Published in Parasite Immunology 19, issue 3, 145-148, 1997
which should be used for any reference to this work

Elimination of *P. berghei* liver stages is independent of Fas (CD95/Apo-I) or perforin-mediated cytotoxicity

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SUMMARY

Immunization of mammals with irradiated malaria sporozoites protects from a subsequent contact with the parasite. Protective immunity is directed against the pre-erythrocytic stages of the parasite, sporozoites and liver stages. Specific antibodies neutralize part of the infectious sporozoites injected by the mosquito vector, while liver stages are the target of a cellular immune response which is mediated by T cells. In this study, we evaluated the T-cell dependent protection induced by the injection of P. berghei *irradiated sporozoites and the contribution of perforin and of the receptor/ligand system CD95/CD95L, two T cell-dependent mechanisms known to mediate elimination of target cells. Wild type, perforin deficient, CD95 mutant, CD95L mutant and perforin deficient/CD95L mutant mice were immunized with* P. berghei *irradiated sporozoites and submitted to a challenge with infectious sporozoites. All mice immunized with* P. berghei *irradiated sporozoites were protected against a sporozoite challenge, including perforin deficient/CD95L mutant animals. These results indicate that T cells do not kill malaria-infected hepatocytes via one of the known pathways, but rather that activated parasite-specific T cells produce cytokines which activate in cascade other mechanisms responsible for the intracellular elimination of the parasite.*

Keywords P.berghei, *perforin, CD95/Apo-I, liver stage, CTL*

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Received: 20 June 1996
Accepted for publication: 29 November 1996

INTRODUCTION

One of the goals of malaria vaccine research is to generate a vaccine against pre-erythrocytic stages of the parasite, sporozoites and liver stages, that mimics the protection induced by injection of attenuated sporozoites. The cellular immune response so generated is mainly directed against the liver stage of the parasite. It has been shown that $CD8^+$ and $CD4^+$ T cells play a role in protection (Nardin & Nussenzweig 1993). However, the mechanisms by which T cells eliminate the parasite are not well defined and it has never been formally proved that T cells lyse the infected hepatocytes.

T cells use two main mechanisms to kill target cells. Upon recognition of a target cell expressing MHC I/peptide complexes, $CD8^+$ T cells release perforin and granzyme. A second mechanism is mediated by the interaction of a receptor and its ligand, Fas (CD95) on the target cell and Fas ligand (CD95L) which is expressed after activation on the effector T cell. Both pathways induce apoptosis of the target cell (Kägi *et al.* 1994, Lowin *et al.* 1994).

In this study, C57BL/6 ($H\text{-}2^b$) mice were used. Wild-type [WT], perforin-deficient [Perf −/−] (Lowin *et al.* 1994), Fas mutant [Lpr], FasL mutant [Gld] (Bhandoola *et al.* 1994) and double deficient mice [Perf−/ − × Gld] (Braun *et al.* 1996) were immunized with irradiated sporozoites to evaluate the involvement of these two systems in T cell-dependent elimination of malaria-infected hepatocytes (White *et al.* 1996).

WT mice were purchased from Harlan (Zeist, NL). Lpr, Gld, perforin deficient and double deficient mice were bred in the animal facilities of the Institute of Biochemistry. Lpr and Gld mice were originally obtained from The Jackson Laboratories (Bar Harbor, ME, USA). Perforin, Lpr and Gld deficient/mutant animals were obtained from parents homozygous for one locus. Double deficient mice were obtained after cross-breeding of parents homozygous for both loci.

Deficient animals are routinely checked as already described elsewhere (Braun *et al*. 1996).

P. berghei (ANKA strain clone 1) sporozoites were produced by feeding laboratory-bred *A. gambiae* mosquitoes on infectious mice. Infected mosquitoes were irradiated (10 000 rads) and then anaesthetized with ether to dissect salivary glands. Sporozoites were counted, resuspended in TBS buffer (pH 7·2) and then injected into the tail vein of C57BL/6. Mice (6–10 weeks old) were boosted eight days after the first injection and one week later challenged by natural (mosquitos) or artificial injection of sporozoites (isolated from salivary glands). Finally, parasitaemia was assessed regularly by Giemsa-stained bloodsmears. Mice were considered protected when no parasites were detected 14 days after the challenge.

We first determined whether all types of mice used in this study could be infected with *P. berghei* sporozoites. Mice were injected intravenously with 400 infectious sporozoites. C57BL/6 mice are susceptible to sporozoite-induced infection. Some mice became positive on day 4 after challenge, while most of them had detectable parasites on day 6. The development of the disease is very similar in all groups of mice tested. Blood forms were detectable approximately at the same time and this was accompanied by acute symptoms of malaria (Table 1).

Mice exhibiting a defect in one or in both mechanisms were then immunized with *P. berghei* irradiated sporozoites to assess the contribution of perforin and Fas in the CTL-dependent killing of the parasite in the hepatocytes. As shown in Table 2, all types of mice were protected by the injection of irradiated sporozoites. Protection was neither abolished by the absence of perforin nor by a defect in Fas/FasL interaction. Additionally, perforin/FasL double deficient animals were also protected.

Elimination of malaria liver stages by parasite-specific T cells is MHC restricted and it has been shown in various experimental models that $CD4^+$ or $CD8^+$ T cells are implicated in protection (Weiss *et al*. 1988, Del Giudice *et al*. 1990, Tsuji *et al*. 1990, Rodrigues, Nussenzweig & Zavala 1993). Upon activation, parasite-specific T cells should be capable of (i) eliminating the parasite and its host hepatocyte or (ii) activating other mechanisms that can kill specifically intracellular parasite in the hepatocyte. Destruction of parasite-infected hepatocytes by activated T cells has only been demonstrated *in vitro* under tissue culture conditions (Hoffman *et al*. 1989, Weiss *et al*. 1990).

On the contrary, there are many evidences suggesting that cytokines secreted by T cells or other cell types (e.g. NK cells, Küpffer cells) might be crucial to maintain protective immunity. Nüssler *et al*. (1991a) have shown that TNF inhibits the growth of *P. yoelii* liver stages by stimulating non-parenchymal liver cells to produce IL-6 and that the effect of IL-6 is L-arginine and NO-dependent (Nüssler *et al*. 1991b, Pied *et al*. 1991). Similarly, injection of recombinant IFN-γ is sufficient to strongly inhibit *P. berghei* infection in A/J mice and in Norway Brown rats (Ferreira *et al*. 1986). In the same experimental model, protection was suppressed by treatment with antibodies directed against IFN-γ or $CD8^+$-T cells. This indicates that parasite-specific cytotoxic T cells produce IFN-γ or that they secrete another cytokine capable of subsequently inducing IFN-γ production (Schofield *et al*. 1987). It has been extensively demonstrated

Table 1 Naive mice were submitted to a parasite challenge with infectious sporozoites to determine their susceptibility to *P. berghei* infection; 400 sporozoites were injected intravenously. The presence of parasites was assessed by Giemsa-stained bloodsmears. Parasitaemia represents the percentage of infected red blood cells (range or single number when all parasitaemia were equal)

Mice	WT	Lpr	Gld	Perf −/−	Gld× Perf −/−
	Infected/exposed				
Day 3	0/5	0/6	0/5	0/6	0/3
Parasitaemia	–	–	–	–	–
Day 4	2/5	1/6	1/5	0/6	1/3
Parasitaemia	< 1	< 1	< 1	–	< 1
Day 5	3/5	4/6	1/5	3/6	1/3
Parasitaemia	< 1	< 1	< 1	< 1	< 1
Day 6	4/5	5/6	4/5	5/6	3/3
Parasitaemia	< 1–1	< 1–1	< 1	< 1–5	< 1–1
Day 7	4/5	5/6	4/5	5/6	2/2*
Parasitaemia	1–13	3–15	1–13	3–20	6–10
Day 8	4/5	5/6	4/5	5/6	2/2
Parasitaemia	20–78	20–70	16–50	24–80	10–50

* 1 mouse died unexpectedly between days 6 and 7.

Table 2 Mice immunized with *P. berghei* irradiated sporozoites were submitted to a parasite challenge with infectious sporozoites; parasitaemia was assessed by Giemsa-stained bloodsmears; mice were considered protected when no parasites were detected 14 days after the challenge. Data represent accumulated results of two experiments

Mice	WT	WT	Gld	Lpr	Perf −/−	Perf −/− × Gld
[1]Irradiated sporozoites	NO	YES	YES	YES	YES	YES
Experiment	1, 2	1, 2	2	2	1	2
[2]Infected/Exposed						
Day 5	11/15	0/12	0/5	0/6	0/5	0/6
Day 6	13/15	0/12	0/5	0/6	0/5	0/6
Day 10	13/15	0/12	0/5	0/6	0/5	0/6
Day 14	13/15	0/12	0/5	0/6	0/5	0/6

[1] Mice were immunized with 72 000 (exp. 2) or 118 000 (exp. 1) irradiated sporozoites.
[2] Mice were challenged with infected mosquitoes (exp. 1, mice were submitted to 4 infected bites) or were injected with 400 spz i.v. (exp. 2).

that IFN-γ mediated protection is NO-dependent. Indeed, addition of different inducible nitric oxide synthase (iNOS) inhibitors reverses the protection generated by injection of attenuated sporozoites (Mellouk *et al.* 1991, Nüssler *et al.* 1993, Seguin *et al.* 1994, Klotz *et al.* 1995). Finally, it has been shown that mice lacking IFN-γ receptor (IFN-$\gamma R^{0/0}$) are not protected by a single injection of *P. yoelii* irradiated sporozoites whereas wild type mice were protected and expressed high levels of iNOS mRNA in their liver. However, a second injection of irradiated sporozoites restored protection in IFN-$\gamma R^{0/0}$ mice. This latter observation suggests that other yet unknown mechanisms play a role against pre-erythrocytic stages of malaria (Tsuji *et al.* 1995).

In this study, we attempted to clarify the mechanism of *P. berghei* liver stage elimination. We were able to demonstrate that Fas and perforin-mediated killing are not involved in the elimination of *P. berghei* liver stages by $CD8^+$ T cells. Thus, $CD8^+$ T cells do not kill parasite-infected hepatocytes via these two pathways. As extensively demonstrated by other investigators, it is likely that parasite-specific T cells produce cytokines among which IFN-γ seems to play a key role. This cytokine is capable of activating intracellular mechanisms like NO-synthase which can eliminate or inhibit the development of the liver stage of malaria (Suhrbier 1991). However, several questions remain open; for instance, the mechanisms by which parasite-specific T cells are activated as well as the contribution of other cell types found in the liver (Küpffer cell, NK cells) are still poorly understood in irradiated sporozoite induced protection.

ACKNOWLEDGEMENTS

We are very grateful to Pascal Petitjean for the production of *A. gambiae* infected mosquitoes. This work is supported by the Swiss National Science Foundation.

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