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Secreted Antibody Is Required for Immunity to *Plasmodium berghei*▽

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Infection with *Plasmodium berghei* is lethal to mice, causing high levels of parasitemia, severe anemia, and death. However, when mice are treated with antimalarial drugs during acute infection, they have enhanced immunity to subsequent infections. With this infection and cure model of immunity, we systematically examined the basis of adaptive immunity to infection using immunodeficient mice. In order to induce adaptive immunity, mice were infected with blood-stage parasites. When the mice developed 2 to 3% parasitemia, they were treated with chloroquine to cure the infection. These convalescent mice were then challenged with homologous blood-stage parasites. Immunized wild-type mice were able to control the level of infection. In contrast, mice lacking mature B cells and T cells were unable to control a challenge infection, indicating the critical role of lymphocytes in immunity to *P. berghei*. Furthermore, mice lacking secreted antibody were unable to control the level of parasitemia following a challenge infection. Our results indicate that secreted antibody is a requirement for immunity to *P. berghei*.

Each year there are approximately 500 million cases of malaria worldwide, resulting in 2 to 3 million deaths, primarily in children in sub-Saharan Africa (42). Malaria is caused by infection with one of four protozoan *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. *P. falciparum* is responsible for the majority of severe disease, which can manifest itself in anemia, cerebral malaria, organ failure, and death. Repeated infection and treatment of individuals in areas of malaria endemicity eventually induce a level of immunity that limits morbidity and results in chronic infection with low levels of parasitemia (41). A fully effective vaccine that reduces parasite burden and severe disease has not been developed.

Murine models of malaria have long been used to examine the immune response to *Plasmodium* parasites and to understand the host factors required for the development of immunity. *P. berghei* infection in mice is lethal, causing high levels of parasitemia, severe anemia, and body weight loss. However, mice can become resistant to subsequent infections by treatment with antimalarial drugs during acute infection (27). This is known as the infection and cure model, and mice that develop this immunity mimic the human experience of disease in that they are reinfected but experience low-level patent parasitemia and survive. However, it takes years to establish this level of immunity in humans (36), while in mice it is accomplished by only one infection and drug cure, which provides long-lasting protection (12, 13, 48). An understanding of the basis of rodent immunity to blood-stage infection will help to direct future vaccine approaches.

The fact that immunity induced by infection and cure is long-lasting suggests that the adaptive immune system is required for immunity. Evidence from previous work indicates a role for B and T cells in immunity. Mice lacking both mature B and T cells (SCID mice) (6), as well as mice deficient in mature B cells (μ-MT mice) (23), were unable to eliminate primary and secondary infection, suggesting that B cells are required for adaptive immunity to *Plasmodium* species (30, 47). Immunity to *P. yoelii*, another rodent parasite, can also be induced by infection and cure. In the *P. yoelii* system, it has been shown that immunity can be passively transferred to naïve recipient mice (17, 21, 34). Hyperimmune serum (from mice infected and challenged multiple times) is most effective; it allowed mice with an active infection to clear blood-stage parasites within 48 h (17). The results from these studies suggest that B cells and antibody are required for immunity, but the requirement for secreted antibody has not been well defined. While all of the studies thus far have relied on mice lacking mature B cells, in this study we were able to examine immunity to *P. berghei* in a mouse with intact B cells, which express surface immunoglobulin M (IgM) but are unable to secrete antibody (25).

Here we use the infection and cure model to examine the requirement for secreted antibody in immunity to *P. berghei* in the murine host. We establish a model of infection and cure with *P. berghei* and demonstrate the pivotal requirement for secreted antibody in adaptive immunity to *P. berghei*.

MATERIALS AND METHODS

Mouse strains. Male C57BL/6 mice (Charles River Laboratories and The Jackson Laboratory), B6.129S7-*Rag1*^{tm1Mom}/J mice (The Jackson Laboratory), and C57BL/6 mice deficient for activation-induced cytidine deaminase (AID) and secretory μ chain (μs), a gift from the Ploegh laboratory (MIT), were housed and bred in a pathogen-free facility at the Harvard School of Public Health. All animal studies were approved by the Institutional Animal Care and Use Committee.

AID$^{-/-}$ μs$^{-/-}$ mice have no serum Ig. The absence of γ1, μ, α, κ, and λ polypeptides in the serum was established by Western blotting, and an enzyme-linked immunosorbent assay confirmed the lack of IgG1, IgG2a, IgG3, and IgA (25). In addition, it was demonstrated by fluorescence-activated cell sorter anal-

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ysis that these mice are unable to maintain terminally differentiated plasma cells (25).

Immune model. In order to obtain parasites for each infection, whether primary or challenge, a source mouse was infected from a frozen stock of parasites. Parasites were harvested from the source mouse when it reached 2 to 3% parasitemia. Experimental groups of mice were infected or mock infected (phosphate-buffered saline [PBS]) with *P. berghei* NK65 by intraperitoneal (i.p.) injection of 1×10^6 infected red blood cells (iRBCs). All mice were treated with eight doses of chloroquine (3 to 4 mg/kg of body weight) over 8 to 10 days, when infected mice reached 2 to 3% parasitemia. Parasitemia was measured by calculating the percentage of iRBCs on a thin tail blood smear stained with Giemsa. Following an incubation of at least 40 days posttreatment, all mice were infected by i.p. injection of 1×10^6 iRBCs from the same stock of parasites as the primary infection. Parasitemia was monitored by thin tail blood smear.

Serum samples. C57BL/6 mice (Charles River Laboratory) were infected or mock infected with *P. berghei* NK65 by i.p. injection of 1×10^6 iRBCs from a source mouse as described above. Infected mice were monitored by tail smear as described above and treated with chloroquine (3 to 4 mg/kg) if parasitemia reached 10%. Blood was collected by cardiac puncture on day 17 postinfection. Blood was allowed to coagulate at room temperature for 1 h, after which it was placed on ice. Blood was centrifuged for 10 min at $16{,}100 \times g$ at 4°C. The serum was removed and stored at −80°C until use.

Passive transfer of serum. C57BL/6 mice were either mock infected or infected with 1×10^6 iRBCs as described above. Nonimmune and immune sera were collected 17 days following mock infection or infection, and 1 ml of serum was transferred into naïve C57BL/6 mice 3 h postinfection by i.p. injection with 1×10^6 iRBCs. Mice were then given 0.5 ml serum on day 3 and day 6 postinfection, and parasitemia was monitored by thin tail blood smear for 7 days.

Statistical analysis. For results shown in Fig. 1, 2, and 4, the significance of differences in parasitemia between two groups of mice at a particular time point was calculated by the Mann-Whitney test (Wilcoxon rank sum test) using GraphPad Prism version 4.03 for Windows. Differences were considered significant at a P value of <0.05. Mice in the experiment represented in Fig. 2 were sacrificed at >20% parasitemia or when they became moribund. Statistically significant data reported for mice at days 18 and 19 postchallenge assume that RAG1$^{-/-}$ mice with high levels of parasitemia that died in the previous days would not have spontaneously cleared parasitemia. For results shown in Fig. 4, statistical analysis was not performed at day 12 since data were missing from two C57BL/6 mice that were removed from the experiment.

For results shown in Fig. 3, the statistical significance of differences in peak parasitemia following serum transfer over the course of infection was calculated using a second-order mixed-effects regression model, which accounts for multiple observations of the same replicate and allows for uneven spacing of sampling times in the two groups (26). P values for between-group differences were calculated using Markov chain/Monte Carlo methods (15) and were considered significant at <0.05. All mixed models were fit in R (R Foundation for Statistical Computing, 2008) using the package lme4 (4).

RESULTS

Immunized C57BL/6 mice control secondary *P. berghei* infection. To explore the requirement for secreted antibody in the development of immunity to *P. berghei*, we first established an infection and cure model in which immunity is defined as the ability to limit the growth of asexual parasites to low levels following challenge with iRBCs. Wild-type mice were immunized by infection with blood-stage parasites and treated with an antimalarial drug to clear parasites from the blood. Concurrently, a control group was mock infected with PBS. At least 6 weeks later, both groups of mice were challenged with a blood-stage infection (Fig. 1). Following challenge, the immunized C57BL/6 mice developed patent parasitemia lasting approximately 1 week, with peaks of approximately 1 to 2%. The immunized group was then able to control parasitemia to low levels for at least 50 days (data not shown). In contrast, the nonimmunized control group experienced significantly higher ($P < 0.05$) parasitemia as early as day 4 postchallenge. These results demonstrate that mice with an intact adaptive immune system are able to limit parasite growth to low levels during secondary *P. berghei* infection following a primary infection resolved with drug treatment.

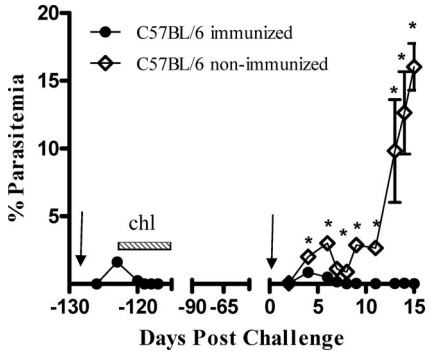

FIG. 1. Course of infection in immunized C57BL/6 mice. C57BL/6 mice in the immunized group ($n = 6$) were infected with *P. berghei* by i.p. injection of iRBCs. Mice in the nonimmunized group ($n = 3$) were mock infected by i.p. injection of PBS. All mice were treated with chloroquine (chl) when parasitemia reached 2 to 3% in the immunized group. Following an incubation of at least 40 days, all mice were challenged, and parasitemia was monitored on the days indicated. Immunized mice developed patent parasitemia following challenge but were able to control the infection. Mice in the nonimmunized group were unable to control the infection. Data are representative of results from seven independent experiments. The asterisks indicate a significant difference in parasitemia ($P < 0.05$) between groups. The arrows indicate infection or challenge at the times indicated.

Immunized mice lacking mature B cells and T cells are unable to control secondary *P. berghei* infection. In order to determine whether lymphocytes are required for adaptive immunity to *P. berghei*, we applied the infection and cure model to RAG1$^{-/-}$ mice, which lack mature B and T cells and therefore have no functional adaptive immune response (32). Previous experiments using SCID mice have been useful in elucidating the importance of B and T cells in immunity to *Plasmodium* species (6); the present study took advantage of mice lacking RAG1, whose defects are confined to the immune system. To obtain immunized mice, groups of RAG1$^{-/-}$ and wild-type mice were infected by injection of iRBCs. When the mice reached 2 to 3% parasitemia, they were treated with an antimalarial drug to clear the parasites from the bloodstream. Both groups were challenged by injection of iRBCs.

As shown in Fig. 2, immunized RAG1$^{-/-}$ mice were unable to control the challenge infection, and parasitemia in these mice continued to increase until they were sacrificed. They developed significantly higher parasitemia ($P < 0.05$) than immunized wild-type mice by day 15 postchallenge (10.6% versus 0.4%, respectively). The immunized wild-type mice were able to maintain parasitemia at <2% for the duration of the experiment. Nonimmunized wild-type controls were unable to control infection and were sacrificed at a parasitemia of >20% (data not shown). These data suggest that lymphocytes are required for immunity to a secondary challenge with *P. berghei*.

Serum from *P. berghei*-infected mice reduces parasite burden in naïve mice. To study the role of antibody generated during immunization in the control of parasitemia during blood-stage *P. berghei* infection, we transferred immune serum

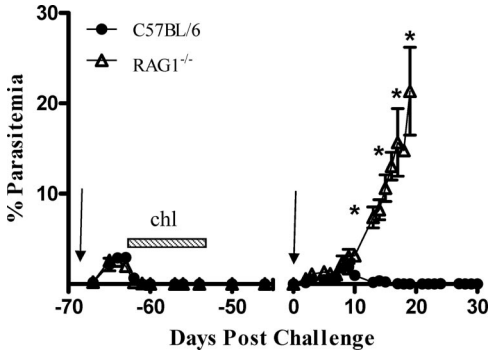

FIG. 2. Course of infection in immunized mice lacking B and T cells. Both C57BL/6 ($n = 5$) and RAG1$^{-/-}$ ($n = 6$) mice were immunized and monitored as described for the immunization model. All mice were treated with chloroquine (chl) when parasitemia reached 2 to 3%. Following challenge, RAG1$^{-/-}$ mice were unable to control infection. Data are representative of results from three independent experiments. The asterisks represent a significant difference in parasitemia ($P < 0.05$) between groups. The arrows indicate infection or challenge at the times indicated.

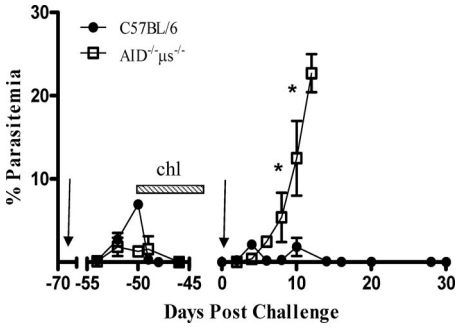

FIG. 4. Course of infection in immunized mice lacking secreted antibody. Both C57BL/6 ($n = 6$) and AID$^{-/-}$ μs$^{-/-}$ ($n = 3$) mice were immunized and monitored as described for the immunization model. All mice were treated with chloroquine (chl) when parasitemia reached 2 to 3%. Following challenge, AID$^{-/-}$ μs$^{-/-}$ mice were unable to control infection. Data are representative of results from two independent experiments. The asterisks represent a significant difference in parasitemia ($P < 0.05$) between groups. The arrows indicate infection or challenge at the times indicated.

into naïve mice. Serum from immunized or nonimmunized mice was passively transferred to naïve C57BL/6 mice following infection with parasites. Mice that received immune serum had significantly increased resistance to *P. berghei* over the course of the experiment ($P < 0.05$) (Fig. 3). Mice that received immune serum had, on average, threefold-lower levels of parasitemia than those receiving nonimmune serum. These results indicate the importance of antibodies in controlling parasitemia and suggest that secreted antibody may be required for immunity.

Immunized mice lacking secreted antibody are unable to control secondary *P. berghei* infection. In order to specifically determine whether secreted anybody is required for adaptive immunity to *P. berghei*, we tested whether mice lacking secreted antibodies (25) could develop immunity to *P. berghei*. AID$^{-/-}$ μs$^{-/-}$ mice have intact T cells; in these mice, B cells have surface IgM but do not undergo class switching due to the disruption of the AID gene (33, 39) and do not secrete antibody due to the disruption of μs (5). With the infection and cure model, groups of AID$^{-/-}$ μs$^{-/-}$ and wild-type mice were immunized by injection of iRBCs. When the mice reached 2 to 3% parasitemia, they were treated with an antimalarial drug to clear the parasites from the bloodstream. Both groups were challenged by injection of iRBCs. In contrast to immunized wild-type mice, immunized AID$^{-/-}$ μs$^{-/-}$ mice were unable to control infection (Fig. 4). By day 10 postchallenge, the parasitemia of immunized AID$^{-/-}$ μs$^{-/-}$ mice was significantly higher ($P < 0.05$) than that of the immunized wild-type group (12.5% versus 1.8%, respectively). All immunized AID$^{-/-}$ μs$^{-/-}$ mice reached levels of parasitemia sufficient enough that they became moribund and were sacrificed by day 12. Nonimmunized control groups of wild-type and AID$^{-/-}$ μs$^{-/-}$ mice were mock infected, treated with chloroquine, and challenged along with the immunized mice. Nonimmunized control mice were also unable to control infection and were sacrificed at a parasitemia of >20% (data not shown). These data demonstrate that secreted antibody is essential for immunity to blood-stage *P. berghei* infection.

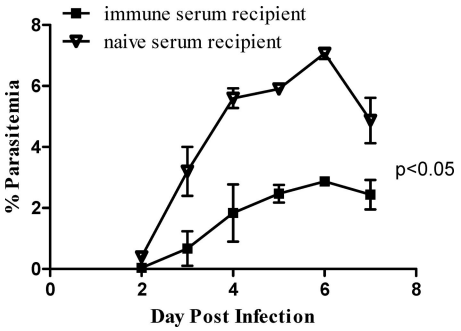

FIG. 3. Passive protection of naïve C57BL/6 mice against *P. berghei*. Serum samples were collected from immunized mice or nonimmunized mice. Serum was injected i.p. into naïve C57BL/6 recipients (three mice per group) on days 0, 3, and 6. Passively immunized mice were challenged i.p. with 1×10^6 iRBCs on day 0, and parasitemia was monitored on the days indicated.

DISCUSSION

We have used an infection and cure model of malaria in mice to examine the basis of immunity to infection. First, we established a model of infection and cure in which immunized wild-type mice are able to control the level of parasitemia of a challenge which would otherwise be lethal. Using this model, we were able to demonstrate that lymphocytes are essential for immunity to *P. berghei* by examining the ability of mice lacking mature B and T cells to develop immunity. Furthermore, we were able to directly test the role of secreted antibody in immunity by using mice lacking secreted antibody in our infection and cure model. Our results indicate that secreted antibody is essential for adaptive immunity to *P. berghei*.

Secreted antibody may function to reduce parasitemia by opsonizing iRBCs to trigger uptake by macrophages or by triggering the complement cascade. Studies with humans have found a correlation between IgG1 and IgG3 levels and protection against severe disease (7, 18, 35); these two antibody isotypes have also been shown to be capable of mediating

opsonization (20). Furthermore, passive transfer of malaria-specific IgG has been shown to reduce parasitemia in humans (8, 11, 40).

We found that secreted antibodies were able to reduce parasitemia following a passive transfer but were not able to clear infection completely. These results suggest that, although secreted antibody is essential for protective immunity to *P. berghei*, other components of the immune system also play important roles. While our results suggest that antibodies are crucial for immunity, they do not exclude a role for T cells. The importance of T cells in immunity to blood-stage malaria has been confirmed by a number of previous experiments, including the adoptive transfer of spleen cells, the depletion of $CD4^+$ T cells, and the transfer of $CD4^+$ T-cell clones specific for malaria (3, 9, 10, 22, 24, 29, 38, 43–46), but when transferred into a mouse lacking B cells, $CD4^+$ T cells are insufficient to clear parasites (30). Similarly, it appears that B cells and antibody play an important role in parasite clearance but are insufficient alone. When B-cell-deficient mice are infected (or drug treated and then challenged), they are able to control acute infection but later develop chronic, low-level parasitemia and cannot completely clear infection. The adoptive transfer of B cells from immune mice during these relapsing peaks of parasitemia allowed mice to clear infection (47).

There is evidence that the requirement for T cells in the clearance of infection depends on the species of parasite. *P. yoelii* infection, which is lethal to mice, can be resolved with humoral immunity (19, 37, 47). Mice infected with *P. chabaudi*, which causes a nonlethal infection, require T cells as well as B-cell-dependent mechanisms to clear an infection (9, 10, 30, 43). Future experiments will attempt to clarify the role of $CD4^+$ T cells in adaptive immunity to *P. berghei*, which may also help to determine which of these models is the most relevant to malaria caused by *P. falciparum* in humans (28).

Although we have established a critical role for secreted antibody in the development of immunity, the target of this antibody is unknown. Variant antigen families in *Plasmodium* species have been shown to be immunogenic (1, 2, 14, 16), and antibodies produced against them are variant specific and thought to play a role in protection (31, 36). One example of a known variant antigen family in *P. berghei* is *bir*, a homologue of the major variant antigen family in malaria. The *bir* genes have been understudied but may prove to be a target of the secreted antibodies necessary for immunity to *P. berghei*.

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