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Immunology

CD8$^+$ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites

(*Plasmodium yoelii*/CD4$^+$ T-helper cells)

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ABSTRACT In recent malaria sporozoite vaccine trials in humans and mice, antibodies to the sporozoite coat protein have given only modest protection against sporozoite challenge. In contrast, irradiated sporozoites can protect mice against massive sporozoite infections. Evidence suggests that immunity in these mice is mediated by T cells. To identify the mechanism of immunity, we used monoclonal antibodies specific for either the CD4 or CD8 molecule to selectively deplete sporozoite-immunized mice of T-cell subsets. Though *in vivo* depletion of CD4$^+$ T cells did not reduce immunity, depletion of CD8$^+$ T cells abolished protection. Monoclonal antibody treatment did not affect anti-sporozoite antibody levels. Our data indicate that cytotoxic T cells are critical for immunity to large numbers of sporozoites and suggest that vaccine development should be reoriented toward stimulating cellular as well as humoral immunity.

In the life cycle of the malaria parasite, sporozoites pass from the mosquito through the blood of the host and invade liver cells where they develop into hepatic-stage parasites. Later, mature parasites are released from the liver to invade erythrocytes. When sporozoites are experimentally irradiated, they still invade liver cells but they are unable to mature to the stage that infects erythrocytes (1). Immunization with irradiated sporozoites can protect mice against infection with several thousand sporozoites (2). Humans can also be successfully immunized with irradiated sporozoites (3, 4), but use of attenuated parasites as a vaccine is impractical. A current sporozoite vaccine strategy is to induce antibodies against the central repetitive sequence of the circumsporozoite (CS) protein, which covers the sporozoite surface (5–7). Several recent trials of such vaccines in humans (8, 9) and mice (10, 11) have had limited success. This suggests that humoral immunity may be less important than previously thought and that cellular immunity may be critical for a highly effective vaccine (10, 12, 13). Indeed, μ-suppressed mice, which lack B cells and circulating immunoglobulins, can be immunized with sporozoites (14), demonstrating that T cells are sufficient for sporozoite immunity.

In this paper we dissect the T-cell response to malaria sporozoites by depleting immune mice of T cells carrying either the CD4 or CD8 surface molecule. CD4 and CD8, previously referred to in the mouse as L3T4 and Lyt2, respectively, are molecules that function together with the T-cell receptor complex (15, 16). Outside of the thymus, most T cells carry only one of these molecules that restrict T-cell activation. T cells carrying the CD8 molecule can only be activated by antigens presented along with class I major histocompatibility complex (MHC) molecules, whereas T cells bearing the CD4 molecule are activated by antigens along with class II MHC molecules (17). In animals, CD4-bearing T cells function mainly as T-helper cells, whereas CD8-bearing T cells are mainly cytotoxic and suppressor cells (18). The *in vivo* injection of monoclonal antibodies to CD8 or CD4 results in the almost complete depletion of T cells with these markers from the spleen and lymph nodes (19). We have used these antibodies to eliminate T-cell subsets in mice immune to malaria sporozoites to determine which T cells are responsible for anti-sporozoite immunity.

MATERIALS AND METHODS

Mice. BALB/c and athymic mice were purchased from Charles River Breeding Laboratories.

Sporozoites. *Plasmodium yoelii* (17X NL) sporozoites in *Anopheles stephensi* mosquitoes were harvested 14–16 days after an infectious blood meal. Harvesting was by a modification of the method of Ozaki *et al* (20). Thirty to 60 mosquitoes were anesthetized with chloroform and placed on a glass slide in a drop of medium 199 with 3% mouse serum. The abdomen was held with fine forceps, and the thorax of each mosquito was cut just anterior to the wing. The collection of upper bodies without further preparation was then spun through glass wool according to the published method. After counting in a hemacytometer, sporozoites were diluted to final concentration in medium 199 with 3% mouse serum.

Antibodies. Monoclonal antibodies were produced in ascites fluid of athymic mice and of BALB/c mice treated with cortisone and irradiation (21). Anti-CD8 antibody came from the anti-Lyt2.2 hybridoma 19/178 (mouse IgG2a) (22). Anti-CD4 antibody came from the anti-L3T4 clone GK1.5 (rat IgG2b) (23). A control antibody came from the anti-*Plasmodium falciparum* gamete clone 1B3 (mouse IgG2a) (45). Control rat immunoglobulin was from normal rat serum purchased from Accurate Chemical and Scientific (Westbury, NY). All immunoglobulins were purified by 50% ammonium sulfate precipitation. The rat anti-CD4 antibody and rat serum immunoglobulin were further purified over an anion-exchange column. Final antibody concentrations were determined by optical density or by enzyme-linked immunosorbent assay (ELISA).

Immunization Protocol. Mice 4–26 weeks old were immunized with three or four doses of live sporozoites that had received irradiation (10,000 rads; 1 rad = 0.01 gray) from a ^{137}Cs source. The first immunization was of 75,000 sporozoites; subsequent immunizations were of 20,000 sporozoites given at 2- to 4-week intervals. All immunizations were administered into a tail vein. Two to 4 weeks after the final immunization, animals were challenged with 5000 in-

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Abbreviations: CS, circumsporozoite; IFA, indirect immunofluorescence antibody; SRBC, sheep erythrocyte(s); PFC, plaque-forming cell(s); MHC, major histocompatibility complex; FMF, flow microfluorometry; FITC, fluorescein isothiocyanate.

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Table 1. Effect of anti-T-cell treatments on sporozoite immunity

Immune status	Treatment	Experiment 1*			Experiment 2†		
		Infected/total	Days to detectable parasitemia: Median	Range	Infected/total	Days to detectable parasitemia: Median	Range
Normal	None	10/10	4	4–5	5/5	5	5–7
Immune	None	0/4	Not detected		0/6	Not detected	
Immune	Anti-CD8 mouse IgG2a	5/5	6	5–6	4/4	5	5–6
Immune	Control mouse IgG2a	ND			0/5	Not detected	
Immune	Anti-CD4 rat IgG2b	ND			0/5	Not detected	
Immune	Control rat serum IgG	ND			0/5	Not detected	

ND, not done.
*Mice received three doses of irradiated sporozoites; blood smears were taken beginning on the fourth day after challenge.
†Mice received four doses of irradiated sporozoites; blood smears were taken beginning on the third day after challenge.

fectious sporozoites administered through a tail vein and followed with blood smears for 14 days. This is a large dose, as the injection of 20 *P. yoelii* sporozoites causes infection half of the time in normal mice. Seventy-two of 74 animals were not infected and were considered immune. Immunity was stage specific, as sporozoite-immunized mice were susceptible to infection by transfusion of *P. yoelii*-infected blood. These immune animals were then used within 2–4 weeks in the studies described below.

Lymphocyte-Depletion Protocol. For $CD8^+$ T-cell depletion, immune mice were injected intraperitoneally (i.p.) with 1 mg of anti-CD8 antibody for 2 successive days and then challenged with 5000 infectious sporozoites by way of the tail vein 2 days later. As a control group, another set of immune mice received 1 mg of mouse IgG2a on the same schedule. For $CD4^+$ cell depletion, immune mice received 0.5 mg of anti-CD4 antibody i.p. for 8 days and were challenged with 5000 sporozoites 2 days later. These mice received additional injections of 0.5 mg of anti-CD4 every 3 days. A control group of immune mice received 0.5 mg of rat immunoglobulin on the same schedule.

Flow Microfluorometry (FMF). The following reagents were used: fluorescein isothiocyanate-conjugated (FITC) anti-Thy1.2 (24) (Dupont/New England Nuclear); goat anti-mouse IgG2a FITC (Southern Biotechnology, Birmingham, AL); mouse-adsorbed goat anti-rat immunoglobulin FITC (Kierkegaard and Perry, Gaithersburg, MD); biotinylated anti-CD8 (clone 53-6.5) (24) (Becton Dickinson); Texas red-avidin and biotinylated anti-CD4 (clone H129.19) (25), gifts of B. J. Fowlkes. A FACS-440 flow cytometer (Becton Dickinson) was used for reading one- and two-color-stained samples. Spleen cells from antibody-treated mice were examined by FMF to quantitate T-cell depletion. Animals were sacrificed following treatment either (*i*) at the time of challenge, (*ii*) when parasites appeared in the peripheral blood, or (*iii*) 11 days after challenge if animals were protected. Single-color FMF was performed by using anti-CD8 (clone 19/178) followed by goat anti-mouse IgG2a FITC or anti-CD4 (clone GK1.5) followed by goat anti-rat immunoglobulin FITC. Two-color FMF was performed by using anti-Thy1.2 FITC (green channel) and either biotinylated anti-CD8 or biotinylated anti-CD4 with Texas red-avidin (red channel).

Plaque-Forming Cell (PFC) Assay. Mice were immunized i.p. with 0.2 ml of a 10% suspension of sheep erythrocytes (SRBC). They were killed 4 days later, and PFC assays were performed according to standard methods (26).

Serology. All animals were bled for serum after monoclonal antibody treatment on the day before sporozoite challenge. Sera were titered by ELISA (11) against a synthetic 24-amino acid peptide, $(Gln\text{-}Gly\text{-}Pro\text{-}Gly\text{-}Ala\text{-}Pro)_4$, corresponding to four copies of the *P. yoelii* CS protein 6-amino acid repeat. Pooled sera were also assayed by indirect immunofluorescent antibody assay (IFA) against air-dried *P. yoelii* sporozoites (27).

Parasitemia. Thin blood films were taken daily starting on the third or fourth day after sporozoite challenge. Blood films were Giemsa stained and 50 oil-immersion fields were scanned for parasites. Mice were considered to be protected if no parasites were detected by day 11 after challenge.

RESULTS

In two experiments we observed that *in vivo* depletion of CD8 T cells completely abolished sporozoite immunity (Table 1). In the first experiment anti-CD8 antibody-treated mice had a slightly longer time to detectable parasitemia than did control animals. In the second experiment no such delay was observed, with treated and control animals developing detectable parasitemia on day 5 (median). In contrast, all immune animals treated with anti-CD4 antibodies were still protected. Immune animals that received control antibodies were also protected from sporozoite challenge. Anti-sporozoite antibody titers by ELISA and IFA were similar in treated and control animals (Table 2).

FMF analysis was performed to quantitate T-cell depletion (Fig. 1). Animals were sacrificed and spleen cells were taken either at the time of challenge or following sporozoite challenge and assessment of immunity. The extent of immunodepletion was the same at both time points. An average of 94% of $CD8^+$ T cells was removed following anti-CD8 antibody treatment, and an average of 97% of $CD4^+$ T cells was removed following treatment with anti-CD4 antibody. Neither antibody depleted T cells of the other phenotype.

Because depletion of $CD4^+$ T cells had no effect on immunity, we wished to assess T-helper cell function in the depleted mice. Anti-CD4-treated mice, normal mice, and nude mice were given SRBC i.p., and 4 days later PFC responses were measured in spleens. As shown in Table 3,

Table 2. Effect of immunodepletion on anti-sporozoite antibodies

Immune status	Treatment	IFA	ELISA: Median	ELISA: Range
Normal	None	<1:10	<1:16	ND
Immune	None	1:560	1:256	1:64–1:1024
Immune	Anti-CD8 mouse IgG2a	1:560	1:512	1:32–1:1024
Immune	Control mouse IgG2a	1:560	1:256	1:128–1:1024
Immune	Anti-CD4 rat IgG2b	1:280	1:256	1:128–1:1024
Immune	Control rat serum Ig	1:560	1:512	1:256–1:1024

Serum was taken the day before sporozoite challenge from animals in experiment 2, Table 1. IFA was performed on pooled serum samples. ELISA was performed on each serum, and results are shown as the median and range for each group. ND, not done.

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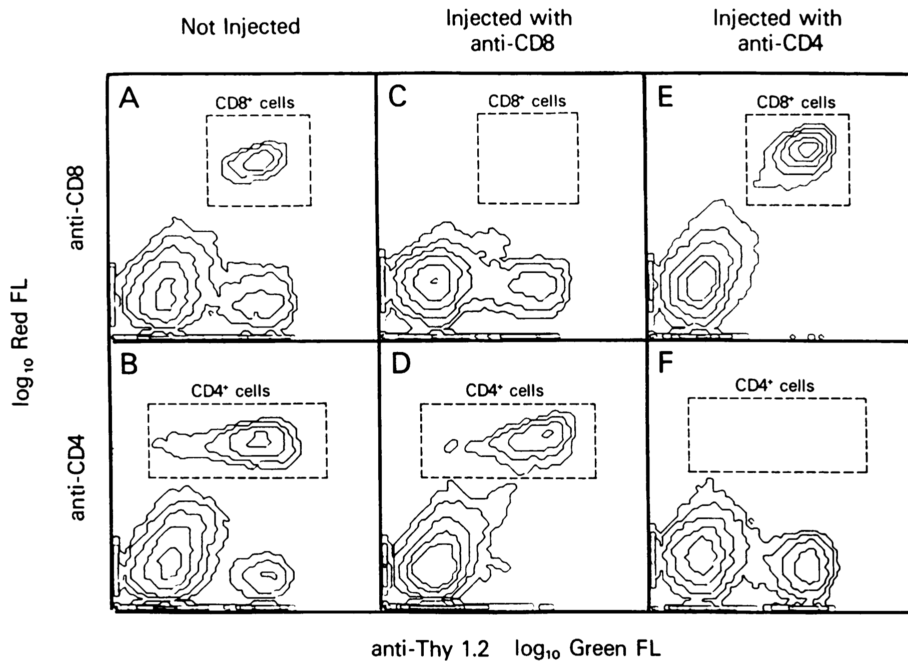

FIG. 1 Spleen cells from sporozoite-immunized mice (Table 1, experiment 2) analyzed by two-color FMF. (*A*) No anti-T-cell treatment, stained for CD8 and Thy1. CD8$^+$ cells are present. (*B*) Same sample as *A*, stained for CD4 and Thy1. CD4$^+$ cells are present. (*C*) Treated with anti-CD8 antibody, stained for CD8 and Thy1 on the day sporozoites produced a detectable parasitemia. CD8$^+$ cells are depleted. (*D*) Same sample as *C*, stained for CD4 and Thy1. CD4$^+$ cells are present. (*E*) Treated with anti-CD4 antibody, stained for CD8 and Thy1, 11 days after sporozoite inoculation. CD8$^+$ cells are present. (*F*) Same sample as *E*, stained for CD4 and Thy1. CD4$^+$ cells are depleted, but animals did not develop parasitemia. FL, fluorescence.

the PFC (IgM) responses of mice injected with anti-CD4 were below those of uninjected mice and were comparable to PFC responses of nude mice, confirming that T-helper cell function was absent.

DISCUSSION

Our data indicate that the CD8$^+$ subset of T cells is necessary for the protection of sporozoite-immunized mice against a challenge of 5000 infectious sporozoites. In contrast, another major subset, the CD4$^+$ T cells, is not required for a protective response in previously immune animals. These conclusions come from depleting live animals of T-cell subsets by using injections of monoclonal antibodies specific for known T-cell-surface molecules. CD8$^+$ T cells have primarily cytotoxic and suppressor cell functions, whereas CD4$^+$ T cells function primarily as T-helper cells (17, 18). Injection of mice with antibody to CD8 removes CD8$^+$ T cells (19) and has been shown to halt the rejection of tumors (28) and to interfere with the clearance of viral infections (29). Injection of antibody to CD4 clears CD4$^+$ T cells (19) and prevents delayed-type hypersensitivity responses (30) and antibody production to T-dependent antigens (31). As it is difficult to imagine how depleting a suppressor cell could reduce an immune response, we believe the critical CD8$^+$ T cells depleted in these experiments are cytotoxic T cells.

How could cytotoxic T cells kill malaria parasites in sporozoite-immunized mice? T cells recognize antigens only after they have been processed and presented on cell surfaces along with MHC molecules (32). Thus, T cells cannot act directly on the free-swimming sporozoite but respond to parasites after sporozoites enter host cells and processed parasite antigens are displayed on the cell membrane. CD8$^+$ T cells can only be activated by cells bearing class I MHC molecules, whereas CD4$^+$ T cells are restricted by class II MHC molecules (18). Since hepatocytes carry only class I MHC molecules (33) they can present parasite antigens only to CD8$^+$ T cells. CD8$^+$ T cells could kill infected hepatocytes by classic cell–cell cytotoxic mechanisms or by the local release of lymphokines. Interferon γ is known to kill hepatic-stage parasites (34–37) and is made by CD8$^+$ T cells (38). Although CD4$^+$ T cells can make interferon γ, they would not be triggered by the infected hepatocytes, which do not carry class II MHC molecules. This is consistent with our observation that anti-CD4 treatment does not alter the effector response of immune mice.

Table 3. Effect of anti-CD4 antibody treatment on the PFC (IgM) response to SRBC

Mice	Treatment	CD4$^+$ splenocytes, %	PFC Per 10^6 cells	PFC Per spleen
BALB/c	None	26.2	1364	261,141
BALB/c	Anti-CD4	0.4	15	2,025
Athymic	None	2.9	68	4,461

The results are the averages of two BALB/c mice without treatment, five BALB/c mice with anti-CD4 treatment, and two athymic nude mice. The percentage of splenocytes that was CD4$^+$ was calculated by single-color FMF.

If we wish to stimulate cellular responses with a synthetic anti-sporozoite vaccine, the first task is to identify the parasite antigens recognized by CD8$^+$ T cells. These antigens must have been presented to the host during immunization with irradiated sporozoites. However, they need not be from the CS or other sporozoite proteins and may be liver-stage antigens (1, 39). From indirect evidence, we speculate that the CS protein is indeed the target for the effector T cells. Any parasite antigen on the hepatocyte surface recognized by cytotoxic cells would be expected to come under selective pressure, as parasites expressing mutations within these T epitopes would not be recognized and killed. A comparison of four sequences of the CS protein from different clones of *P. falciparum* (40) reveals that the polymorphic segments of the molecule correspond to the human immunodominant T-cell epitopes (44). This implies that these epitopes are important to parasite survival, and they may be the target antigens for cytotoxic T cells.

CD8$^+$ T cells are necessary for effector immunity in sporozoite-immunized animals, but alone they may not provide the optimal immune response. Ultimately, the best vaccine may induce humoral and T-cell immunity. Since cytotoxic T cells are generally not activated by exogenous protein antigens (32, 41, 42), other approaches such as recombinant viruses or liposomes (43) may need to be tried if synthetic vaccines are to mimic the cellular immunity stimulated by irradiated sporozoites.

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