

Heterologous Immunity in the Absence of Variant-Specific Antibodies after Exposure to Subpatent Infection with Blood-Stage Malaria

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We examined immunity induced by subpatent blood-stage malaria (undetectable by microscopy) using the rodent malaria parasite, *Plasmodium chabaudi chabaudi*, postulating that limited infection may allow expansion of antigen-specific T cells that are normally deleted by apoptosis. After three infections drug cured at 48 h, mice were protected against high-dose challenge with homologous or heterologous parasites (different strain or variant). Immunity differed from that generated by three untreated, patent infections. Subpatently infected mice lacked immunoglobulin G (IgG) to variant surface antigens, despite producing similar titers of total malaria-specific IgG to those produced by patently infected mice, including antibodies specific for merozoite surface antigens conserved between heterologous strains. Antigen-specific proliferation of splenocytes harvested prechallenge was significantly higher in subpatently infected mice than in patently infected or naive mice. In subpatently infected mice, lymphoproliferation was similar in response to homologous and heterologous parasites, suggesting that antigenic targets of cell-mediated immunity were conserved. A Th1 cytokine response was evident during challenge. Apoptosis of CD4⁺ and CD8⁺ splenic lymphocytes occurred during patent but not subpatent infection, suggesting a reason for the relative prominence of cell-mediated immunity after subpatent infection. In conclusion, subpatent infection with blood stage malaria parasites induced protective immunity, which differed from that induced by patent infection and targeted conserved antigens. These findings suggest that alternative vaccine strategies based on delivery of multiple parasite antigens at low dose may induce effective immunity targeting conserved determinants.

In regions where *Plasmodium falciparum* malaria transmission is high, individuals eventually acquire nonsterile immunity, where low parasitemia may occur in the absence of clinical symptoms. Immunity to blood-stage malaria appears to be predominantly antibody mediated. Transfusion of gamma globulin from immune adults to children with severe malaria is associated with reduced parasitemia and clinical improvement (5). Merozoite surface antigens (2, 6, 32) and variant antigens expressed on the surface of malaria-infected erythrocytes (including PfEMP1) (3, 23) are both targets of protective antibody responses.

It is still unclear whether cell-mediated (antibody-independent) immune responses play a major role in naturally acquired immunity to blood-stage malaria in humans (reviewed in reference 9). Loss or suppression of malaria-specific T cells during the course of natural infection may limit the contribution of inflammatory T cells to protective immunity. Reduced numbers of peripheral blood T lymphocytes and diminished proliferative responses to *in vitro* stimulation with malarial antigens are observed during acute *P. falciparum* infection (15–17). Animal studies indicate that malaria-specific effector and helper CD4⁺ T cells are deleted by apoptosis during infection (12, 41, 42), and this may also be the mechanism in humans (39).

We postulated that limited exposure to blood-stage malaria may allow the expansion of helper and effector CD4⁺ T cells,

which are normally eliminated during untreated infection. Recently, we showed that repeated exposure of human volunteers to extremely low doses of *P. falciparum* induced immunity to a low-dose challenge with homologous parasites in the absence of detectable malaria-specific antibodies (31), consistent with this hypothesis. For ethical reasons, we were unable to challenge human volunteers with realistic parasite doses or to challenge with heterologous parasite strains or variants to determine the specificity of this immunity.

In the present study, we have used the rodent malaria model *Plasmodium chabaudi chabaudi* in a resistant mouse strain to clearly demonstrate that repeated subpatent infection with blood-stage malaria, drug cured before parasites were detectable by microscopy, could induce effective immunity against high-dose challenge with homologous or heterologous parasites. Mice exposed to subpatent infection lacked variant-specific antibodies but had antibodies to merozoite antigens and prominent cell-mediated immune responses, and this was associated with protection of CD4⁺ and CD8⁺ splenic lymphocytes from apoptosis that occurred during untreated patent parasitemia.

MATERIALS AND METHODS

Mice. Female C57BL/6j mice, 8 to 12 weeks old, were obtained from the Animal Resources Centre (Willeton, Australia). Experiments were approved by the Institute Ethics Committee.

Parasites. *P. c. chabaudi* AS and *P. c. chabaudi* CB were supplied by Richard Carter, University of Edinburgh (4, 24, 36). Parasites were cryopreserved in glycerolyte 57 (Baxter Healthcare Corp.). Parasitemias were monitored by performing Giemsa-stained thin tail blood smears.

Infection protocol. Mice were given three intravenous (*i.v.*) infections with 10⁵ parasitized red blood cells (pRBC) of *P. c. chabaudi* AS at 4-weekly intervals to allow drug clearance. Parasites were derived from a single collection during peak parasitemia in one mouse. For each subpatent infection, mice were drug cured

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48 h after infection (0.2 mg of atovaquone and 0.08 mg of proguanil in 100 μ l of water by oral gavage daily for 4 days). Preliminary experiments confirmed that this protocol was highly effective in preventing the development of microscopically detectable parasitemia. Control patently infected mice self-cured. Naive mice were injected with phosphate-buffered saline (PBS) and received drug treatment at the same time as subpatently infected mice. All mice were drug treated 15 to 20 days before being subjected to a challenge infection with 10^6 pRBC.

ELISA. To prepare crude parasite antigen for enzyme-linked immunosorbent assay (ELISA), blood from mice infected with *P. c. chabaudi* AS or *P. c. chabaudi* CB (30 to 40% parasitemia) was collected, washed in PBS, and incubated with 0.01% (wt/vol) saponin (Aldrich) at 37°C for 20 min. The pellet was washed in PBS, resuspended in 1.5 ml of PBS, and sonicated.

MaxiSorp Nunc immuno plates, with 96 wells (Nalge Nunc Int.), were coated overnight at 4°C with 5 or 10 μ g of parasite antigen per ml in bicarbonate coating buffer (pH 9.6). The details of the assay have been described previously (13).

Staining variant antigens on the surface of pRBC. The staining procedure was based on previously described methods (8, 37). Mice were kept in a reverse light cycle (20:00 h to 08:00 h) for at least 1 week before infection so that late-stage parasites expressing variant surface antigens could be collected in the morning. The mice were infected from frozen aliquots of *P. c. chabaudi* AS (the same stabilate used during the subpatent infection protocol) or *P. c. chabaudi* CB. At 10 to 20% parasitemia, the mice were sacrificed at 10:00 h and blood was collected. After two washes, blood was resuspended at 5% hematocrit in RPMI-HEPES supplemented with 0.2% (wt/vol) NaHCO₃ and 10% fetal calf serum (FCS) and cultured at 37°C for 3 to 4 h in 5% CO₂-5% O₂ until late trophozoites were evident. After three washes in PBS-1% FCS, a 100- μ l volume of cells (0.2% hematocrit) was stained using a three-step method and sequentially incubated with test serum (1/10 dilution), goat anti-mouse immunoglobulin G (IgG) (1/50 dilution; Caltag), and fluorescein isothiocyanate (FITC)-conjugated swine anti-goat IgG (1/20 dilution; Caltag) plus ethidium bromide (20 μ g/ml). Incubations were carried out for 30 min at room temperature, and cells were washed in PBS-1% FCS between each step. Fluorescence was measured on a FACSCalibur (BD), and data were analyzed using CellQuest software (BD). Identification of pRBC in blood from infected mice using DNA-RNA stains is confounded by the presence of reticulocytes. We overcame this problem by gating on a subpopulation of RBC with higher forward scatter and side scatter that contained a high proportion of late-stage trophozoites (confirmed by cell sorting) and counted 1,000 events.

Immunofluorescence assay. Late-stage parasites were obtained by culturing as described above, but for the immunofluorescence assay (IFA), pRBC were cultured for approximately 8 h until schizonts were visible on a blood smear. Cells were then washed twice in PBS and resuspended at 1% hematocrit. A 4- μ l volume of cells was applied to each well on a 10-well multitest slide (ICN Biomedicals Inc.) and allowed to dry. The slides were wrapped in a tissue, sealed inside a plastic bag containing silica beads, and stored at -20°C.

For staining, the slides were fixed in 100% acetone for 10 min. After they were washed for 10 min in a large volume of PBS, excess PBS was removed and an ImmEdge pen (Vector Laboratories) was used to draw around individual wells. After blocking with 80 μ l of 1% bovine serum albumin (BSA)-PBS for 15 min, 60 μ l of test serum (diluted 1/100 in 1% BSA-PBS) was added and the mixture was incubated for 1 h (humid container). The wells were washed five times with 1% BSA-PBS. Texas Red-conjugated goat anti-mouse IgG (heavy plus light chains) (Jackson ImmunoResearch Laboratories) (1/75 dilution) and Hoechst (1/500 dilution) in 1% BSA-PBS were added, and the slides were incubated for 1 h in the dark (humid container). The slides were then washed for 10 min in a large volume of PBS. VectaShield and coverslips were applied, and the slides were examined by fluorescence microscopy.

Isolation of splenic mononuclear cells. Spleens were harvested, and single-cell suspensions were prepared. RBC were lysed using Gey's erythrocyte lysis buffer (21), and mononuclear cells were isolated by density centrifugation over Nycoprep 1.077 (Axis-Shield PoC AS, Oslo, Norway).

Proliferation assays of splenic mononuclear cells. Spleen cells were diluted to 2×10^6 cells/ml in culture medium (Eagle's minimum essential medium [Trace Scientific] with 5% heat-inactivated FCS, 50 μ g of streptomycin [CSL] per ml, 100 μ g of penicillin [CSL] per ml, and 55 μ M 2-mercaptoethanol [Gibco BRL]), and 200 μ l/well was aliquoted into 96-well flat-bottom tissue culture plates (Corning Life Sciences). Triplicate wells were cultured with concanavalin A (ConA) (10 μ g/ml), *P. c. chabaudi* AS or *P. c. chabaudi* CB pRBC (2.5×10^6 to 10×10^6 /ml), or normal mouse RBC adjusted to give equivalent concentrations of total RBC (from frozen aliquots). After 3 days, the wells were pulsed with 0.25 μ Ci of [³H] thymidine (NEN) per well for a further 18 to 24 h. The cells were harvested onto fiberglass filter mats, and radioactivity was measured.

Immunofluorescence staining and flow cytometric analysis of mononuclear cells. Incubations with monoclonal antibodies were performed on ice in the dark for 30 min. Cells were washed in PBS-1% FCS-0.05% NaN₃. Fluorescence was measured using a FACSCalibur (BD). Ten thousand events were counted, and data were analyzed using CellQuest software.

(i) **Splenic mononuclear cell subpopulations.** Cells were single stained with CD19-phycoerythrin (PE) (Caltag) or double stained with CD3-FITC (Pharmingen) and CD4-PE (Caltag) or CD3-FITC and CD8-PE (Caltag).

(ii) **Annexin V staining.** Cells were single stained with CD4-PE, CD8-PE, or CD19-PE. After two washes, the cells were stained with the Annexin V-Fluores staining kit (Roche Diagnostics) and washed before being subjected to analysis.

(iii) **Intracellular cytokine staining of whole blood.** Cells were cultured with 1.32 μ l of GolgiStop (Pharmingen) per ml with or without 20 ng of phorbol 12-myristate 13-acetate (PMA) (Sigma) per ml and 1 μ g of calcium ionophore (Sigma) per ml for 4 h at 37°C by previously described methods (34, 38). The cells were washed, resuspended in 200 μ l of culture medium, and stained with CD4-FITC (Caltag). After lysis of RBC (21), the cells were fixed and permeabilized using a Cytotfix/Cytoperm kit (Pharmingen). Gamma interferon (IFN- γ)-PE (Pharmingen), interleukin-4 (IL-4)-PE (Pharmingen), or PE-conjugated isotype-matched negative control (Pharmingen) diluted 1/50 in PermWash Buffer was added, and the mixture was incubated for 30 min, washed in fluorescence-activated cell sorter buffer and analyzed.

Statistical analysis. Protective immunity was determined by comparing peak parasitemia during challenge. Parasitemia data from two challenge experiments were pooled for analysis after establishing there were no significant differences between experiments. Nonparametric tests (Mann-Whitney or Kruskal Wallis) were used to compare groups ($\alpha = 0.05$).

RESULTS

Specificity of immunity induced by subpatent infection.

Mice exposed to three subpatent infections (drug treated 48 h postinfection) or three patent infections (self-cured) with *P. c. chabaudi* AS, together with naive controls, were challenged with identical parasites or with a different parasite strain or variant.

Mice exposed to subpatent infections were protected against high-dose challenge with homologous parasites compared with naive mice (mean peak parasitemia \pm standard error of the mean, 1.6% \pm 0.7% and 36.5% \pm 1.2%, respectively [$P < 0.001$]) (Fig. 1A). However, patent infections induced better protection against homologous challenge than did subpatent infections (mean peak parasitemia, 0.02% \pm 0.01% and 1.6% \pm 0.7, respectively [$P < 0.001$]). Reduced parasitemia in subpatently infected mice was associated with protection against overt signs of clinical disease, including ruffled hair and reduced activity and, in separate experiments, was shown to correlate with prevention of significant weight loss (mean weight loss \pm SEM: 0.7% \pm 1.1% compared with 8.6% \pm 1.5% for naive mice [$P < 0.01$] and 0.002% \pm 1.0% [$P = 0.6$] for patently infected mice [$n = 5$ per group]).

Following subpatent infection with *P. c. chabaudi* AS, mice were also strongly protected against heterologous challenge with *P. c. chabaudi* CB, in contrast to naive mice (3.3% \pm 1.4% and 22.2% \pm 2.9%, respectively [$P < 0.01$]) (Fig. 1A). In subpatently infected mice, there was no significant difference in peak parasitemia during homologous (AS) and heterologous (CB) challenge ($P = 0.51$).

During heterologous challenge with *P. c. chabaudi* CB, patently infected mice had significantly lower peak parasitemias (0.08% \pm 0.03%) than did naive mice (22.2% \pm 2.9% [$P < 0.001$]) (Fig. 1A). However, peak parasitemias were modestly but statistically significantly higher during heterologous (CB) challenge compared with homologous (AS) challenge ($P < 0.05$). Peak parasitemias were significantly higher in subpa-

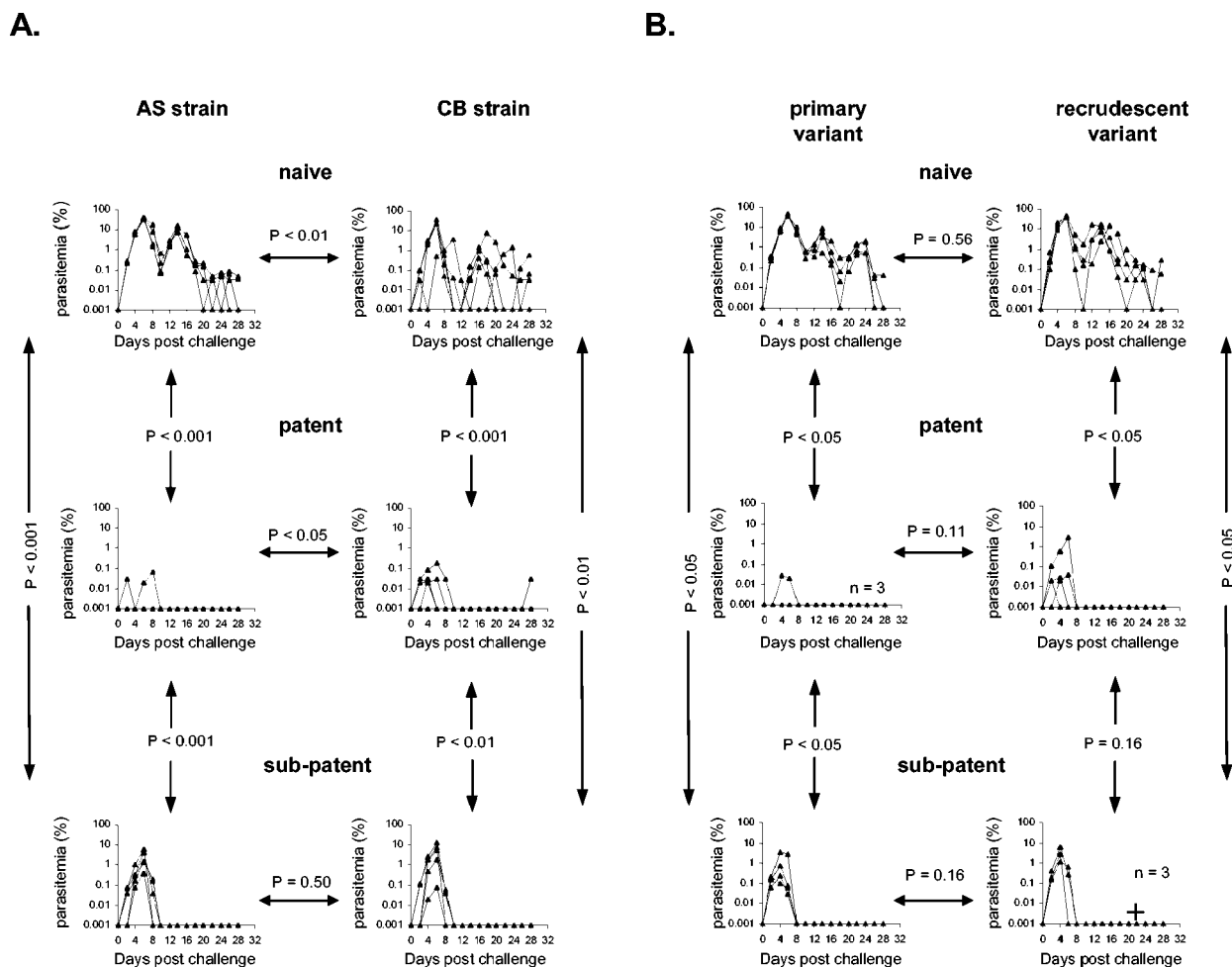


FIG. 1. Specificity of immunity induced by subpatent infection. Subpatently and patently infected mice were infected three times i.v. at monthly intervals with 10^5 *P. c. chabaudi* AS pRBC. Naive mice were injected i.v. with PBS. Subpatently infected mice were drug cured 48 h postinfection, and naive mice received the drug at the same time. Patently infected mice were allowed to self-cure. (A) Mice were challenged i.v. with 10^6 homologous *P. c. chabaudi* AS or heterologous *P. c. chabaudi* CB pRBC. Data for individual mice are shown ($n = 5$). This is representative of two similar experiments. Statistical comparisons between groups are indicated. (B) Mice were challenged i.v. with 10^6 *P. c. chabaudi* AS homologous primary variant parasites or heterologous recrudescence variant parasites (derived from recrudescence in the same donor mouse). Individual mice are shown ($n = 4$ except where indicated). The cross indicates the death of one mouse. Statistical comparisons between groups are indicated.

tently infected mice than in patently infected mice challenged with CB parasites ($P < 0.01$).

Previous studies have shown that during *P. c. chabaudi* AS infection, recrudescence parasites express different variant surface antigens from those expressed by parasites that dominate the primary peak (25, 26, 30). To compare immunity to parasites expressing homologous and heterologous variant surface antigens, parasites were collected during the primary peak ("primary variant") or recrudescence peak ("recrudescence variant") of a *P. c. chabaudi* AS infection in a single mouse and frozen in multiple aliquots. Three patent and subpatent infections with primary variant parasites were followed by challenge with either homologous primary variant or heterologous recrudescence variant parasites (Fig. 1B). The results were similar to those from experiments using *P. c. chabaudi* AS and CB strains. The results of these experiments collectively showed that subpatent infections, like patent infections, could induce heterologous immunity against high-dose challenge with blood stage malaria parasites.

Magnitude and specificity of the antibody response induced by subpatent infection. Mice exposed to subpatent or patent infections with *P. c. chabaudi* AS had significant titers of IgG against homologous *P. c. chabaudi* AS and heterologous *P. c. chabaudi* CB antigen by ELISA, compared to naive mice ($P < 0.001$) (Fig. 2). Subpatently and patently infected mice had similar titers of IgG recognizing AS and CB antigen, suggesting that most IgG was specific for epitopes that were commonly expressed in both strains.

In sera from subpatently and patently infected mice, immunofluorescence assays of fixed parasites detected IgG that was bound to the surface of merozoites and other intracellular antigens in homologous AS and heterologous CB pRBC. This suggested that conserved merozoite surface antigens were targets of antibody responses induced by both subpatent and patent infections (Fig. 3).

Interestingly, subpatently infected mice lacked IgG specific for variant antigens on the surface of homologous *P. c. chabaudi* AS pRBC (Fig. 4). In contrast, serum from patently infected

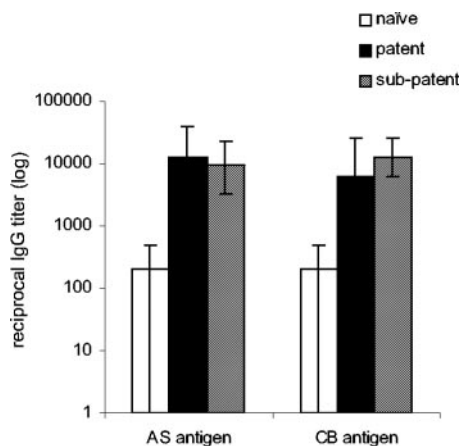


FIG. 2. Specificity of antibodies induced by subpatent infection, measured by ELISA. Following three infections with *P. c. chabaudi* AS and prior to challenge infection, serum was collected from patently and subpatently infected mice as well as naive controls (Fig. 1A). Titers of parasite-specific IgG against homologous *P. c. chabaudi* AS and heterologous *P. c. chabaudi* CB crude parasite antigens were measured by ELISA. Reciprocal median IgG titers (and interquartile range) are shown ($n = 10$). These data are representative of two experiments.

mice had significant levels of IgG that bound to the surface of AS but not CB live pRBC ($P < 0.01$ compared to serum from naive mice), indicating that these antibodies were variant specific. Since serum from patently and subpatently infected mice had similar levels of total IgG by ELISA (Fig. 2), this could not be attributed to nonspecific binding.

Antigen-specific proliferation of splenic lymphocytes. Spleen cells collected from mice 6 weeks after the third subpatent infection with *P. c. chabaudi* AS (2 weeks after drug treatment) showed significant antigen-specific proliferation in response to stimulation with freeze-thawed AS pRBC (Fig. 5A) or CB pRBC (Fig. 5B) ($P < 0.05$ compared with naive mice), suggesting priming of T cells specific for antigens commonly expressed in both strains. In contrast, proliferation of splenic lymphocytes collected from patently infected mice (6 weeks after the third infection, 2 weeks after drug treatment) was not significantly greater than proliferation of splenic lymphocytes from naive mice in the presence of AS or CB pRBC. The proportions of CD3⁺ CD4⁺ and CD3⁺ CD8⁺ splenic lymphocytes were not significantly different among naive, patently infected, and subpatently infected mice (results not shown), consistent with comparable proliferative responses to ConA stimulation observed in all groups (Fig. 5). This suggests that differences in the proliferative responses to malaria parasite antigen were attributable to differences in the relative numbers of antigen-specific T cells rather than to a global change in splenic lymphocyte subpopulations after patent infection.

Cytokine production by peripheral blood lymphocytes. To characterize the cytokine profile of the dominant CD4⁺ T lymphocyte population present during challenge, intracellular cytokine staining was performed on peripheral blood lymphocytes collected on day 6 post-challenge (pooled from mice in each group [$n = 4$]). There were higher proportions of IFN- γ ⁺ than IL-4⁺ CD4⁺ lymphocytes in naive, patently infected, and subpatently infected mice (Fig. 6), suggesting a predominantly Th1-type response.

Subpatent infection primed antigen-specific splenic lymphocytes without inducing lymphocyte apoptosis. Mice were given a single infection with 10⁵ *P. c. chabaudi* AS pRBC. On day 2, a group of infected mice and naive controls were sacrificed and the splenic lymphocyte subsets (CD4⁺, CD8⁺, and CD19⁺) were examined for evidence of apoptosis. Antigen-specific proliferation was also examined. Lymphocytes from infected mice showed no higher levels of annexin V staining than did naive mice on day 2 (Fig. 7). Lymphoproliferative responses to *P. c. chabaudi* AS antigen were similar in naive and infected mice (Fig. 7).

From day 2, one group of the remaining mice was drug cured (subpatent) while another group developed patent parasitemia (patent). When patently infected mice reached peak parasitemia on day 8, both groups, along with naive control mice, were sacrificed. A significantly higher percentage of CD4⁺ and CD8⁺ splenic lymphocytes from patently infected mice were positive by annexin V staining compared with naive mice ($P < 0.05$) (Fig. 7). The proportions of CD4⁺ and CD8⁺ lymphocytes in the spleen were significantly reduced in patently in-

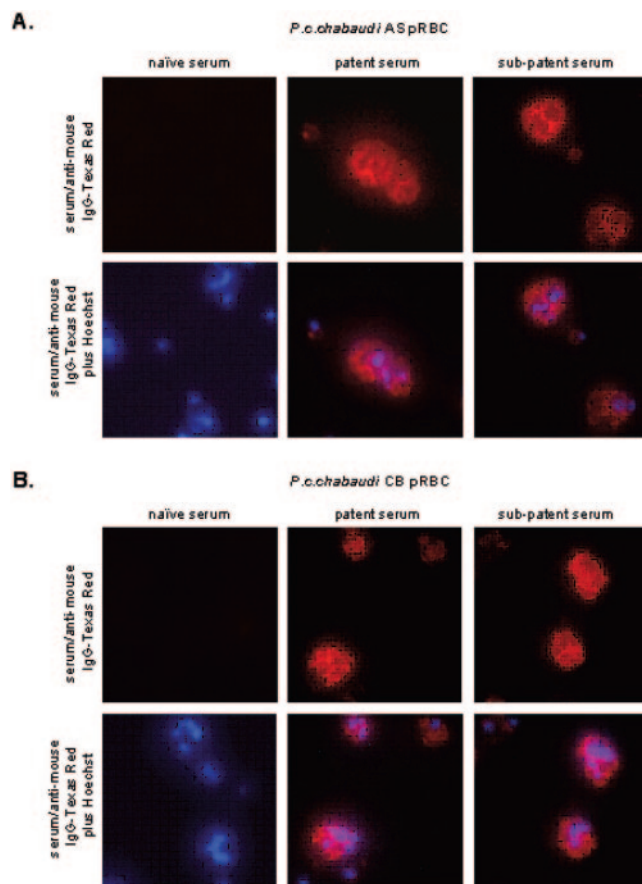


FIG. 3. Specificity of antibodies induced by subpatent infection, measured by IFA. Serum was collected from naive, patently infected, and subpatently infected mice prior to the challenge infection (Fig. 1A). Binding of serum IgG to homologous *P. c. chabaudi* AS (A) and heterologous *P. c. chabaudi* CB (B) acetone-fixed schizonts was assessed by immunofluorescent staining with Texas Red-conjugated anti-mouse IgG and Hoechst ($n = 10$). Representative images are shown. Similar results were obtained in two separate experiments.

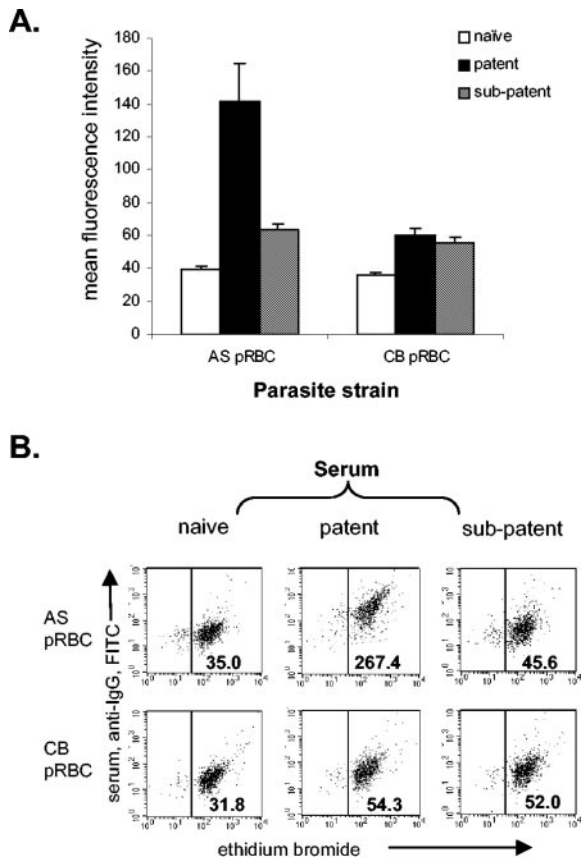


FIG. 4. Antibodies to variant parasite antigens on the RBC surface. Serum was collected from naive, patently infected, and subpatently infected mice prior to challenge infection (Fig. 1A). Binding of serum IgG to the surface of homologous *P. c. chabaudi* AS and heterologous CB pRBC (trophozoite stage) was determined by flow cytometry. (A) Means and SEMs of the mean fluorescence intensity of FITC labeling (ethidium bromide-positive cells) are shown for each group of mice ($n = 10$). These data are representative of two similar experiments. (B) Representative dot plots of AS and CB pRBC stained with serum, goat anti-mouse IgG, swine anti-goat IgG-FITC, and ethidium bromide are shown, with the mean fluorescence intensity of FITC labeling (ethidium bromide-positive cells) indicated.

ected mice compared with the levels in naive mice ($P < 0.05$) (results not shown). The proportion of apoptotic splenic lymphocytes in subpatently infected mice was similar to that of naive controls, and there was no alteration of CD4⁺ and CD8⁺ lymphocyte subsets. Despite limited exposure to blood stage parasites during a single infection, splenic lymphocytes from subpatently infected mice showed significant proliferation in response to all doses of parasite antigen, compared with lymphocytes from naive mice ($P < 0.05$). In contrast, cells from patently infected mice showed no greater response to *P. c. chabaudi* AS freeze-thawed pRBC than did cells from naive control mice, and the response to ConA was significantly lower ($P < 0.05$), consistent with the reduced proportions of CD4⁺ and CD8⁺ lymphocytes in the spleen during acute patent infection.

DISCUSSION

We hypothesized that limited blood stage malaria infection may allow an expansion of antigen-specific CD4⁺ T lympho-

cytes that are normally deleted by apoptosis (12, 41, 42), leading to a modified but effective immune response. In an earlier study of human volunteers, three infections with approximately 30 *P. falciparum*-infected RBC that were drug treated after 8 days generated immunity against a short-term challenge with a similar number of homologous parasites (31). Here we have extended these studies by using the mouse malaria model, *P. c. chabaudi*, to demonstrate that repeated subpatent blood stage infection could induce significant protective immunity against high-dose homologous and heterologous parasite challenge. Protection against heterologous parasites was almost as effective as protection against homologous parasites, suggesting that the main targets of this immunity are conserved antigens. Consistent with this, lymphoproliferation and IFA data showing responses to both homologous and heterologous antigens in vitro indicated that antibody and cell-mediated components of immunity induced by subpatent infection targeted conserved determinants. Conservation of antigenic targets may reflect the

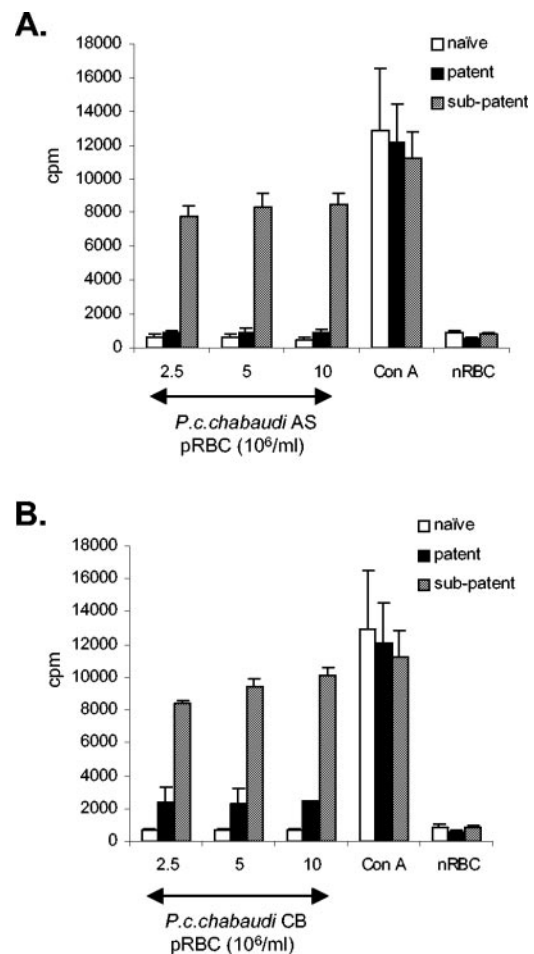


FIG. 5. Antigen-specific proliferation of splenic lymphocytes. Subpatently and patently infected mice were sacrificed after completion of three infections with *P. c. chabaudi* AS, together with naive controls. Splenic lymphocytes from all mice were stimulated for 3 days with freeze-thawed *P. c. chabaudi* AS (A) or *P. c. chabaudi* CB (B) pRBC, ConA, and freeze-thawed uninfected mouse RBC (nRBC) and pulsed with [³H] thymidine for a further 18 to 24 h ($n = 4$). This is representative of two similar experiments.

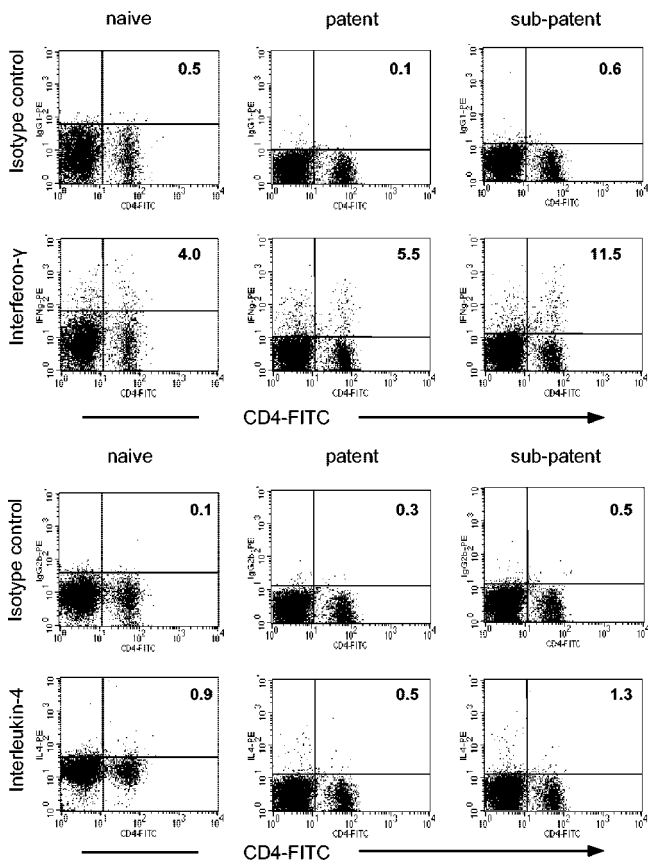


FIG. 6. Intracellular cytokine staining of peripheral blood lymphocytes day 6 post-challenge. Peripheral blood was collected and pooled from mice in each group ($n = 4$) on day 6 post-challenge. After a 4-h stimulation of whole blood with PMA and calcium ionophore, intracellular cytokine staining for IFN γ and IL-4 production by CD4⁺ lymphocytes was performed. Dot plots from each group are shown. Numbers indicate the percentage of CD4⁺ lymphocytes producing each cytokine and nonspecific staining with isotype control monoclonal antibody.

absence of immune pressure in a natural setting. Although there is evidence for selective pressure on preerythrocytic T-cell epitopes (11), this has not been shown for blood stage antigenic targets of CD4⁺ T cells.

Despite differences in parasite dose and duration of infection between the previous study with *P. falciparum* (31) and the present study with *P. c. chabaudi*, there were similarities in the nature of the immunity induced by limited exposure to blood stage malaria. In both studies, in vitro correlates of cell-mediated immunity, lymphoproliferation and Th1 cytokine secretion, were prominent. Higher cellular immune responses have also been observed in children in malaria-endemic areas after long-term chemoprophylaxis, than in placebo controls (7, 28), suggesting that our observations with the *P. c. chabaudi* model are relevant to human malarial immunity.

Whereas the human volunteers infected with very low doses of *P. falciparum* failed to produce detectable malaria-specific antibodies (31), after subpatent *P. c. chabaudi* infections mice produced similar levels of total malaria-specific antibodies to those in mice exposed to patent infections. However, a striking feature of immunity induced by subpatent infections with *P. c.*

chabaudi was the absence of antibodies specific for variant antigens on the surface of pRBC, suggesting that these proteins are less immunogenic than other malarial antigens. Structurally complex proteins may not undergo optimal antigen processing and thus may slow the generation of helper CD4⁺ T cell responses (18). Subpatent infection, by reducing antigen concentrations, may prevent the generation of adequate T-cell help for effective antibody responses to such proteins (43). Alternatively, if variant surface antigens are expressed at relatively low levels compared to merozoite antigens, a higher parasitemia may be necessary to generate a detectable variant specific antibody response.

Despite their lack of variant-specific antibodies, subpatently infected mice quickly controlled their parasitemia during high-dose challenge and were protected against overt clinical signs of infection, suggesting that variant-specific antibodies are not necessary for protection against severe disease. The presence of variant-specific antibodies prechallenge probably explains why patently infected mice were better protected than subpatently infected mice against homologous challenge.

We explored the mechanism of the modified immune response observed after subpatent infections. Adoptive-transfer

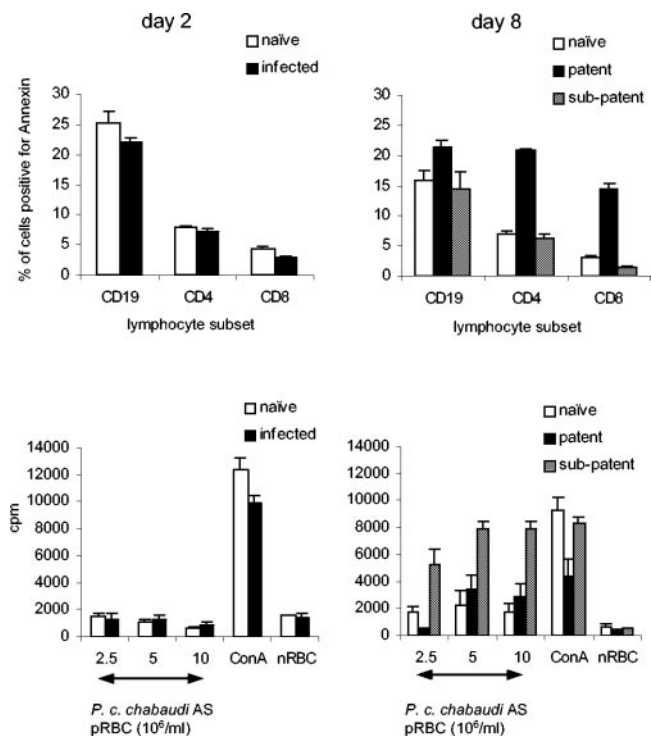


FIG. 7. A single subpatent infection primed antigen-specific splenic lymphocytes without inducing lymphocyte apoptosis. Mice were infected with 10^5 *P. c. chabaudi* AS pRBC i.v. on day 0. On day 2, the first group of infected mice was killed, along with naive controls ($n = 4$). Subpatently infected mice were drug-cured on day 2, while patently infected mice were allowed to develop detectable parasitemia. Naive, patently infected, and subpatently infected mice were killed on day 8 ($n = 4$). Apoptosis of splenic lymphocyte subsets was assessed by staining with annexin V, and proliferation of lymphocytes, stimulated with freeze-thawed *P. c. chabaudi* AS pRBC was examined on days 2 and 8. Means and SEM are shown. This is representative of two similar experiments.

studies have shown that malaria-specific effector CD4⁺ T cells (12, 42) and helper CD4⁺ T cells (41) are deleted by apoptosis during acute infection. This process is antigen specific and does not affect bystander CD4⁺ T cells specific for unrelated antigens (42). In the present study, apoptosis of CD4⁺ and CD8⁺ splenic lymphocytes associated with patent infection was prevented by drug cure when the infection was at the subpatent level. Splenocytes harvested after a single subpatent infection showed significantly higher antigen-specific proliferation than did those harvested after patent infection. The reduced response to ConA observed in patent mice was probably also attributable to apoptotic deletion of antigen-specific T lymphocytes, which would have constituted a large proportion of splenic T lymphocytes during acute infection. When spleens were harvested from mice that had experienced three patent infections, after resolution of infection the responses to ConA were similar to those of naive and subpatently infected mice, indicating that T cells specific for nonmalarial antigens were probably not affected (Fig. 5). It could be argued that apoptosis of T lymphocytes during patent infection reflects homeostatic mechanisms (reviewed in references 10 and 22). However, T-cell responses in patent mice were often similar to those in naive mice, suggesting overcorrection.

Modulation of dendritic cell function or induction of regulatory T cells might also affect lymphoproliferation and cytokine secretion after exposure to patent infection. Maturation of human monocyte-derived dendritic cells in vitro is impaired by prior exposure to *P. falciparum* schizonts (40), and blood stage *P. yoelii* parasites inhibit the maturation of mouse bone marrow-derived dendritic cells (27). However, *P. c. chabaudi* AS-infected RBC have been shown to directly activate dendritic cells in vitro (33), *P. c. chabaudi* AS infection induces the maturation of splenic dendritic cells (20), and functional dendritic cells are induced in vivo by *P. yoelii* infection (29). Thus, it has not been clearly established that dendritic cell function is impaired during acute malaria infection in vivo (reviewed in reference 18), and there is certainly no evidence that this occurs in the *P. c. chabaudi* model.

Depletion of CD4⁺ CD25⁺ regulatory T cells protects mice against a lethal strain of *P. yoelii*, associated with improved in vitro proliferative responses to parasitized RBC (14). Although regulatory T cells may also have contributed to the poor proliferative responses of splenocytes from patent mice in the present study, our previous observations that antigen-specific CD4⁺ T cells are deleted during infection (42) are more consistent with apoptosis as the primary process responsible for impaired cellular immune responses after patent infection.

Despite evidence of T-cell apoptosis and impaired antigen-specific cellular responses after untreated malaria infection, patent mice were still effectively protected against homologous and heterologous challenge. Although it is well established that Th1 effector CD4⁺ T cells are required for the control of parasitemia early in primary *P. c. chabaudi* infection (19), these data suggest that inflammatory CD4⁺ T-cell responses are unlikely to contribute significantly to protection against repeat infection. In contrast, antibody boosting during challenge in patent mice (results not shown) suggested that antibody responses were preserved, consistent with

the reduced requirements for CD4⁺ T-cell help during secondary antibody responses (1, 35).

Although subpatently infected mice had increased antigen-specific T-cell responses, they were not better protected than patent mice. There are two possible explanations. First, the protective immunity induced by both patent and subpatent infection is largely mediated by preexisting antibodies and antibodies generated during secondary response to challenge, and the contribution of antibody-independent cell-mediated immunity is minor. It is more likely that enhanced cell-mediated immunity is induced by subpatent infection, as suggested by our observations, but this is offset by the effects of reduced antigen exposure on other aspects of immunity. Protective antibody responses to specific antigens might have been quantitatively or qualitatively inferior because of limited antigen exposure during subpatent infection, even though total malaria-specific antibody levels were unaffected.

In conclusion, we have shown that limited exposure to blood stage parasites is sufficient to induce protective immunity against homologous and heterologous parasites. Antibodies to conserved determinants, including merozoite antigens, coupled with a prominent Th1 cell-mediated immune response contributed to protection. Although the animals were well protected against challenge, antibodies to variant surface antigens were not detected, suggesting that these antibodies are not required for immunity to severe disease. The enhanced cell-mediated immunity induced by subpatent infection was associated with protection of T lymphocytes from the apoptosis normally associated with patent infection.

In our previous study of human volunteers (31), the absence of detectable malaria-specific antibodies probably reflects the extremely low parasite doses used for immunization. Given the role of antibody in immunity induced by subpatent infection with *P. c. chabaudi* demonstrated in the present study, these individuals might not have been protected against high-dose challenge. However, the findings of the present study suggest that administration of a parasite dose sufficient to induce both antibody and cell-mediated immunity is protective and largely targets conserved determinants. Induction of this type of immunity by immunizing with low doses of purified antigens from whole parasites may be an alternative but highly effective vaccine strategy. These observations have important implications for vaccine approaches and understanding of immunity associated with different levels of parasite exposure in malaria-endemic settings.

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