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# Absolute Requirement for an Active Immune Response Involving B Cells and Th Cells in Immunity to *Plasmodium yoelii* Passively Acquired with Antibodies to the 19-kDa Carboxyl-Terminal Fragment of Merozoite Surface Protein-1<sup>1</sup>

Chakrit Hirunpetcharat,\* Peter Vukovic,\* Xue QinLiu,\* David C. Kaslow,† Louis H. Miller,† and Michael F. Good<sup>2\*</sup>

Vaccination of mice with the leading malaria vaccine candidate homologue, the 19-kDa carboxyl terminus of merozoite surface protein-1 (MSP1<sub>19</sub>), results in sterile immunity to *Plasmodium yoelii*, with no parasites detected in blood. Although such immunity depends upon high titer Abs at challenge, high doses of immune sera transferred into naive mice reduce parasitemia (and protect from death) but do not result in a similar degree of protection (with most mice experiencing high peak parasitemias); this finding suggests that ongoing parasite-specific immune responses postchallenge are essential. We analyzed this postchallenge response by transferring Abs into manipulated but malaria-naive mice and observed that Abs cannot protect SCID, nude, CD4<sup>+</sup> T cell-depleted, or B cell knockout mice, with all mice dying. Thus, in addition to the Abs that develop following MSP1<sub>19</sub> vaccination, a continuing active immune response postchallenge is required for protection. MSP1<sub>19</sub>-specific Abs can adoptively transfer protection to strains of mice that are not protected following vaccination with MSP1<sub>19</sub>, suggesting that the Ags targeted by the immune response postchallenge include Ags apart from MSP1<sub>19</sub>. These data have important implications for the development of a human malaria vaccine. *The Journal of Immunology*, 1999, 162: 7309–7314.

Although immunity to malaria can be induced by repeated exposure to malaria parasites, the quest to develop a subunit vaccine has not yet been successful (1). A few leading candidates are currently being tested in animal systems. The 19-kDa carboxyl-terminal fragment of the merozoite surface protein-1 (MSP1<sub>19</sub>)<sup>3</sup> is the most promising (2–8). Mice immunized with MSP1<sub>19</sub> from *Plasmodium yoelii* are completely protected, with parasites never detected in the peripheral blood (7). Control animals die within 8 days of challenge. However, the mechanism of protection is not well understood.

Studies have implicated Abs in particular in MSP1<sub>19</sub>-induced immunity (7). However, serum from MSP1<sub>19</sub>-immunized mice can adoptively transfer only partial protection to naive recipients (6); all mice developed patent infection postchallenge, with mice ultimately curing if the amount of transferred Ab is sufficient. The ability of these mice to eradicate parasites must be due to factors other than the transferred Abs per se, because the parasites are

being cleared when the level of Ab is less than the level prechallenge. This finding, when taken together with the observation that a depletion of CD4<sup>+</sup> T cells from MSP1<sub>19</sub>-immunized mice can abrogate immunity (4, 7), suggests that T cells are required for immunity postchallenge. To address the nature of the postchallenge immune response, we compared the ability of MSP1<sub>19</sub> immune serum to transfer immunity to normal, SCID, nude, CD4<sup>+</sup> T cell-depleted, and B cell-deficient ( $\mu$ MT) mice (10, 11).

## Materials and Methods

### Mice and parasites

We used 6- to 8-wk-old C3H/HeJ, BALB/c, BALB/c nude (nu/nu), SCID, C57BL/6 (B6), and  $\mu$ -chain knockout (KO) mice (10, 11). The KO mice, which were kindly provided by Barbara Fazekas de St Groth, were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and were backcrossed to the B6 background for  $\geq 10$  generations. These mice have neither B cells nor Ab. *P. yoelii* YM (lethal) (12) was used.

### Recombinant MSP1<sub>19</sub>

MSP1<sub>19</sub> of *P. yoelii* was produced in *Saccharomyces cerevisiae* (yMSP1<sub>19</sub>) as described previously (6).

### Preparation of MSP1<sub>19</sub> immune sera

BALB/c mice were immunized with MSP1<sub>19</sub> using a parenteral immunization protocol described previously as protocol A (7).

### Ab depletion

Serum was depleted of Ab by passage over an immobilized protein A agarose column (Pierce, Rockford, IL) according to the manufacturer's instructions. Analysis of immune serum from mice optimally vaccinated with MSP1<sub>19</sub> (7) revealed a 150-fold reduction in MSP1<sub>19</sub>-specific titer following treatment.

### Passive transfer study

Mice were injected i.p. with 0.5 ml of MSP1<sub>19</sub> immune serum at days -1, 0, and 1, relative to the day of challenge infection (resulting in a titer of

\*Queensland Institute of Medical Research, Brisbane, Australia; and †Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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<sup>2</sup> Address correspondence and reprint requests to Dr. Michael F. Good, Queensland Institute of Medical Research, P.O. Royal Brisbane Hospital, Brisbane 4029, Australia. E-mail address: michaelG@qimr.edu.au

<sup>3</sup> Abbreviations used in this paper: MSP1, merozoite surface protein-1; B6, C57BL/6; KO, knockout; NR1g, normal rat Ig; NMS, normal mouse serum; pRBC, parasitized RBC.

$\sim 2 \times 10^6$  in the recipient). Mice were challenged i.v. with  $10^4$  *P. yoelii* YM parasitized RBCs (pRBCs) on day 0. Parasitemias were monitored as described previously (7).

#### *In vivo* CD4<sup>+</sup> T cell depletion

Mice were depleted of CD4<sup>+</sup> T cells by three daily treatments with 1 mg of rat anti-CD4 (GK1.5) mAb before challenge with parasite (7).

#### Ab assay

An MSP1<sub>19</sub>-specific ELISA was performed as described previously (7).

## Results

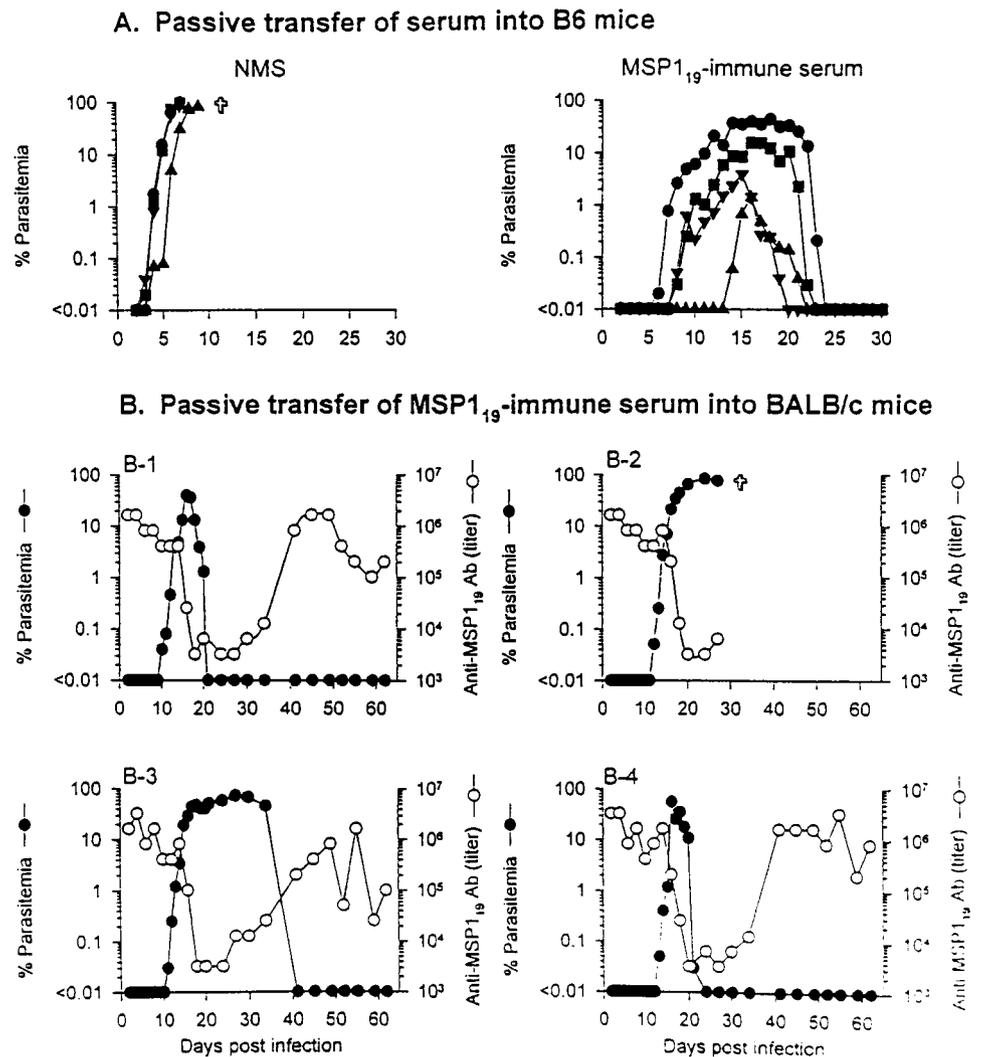
### MSP1<sub>19</sub>-specific passive immunity in normal mice

First, we confirmed that three injections each of 0.5 ml of immune serum around the time of challenge (resulting in an immediate titer of  $\sim 2 \times 10^6$  in the recipient) can delay patency of infection and ultimately enable normal immunocompetent mice to resolve their infection after a peak parasitemia of 1–44% (Fig. 1). Although the serum donors themselves were not challenged before taking serum, other mice similarly immunized were challenged and were solidly immune (parasites not detected postchallenge), with titers at the time of challenge ranging from  $0.5 \times 10^6$  to  $6 \times 10^6$ . Immune sera passively transfer protection in a dose-dependent manner. Normal mice given three doses of 0.5 ml of pooled sera were protected after a patent infection, whereas two of three animals given three doses of 0.25 ml of sera showed a delayed patency before suc-

cumbing; mice given three doses of 0.1 ml of sera or three doses of PBS before challenge were not protected at all. Membrane filtration of immune sera, excluding molecules of  $>30$  kDa, removed all protective effect from the serum, excluding the possibility that Ag (19 kDa) may be present in the serum and responsible for protection (data not shown). Furthermore, depletion of Ig from immune serum by passage over a protein A column (*Materials and Methods*) completely abolished the ability of the serum to protect C3H recipients (data not shown).

We also show that the titer of MSP1<sub>19</sub>-specific Abs falls during the ascent of parasitemia, which is consistent with the passively transferred Abs being adsorbed by merozoites (Fig. 1B). To exclude the possibility that the fall in Ab may be due to simple physiological turnover, we conducted experiments in which mice received either MSP1<sub>19</sub>-specific immune serum or serum from mice immunized with an irrelevant Ab specific for peptide 145 of the M protein of group A streptococcus (13). Following infection, we observed a fall in MSP1<sub>19</sub>-specific titer from  $10^6$  to  $\sim 10^3$  in mice that received these Abs and that were challenged, coinciding with the increase in parasitemia. Unchallenged mice had a very small drop (to  $3 \times 10^5$ ) over the course of the experiment. There was no change in the titer of p145-specific Abs over the course of the experiment in either the challenged or nonchallenged group.

Parasitemia subsequently falls in the passively immunized mice at a time when the titer of MSP1<sub>19</sub>-specific Abs is low, which is



**FIGURE 1.** Protection and Ab response induced following passive transfer of MSP1<sub>19</sub> immune serum into mice followed by challenge infection. B6 (A) and BALB/c (B) mice were administered three i.p. daily injections of 0.5 ml of NMS or MSP1<sub>19</sub> immune serum on days -1, 0, and 1, relative to the day of parasite challenge. Mice were challenged i.v. with  $10^4$  *P. yoelii* YM pRBCs on day 0. Sera were collected every 2 days and assayed for Abs to MSP1<sub>19</sub> by ELISA. The panels in B refer to individual mice.

suggestive of an active immune response. Furthermore, after clearance of parasites, the level of MSP1<sub>19</sub>-specific Abs subsequently rises again, which is consistent with active Ab production.

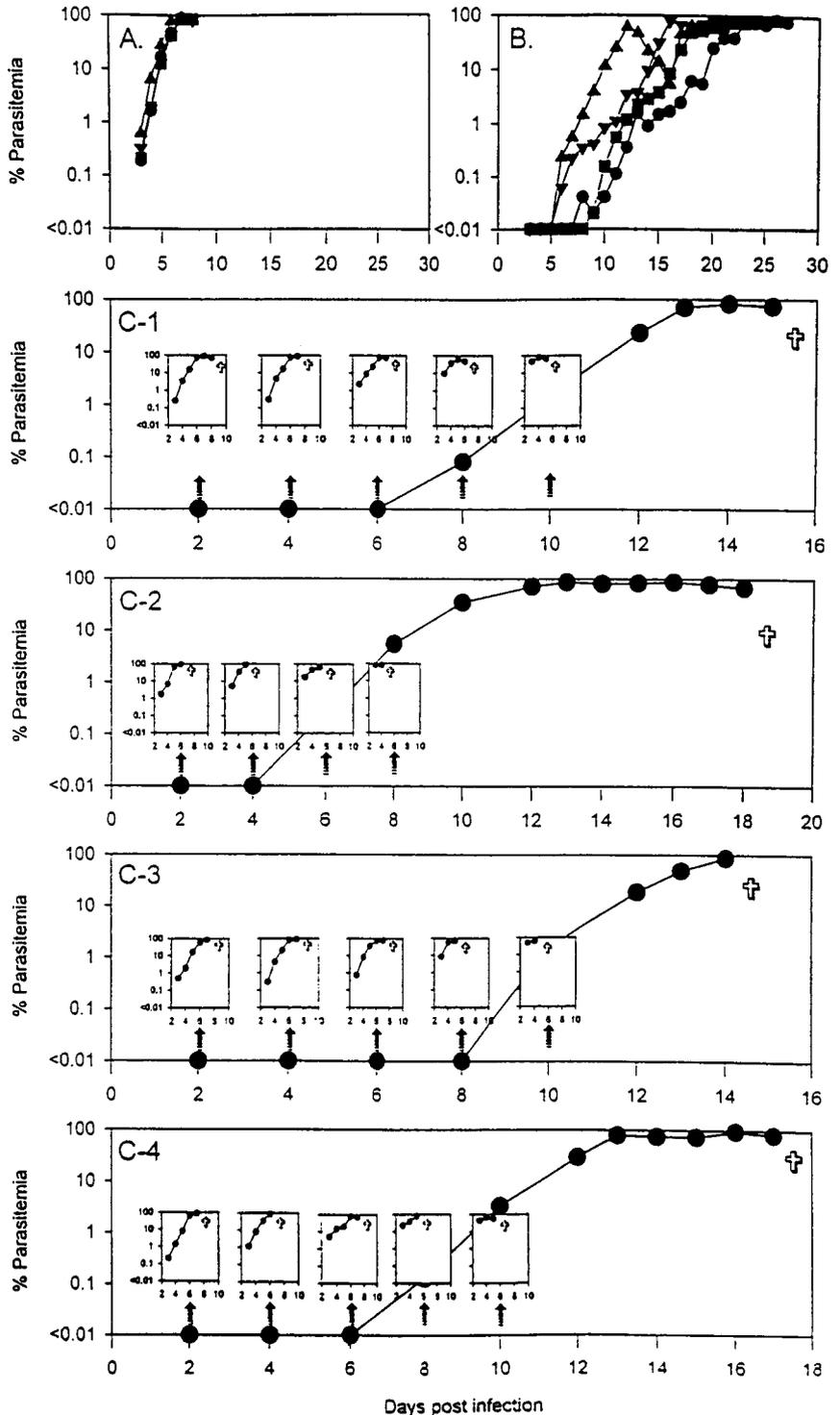
*Passively transferred MSP1<sub>19</sub>-specific Abs cannot eliminate parasites*

To demonstrate that the ultimate clearance of the parasite was not due to the transferred Abs per se, we transferred immune serum to SCID mice. Although these mice were able to control infection for  $\leq 8$  days, they developed patent infection that did not resolve (Fig. 2). To exclude the possibility that parasites were not sequestered

away from Ab for the 8-day prepatent period, we transferred blood from infected mice to naive reporter mice at days 2, 4, 6, and 8 postchallenge and observed that reporter mice developed malaria infection in all cases (Fig. 2C). Thus, an active immune response was required to clear parasites from mice that were passively administered MSP1<sub>19</sub>-specific Abs.

*Requirement for T cells and Th cells*

To determine a requirement for naive T cells at the effector stage, serum from MSP1<sub>19</sub>-immunized mice was transferred into normal



**FIGURE 2.** Subpatent parasitemias in mice that were passively transferred MSP1<sub>19</sub> immune serum. SCID mice were administered three daily 0.5-ml injections of NMS (A) or MSP1<sub>19</sub> immune serum (B and C) on days -1, 0, and 1, relative to the day of parasite challenge. Mice were challenged i.v. with 10<sup>4</sup> *P. yoelii* YM pRBCs on day 0. Blood was collected every 2 days after challenge from the mice in group C and transferred into naive BALB/c mice; parasitemias were examined (small boxes as indicated by arrows).

and athymic (nu/nu) naive BALB/c mice (Fig. 3, A–D). Postchallenge, athymic recipients of MSP1<sub>19</sub>-specific Abs or normal mouse serum (NMS) developed high parasitemia and died. We assessed the contribution of CD4<sup>+</sup> T cells by depleting normal B6 mice with either GK1.5 (anti-CD4) Abs or normal rat Ig (NRIg) before the administration of MSP1<sub>19</sub>-specific serum and challenge (Fig. 3, E and F). GK1.5 treatment, as assessed by FACS analysis, destroyed 98.8% of splenic CD4<sup>+</sup> T cells in nonchallenged littermates. Postchallenge, we observed that the NRIg group survived after a patent parasitemia of 2–24%, whereas all mice in the GK1.5-treated group died. Thus, naive CD4<sup>+</sup> T cells are required for immunity in mice administered MSP1<sub>19</sub>-specific Abs. Next, we performed passive transfer studies in B cell-deficient ( $\mu$ MT) mice (which have T cells that are capable of reacting by proliferation to MSP1<sub>19</sub> following vaccination (7)). Although patent parasitemia was delayed in the group that received anti-MSP1<sub>19</sub> immune serum, these mice were unable to control their parasitemias (Fig. 4).

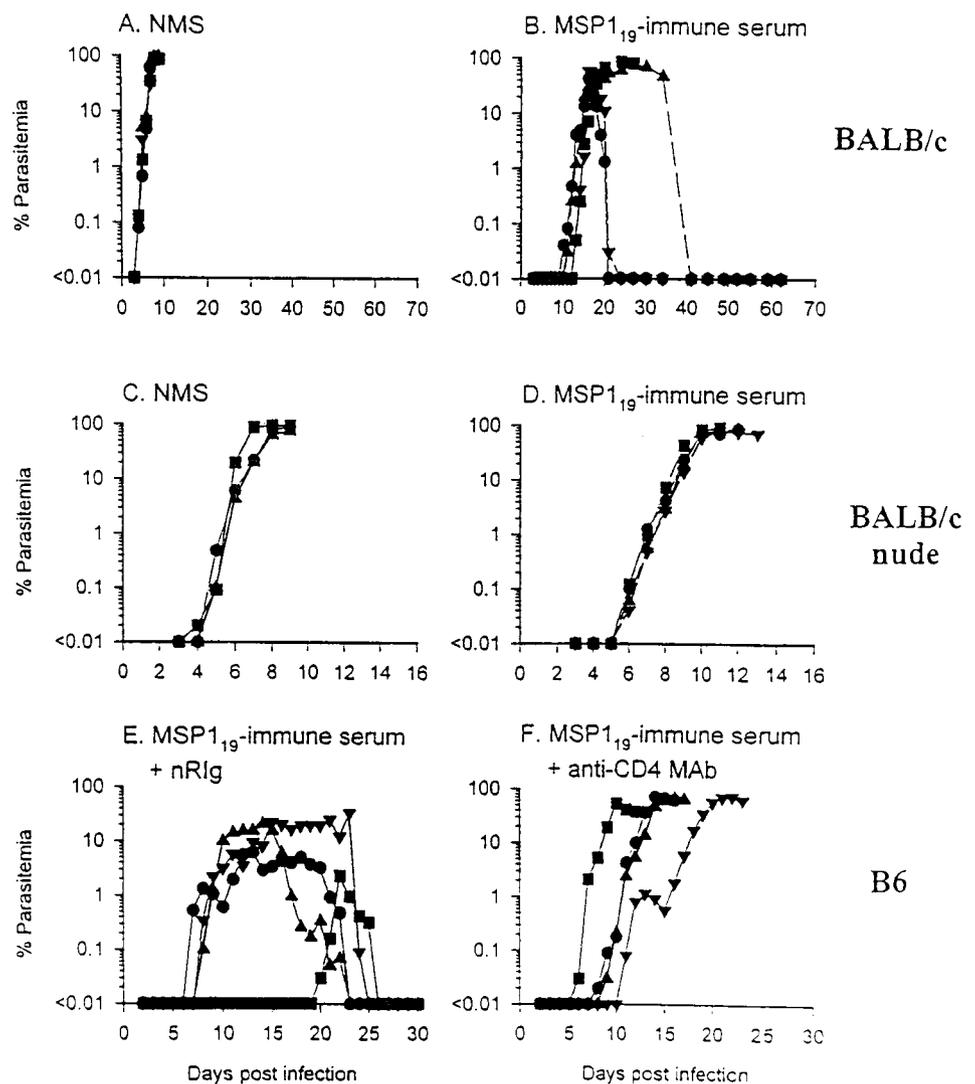
*Ability of MSP1<sub>19</sub>-specific Abs to transfer protection to strains of mice not protected following MSP1<sub>19</sub> vaccination*

The above data strongly suggest that an active immune response is required postinfection for MSP1<sub>19</sub> vaccination to be effective. The stimulus for that immune response must obviously be the parasite, not the vaccine. The target Ag or Ags within the parasite are not known. If MSP1<sub>19</sub> itself was the principal target, then it may be

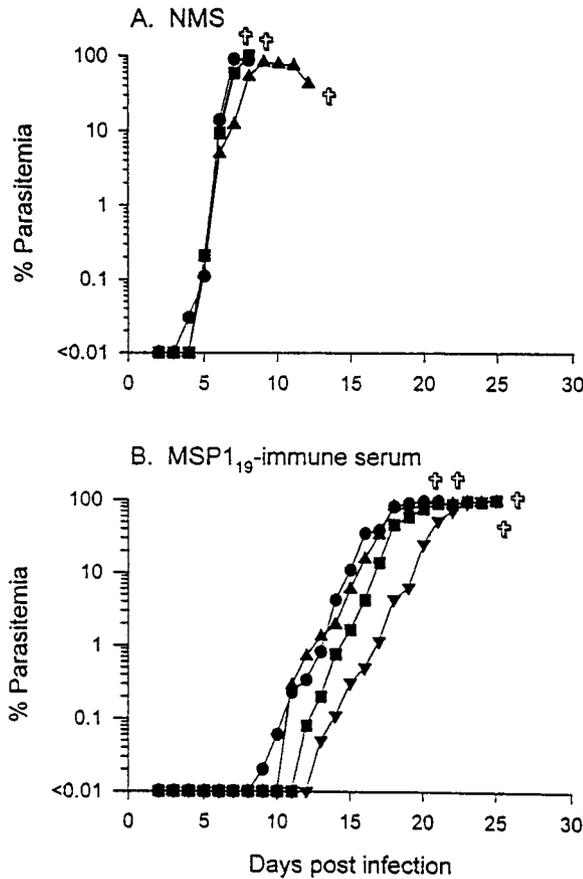
expected that strains of mice that were poor responders to MSP1<sub>19</sub> would not be protected by adoptively transferred Abs. To address this issue, we transferred Abs into three strains of normal immunocompetent mice (C57BL/10 (H-2<sup>b</sup>), B10.BR (H-2<sup>k</sup>), and B10.D2 (H-2<sup>d</sup>)) and subsequently challenged these mice with *P. yoelii*. We have shown previously that B10 mice are strongly protected following GST-MSP1<sub>19</sub> vaccination, but that B10.BR mice are not protected at all (6). The ability of MSP1<sub>19</sub> to protectively immunize B10.D2 mice has not been ascertained. As shown in Fig. 5 however, all three strains of mice were equally protected by MSP1<sub>19</sub>-specific Abs, with peak parasitemias of <40% in all animals. Control mice that received NMS instead of MSP1<sub>19</sub>-specific Abs either died or suffered a peak parasitemia of between 60% and 80% postchallenge.

## Discussion

The results presented here show for the first time that an active immune response postchallenge is required for protection against malaria even if protective Abs are present prechallenge at high titer. It is important to note that this active postchallenge immune response is not a classical boosting response. Boosting implies an augmentation of the numbers of T and B cells initially induced by the vaccine itself following parasite challenge. The animals challenged in this study were naive with respect to the presence of



**FIGURE 3.** Requirement of CD4<sup>+</sup> T cells for recovery from infection. Normal BALB/c, BALB/c nude, and B6 mice were administered three daily i.p. injections of 0.5 ml of NMS (A and C) or MSP1<sub>19</sub> immune serum (B and D–F) on days –1, 0, and 1, relative to the day of parasite challenge. B6 mice were treated with NRIg (E) or anti-CD4 mAb (F) as described in *Materials and Methods*. Mice were challenged i.v. with 10<sup>4</sup> *P. yoelii* YM pRBCs on day 0.

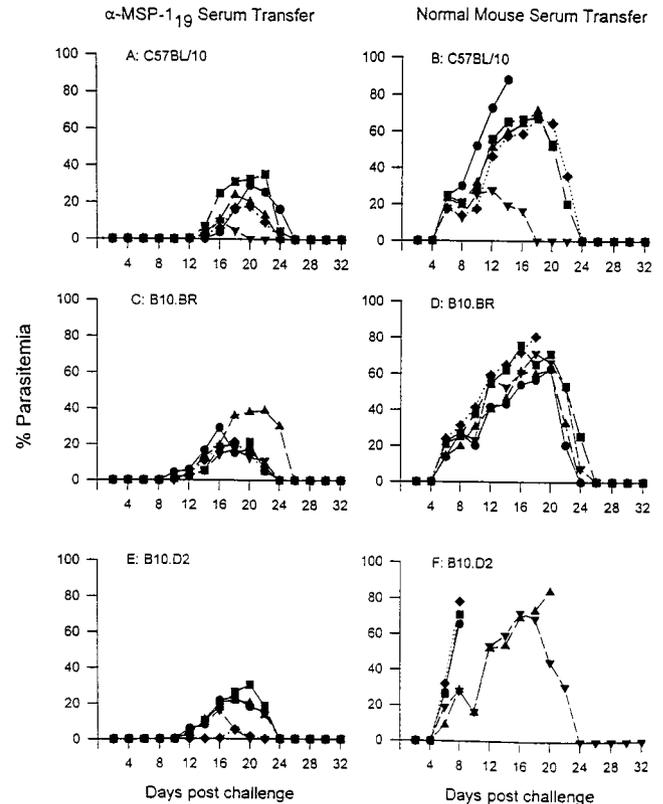


**FIGURE 4.** Passive transfer of MSP1<sub>19</sub> immune serum into B cell-deficient mice. B cell KO mice were administered three daily i.p. injections of 0.5 ml of NMS (A) or MSP1<sub>19</sub> immune serum (B) on days -1, 0, and 1, relative to the day of parasite challenge. Mice were challenged i.v. with  $10^4$  *P. yoelii* YM pRBCs on day 0.

vaccine-specific T and B cells. Rather, the immune response required postchallenge is a naive immune response targeting the parasite per se. Our system of passive transfer is clearly different from that in which animals are actively vaccinated. In that situation, classical boosting of the vaccine-induced immune response is likely to be very important. However, such boosting would significantly increase the titer of the vaccine-specific Abs only if the animal was able to respond to the vaccine Ag as present in the native parasite. We know from a number of malaria studies that this requirement cannot always be relied upon to occur (14); thus, such animals following active vaccination may be very similar to the animals in our system that adoptively received MSP1<sub>19</sub>-specific Abs. Thus, although classical boosting of a vaccine-induced immune response will be important, it may not always occur; in such situations, and perhaps in all situations, our data argue that an additional immune response is required.

The mechanism by which the passively transferred Abs delay the patency of the infection was not studied here. However, our data strongly suggest that during infection, specific Abs, but not Abs of an irrelevant specificity, are consumed. Abs may function by blocking the processing of the larger mature MSP1 protein on the merozoite surface (15) or may simply sterically hinder the merozoite invasion of erythrocytes.

The data presented here are particularly encouraging for the likely efficacy of subunit malaria vaccines. Although we have shown that an active de novo immune response postchallenge is required for protection as well as the vaccine-induced Ab response



**FIGURE 5.** Passive transfer of MSP1<sub>19</sub> immune serum or NMS into different strains of H-2 congenic mouse strains (as indicated). Mice received three daily i.p. injections of 0.5 ml of serum on days -1, 0, and 1, relative to the day of parasite challenge. Mice were challenged i.v. with  $10^4$  *P. yoelii* YM pRBCs on day 0.

prechallenge, the data also show that mice that cannot be protected following vaccination with a particular subunit preparation can be passively protected by adoptively transferred Abs. B10.BR mice are not protected following vaccination with GST-MSP1<sub>19</sub> (6). The reasons for this are not clear, but may relate to the titer of Ab induced by vaccination or to other factors such as the fine specificity of the Ab response. However, these factors may be overcome, for example by conjugation to a different carrier protein, which may provide more T cell help. It would then be expected that these mice would be protected postchallenge, because the active immune response postchallenge is not restricted to MSP1<sub>19</sub> per se but to other, possibly multiple, parasite Ags. If this was not the case, B10.BR mice would not be expected to be protected following adoptive transfer of MSP1<sub>19</sub>-specific Abs; however, these mice were in fact protected as well as B10 mice (Fig. 5) (a strain that is strongly protected following vaccination) (6). If the mechanism of protection induced by other merozoite surface proteins, for example apical membrane Ag-1, is similar to that mediated by MSP1<sub>19</sub>, then this has obvious and important implications for designing a human malaria vaccine, particularly small subunit vaccines based on merozoite surface proteins. If Ab is the principal mechanism of protection, then provided that a satisfactory Ab response is induced by vaccination, it is likely that the Ab response required postchallenge will not be restricted to the small subunit Ag, but will involve other proteins, thus increasing the likelihood of protection.

Our data provide an explanation as to why immunized mice can be solidly protected (no patent parasitemia), whereas normal mice passively given Abs develop a patent infection before cure. An

active immune response postchallenge is critical for protection. The nature of the immune response could be humoral or cellular (or both). Infection of normal mice that have passively received anti-MSP1<sub>19</sub> Abs would result in a slower primary antiparasite immune response, which would take time to achieve protective levels. Meanwhile, the passively transferred Abs are being consumed; until the active response is sufficient, parasitemia increases. In contrast, vaccinated mice will have a rapid secondary immune response postchallenge (16).

The data in this paper also raise the possibility that anti-MSP1<sub>19</sub>-specific Abs could be used in the treatment of clinical malaria. In the experiments described here, mice were challenged posttransfer. However, we have shown that adoptive transfer of serum postchallenge can also temporarily reduce parasitemia (our unpublished data). Such Abs might be considered as adjunct therapy to be given in conjunction with chemotherapeutic agents in cases in which the efficacy of the chemotherapy might be in doubt due to the prevalence of drug resistance.

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