozoite and liver-stage antigens occurs upon immunization with irradiated sporozoites in BALB/c mice, due to continued expression of antigens by the blocked hepatic forms in the liver. Such memory cells can be expanded upon *in vitro* restimulation with peptide antigens. Thus both in the case of live sporozoite infection or irradiated sporozoite immunization in BALB/c mice, the persistent, developmentally young hepatic-stage parasites may be responsible for the high resistance and long-lasting protective memory, further stressing the need for hepatic-stage antigens to generate resistance and the presence of live parasites to confer protection.

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