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$CD4^+$ and $CD8^+$ T Lymphocytes Both Contribute to Acquired Immunity to Blood-Stage *Plasmodium chabaudi* AS

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In the present study, the contribution of $CD4^+$ and $CD8^+$ T lymphocytes to acquired immunity to blood-stage infection with the murine malaria species *Plasmodium chabaudi* AS was investigated. C57BL/6 mice, which are genetically resistant to infection with this hemoprotozoan parasite and exhibit a transient course of infection, were treated intraperitoneally with monoclonal antibodies to T-cell epitopes, either anti-Thy-1, anti-CD4, or anti-CD8. After intraperitoneal infection with 10^6 parasitized erythrocytes, control C57BL/6 mice exhibited a peak parasitemia on day 9 of approximately 35% parasitized erythrocytes and eliminated the infection within 4 weeks. Mice depleted of Thy-1^+ or $CD4^+$ T cells had significantly higher parasitemias on day 7 as well as significantly higher peak parasitemias. These mice were unable to control the infection and developed a persistent, high parasitemia that fluctuated between 40 and 60% until the experiment was terminated on day 56 postinfection. Depletion of $CD8^+$ T lymphocytes was found to have no effect on the early course of parasitemia or on the level of peak parasitemia. However, mice depleted of $CD8^+$ T cells experienced two recurrent bouts of parasitemia during the later stage of the infection and required more than 5 weeks to eliminate the parasites. After the peak parasitemia, which occurred in control and experimental animals on day 9, there was a sharp drop in parasitemia coinciding with a wave of reticulocytosis. Therefore, the contribution of the influx of reticulocytes, which are not the preferred host cell of this hemoprotozoan parasite, to limiting the parasitemia was also examined by determining the course of reticulocytosis during infection in control and T cell-depleted animals. Early in infection, there was a marked and comparable reticulocytosis in the peripheral blood of control and T cell-depleted mice; the reticulocytosis peaked on day 12 and coincided with the dramatic and sudden reduction in parasitemia occurring in all groups. In both control and CD8-depleted mice the percentage of reticulocytes decreased as the infection was resolved, whereas in CD4-depleted mice marked reticulocytosis correlated with high, persistent parasitemia. These results thus demonstrate that both $CD4^+$ and $CD8^+$ T cells are involved in acquired immunity to blood-stage *P. chabaudi* AS and that the influx of reticulocytes into the blood that occurs just after the peak parasitemia may contribute temporarily to limiting the parasitemia.

Evidence has accumulated to suggest that both humoral and cell-mediated mechanisms are involved in acquired immunity to blood-stage malaria. Protective immunity to the blood stage of *Plasmodium berghei* and *P. yoelii*, which appears to occur by an antibody-dependent mechanism, fails to develop in athymic nude mice (7, 40). Adoptive transfer studies have confirmed the importance of T cells in the development of immunity to these parasites (3, 4, 12, 18). Helper T cells aiding B cells to produce antimalarial antibodies appear to be the mechanism of T cell-dependent immunity to *P. yoelii* and *P. berghei* (16). Studies have shown that antibody-independent mechanisms of immunity also exist for some murine malaria parasites. Grun and Weidanz (14) were the first to demonstrate such a mechanism for *Plasmodium chabaudi adami*. This observation has recently been extended to include *Plasmodium vinckei petteri*, *P. chabaudi* CB, and a related hemoprotozoan parasite, *Babesia microti* (6), as well as *P. chabaudi* AS, the parasite used in the present study (36a).

Although $CD8^+$ T lymphocytes are clearly involved in protecting mice during the sporozoite stage of infection with *P. berghei* and *P. yoelii* (30, 41), their role during the erythrocytic stage of malaria is less clear. In the model of blood-stage *P. chabaudi adami* infection, protection was found to be transferred most efficiently to recipient nude mice by $CD4^+$-enriched immune spleen cells; $CD8^+$-enriched T cells also were found to transfer protection, albeit to a lesser extent (5). Mogil and her colleagues (23) demonstrated that adoptive transfer of $CD8^+$ T cells from *P. yoelii*-immune animals into naive mice accelerated recovery, whereas Vinetz and his colleagues (39) were unable to demonstrate a protective effect of $CD8^+$ T cells. Recent evidence suggests that $CD4^+$ T cells but not $CD8^+$ T cells are required for the development of immunity to *P. chabaudi* AS (37).

In the experiments described here, we examined the contribution of CD4- and CD8-bearing T cells to acquired immunity to blood-stage *P. chabaudi* AS. C57BL/6 mice treated with monoclonal antibodies (MAbs) to specifically deplete T-cell subsets in vivo were observed for aberrations in the course of infection with *P. chabaudi* AS. These studies, which were in progress when the results of Süss et al. (37) were published, utilized the same experimental model, that is, C57BL/6 mice infected intraperitoneally with *P. chabaudi* AS.

The results of the present studies confirm the importance of CD4-bearing T cells in acquired immunity to infection with *P. chabaudi* AS and demonstrate a role for $CD8^+$ T cells in the resolution of infection. In addition, we present

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evidence that the influx of reticulocytes into the blood that occurs just after the peak parasitemia may contribute temporarily to limiting the parasitemia.

MATERIALS AND METHODS

Mice. Female C57BL/6NCrlBR (C57BL/6) mice 6 to 8 weeks of age were purchased from Charles River, Inc., St. Constant, Quebec. Male nude mice (Hsd: *nu/nu*) 6 weeks old were purchased from Harlan Sprague Dawley, Inc., Indianapolis, Ind. BALB/c mice were bred in our facility.

Parasite. The AS strain of *P. chabaudi* was obtained from D. Walliker (University of Edinburgh, Edinburgh, Scotland). Aliquots of parasitized blood were stored in liquid nitrogen. Parasites were kept viable by weekly passage in C57BL/6 mice. After 12 consecutive passages, a fresh inoculum was prepared from the frozen stock. For passage or infection, blood was collected from two C57BL/6 mice by bleeding via the retroorbital plexus and pooled. Total erythrocyte (RBC) counts were determined. The percent parasitemia was determined by counting the percentage of parasitized RBC (PRBC) per 100 RBC on duplicate, Dif-Quik (American Scientific Products, McGaw Park, Ill.)-stained thin blood smears. RBC, diluted in sterile phosphate-buffered saline, were adjusted to the desired concentration of parasitized RBC and injected intraperitoneally (i.p.) into passage or experimental mice. Infection was initiated with a dose of 10^7 PRBC for passage mice and 10^6 PRBC for experimental mice.

Determination of parasitemia. Blood samples were collected from experimental mice by bleeding via the tail vein at the times indicated. Duplicate thin blood smears were prepared and stained with Dif-Quik. Parasitemias were determined by counting the percentage of infected cells per 100 RBC per slide. The parasitemia is expressed as mean percent PRBC ± the standard error of the mean (SEM) for each group of mice.

Determination of percentage of reticulocytes. A total of 200 RBC per duplicate Dif-Quik-stained thin blood smears was examined to determine the percentage of reticulocytes present in the peripheral blood. Reticulocytes were distinguished on the basis of size and staining characteristics. The results are expressed as the mean percent reticulocytes ± SEM for each group of mice.

MAbs. Three hybridomas were used to obtain rat anti-mouse MAbs. Both GK1.5 (anti-CD4; rat immunoglobulin G2b [IgG2b]) and 30-H12 (anti-Thy-1.2; rat IgG2b) hybridomas were kindly provided by T. Owens, Centre for the Study of Host Resistance, McGill University, Montreal, Quebec (10, 22). The hybridoma line 2.43 (anti-CD8; rat IgG2b) was purchased from the American Type Culture Collection, Rockville, Md. (29). MAbs from the hybridoma lines 30-H12 and 2.43 were obtained as ascites fluids from pristane-primed nude mice injected i.p. with the relevant hybridoma. Anti-CD4 MAb was obtained as ascites fluid from sublethally irradiated, pristane-primed BALB/c mice injected i.p. with the hybridoma. Ascites fluids were delipified by centrifugation, and aliquots were frozen at −20°C until required. The concentration of antibody was determined from pooled ascites fluid by using an enzyme-linked immunosorbent assay with specificity for rat IgG and calculated by using a commercially available standard (Becton Dickinson, Mississauga, Ontario).

In vivo treatment with MAbs. Aliquots of MAbs were thawed and diluted in phosphate-buffered saline to the desired concentration. Twenty C57BL/6 mice in one experiment and 32 mice in a second experiment were divided into four equal groups. Each group was treated with either anti-Thy-1 MAb, anti-CD4 MAb, anti-CD8 MAb, or normal rat serum. Mice were given one pretreatment of 500 μg of the appropriate anti-T-cell MAb injected i.p. 4 days before infection with 10^6 PRBC. Unless otherwise indicated, mice were treated i.p. with 200 μg of MAb every 3 days beginning on the day of infection (day 0) until the experiment was terminated on day 56 postinfection. Control mice were treated with normal rat serum containing a concentration of IgG equivalent to that of the MAb preparations administered according to the schedule used for the experimental groups. In one experiment, one mouse per group was sacrificed on day 34 postinfection, and in the other experiment, one mouse per group was sacrificed on the day of infection (day 0) and on days 9 and 56; the spleens were removed and prepared for fluorocytometric analysis.

Spleen cell preparation. Spleens were aseptically removed and perfused with 10 ml of RPMI 1640 (Flow Laboratories, Inc., Mississauga, Ontario) supplemented with 5% heat-inactivated fetal calf serum (Hyclone Laboratories, Inc., Logan, Utah), 2% HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Flow), and 0.12% gentamicin. Cell suspensions were centrifuged at 350 × *g* for 10 min. RBC were lysed with cold NH_4Cl (0.17 M), and the cells were washed two times in fresh medium. Membrane debris was removed by filtering the cell suspension through sterile gauze. The viability was determined by trypan blue exclusion and was always greater than 90%.

Fluorocytometric analysis. Spleen cell suspensions were diluted to a concentration of 2×10^6 cells per ml. Aliquots of 0.5 ml were transferred to microtubes and centrifuged for 6 s at 13,000 rpm in a Microcentrifuge (Accurate Chemical and Scientific Corp., Westbury, N.J.). The pelleted cells were incubated in 50 μl of Hanks balanced salt solution (Gibco, Burlington, Ontario) supplemented with 2% fetal calf serum and 0.1% sodium azide containing 50 μg of either anti-Thy-1, anti-CD4, or anti-CD8 MAbs per ml. Control cells were incubated in medium alone. After incubation on ice for 20 min, the cells were washed twice, treated with dichlorotriazinyl amino fluorescein-labeled goat anti-rat IgG (H & L chain; Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.), and incubated on ice for 20 min. Cells were washed twice and fixed in 0.5 ml of 0.1% paraformaldehyde in phosphate-buffered saline (pH 7.4). Labeled cells were analyzed by flow cytometry (FACSCAN; Becton Dickinson).

Functional studies. Normal, uninfected mice were treated with MAbs or normal rat serum according to the treatment schedule described above and were sacrificed 1 day after receiving the fourth injection of antibody. T-cell function was measured by the following in vitro assays.

(i) Th cells. The presence of $CD4^+$ T cells in splenic lymphocytes was determined by their ability to help B cells make a primary antibody response in vivo to the T cell-dependent antigen sheep RBC (SRBC). Mice were immunized by intravenous injection with 0.3 ml of a 30% suspension of SRBC (approximately 5×10^8 cells), which were obtained as a 50% sterile suspension in Alsever solution (Institut Armand Frappier, Laval des Rapides, Quebec). Four days later, spleens were removed from immunized mice and weighed. Single-cell suspensions of splenic leukocytes were prepared as described above. Total and viable leukocyte counts were determined on individual spleen cell suspensions. The frequency of direct plaque-forming cells (PFC) was determined by using the slide method of Cunningham and Szenberg (9). Briefly, 50-μl aliquots of spleen cell

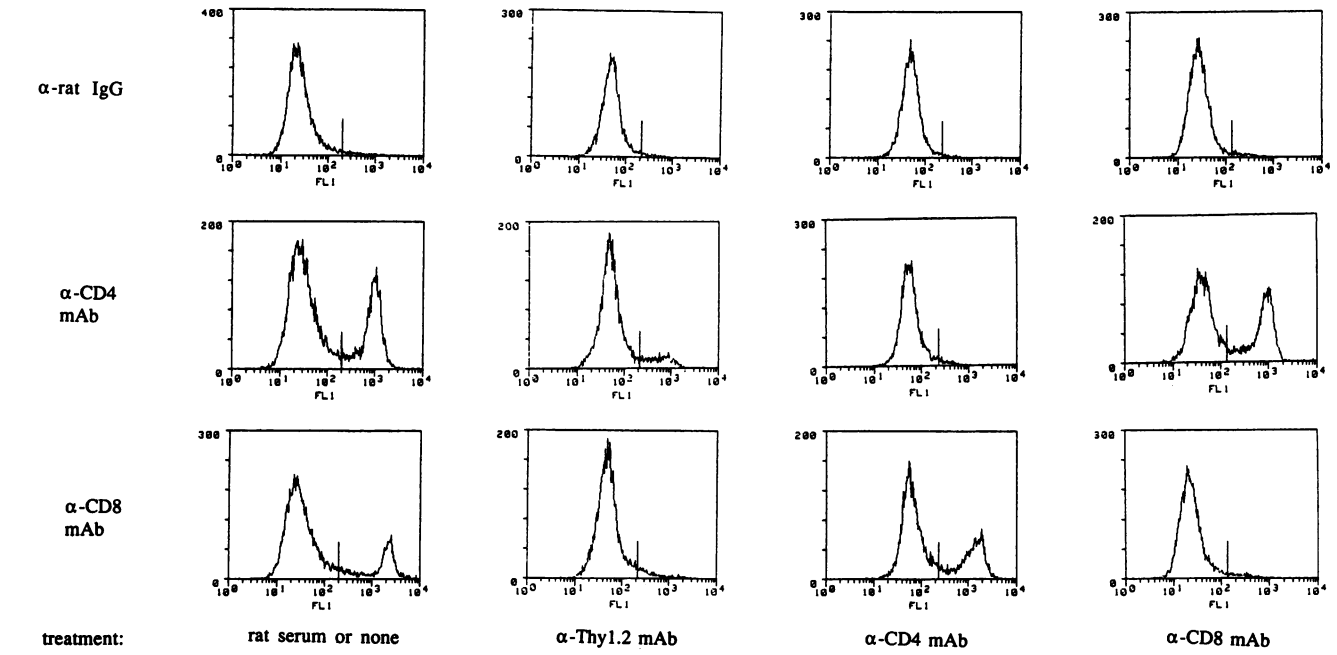

FIG. 1. Fluorocytometric analysis of spleens from mice treated with either anti-Thy-1 MAb, anti-CD4 MAb, anti-CD8 MAb, or normal rat serum. One mouse per group was sacrificed on day 34 after infection with *P. chabaudi* AS. The three vertical panels represent the different antibody treatments of mice as indicated at the bottom. The four horizontal panels represent fluorescent staining with goat anti-rat antibody. Cells were incubated with the MAb indicated on the left side before incubation with the fluorescein-labeled goat anti-rat antibody. Mice treated with normal rat serum or those which were untreated gave essentially identical results. Only the results from mice treated with normal rat serum are shown.

suspensions in Hanks balanced salt solution were incubated for 1 min in a 37°C water bath with 75 μl of SRBC and 0.5 ml of guinea pig C (Gibco) in appropriate concentrations. The mixture was then transferred to four to five Cunningham chambers, which were sealed with a mixture of Parafilm and petrolatum. The number of PFC per spleen was determined after 1 h at 37°C in 5% CO_2.

(ii) Proliferation assay. Spleen cells prepared as described above were adjusted to a final concentration of 2.5×10^6 cells per ml in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum, 2% HEPES buffer, and 0.12% gentamicin. Aliquots of 0.1 ml were incubated for 72 h in 96-well flat-bottom tissue culture plates (Linbro; Flow), and 0.1 ml of medium alone or medium containing 1 μg of concanavalin A (Calbiochem, La Jolla, Calif.) per ml, 50 μg of *Escherichia coli* lipopolysaccharide (Difco, Detroit, Mich.) per ml, or a 1:20 dilution of phytohemagglutinin (Difco) was added to triplicate wells. During the last 16 h of culture, 1 μCi of [^{3}H]thymidine (specific activity, 1 Ci/mmol) in 10 μl of complete medium was added to each well. Cells were harvested with an automatic cell harvester (Flow Laboratories). Incorporated radioactivity was measured in a liquid scintillation spectrometer (Packard, Scarborough, Ontario).

(iii) Cytotoxic cells. Peritoneal exudate cells were harvested from groups of two C57BL/6 mice treated with rat serum or MAbs as described above 4 days after mice were injected i.p. with 10^7 irradiated P815 mastocytoma cells (kindly provided by W. Lapp, Department of Physiology, McGill University). Peritoneal cells from each group were pooled and adjusted to a concentration of 2×10^7 cells per ml in RPMI 1640 supplemented with 5% fetal calf serum, 1% sodium pyruvate (Flow), 1% L-glutamine (Flow), and 1% minimal nonessential amino acids (Flow). Twofold serial dilutions were made in 96-well round-bottom plates (Linbro) in a volume of 100 μl. All samples were prepared in triplicate. To each well, 10^5 P815 tumor cells labeled with ^{51}Cr (5 μCi/ml; specific activity, 224 mCi/mg of Cr; ICN Biochemicals Canada, Limited, Montreal, Quebec) were added in 100 μl. Plates were centrifuged at 250 × *g* after incubation for 4 h at 37°C in 5% CO_2. The ^{51}Cr release in 100 μl of supernatant was determined by using a gamma counter. To determine the spontaneous release, labeled tumor cells were incubated with medium alone. Maximum release was determined by lysing 100 μl of target cells in 100 μl of 5% sodium dodecyl sulfate. Cytotoxicity was assessed by the following formula: percent specific ^{51}Cr release (cytotoxicity) = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. The results are presented as the mean percent specific ^{51}Cr release ± SEM for triplicate samples of supernatants from wells containing the optimal effector/target cell ratio of 10:1.

Statistical analysis. Differences in the mean percentage of PRBC between control mice and animals treated with the MAbs were analyzed by the Student *t* test. A probability of less than 0.05 was considered to be significant.

RESULTS

Fluorocytometric analysis. Individual mice from each group were sacrificed, and the efficacy of the MAbs at depleting the various T-cell subsets was determined fluorocytometrically. The results of a representative fluorocyto-

TABLE 1. PFC responses and mitogenic responsiveness of spleen cells from C57BL/6 mice treated in vivo with MAbs against T-cell subsets

Treatment[a]	PFC/10^6 spleen cells[b] (mean ± SEM)	Mitogenic responsiveness[c] (mean cpm ± SEM)			
		Medium	ConA	PHA	LPS
Normal rat serum	1,086 ± 167	663 ± 50	57,895 ± 4,422	5,674 ± 520	38,386 ± 1,996
Anti-Thy-1 MAb	31 ± 14[d]	869 ± 339	1,528 ± 276[d]	1,091 ± 524[d]	37,887 ± 5,871
Anti-CD4 MAb	13 ± 11[d]	457 ± 46	11,582 ± 1,543[d]	745 ± 66[d]	44,272 ± 3,243
Anti-CD8 MAb	653 ± 82	665 ± 56	51,254 ± 4,209	7,421 ± 1,045	34,512 ± 2,369

[a] Groups of four mice for the PFC assay and two mice for the proliferation assay in two separate experiments were treated intravenously with equivalent amounts of either MAb or rat serum administered four times.

[b] Mice were injected intravenously with approximately 5×10^8 SRBC 4 days before the number of PFC on single-cell suspensions of spleen cells was determined.

[c] Aliquots of 2.5×10^5 spleen cells in 0.1 ml in triplicate were incubated with 0.1 ml of medium alone or 0.1 ml of medium containing 1 μg of concanavalin A (ConA) per ml, a 1:20 dilution of phytohemagglutinin (PHA), or 50 μg of lipopolysaccharide (LPS) per ml. Results are expressed as means ± SEM of 6 to 12 culture wells.

[d] $P \leq 0.001$.

metric analysis of spleen cells recovered on day 34 postinfection are shown in Fig. 1, which demonstrates that there was depletion of the appropriate T-cell subset in the MAb-treated animals.

Functional studies. Examination by three different in vitro assays of the functional ability of spleen cells from T cell-depleted mice that were not infected with *P. chabaudi* AS supported the results of the fluorocytometric analysis; the appropriate T-cell subset was depleted. First, animals depleted of helper T cells by treatment with either anti-Thy-1 or anti-CD4 MAbs were unable to respond to the T cell-dependent antigen SRBC (Table 1). Such animals exhibited an almost total elimination of the in vivo primary antibody response to this antigen as determined by the number of direct PFC per 10^6 spleen cells. Second, measurement of the in vitro mitogenic responses of spleen cells from the MAb-treated animals showed marked and significant decreases in the responsiveness of cells obtained from mice depleted of $CD4^+$ T cells to the T-cell mitogens phytohemagglutinin and concanavalin A but not to the B-cell mitogen lipopolysaccharide (Table 1). Third, the cytotoxic activity of peritoneal exudate cells, mediated by the $CD8^+$ subset of T cells, against the murine tumor cell line P815 was significantly decreased in mice depleted of $CD8^+$ T cells by treatment with either anti-Thy-1 or anti-CD8 MAbs (Fig. 2).

Course of *P. chabaudi* AS infection in T cell-depleted mice. Control rat serum-treated C57BL/6 mice experienced a course of infection essentially identical to that of untreated animals (Fig. 3). In each case, mice experienced a peak parasitemia of approximately 35% PRBC on day 9 postinfection. All the animals subsequently cleared the infection. By day 22, the parasitemia was reduced to less than 2%, and the infection was eliminated from the peripheral blood 4 weeks after initiation of the infection. No recrudescence of parasitemia was observed in these mice throughout the 56 days of the experiment.

To confirm the role of T cells in acquired immunity to *P. chabaudi* AS, C57BL/6 mice were treated with anti-Thy-1 MAbs (data not shown). Depletion of T cells in normal, intact euthymic animals resulted in a course of infection similar to that exhibited by C57BL/6 *nu/nu* mice (36a). There was a significant difference in the level of parasitemia between Thy-1-depleted mice and control mice as early as day 7 (34.6% ± 2.5% and 25.5% ± 1.9% PRBC, respectively; $P \leq 0.01$), and the peak parasitemia of Thy-1-depleted animals was significantly higher than that exhibited by control mice (58.7% ± 3.4% and 35.4% ± 1.6% PRBC, respectively; $P \leq 0.001$). Mice depleted of T cells by treatment with anti-Thy-1 MAbs were unable to control the infection and suffered from a persistent, high parasitemia. There was 36.4% mortality (4 of 11 animals) among these animals. As described below, the high, persistent parasitemia in these animals coincided with a marked and sustained reticulocytosis.

The prepatent and early patent periods of CD4-depleted mice were similar to those of control animals (Fig. 4). In two separate experiments, mice depleted of $CD4^+$ T cells had a significantly higher parasitemia on day 7 than did control animals. In the results of the experiment shown here, the

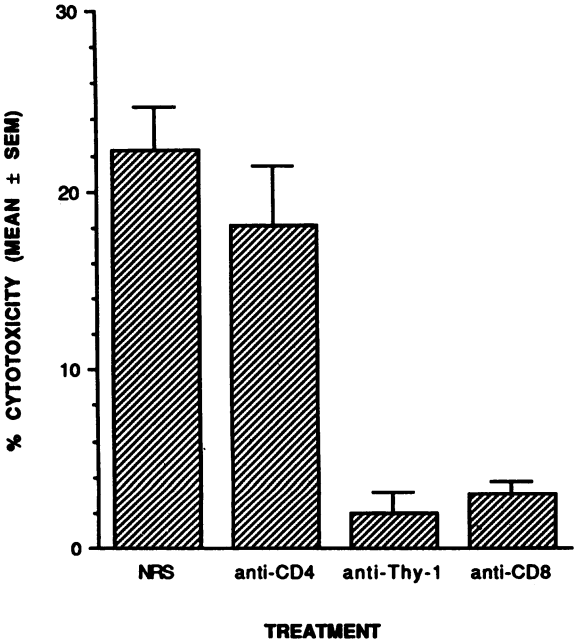

FIG. 2. Cytotoxic responses of peritoneal cells from mice treated in vivo with MAbs against T-cell subsets. The percentage of specific ^{51}Cr-release was determined on triplicate samples of cells from groups of two mice treated as indicated at an effector/target cell ratio of 10:1. Values are expressed as the mean ± SEM ($n = 6$). Responses of cells from mice treated with anti-Thy-1 or anti-CD8 MAb are significantly less than those of mice treated with normal rat serum ($P \leq 0.001$).

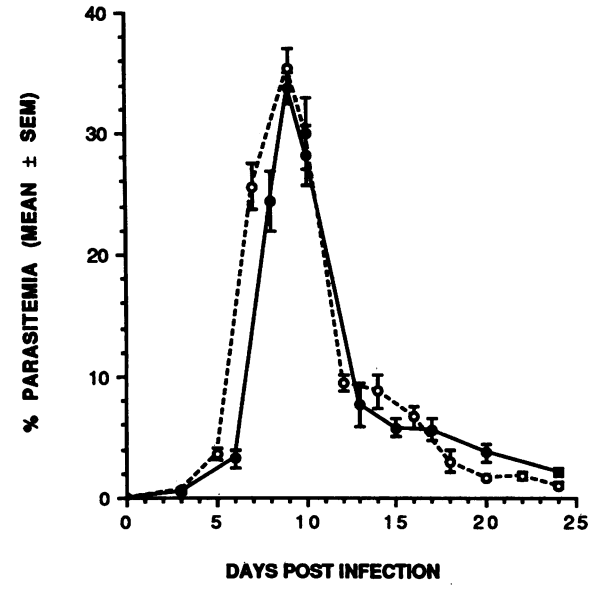

FIG. 3. Course of *P. chabaudi* AS infection in untreated mice (○) or in mice treated with normal rat serum (●). Parasitemia was determined on the days indicated on duplicate blood smears collected from groups of five mice.

parasitemia of CD4-depleted mice on day 7 was 37% ± 1.3% PRBC ($P \leq 0.001$ in comparison with control mice). On day 9, the parasitemia had risen to a peak of 61.3% ± 1.6% PRBC, a level significantly greater than the control parasitemia level of 35.4% ± 1.6% PRBC ($P \leq 0.001$). Parasitemia declined in the CD4-depleted animals in a manner similar to that in control C57BL/6 mice; by day 12, parasitemia was 10% ± 1.1%. The percentage of PRBC present in the peripheral blood of CD4-depleted mice, however, quickly rebounded to a level comparable to that of the peak parasitemia. The CD4-depleted mice were unable to control the infection and developed a high, persistent parasitemia that fluctuated between 40 and 60% PRBC until the experiment was terminated on day 56. In contrast to Thy-1-depleted animals, CD4-depleted mice survived until the experiment was terminated despite suffering similar high, persistent parasitemia levels that coincided with sustained reticulocytosis. Similar results were obtained in a second experiment.

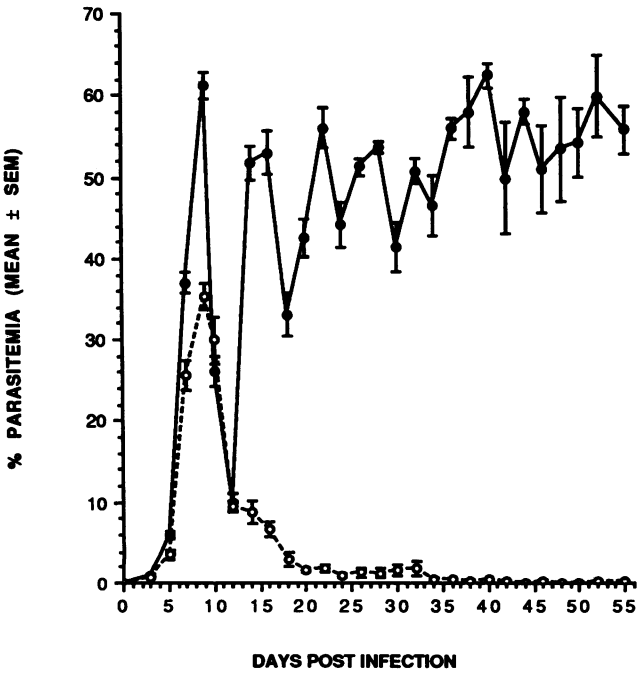

FIG. 4. Course of *P. chabaudi* AS infection in mice treated with anti-CD4 MAb (●) or normal rat serum (○). Parasitemia was determined beginning on day 3 postinfection and every 2 days until day 56 on duplicate blood smears collected from groups of eight mice.

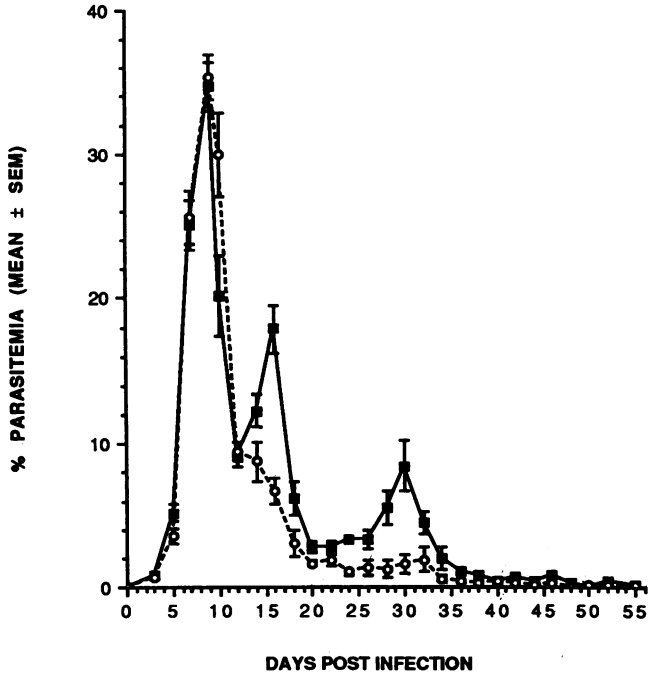

FIG. 5. Course of *P. chabaudi* AS infection in mice treated with anti-CD8 MAb (●) or normal rat serum (○). Parasitemia was determined beginning on day 3 postinfection and every 2 days until day 56 on duplicate blood smears collected from groups of eight mice.

In two separate experiments, depletion of CD8+ T cells had no effect on the course and level of parasitemia during the prepatent and patent periods or at the time of the peak parasitemia. The results of one of the experiments with 8 mice per group are shown in Fig. 5. The elimination phase was initially identical to that in control animals. However, two recurrent bouts of parasitemia were consistently observed in both experiments in CD8-depleted mice. In the case of the experiment shown here, the first reached a peak of 17.9% ± 1.6% PRBC ($P \leq 0.001$) on day 16. The parasitemia levels declined and remained at approximately 3% before a second smaller but still significant peak was observed on day 30 (8.5% ± 1.8% PRBC; $P \leq 0.01$). The plateau (3% PRBC) level between these two peaks was also found to be statistically significantly different from that of control mice, which at this time experienced a parasitemia of less than 1%. The CD8-depleted mice differed from control animals in the developmental stages of parasites present in the blood during the elimination phase. The parasites observed in control mice were generally crisis forms, whereas

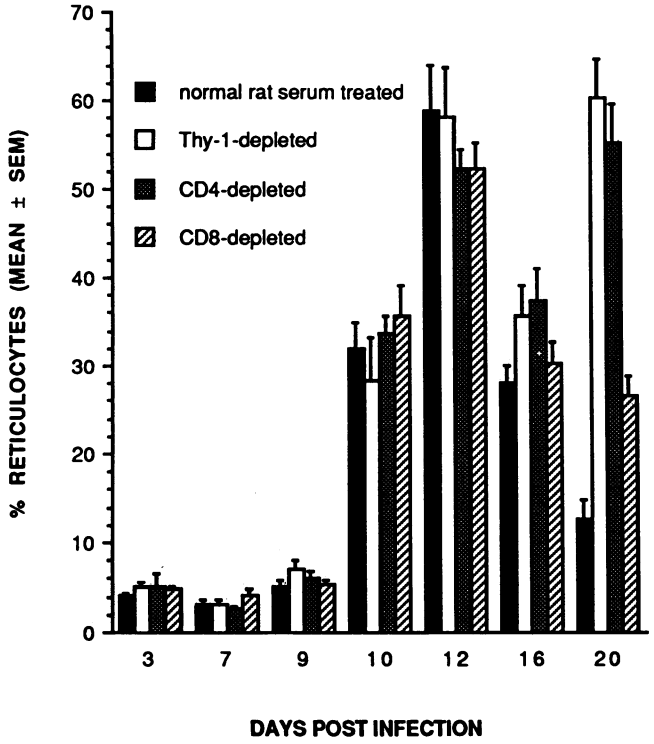

FIG. 6. Course of reticulocytosis during *P. chabaudi* AS infection in T cell-depleted mice. On the days indicated, the percentage of reticulocytes present in the peripheral blood was determined on duplicate blood smears collected from groups of eight mice.

parasites present in the peripheral blood during the two recurrent parasitemia peaks of the CD8-depleted groups were healthy, dividing forms. Moreover, elimination of the parasite in CD8-depleted animals required more than 5 weeks instead of the 3 to 4 weeks characteristic of C57BL/6 mice.

Course of reticulocytosis in T cell-depleted mice. The contribution of reticulocytosis to limiting the level of parasitemia during infection with *P. chabaudi* AS, hemoprotozoan parasites which preferentially invade mature erythrocytes, was investigated by determining the percentage of reticulocytes present in the peripheral blood during the course of infection in control and T cell-depleted animals. Reticulocytes were found to represent approximately 5% of the total number of RBC in all experimental groups until day 10, when there was a dramatic influx of reticulocytes (Fig. 6). This influx coincided with severe anemia, and reticulocytes comprised approximately 30% of the total RBC in both the experimental and control groups of mice. On day 12 of infection, when the parasitemia levels had fallen to approximately 10% in control and experimental animals, the percentage of reticulocytes was 50 to 60% in all groups. These results suggest that the influx of reticulocytes, which are not the preferred host cell of this parasite (21), that occurs just after the peak parasitemia temporarily limited the parasitemia. By day 20 after infection, control mice were successfully eliminating the parasites, and the percentage of reticulocytes had decreased to 12.8% ± 2.1% (mean ± SEM). CD8-depleted mice, which had difficulty in resolving the infection and experienced two recurrent bouts of parasitemia, had a significantly higher percentage of reticulocytes on day 20 compared with control animals (26.6% ± 2.1%; $P \leq 0.001$). These animals, however, did eventually clear the parasite, and the percentage of reticulocytes decreased to normal levels (data not shown). In contrast to results obtained with control and CD8-depleted animals, in which the percentage of reticulocytes was decreasing as the infection was resolved, the percentage of reticulocytes decreased on day 16 but was again significantly increased on day 20 in both Thy-1- and CD4-depleted mice (60.3% ± 4.4% and 55.2% ± 4.4%, respectively; $P \leq 0.001$). This high level of reticulocytes in the peripheral blood was maintained until the experiment was terminated on day 56 and coincided with the high, persistent parasitemia of these animals. It is of interest to point out that, late in infection in both Thy 1-depleted and CD-4-depleted animals, numerous parasitized reticulocytes were observed.

DISCUSSION

The results of the experiments presented in this study demonstrate the importance of both $CD4^+$ and $CD8^+$ T lymphocytes in the development of acquired immunity to blood-stage infection with *P. chabaudi* AS. For these studies, we used C57BL/6 hosts, which we have previously characterized as resistant to infection with this parasite (34–36). Depletion of T cells, specifically $CD4^+$ T cells, resulted in a significantly higher parasitemia as early as 7 days postinfection. The peak parasitemia in these animals was also significantly higher than that in controls, and a persistent, high parasitemia developed that the mice were unable to clear. In contrast, in the absence of $CD8^+$ T cells, acute infection with *P. chabaudi* AS was ultimately resolved. However, two recurrent bouts of parasitemia were consistently observed during the elimination phase, which required more than 5 weeks instead of the 4 weeks characteristic of this infection in resistant C57BL/6 mice. This observation demonstrates that $CD8^+$ T cells also contribute to acquired immunity to this parasite.

Our results confirm previous studies utilizing adoptive transfer of purified and enriched T-cell populations that demonstrated the importance of $CD4^+$ T lymphocytes in protective immunity to *P. yoelii* (3, 18, 23, 39) and *P. chabaudi* (5, 37). Studies by Brake and his colleagues (2) demonstrated that T-cell lines obtained from *P. chabaudi adami*-infected mice and propagated in vitro can transfer protection in vivo. These investigators have further shown that a $CD4^+$ T-cell clone, which is specific for *P. chabaudi adami*, can transfer protection to nude mice and can produce gamma interferon and interleukin-2 in response to specific antigens (1).

While the experiments described here were in progress, Süss et al. (37) published results of similar experiments with C57BL/6 mice infected with *P. chabaudi* AS. They concluded that $CD4^+$ T cells are the predominant lymphocyte involved in the development of protective immunity to *P. chabaudi* AS and that $CD8^+$ T cells do not contribute significantly. Differences in the origin and virulence of the parasites and the size of the inoculum, as well as differences in the hybridoma source of the MAbs and the experimental protocol, may have contributed to the conflicting results. A similar difference regarding the protective effects of $CD8^+$ T cells in immunity to *P. yoelii* has recently been appreciated. Mogil and her colleagues (23) demonstrated that adoptive transfer of $CD8^+$ T cells from *P. yoelii*-immune animals into naive mice accelerated recovery, whereas Vinetz and his colleagues (39) were unable to demonstrate a protective effect of $CD8^+$ T cells.

The role of $CD4^+$ T cells in cell-mediated immunity against intracellular bacteria, fungi, and protozoa is generally accepted to be via the production of lymphokines that activate macrophages to express powerful antimicrobial activity. This mechanism has been hypothesized for the elimination of some plasmodium species, including *P. chabaudi* AS (33). $CD4^+$ T cells may also provide help to expand effector $CD8^+$ T-cell populations via the production of interleukin-2. Interleukin-2 produced by $CD4^+$ T cells has been demonstrated not only to enhance the effectiveness of cytotoxic T cells but also to induce the production of gamma interferon by these cells (11, 38).

It is becoming increasingly evident that $CD8^+$, cytotoxic T cells also play an important role in host resistance to intracellular microbial pathogens (reviewed in reference 19). $CD8^+$ T cells have been shown to be capable of lysing macrophages infected with either *Listeria monocytogenes* or *Mycobacterium tuberculosis* in a parasite-specific manner that can be either major histocompatibility complex (MHC) restricted or nonrestricted.

Whereas $CD4^+$ T lymphocytes recognize foreign antigen in association with class II MHC molecules, $CD8^+$ T cells recognize virally infected cells that display foreign antigens in association with class I MHC antigens. Recent studies by two groups have demonstrated that protective immunity to the exoerythrocytic stage of infection with *P. berghei* and *P. yoelii* sporozoites is mediated by $CD8^+$ T cells (30, 41). $CD8^+$ cells are believed to recognize sporozoite-infected hepatocytes that express class I MHC molecules.

$CD8^+$ T lymphocytes have generally been viewed as playing little or no role in immunity to the erythrocyte stage of plasmodium infection. As discussed above, protective immunity to *P. yoelii* could be adoptively transferred by $CD8^+$ T cells (23). $CD8^+$ T-cell clones isolated from individuals infected with *P. falciparum* have been demonstrated to respond to specific antigen in vitro (32). Further studies have shown that these MHC-restricted *P. falciparum*-specific clones are capable of lysing unrelated tumor target cells when stimulated with anti-CD3 antibodies, suggesting that these cells possess cytotoxic potential (31). In addition, unfractionated murine splenic lymphocytes, depleted of macrophages, have been shown to lyse *P. berghei*-infected erythrocytes, both in the presence and in the absence of immune serum (8, 26).

Since mature RBC express little class I MHC, it is difficult to reconcile how cytotoxic T lymphocytes can exert a direct effect on parasitized erythrocytes. Although it is true that mature RBC do not express MHC antigens, it is clear in humans that reticulocytes express a significant number of these molecules (15, 20). Studies by Jayawardena et al. (17) have shown that in mice there is increased expression of H-2K and H-2D molecules on *P. yoelii*-infected reticulocytes; these authors suggested that this may be a factor contributing to immunogenicity. In the same study, *P. chabaudi*-infected erythrocytes also showed a slight enhancement of class I MHC expression. Alternatively, $CD8^+$ T cells may exert an antiplasmodial effect indirectly via the production of gamma interferon. This lymphokine is necessary for activating macrophages to express enhanced antimicrobial activity against both human and murine *Plasmodium* species in vitro and to destroy the intraerythrocytic parasite by the production of H_2O_2 (24, 25).

A phenomenon of interest to us and one that was also observed by Süss et al. (37) was the dramatic and sudden reduction in parasitemia that occurred immediately after the peak parasitemia in all experimental groups, including the control mice. In light of effective T-cell depletion, it seems unlikely that a specific T cell-dependent immune mechanism contributed to this sudden reduction. Nonspecific immune mechanisms may have contributed to this drop. Erythropoiesis in response to the malaria-induced anemia may account for this observation. Anemia was apparent in both the T cell-depleted and control mice on day 10 of infection. This resulted in a sudden influx of reticulocytes, which constituted approximately 30% of the total peripheral RBC. The rapid reduction in the number of mature erythrocytes, the host cells preferred by *P. chabaudi* AS, as a result of lysis of infected cells and the influx of reticulocytes may therefore account for the sharp drop in parasitemia. A similar conclusion was reached by Ott (28) on the basis of the observation that stimulation of reticulocytosis by injection of phenylhydrazine resulted in a diminished level of parasitemia during infection with *P. chabaudi*. Although *P. chabaudi* has a preference for the mature erythrocyte, it would appear that the parasite is not exclusively restricted to infecting this host cell type. The marked and sustained increase in the numbers of reticulocytes in Thy-1- or CD4-depleted mice resulted in infected reticulocytes late in infection. This may explain why, just after the peak parasitemia and initial drop in parasitemia in mice depleted of $CD4^+$ T cells, the parasite invaded reticulocytes whose numbers appeared to be maintained as a result of a sustained reticulocytosis and why a persistent, fulminant parasitemia ensued.

Thus, both CD4- and Thy-1-depleted C57BL/6 mice appeared to compensate for the lack of immune function by mounting a marked and sustained erythropoietic response, with the spleen becoming the major site of erythropoiesis. Spleens from these animals were grossly enlarged, varying in weight from 10 to 15 times that of a normal mouse. We have previously demonstrated that C57BL/6 mice have a superior erythropoietic system and respond to injection with phenylhydrazine with a prompt and marked reticulocytosis (34). During infection with *P. chabaudi* AS, susceptible A/J mice develop marked anemia that appears to culminate in their death, whereas resistant C57BL/6 mice suffer only mild anemia (34). In addition, *P. chabaudi* AS-resistant mouse strains, such as C57BL/6, develop marked splenomegaly, a trait which is either identical to or closely linked to that conferred by the gene controlling resistance to infection with this murine hemoprotozoan parasite (35, 36). The importance of an architecturally intact spleen in the control and elimination of an acute malaria infection has been well documented (13, 27).

In conclusion, our results demonstrate that both $CD4^+$ and $CD8^+$ T cells play a role in acquired immunity to *P. chabaudi* AS. Furthermore, the influx of reticulocytes into the blood just after the peak parasitemia may contribute temporarily to limitation of the parasitemia.

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