Plasmodium berghei development in irradiated sporozoite-immunized C57BL6 mice

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SUMMARY

The C57BL6 strain of mice is highly susceptible to *Plasmodium berghei* sporozoite infections and consequently requires repeated immunizations with irradiated sporozoites to obtain protective immunity. After a live sporozoite challenge in the immunized hosts, hepatic-stage parasites found in the liver after 48 h are of different sizes – small schizonts corresponding to blocked forms (derived from irradiated sporozoites), and schizonts of intermediate size (derived from live sporozoites). Large schizonts corresponding to mature hepatic forms are found only in unimmunized but challenged C57BL6 mice. Using monoclonal and polyclonal antibodies directed to liver-stage parasites, different patterns of binding reactivity to the above forms are observed. More than 20% of the irradiated sporozoites transform into blocked forms after immunization and persist in the liver. Upon sporozoite challenge in such immunized animals the rate of transformation of sporozoites into hepatic parasites is less than 2%. These observations shed light on the fate of live sporozoite development in irradiated sporozoite-immunized C57BL6 mice.

Key words: *Plasmodium berghei*, C57BL6 mice, sporozoites, immunity.

INTRODUCTION

Immunization with irradiation-attenuated *Plasmodium berghei* sporozoites protects mice strains to a live sporozoite challenge (Nussenzweig *et al*. 1967). The C57BL6 strain of mice is highly susceptible to *P. berghei* sporozoite infections and requires at least 3 immunizations with 30000 irradiated sporozoites each time, to be protected against a live sporozoite challenge (Jaffe, Lowell & Gordon, 1990). In the immunized host immature liver-stage parasites derived from irradiated sporozoites persist in the liver for a period of time (Scheller, Stump & Azad, 1995). These persistent pre-erythrocytic forms in the immunized host are thought to play an important role in the induction and maintenance of immunity (Scheller & Azad, 1995). The immune response induced by irradiated sporozoites and mediated by antibodies, T cells and gamma-interferon (Nardin & Nussenzweig, 1993; Schofield *et al*. 1987; Chatterjee *et al*. 1996), influences the evolution of live sporozoites in the immunized host. However, this process is still largely unknown. We report here the results of a study on the hepatic forms observed in C57BL6 mice after the injection of irradiated and live sporozoites. Groups of susceptible C57 black mice were immunized with irradiated sporozoites. Immunized mice were challenged with high doses of *P. berghei* sporozoites. The development of hepatic forms originating from irradiated and live sporozoites was studied in liver samples using a panel of different antibodies.

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MATERIALS AND METHODS

Parasite

The *Plasmodium berghei* ANKA strain (Vincke *et al*. 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 in mice by cyclical transmission through *Anopheles stephensi* mosquitoes in our insectarium.

Maintenance of the vector and sporozoite production

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

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 Mr Schaeken, Middelheim Hospital, Antwerp, Belgium). The

Table 1. The size of *Plasmodium berghei* liver-stage parasites developing from irradiated and live sporozoites at 48 h post-challenge in immunized C57BL6 mice

Experimental group:	In Giemsa	In IFAT
1	8 μm	6–8 μm
2 and 3	8, 15 μm	8, 15 μm
4 and 5	30 μm	30 μm

method of irradiation has been described before (Chatterjee *et al*. 1996). An irradiation dose of 12 krad. was selected for our experiments.

Animals and immunization protocol

Female, 6-week-old C57BL6 mice (IFFA Credo, Brussels) were used for immunizations. Fifteen mice were immunized every 2 weeks by intravenous injection of 30000 irradiated sporozoites of *P. berghei* into the tail vein. In total, 3 immunizations were given. One week after the last immunization the mice were divided into 3 groups. Group 1 remained unchallenged, groups 2 and 3 were challenged with 200000 and 1 million live sporozoites respectively. In addition 2 groups of 5 immunologically naive mice were infected with 200000 (Group 4) and 1 million (Group 5) live sporozoites respectively. The sporozoite viability was checked by injecting 2 naive mice with 100 live sporozoites. Thin blood films were made from the tail blood starting from day 5 p.i., fixed with methanol and Giemsa stained. The number of parasites per 10000 erythrocytes was counted and the mean parasitaemia calculated every 2 days.

Liver dissection and detection of pre-erythrocytic stages

Forty-eight hours after the live sporozoite challenge all immunized mice were sacrificed and their livers removed and fixed in Carnoy's solution or quick frozen in liquid nitrogen. Serial frozen or paraffin sections (4 μm) were cut. The presence of *P. berghei* hepatic stages in liver sections was determined using the Giemsa collophonium staining technique as described before (Wéry, 1968), and by an immunofluorescence antibody test (IFAT). For IFAT the following antibodies were used – a monoclonal antibody specific for the *P. berghei* circumsporozoite (CS) protein repeat sequence (3D11) (Del Giudice *et al*. 1987), a monoclonal antibody specific for the *P. berghei* liver-stage protein (anti-PbL1) (Suhrbier *et al*. 1990), a polyclonal antibody generated to *P. berghei* sporozoite and liver stages in rabbit and a polyclonal antibody generated to *P. berghei* blood stages in mice.

IFAT

Slides with frozen liver sections were rehydrated with milk powder solution (5%) and rinsed 4 times with phosphate-buffered saline (PBS). Fifty μl of normal mouse serum diluted 1/10, anti-sporozoite and liver-stage serum (generated in rabbit and diluted 1/10), anti-blood stage serum (generated in mouse and diluted 1/100), and 1:100 diluted monoclonal antibodies (anti-PbL1 and 3D11 at 50 μg/ml) were added on different slides and the slides were incubated for 30 min at 37 °C in a humid chamber. After 4 washes with PBS, slides were incubated with fluorescein isothiocyanate-labelled anti-mouse immunoglobulin G (Diagnostics Pasteur) or anti-rabbit immunoglobulin G (Cappel) diluted 1:100 in Evans blue solution, incubated for a further 30 min and rinsed again with PBS. Slides were mounted with a glass cover-slip using glycerol medium and the hepatic stages were observed under a fluorescent microscope ($\times$ 50 magnification).

Evaluation of the number of hepatic forms

The number of hepatic stages in the whole liver was calculated with the following formula: (mean hepatic stages per section $\times$ volume of mouse liver)/volume of section.

The volume of a C57BL6 liver was estimated as 1·3 cm^3.

RESULTS

The size of liver-stage parasites developing from irradiated and live sporozoites at 48 h post-challenge in immunized C57BL6 mice

The mean size of hepatic schizonts was different according to the groups of mice. Small schizonts (mean diameter 8 μm, corresponding to blocked hepatic forms) were found in mice immunized with irradiated sporozoites but not challenged (Group 1). Schizonts of intermediate size (mean diameter 15 μm, corresponding to live sporozoites developed in immunized mice), as well as small schizonts were found in the livers of immunized and challenged mice (Groups 2 and 3). Large schizonts (30 μm) were found only in non-immunized mice (Groups 4 and 5). Similar observations were observed in liver sections stained by IFAT (Table 1).

The number of liver-stage parasites developing from irradiated and live sporozoites at 48 h post-challenge in immunized C57BL6 mice

Anti-PbL1 staining allowed the detection of hepatic 'blocked' forms, thus the quantitative evaluation of the total number of liver-stage parasites after immunization and 48 h post-challenge was performed using this antibody (Table 1). The blocked

Table 2. The number of *Plasmodium berghei* LS (liver stage) parasites derived from irradiated and live sporozoites (mean ± S.D.) at 48 h post-challenge in immunized mice livers

	Group 1	Group 2	Group 3	Group 4	Group 5
Total number of LS/liver	24679 ± 3498·7	27112 ± 4925·61	28218 ± 4822·86	5195 ± 1544·96	9567 ± 398·1
Number of LS/cm² counted with:					
3D11	4·5 ± 0·6	5·0 ± 1·0	7·7 ± 0·5	2·0 ± 0·5	3·9 ± 0·4
anti-PbL1	7·8 ± 1·2	7·4 ± 0·7	9·0 ± 0·8	1·5 ± 0·3	2·9 ± 0·4
RS*	2·7 ± 0·6	2·9 ± 0·8	3·8 ± 0·5	1·3 ± 0·2	1·7 ± 0·2
BS†	0	1·0 ± 0·2	2·0 ± 0·3	1·7 ± 0·6	3·8 ± 0·2

* Serum antibodies raised in rabbit to sporozoite and liver-stage parasites.
† Serum antibodies raised in mice to blood stages of the parasite that can label only mature LS.

forms (developing from irradiated sporozoites) detected by anti-PbL1 antibody, were present in greater numbers in immunized mice liver as compared to the number of schizonts developing from live sporozoites in control groups. In Group 1, 28 % of irradiated sporozoites developed into schizonts whereas in the control groups (4 and 5) only 1–3 % of live sporozoites developed into schizonts. More challenge-derived schizonts were found in control Groups 4 and 5 than in Groups 2 and 3. The rate of transformation of sporozoites into hepatic stages was 1·9 % for Group 2, 0·8 % for Group 3, 3·3 % for Group 4 and 1·3 % for Group 5.

Binding reactivity of antibodies to hepatic-stage parasites

The number of hepatic forms identified in liver sections at 48 h post-challenge is shown in Table 2. Not all the antibodies showed similar reactivity to hepatic-stage parasites. Using a panel of antibodies directed to the sporozoite, liver and blood stage of the parasite the following binding patterns were observed. (1) Irradiated sporozoite-derived hepatic forms – 3D11 showed peripheral reactivity, the fluorescence was homogeneous using anti-PbL1, and uneven using serum raised to sporozoite inoculation in rabbit. The blocked forms were not labelled by the polyclonal blood-stage serum. (2) Challenge-derived hepatic forms – a peripheral fluorescence with anti-PbL1, uneven using rabbit serum and homogeneous with 3D11. These parasite stages were labelled by anti-blood stage serum and showed peripheral fluorescence. (3) Mature hepatic forms – the fluorescence pattern using anti-PbL1 and 3D11 was peripheral, using anti-blood stage serum and rabbit serum it was uneven.

DISCUSSION

The fate of live sporozoites developing in irradiated sporozoite immunized C57BL6 mice

In this study, using IFAT and Giemsa-collophonium staining, we found different liver-stage parasites in irradiated sporozoite-immunized and subsequently challenged mouse liver that show differences regarding size, antigen expression, fluorescent pattern and number. In immunized mice we found small schizonts 9 days after the last boost. These forms can thus be termed 'blocked', since during normal sporozoite development the mature liver stages leave the liver after about 54 h. These immature liver-stage parasites were stained using anti-PbL1 in IFAT, but not with anti-blood stage antibodies. Anti-PbL1 monoclonal antibody is known to show reactivity to antigens expressed on early liver stages (at 24 h of maturity) (Suhrbier *et al*. 1990), whereas anti-blood stage antibodies label antigens present on mature liver stages (Suhrbier *et al*. 1989).

In immunized and challenged mice we observed 'blocked forms' and forms of intermediate size, whereas in the control unimmunized groups only mature hepatic forms were observed. The number of 'blocked forms' counted was greater as compared to the number of schizonts derived from live sporozoites in control groups. In fact, 28 % of the irradiated sporozoites developed into schizonts whereas only 1–3 % of live sporozoites developed into mature schizonts in control groups. Normally a proportion of the live sporozoites that penetrate into the liver are destroyed during the hepatic development. Only a few sporozoites (2 % in C57BL6) reach the mature forms and generate the subsequent blood infection. Irradiated sporozoites do not develop into mature hepatic-stage parasites, but persist as immature forms in the liver. Possibly these immature forms derived from irradiated sporozoites, express antigens that are crucial for inducing host protection. In immunized mice the challenge-derived schizonts develop to a size intermediate between 'blocked forms' and mature schizonts, and represent a number that was smaller as compared to the number of schizonts developing in control groups. The immune response generated from irradiated sporozoites can probably initiate either (1) the reduction of live sporozoite penetration into the liver, (2) the decrease in the number of challenge-

derived schizonts and/or (3) the inhibition of the complete development of schizonts derived from live sporozoites.

Our previous experiments have shown the presence of an antibody boost upon live sporozoite challenge in irradiated sporozoite-immunized C57BL6 mice (Chatterjee, Druilhe & Wery, 1999; Ngonseu, Chatterjee & Wery, 1998). This antibody boost was observed only after challenge and not after immunization, and was specific for liver-stage parasites. Repeated immunizations with a large number of irradiated sporozoites can generate anti-liver stage responses, indicating T–B cell interactions and generation of antibodies and memory T cells to antigens expressed by blocked liver forms. Upon liver sporozoite challenge in such mice, novel B cell epitopes may be expressed by liver forms developing from live sporozoites which, in cooperation with the memory T cells generated during immunizations, could generate an antibody boost. Further studies will be necessary to find out if the intermediate hepatic-stage parasites developing in irradiated sporozoite-immunized C57BL6 mice after live sporozoite challenge are indeed expressing the epitopes that are inducing this liver-stage specific antibody boost.

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