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Inhibition of Nitric Oxide Interrupts the Accumulation of CD8$^+$ T Cells Surrounding *Plasmodium berghei*-Infected Hepatocytes

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The elimination of liver-stage malaria parasites by nitric oxide (NO)-producing hepatocytes is regulated by T cells. Both CD8$^+$ and CD4$^+$ T cells, which surround infected hepatocytes, are evident by 24 h after sporozoite challenge in Brown Norway rats previously immunized with irradiated *Plasmodium berghei* sporozoites. While the number of CD4$^+$ T cells remained the same beyond 24 h postchallenge, the number of CD8$^+$ T cells increased three- and sixfold by 31 and 44 h, respectively. This increase in the number of CD8$^+$ T cells correlated with a decrease in the number of intrahepatic parasites. In immunized rats, intrahepatic parasites were reduced in number by 31 h after sporozoite challenge and cleared from the liver by 44 h, as visualized by *P. berghei*-specific DNA in situ hybridization. If immunized rats were treated with aminoguanidine, a substrate inhibitor of NO synthase, at the time of challenge, liver-stage protection was blocked, as shown by the increase in parasite liver burden. Further histological examination of infected livers from immunized animals treated with aminoguanidine revealed fewer and smaller cellular infiltrates surrounding the infected hepatocytes, and the number of CD8$^+$ T cells that normally accumulate within the infiltrates was drastically reduced. Consequently, the infected hepatocytes were not cleared from the liver. We hypothesize that the early production of NO may promote the influx and/or enhance local proliferation of malaria parasite-specific CD8$^+$ T cells or a CD8$^+$ T-cell subset which is required for parasite clearance.

The protective immunity induced by irradiated sporozoites is dependent on CD8$^+$ T lymphocytes (21, 30) which recognize malaria peptides on the surface of infected hepatocytes in association with class I major histocompatibility complex molecules (6, 7, 28, 30). Upon activation, these CD8$^+$ T cells may secrete gamma interferon (IFN-γ) (30), which inhibits the development of the exoerythrocytic (EE) forms of the malaria parasite (21, 22, 29). The in vitro development of EE forms of the malaria parasite has been shown to be inhibited by IFN-γ (3, 14), and the protective immunity induced by irradiated sporozoites is eliminated by the in vivo treatment of some strains of mice with anti-IFN-γ (21, 22).

Presumably, IFN-γ-mediated induction of nitric oxide (NO) by malaria-infected hepatocytes contributes to liver-stage protection in animals previously immunized with irradiated *Plasmodium berghei* sporozoites (4, 10, 13, 16, 22, 24) or with a *Plasmodium yoelii* DNA vaccine (2). Immunized rats and mice are no longer protected against a malarial sporozoite challenge when treated with a substrate inhibitor of nitric oxide synthase (NOS), such as aminoguanidine or N^G-monomethyl-L-arginine (L-MNA). When aminoguanidine or L-MNA is administered orally at the time of challenge and for 4 days thereafter, parasitemia is evident within 4 to 5 days in immunized animals. In contrast, immunized animals that are not treated with NOS inhibitors are fully protected against blood-stage malaria (2, 10, 16, 22).

Both CD8$^+$ T cells and IFN-γ are necessary for the induction of NO synthesis in infected hepatocytes. Livers from immunized mice express mRNA for inducible NOS (iNOS) between 12 and 24 h after challenge with sporozoites. Depletion of CD8$^+$ T cells or neutralization of IFN-γ at the time of challenge blocks both expression of iNOS mRNA and protection in immunized animals (22). Our previous studies of Brown Norway rats show that iNOS expression in livers following sporozoite challenge is restricted to infected hepatocytes (10). This iNOS activity is present in 18% of the infected hepatocytes in immune rats by 24 h and reaches 81% by 31 h, while <10% of the infected hepatocytes in naive rats display iNOS activity (4, 10).

The expression of iNOS is also found to be dependent on the persistence of the irradiated parasite in livers of immunized animals (10, 18). Developmentally arrested parasites that result in the livers of animals following a preexposure to irradiated sporozoites maintain the protective immunity of the host (18). By accelerating the removal of preexisting irradiated parasites from the hepatocytes in immunized animals with the drug primaquine, the ability of the immunized animals to express iNOS in response to sporozoite challenge decreases. Subsequently, there is a corresponding loss of liver-stage protection (10, 18).

The cellular response to a challenge has been histologically demonstrated by the presence of inflammatory cell infiltrates in the livers of irradiated sporozoite-immunized mice at 43 h postchallenge (6). However, the series of events that occur in the liver during the time the parasites are cleared remains to be demonstrated. Since the appearance of lymphocyte-rich infiltrates surrounding malaria-infected hepatocytes is an important component in liver-stage immunity against malaria, we further investigated the mechanisms involved in this clearance. We first demonstrate the histological events that occur from 24 to 44 h after challenge of immune rats with sporozoites. We also demonstrate that treatment of immune rats with an NO inhibitor at the time of a challenge with sporozoites results in the production of fewer and smaller cellular infiltrates surrounding the infected hepatocytes. In addition, a reduction of

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the number of CD8$^+$ T cells that accumulate within the infiltrates is observed. Consequently, the parasites are not eliminated from the liver. A role for NO in influencing the recruitment and/or local proliferation of CD8$^+$ T cells which are necessary for the activation of iNOS in infected hepatocytes and the clearance of the intrahepatic parasite in immunized animals is discussed.

MATERIALS AND METHODS

Immunization and challenge. *P. berghei* sporozoites were isolated from irradiated (used for immunization) and nonirradiated (used for challenge) *Anopheles stephensi* mosquitoes on a biphasic gradient with Renografin-60 (Squibb Diagnostics, New Brunswick, N.J.) and 5% rat serum (Rockland, Inc., Gilbertsville, Pa.) as previously described (18, 19). For immunization, salivary gland-infected mosquitoes were irradiated (10,000 rads) by using a ^{137}Ce source at a dose rate of 833 rads per min (Shepherd irradiator Mark I, model 68; J. L. Shepherd and Associates, San Fernando, Calif.). Six- to eight-week-old Brown Norway rats (Jackson Laboratory, Bar Harbor, Maine) were immunized with three doses of 50,000 irradiated sporozoites, administered intravenously at biweekly intervals. They were challenged 2 weeks later by hepatic portal branch inoculation (12, 19, 26) of 10^6 sporozoites, an inoculum that was also given to the naive rats (injected with salivary lysates from uninfected mosquitoes). This method of inoculation increases liver-stage parasite yields to effectively concentrate parasites to the right and caudate liver lobes. To ensure that the results were reproducible, all experiments were performed twice. The animals used in this study were cared for and used strictly in accordance with the Public Health Service guidelines (1).

In vivo administration of aminoguanidine. To inhibit NO production, groups of immunized rats were treated by gastric instillation for 4 days with aminoguanidine, a substrate inhibitor of NOS (50 mg in water/kg of body weight; Sigma Chemical Co., St. Louis, Mo.) (23). As previously described, treatment was initiated 24 h before sporozoite challenge and then administered twice a day for 72 h thereafter (10, 22).

Histology and immunocytochemistry. Rats were euthanized by CO_2 inhalation, and livers were removed at 24, 31, or 44 h after sporozoite challenge. Livers were fixed in 10% buffered formalin. After dehydration with graded alcohols and xylene, livers were embedded in paraffin, sectioned, and Giemsa stained. Tissue sections were first Giemsa stained to assess the histopathology and then restained by the DNA in situ hybridization method to verify the presence of *P. berghei*. As previously described, a digoxygenin-labeled probe specific for the subtelomeric repeat region of DNA from *P. berghei* was used to locate the parasite in liver sections (19, 26).

For immunofluorescence analysis, livers were embedded in OCT (Miles Scientific, Elkhart, Ind.) and quickly frozen in 2-methylbutane cooled with liquid nitrogen. Frozen liver sections (4 μm) were placed on glass microscope slides, air dried, and then fixed in −70°C methanol for 20 min. Fixed slides were rehydrated in phosphate-buffered saline (PBS) and blocked for nonspecific binding sites with 10% fetal bovine serum in PBS prior to antibody staining. To determine the number and type of T cells that comprised the infiltrates, monoclonal antibodies (MAbs) that recognize CD4 (RIB611) and CD8 (W3125) antigens on the surface of T cells (Accurate Chemical and Scientific Corp., Westbury, N.Y.) were used. Trimethyl rhodamine isothiocyanate-conjugated goat antibodies against rat immunoglobulin G (IgG) and fluorescein isothiocyanate-conjugated goat antibodies against rat IgG (Southern Biotechnology Associates, Birmingham, Ala.) were used to react with tissue-bound antibodies RIB611 and W3125. Tissue sections were mounted with Fluoromount G mounting medium (Southern Biotechnology) and examined under a fluorescence microscope.

Analysis of liver sections. Microscope slides of liver sections from each group were assigned a code. In a blinded study, they were analyzed for the number of intrahepatic parasites and infiltrates, diameter of infiltrates, and number of T cells per infiltrate. Slides were decoded at the end of screening. Each slide containing a tissue section was overlaid with a plastic sheet with a window measuring 0.25 cm^2. This window was superimposed on the section, and the parasites and infiltrates within this area were counted. A total of 100 0.25-cm^2 areas in each animal (equivalent to 25 cm^2) were then screened. There were three animals per treatment per time point (25 cm^2 × 3 rats/group = 75-cm^2 area screened per treatment per time point). Each time point represented 24, 31, and 44 h after challenge with sporozoites. The mean number of parasites and infiltrates per 1 cm^2 ± the standard deviation (SD) was calculated for each time point. The mean diameter of an infiltrate at a given time point was determined by the average diameter of 300 infiltrates recorded from each group (recorded the diameter of 100 infiltrates in each of three rats per group). The mean numbers of CD4$^+$ and CD8$^+$ T cells within these infiltrates were determined in a similar manner. The number of each type of T cell was counted in each of 25 infiltrates per three animals per time point (75-cm^2 area). The mean numbers obtained were compared for different treatments by using analysis of variance, adjusting for the other treatments and weighing by the inverses of the variances of the means at different time points.

RESULTS

The development of *P. berghei* in rat liver has previously been characterized (12). When a sporozoite invades a hepatocyte, it transforms into a uninuclear trophozoite. At 24 h this trophozoite is approximately 4 to 5 μm in diameter, after several rounds of replication the EE form at 31 h is 15 μm, and at 44 h the mature EE form is approximately 30 μm. At approximately 46 h, the merozoites formed within the EE form begin to be released into the bloodstream. Well-developed liver-stage parasites at 44 h were readily observed in Giemsa-stained liver sections from nonimmune rats challenged with sporozoites (Fig. 1A). Because it was difficult to definitively locate earlier liver stages of the parasite by Giemsa staining, we used DNA in situ hybridization as an alternative (Fig. 1B [a 44-h EE form]).

In rats immunized with irradiated sporozoites, some of the sporozoites inoculated at the challenge invaded the rat liver, but they were subsequently cleared from the liver. Histological examination of livers from rats immunized with irradiated sporozoites and challenged with viable sporozoites revealed that cellular infiltrates were closely associated with malaria-infected hepatocytes at 31 h (Fig. 1C). Prior to this time, scattered immune cells populated the liver, but few distinct infiltrates were observed. Figure 1C demonstrates a characteristic Giemsa-stained section of a 31-h liver stage parasite surrounded by a cellular infiltrate. Figure 1D demonstrates a cellular infiltrate completely enveloping a parasite at 31 h. DNA in situ hybridization was performed on the same frozen liver section as observed in Fig. 1C, and the *P. berghei*-specific DNA probe confirmed that a malarial parasite was located within the center of the cellular infiltrate beginning at about 31 h after inoculation with sporozoites (Fig. 1E). Between 31 and 44 h, the parasites began to be degraded. Because a 44-h parasite is in the process of being cleared from the host liver, it was difficult to observe the degraded parasite by using standard Giemsa staining. Therefore, we applied DNA in situ hybridization, which stains only the parasite, not the surrounding infiltrates. Figure 1F demonstrates the remnants of a parasite at 44 h.

Further microscopic evaluation of the liver sections of challenged rats showed that the number of intrahepatic parasites (Fig. 2), the number of focal infiltrates (Fig. 3A), and the size of the infiltrates (Fig. 3B) varied between naive and immune mice at 24, 31, and 44 h. Approximately 80 to 85 EE forms/cm^2 developed in the host liver (24 to 44 h) in naive mice inoculated with 1×10^6 sporozoites (Fig. 2). However, when immune mice were challenged with the same amount of sporozoites, by 24 h, approximately 40 parasites/cm^2 had successfully transformed into trophozoites. The decrease in number of parasites that result in the liver of immune rats compared with naive rats may be due to circulating antibodies that may have inhibited sporozoites from invading. It is also possible that some of the trophozoites were cleared by 24 h post-challenge. At 24 h, infiltrates began recognizing infected hepatocytes, and about four focal infiltrates/cm^2 were observed (Fig. 3). There was a slight decrease from 40 EE forms/cm^2 at 24 h to 20 EE forms/cm^2 at 31 h (Fig. 2), while the infiltrates increased from 4 to 25/cm^2 from 24 to 31 h (Fig. 3A). The number of parasites decreased from the original 40 EE forms/cm^2 at 24 h to 20 EE forms/cm^2 at 31 h to 0 EE forms/cm^2 at 44 h (Fig. 2), while the number of focal infiltrates increased from 4 to 25 to 32/cm^2 (Fig. 3A), respectively. There was no significant increase in the number of focal infiltrates that were observed between 31 and 44 h. By this time all of the EE forms were already targeted by the infiltrates, and they just pro-

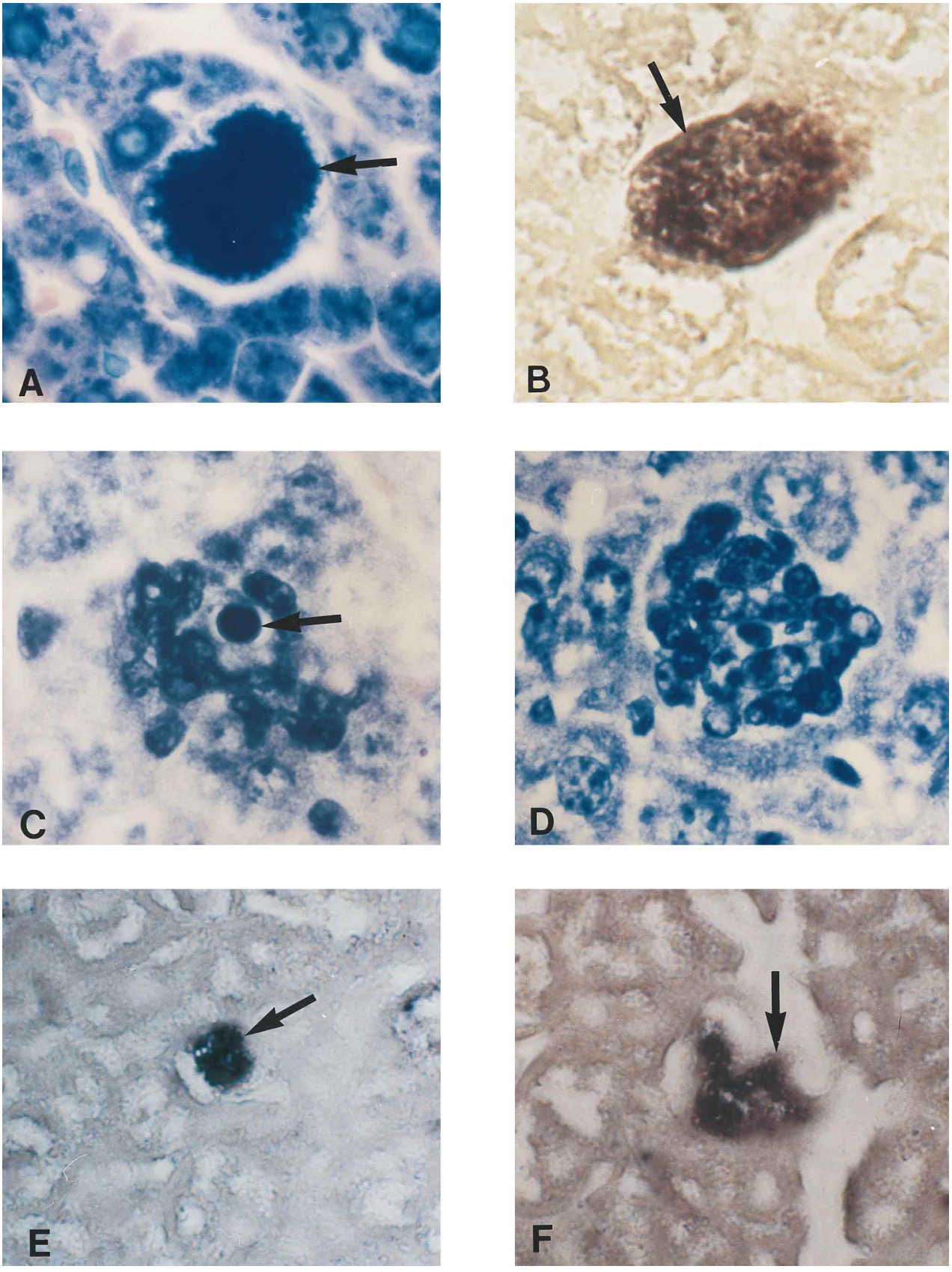
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FIG. 1. (A and B) High-power photomicrograph illustrating a liver section through a 44-h intact parasite (arrow) within a hepatocyte from a nonimmunized rat challenged with sporozoites. (A) Giemsa stain; (B) DNA in situ hybridization using a digoxygenin-labeled probe specific for the subtelomeric repeat region of DNA from *P. berghei*. (C to F) Histopathological changes in the livers of Brown Norway rats inoculated with irradiated *P. berghei* sporozoites. All immunized rats received three doses of 50,000 irradiated sporozoites, administered intravenously at biweekly intervals. They were challenged 2 weeks later by hepatic portal inoculation of 10^6 sporozoites, an inoculum that was also given to the naive rats (injected with salivary lysates). (C and D) Tissue sections that were first Giemsa stained to assess histopathology. Histological examination of this section revealed a cellular infiltrate closely associated with a malaria-infected hepatocytes (arrow). (E) Giemsa-stained slides were subsequently restained by the DNA in situ hybridization method to verify the presence of *P. berghei* parasites in the center of the infiltrates at 31 h (same parasite as in panel C; arrow). (F) Image taken 44 h after challenge with sporozoites, demonstrating the remnants of parasite DNA (arrow).

ceeded to increase in size from 50 to 90 μg in diameter (Fig. 3B). Therefore, the data show a decrease in the number of liver stages of the parasite and a concurrent increase in the number and size of focal infiltrates that recognize them.

Treatment of immune rats with aminoguanidine, an inhibitor of NOS, blocked protection in immunized animals following a challenge with sporozoites (10). Here, aminoguanidine treatment of immunized rats blocked the clearance of liver stages of the parasite, as shown by an increase parasite load (Fig. 2). There was no significant difference between the number of parasites in the immunized animals that were treated with aminoguanidine and the nonimmune or naive animals at either 31 or 44 h after sporozoite challenge. The increase in parasite tissue burden in immune animals, after treatment with aminoguanidine and exposure to viable sporozoites, resulted in parasitemia.

During the assessment of the number of parasites in liver tissue sections, the cellular infiltrates observed at the same time points were fewer in number (Fig. 3A) and smaller in diameter (Fig. 3B) than in the infected livers of immune rats treated with aminoguanidine. While the diameter of the cellular infiltrates detected in the livers of the immune rats nearly doubled in size from 31 to 44 h after challenge (Fig. 3A), the size of the infiltrates in the livers of the immune rats treated with aminoguanidine at 31 and 44 h remained the same size as at 24 h after challenge (Fig. 3B). In a similar fashion, the number of focal cellular infiltrates increased from 24 to 44 h after challenge in immune animals, yet there was no significant increase in the number of cellular infiltrates beyond 24 h after challenge in immune rats treated with aminoguanidine (Fig. 3A).

Because malaria-specific $CD4^+$ and $CD8^+$ T cells are involved in the induction and maintenance of liver-stage immunity (31), the number and type of lymphocytes per cellular infiltrate were then determined. Double-immunofluorescence labeling with MAbs that recognize CD4 and CD8 antigens on the surface of T cells were used to label the lymphocytes associated with malaria-positive cellular infiltrates in frozen liver sections. We found four times as many $CD8^+$ as $CD4^+$ T cells per infiltrate at 31 h after sporozoite challenge in immunized rats; by 44 h, a sixfold increase in the number of $CD8^+$ T cells was observed, whereas there was no additional increase in the number of $CD4^+$ T cells (Fig. 4). Although the inhibition of NO had no effect on the number of $CD4^+$ T cells per infiltrate in immune animals at any time after challenge, the number of $CD8^+$ T cells per infiltrate was dramatically affected. As shown in Fig. 4, aminoguanidine treatment resulted in an inhibition of accumulation of $CD8^+$ T cells in malaria-positive infiltrates beyond 24 h after challenge in immune animals. Interestingly, aminoguanidine treatment had no effect on the initial accumulation of $CD8^+$ T cells within the first 24 h after sporozoite challenge. In addition, aminoguanidine treatment of naive rats had no effect on the infiltrates commonly observed at the time of schizont release from hepatocytes around 48 h (9), suggesting that aminoguanidine did not cause immunosuppression in the rats.

DISCUSSION

This report illustrates the events that lead to the elimination of malaria parasites from the livers of irradiated sporozoite-immunized rats following a challenge with sporozoites. Scattered cellular infiltrates in the liver were observed within 1 day after challenge, and distinct lymphocyte-rich infiltrates surrounding infected hepatocytes were evident at 31 h and progressed in size through 44 h postchallenge. Such infiltrates contain predominately $CD8^+$ and to a lesser extent $CD4^+$ T cells. These lymphocytes were found to be abundant in the livers of animals immunized with irradiated sporozoites and challenged with viable sporozoites, whereas no focal infiltrates

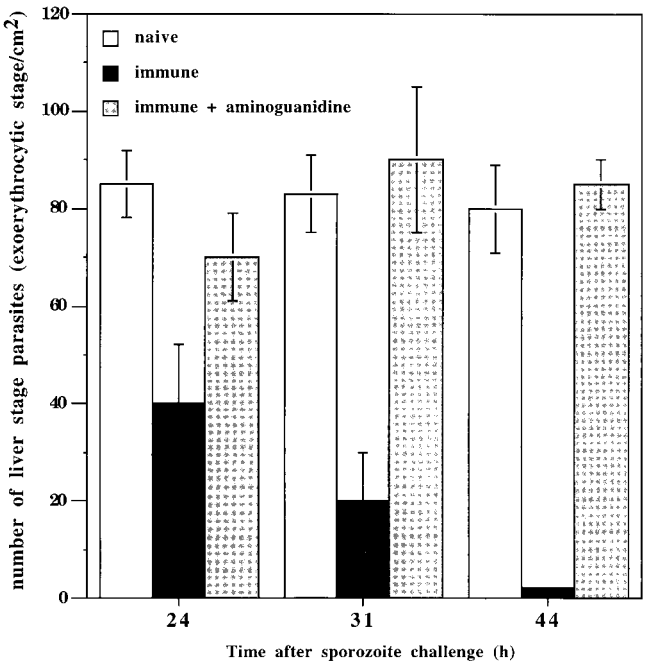

FIG. 2. Effect of aminoguanidine on the number of intrahepatic parasites observed in immunized rats following a challenge with 10^6 sporozoites. To inhibit NO production, immunized rats were treated by gastric instillation for 4 days with a substrate inhibitor of NOS, aminoguanidine (50 mg in water/kg of body weight 24 h before sporozoite challenge and then twice a day for 72 h thereafter). Livers were fixed in 10% buffered formalin, processed, sectioned, and Giemsa stained. Slides of liver sections were placed on the microscope stage and overlaid with a plastic sheet with a window measuring 0.25 cm^2. This window was superimposed on the section, and parasites within this area were counted. A total of 100 0.25-cm^2 areas in each animal, equivalent to 25 cm^2, were screened. There were three rats per treatment per time point. The mean number of intrahepatic forms per 1 cm^2 ± SD is expressed for each treatment for each time point. Microscopic evaluation of liver sections showed a fourfold decrease in the amount of intrahepatic parasites in the immunized rats compared to naive rats at 31 h after sporozoite challenge. Significant differences ($P = 0.0001$) in the number of intrahepatic parasites were observed between immune, aminoguanidine-treated rats (grey bars) and immune, non-aminoguanidine-treated rats (black bars) at both 31 and 44 h postchallenge. Aminoguanidine treatment of immunized rats blocked liver-stage protection, as shown by an increase in parasite load and a subsequent result in parasitemia (data not shown).

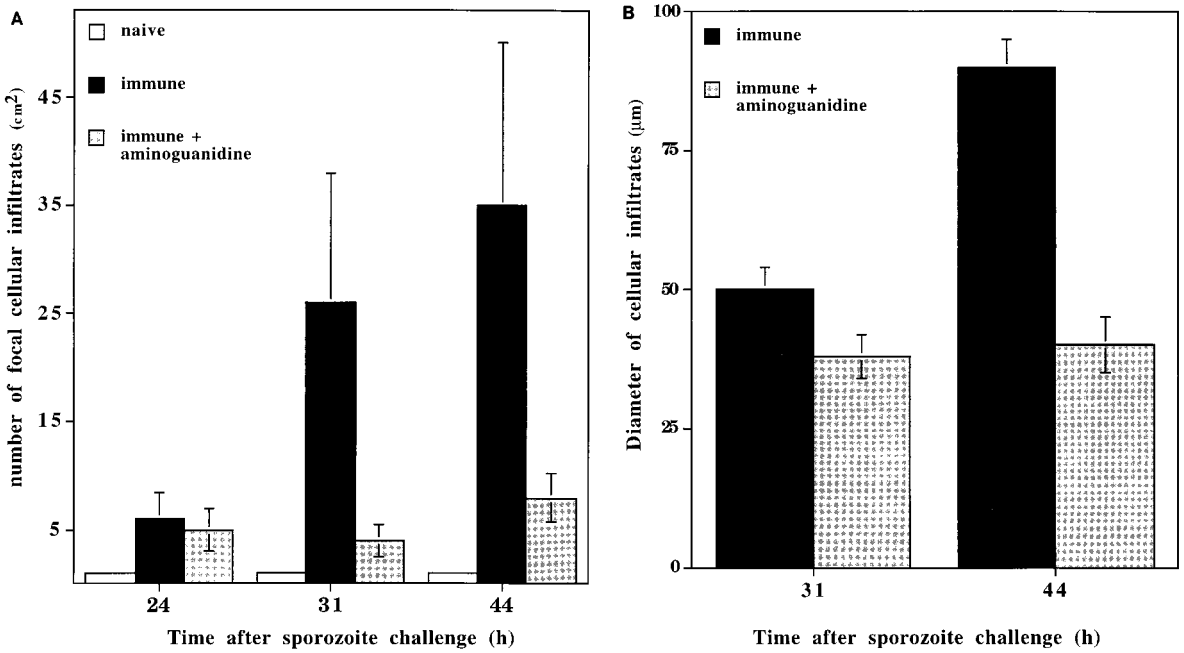

FIG. 3. Effect of aminoguanidine on the number (A) and diameter (B) of focal cellular infiltrates in immunized rats following a challenge with 10^6 sporozoites. The number of focal infiltrates in three mice per treatment for each time point is expressed as the mean ± SD in a 1-cm^2 area. The mean diameter of an infiltrate at a given time point was determined by measuring the diameter of each of 100 infiltrates in each of the three rats (total of 300 infiltrates) and is expressed as the mean ± SD. Analysis of variance revealed a significant difference ($P = 0.0001$) in the diameter of the focal infiltrates between immune, aminoguanidine-treated rats (grey bars) and immune, non-aminoguanidine-treated rats (black bars) only at 44 h postchallenge (A). Significant differences ($P = 0.0001$) were also observed in the number of focal infiltrates between immune, aminoguanidine-treated rats and immune, non-aminoguanidine-treated rats at both 31 and 44 h postchallenge.

were found in nonimmunized animals at 24 to 44 h postchallenge. Further data presented demonstrated that when NOS was inhibited in immune rats treated with aminoguanidine, the number and size of the infiltrates and the number of $CD8^+$ T cells within the infiltrates decreased. Consequently, the parasites were not cleared from the liver.

Previously, immunity induced by irradiated sporozoites has been shown to be mediated by $CD8^+$ T cells (21, 30). From this study and others (6), it is clear that the $CD8^+$ T-cell infiltrates are associated with liver-stage immunity to malaria. $CD4^+$ T cells are believed to be involved in the induction of liver-stage immunity (31), since they are required at the time of immunization for protection against sporozoites, while $CD8^+$ T cells are thought to be involved in the effector response (6, 30). Presumably, $CD8^+$ T cells participate as cytotoxic T cells and/or facilitate immunity by providing IFN-γ, which stimulates iNOS activity in infected hepatocytes (4, 10, 13, 16). Induction of iNOS activity in infected hepatocytes results in the destruction of either the intrahepatic parasite or the cell itself.

We and others have previously shown that the inhibition of NO production in rodents immunized with irradiated *P. berghei* sporozoites results in parasitemia (4, 10, 16, 22). Here, we report that the inhibition of NO production also interrupts the accumulation of $CD8^+$ T cells surrounding liver-stage parasites in immunized rats beyond 24 h after challenge with sporozoites. This decrease in the number of lymphocytes was associated with an increase in the number of intrahepatic parasites. Therefore, NO may serve as both an antimalarial agent and a mediator of lymphocyte activity and behavior by either promoting the influx or enhancing local proliferation of malaria-specific $CD8^+$ T cells or a $CD8^+$ T-cell subset.

In one possible hypothesis, NO may play a role in a cascade of recruitment from the periphery of lymphocyte-rich infiltrates which results in the attack of malaria-infected hepatocytes in immunized animals. Alternatively, the accumulation of $CD8^+$ T cells surrounding infected hepatocytes may be due to the effect of NO on the expansion of antigen-specific $CD8^+$ T cells resident in the liver. In this study, we have provided no evidence for either hypothesis. However, evidence to support the hypothesis that trafficking is important for the accumulation of $CD8^+$ T cells at the site of infection was previously demonstrated by Rodrigues et al., who showed that CD44 expression correlated with ability of malaria-specific $CD8^+$ T-cell clones to traffic to the liver and mediate protection (17).

There are no published studies that support the alternative hypothesis that the accumulation of $CD8^+$ T cells surrounding infected hepatocytes may be due to the effect of NO on the expansion of antigen-specific $CD8^+$ T cells resident in the liver. However, the influence of NO on lymphocyte proliferation has been addressed in the *Plasmodium chaubaudi* blood-stage model. Very high concentrations of NO have been shown to regulate lymphocyte proliferation during the blood stages of infection in mice by exerting an antiproliferative effect, indicating that NO acts as an autoregulatory molecule, preventing the overexpansion of Th1 and $CD8^+$ T cells (23). However, these findings do not correlate with our findings of an increase of $CD8^+$ T cells surrounding EE forms within the liver stages of the parasite.

The effects of NO on antigen-specific $CD4^+$ and $CD8^+$ T-cell adhesion to endothelium and emigration from the vasculature to the site of infection have received little consideration. Thus far, the emphasis has been on the role of NO in heterotypic interaction between neutrophils and endothelium in ischemia-reperfusion and inflammatory models of a noninfectious disease nature. Such reports have convincingly dem-

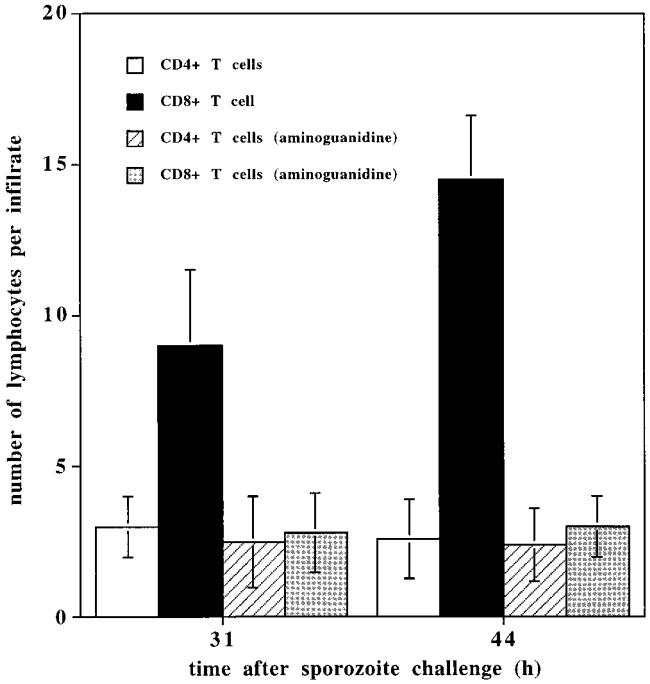

FIG. 4. Effect of aminoguanidine on the number and type of lymphocytes observed in an infiltrate. Frozen sections were fixed with methanol, blocked with 10% fetal bovine serum in PBS, and first incubated with MAb that recognizes CD4 or CD8 antigens on the surface of T cells. A double-immunofluorescence labeling assay was performed by subsequently incubating cells with tetramethyl rhodamine isothiocyanate-conjugated goat antibodies against rat IgG or fluorescein isothiocyanate-conjugated goat antibodies against rat IgG. The specimens were observed under fluorescence microscopy. The mean number of $CD4^+$ and $CD8^+$ T cells within the infiltrates at a given time point was determined by measuring the number of each of each type ($CD4^+$ or $CD8^+$) in 100 infiltrates in each of the three rats (total of 300 infiltrates) and is expressed as the mean ± SD. Analysis of variance revealed significant differences ($P = 0.0001$) in the numbers of $CD8^+$ and $CD4^+$ T cells observed in infiltrates in immune rats at 31 h (four times more $CD8^+$ T cells) and at 44 h (six times more $CD8^+$ T cells) (black bars). There was no additional increase in $CD4^+$ T cells at 31 and 44 h. Although the inhibition of NO had no effect on the number of $CD4^+$ T cells per infiltrate in immune rats at any time after challenge, the accumulation of $CD8^+$ T cells was selectively blocked.

onstrated that NO has profound antiaggregative and antiadhesive properties for leukocytes, primarily if not exclusively neutrophils (11). There is also strong evidence that platelet adhesion to vascular endothelium is blocked by NO (20). However, others have shown that NO production may stimulate the recruitment of other types of inflammatory cells. Mulligan et al. demonstrated that the inhibition of NOS reduced macrophage influx into the lungs of rats with immune complex-induced injury (15). Another explanation for the decrease in cellular infiltrates by NO inhibition is low hepatic flow (5). Since the absence of NO reportedly promotes the development of thrombi within the liver circulation, the reduction in hepatic blood flow may diminish the trafficking of lymphocytes.

Potentially the most intriguing aspect to these observations is that local NO production may selectively regulate the recruitment, retention, and/or proliferation of a cell type or cell subset at the site of a replicating pathogen (i.e., malaria-infected hepatocyte). The fact that the inhibition of NO did not diminish the size or the number of cellular infiltrates that are established at 24 h after challenge, but instead halted further accumulation of lymphocytes (at 31 and 44 h after challenge), suggests that some lymphocytes are NO responsive whereas others are NO nonresponsive. It is tempting to speculate that the initial influx of lymphocytes into the livers of immune animals either provides the appropriate signals (i.e., IFN-γ) to stimulate early NO production or generates NO in response to the parasite (25). Liew and collaborators have shown that an antigen-specific $CD4^+$ Th1 clone can be activated to produce NO and that the amount of NO may be critical in regulating cell behavior and the immune response (23, 27).

The low amounts of NO generated early in the response (12 to 24 h after challenge), either through expression of iNOS or via the constitutive pathway, may contribute to the recruitment of other effector cells (i.e., $CD8^+$ T cells) or possibly stimulate a local proliferative response. Low concentrations of NO have been suggested to promote the proliferation of T cells (23, 27). The generation of low amounts of NO has also been shown to be involved with directed-cell movement toward a chemoattractant. For example, L-NMA reportedly blocks neutrophil chemotaxis through the activation of cyclic GMP (8). It is difficult to know with certainty if this early source of NO is from the inducible or constitutive pathway, since aminoguanidine, like all NOS substrate inhibitors, is not entirely isotype specific. Regardless, the inhibition of NO generated early in the host response appears to influence recruitment, retention, turnover, and/or proliferation of $CD8^+$ T cells required for effector activity in the late response.

Late in the host response (31 to 44 h), high amounts of NO are likely produced locally via IFN-γ stimulation of hepatocytes by malaria-specific $CD8^+$ T cells (22). The fact that the number of $CD4^+$ T cells does not increase beyond 24 to 31 h after challenge, while the number of $CD8^+$ T cells continues to increase with time, leads us to hypothesize that the inhibition of NO during the early phase may set the stage for the subsequent recruitment and/or local proliferation of $CD8^+$ T cells, while inhibition of NO during the late response may block NO-mediated destruction of the intrahepatic parasite. The importance of iNOS in host defense against *Leishmania* and *Listeria* infection was confirmed with the use of knockout mice lacking NOS; however, evaluation of cell types was limited (11, 27). Further studies are warranted to determine if such a phenomenon exists. The use of mice lacking iNOS will determine whether distinct population of cells (or subsets) that make up the cellular infiltrate at various times after sporozoite challenge in the immunized animals are influenced by NO. If this observation proves to be correct, it may provide additional insight into how NO controls an infectious disease process via the selective regulation of cell types or subsets.

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