involved in HIV-induced cell fusion, an important mechanism in the pathogenesis of AIDS, and may pave the way for broader therapeutic strategies than those currently in development.

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Sporozoite Vaccine Induces Genetically Restricted T Cell Elimination of Malaria from Hepatocytes

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The target of the CD8$^+$ T cell–dependent immunity that protects mice immunized with irradiation-attenuated malaria sporozoites has not been established. Immune BALB/c mice were shown to develop malaria-specific, CD8$^+$ T cell–dependent inflammatory infiltrates in their livers after challenge with *Plasmodium berghei* sporozoites. Spleen cells from immune BALB/c and C57BL/6 mice eliminated hepatocytes infected with the liver stage of *P. berghei* in vitro. The activity against infected hepatocytes is not inhibited by antibodies to interferon-γ and is not present in culture supernatants. It is genetically restricted, an indication that malaria antigens on the hepatocyte surface are recognized by immune T effector cells. Subunit vaccine development will require identification of the antigens recognized by these T cells and a method of immunization that induces such immunity.

AFTER INOCULATION, MALARIA SPOROZOITES are in the peripheral circulation for less than an hour before they enter the liver where, during several days, they develop to mature liver-stage parasites that rupture and release merozoites that invade erythrocytes. Immunization with irradiation-attenuated malaria sporozoites (irr-spz) protects animals (*1*) and humans (*2*) against challenge with normal sporozoites. In some strains of mice the effector arm of this protective immunity has been shown to require CD8$^+$ lymphocytes and interferon-γ (*3, 4*). Such immunity does not protect against challenge with erythrocyte-stage parasites (*1*) or liver-stage merozoites (*5*), and circulating sporozoites are an unlikely target for a protective CD8$^+$ T cell–dependent immune response that would require cell surface presentation of antigen in combination with class I major histocompatibility (MHC) proteins. The infected hepatocyte is the likely target for such immunity. However, most workers still refer to sporozoite vaccine development, since there is little or no inflammatory reaction around most mature parasites in the livers of naïve animals after infection (*6, 7*) (Fig. 1A), and the presence of malaria antigens on the surface of hepatocytes has not been demonstrated. In the current studies we show that multiple, malaria-specific inflammatory reactions occur in the livers of immune mice and that T cells from immune mice eliminate liver-stage parasites from hepatocyte cultures.

We immunized BALB/c mice with a single dose of *Plasmodium berghei* irr-spz. Two weeks later the mice were infected with sporozoites, and 43 hours later they were killed. Multiple inflammatory infiltrates (Fig. 1B) were found in the livers of mice immunized with irr-spz and challenged with normal sporozoites, but few infiltrates were found in controls (*8*). In addition, the response was specific for malaria (Table 1). The infiltrates contained numerous macrophages, polymorphonuclear leukocytes, eosinophils, and CD8$^+$ lymphocytes (Fig. 1C). CD4$^+$ lymphocytes were present in lower concentration, and the number of Kupffer cells recognized by monoclonal antibody SER-4 was similar in infiltrates and normal hepatic tissue (*8*). To further define the infiltrates, we adoptively transferred spleen cells from immune or naïve donors into naïve recipients, challenged the recipients, and examined their livers 43 hours later (*9*). Infiltrates were present in the livers of mice that received unfractionated immune spleen cells (ISCs) (5.2 ± 4.19 infiltrates per 20 low-power fields, mean ± SD) and CD4$^-$ ISCs (5.5 ± 2.12), but not in those that received CD8$^-$ ISCs (0) or normal spleen cells (0.3 ± 0.57). The numbers of infiltrates in these experiments exceeded the numbers of mature schizonts expected after such a sporozoite challenge.

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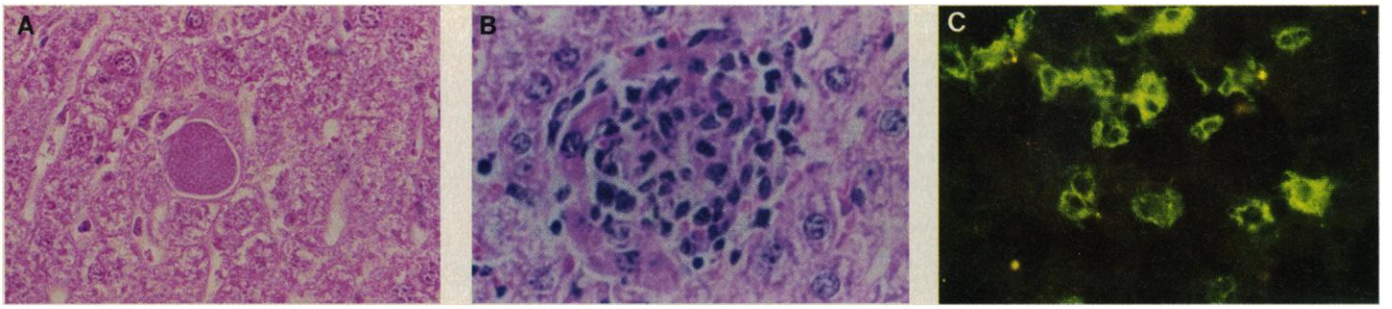

Fig. 1. Histopathology of the livers of naïve and immune BALB/c mice 43 hours after challenge with 7.5×10^4 normal NK-65 *P. berghei* sporozoites. (**A**) A single mature liver-stage schizont without a surrounding inflammatory response in a naïve mouse (×500, hemotoxylin and eosin). (**B**) An infiltrate, but no parasites in the liver of a mouse immunized 2 weeks before challenge with 7.5×10^4 irr-spz (×1250, hemotoxylin and eosin). (**C**) $CD8^+$ lymphocytes in an infiltrate [×500, immunofluorescence (*8*)].

To reach hepatocytes, sporozoites must pass through or between the Kupffer and endothelial cells of the liver sinusoids. The development of the malaria-specific infiltrates could have been the result of an immune response against sporozoites that did not reach hepatocytes, or, considering the $CD8^+$ T cell dependence of the infiltrates, may have been elicited by sporozoite or early liver-stage antigens on hepatocytes.

To determine whether immune T cells could recognize infected hepatocytes, we developed an in vitro assay. When ISCs are added to cultures infected with sporozoites 24 hours earlier, they eliminate parasites from the cultures in a dose-dependent manner (Fig. 2A). Interferon-γ (IFN-γ) inhibits the development of liver-stage parasites in vitro (*10*). We therefore used an antiserum directed against IFN-γ (anti–IFN-γ) to determine whether IFN-γ mediated the elimination of parasites. Mouse recombinant IFN-γ (rIFN-γ) (1000 units) inhibited parasite development by 60%, and this effect could be blocked by adding ammonium sulfate–precipitated polyclonal rabbit anti-rIFN-γ (Fig. 3A). When the antibody to rIFN-γ was added to the ISCs it had no effect on the elimination of parasites (Fig. 3B). This was consistent with our finding that supernatants from cultures to which ISCs are added contain levels of IFN-γ similar to those in cultures to which normal spleen cells are added. Because treatment of immune A/J mice with 1 mg of the mouse monoclonal antibody DB-1 against IFN-γ (*11*) abrogates protective immunity (*3*), we studied the effect of DB-1 in BALB/c mice. Groups of five mice were immunized with 7.5×10^4 irr-spz, and a booster consisting of 2.0×10^4 irr-spz was given after 21 days. Seventeen days later these mice were challenged with 10^4 sporozoites, and 5 min after challenge an immunized group and a control group were given 1 mg of DB-1 intravenously. DB-1 had no effect on protection in immunized mice or infectivity in naïve mice.

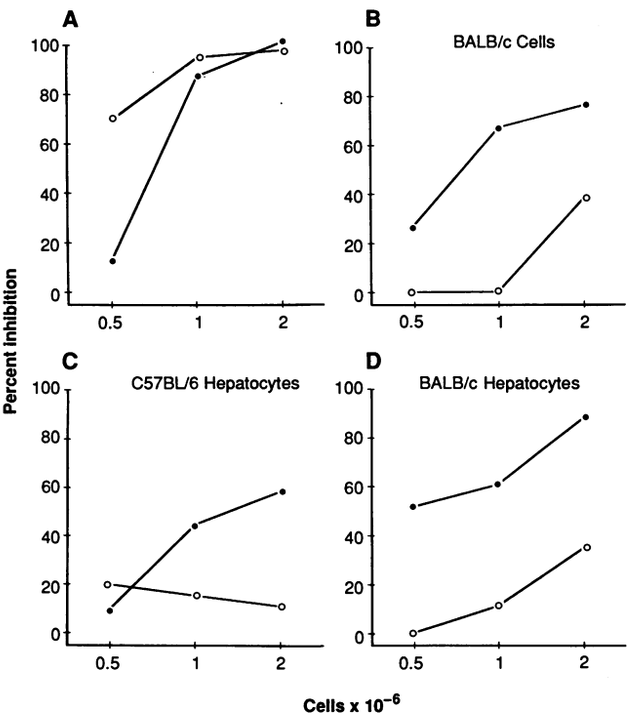

Fig. 2. Spleen cells from immune mice eliminate the liver stages of *P. berghei* from hepatocyte culture, and the effect on parasites is genetically restricted. Mice were immunized with three doses of irr-spz (7.5×10^4, 2.0×10^4, and 2.0×10^4 irr-spz at 2-week intervals) and given a booster dose of 2.5×10^5 irr-spz. Five days later one to three mice were killed; ISCs were isolated and added to primary hepatocyte cultures (*5*) that had been infected with an average of 5×10^4 normal sporozoites 24 hours earlier. The medium was changed 3 and 17 hours later (27 and 41 hours after infection), and the number of liver-stage parasites per culture was counted 24 hours after addition of ISCs (48 hours after sporozoites were added to the culture). The percent inhibition for each concentration of ISC was calculated in relation to the number of parasites present in cultures to which the same number of spleen cells from normal mice (NSC) had been added [percent inhibition = {1 − (average number of parasites in two ISC cultures/average number of parasites in two NSC cultures)}{100}]. If the percent inhibition was negative, it was considered to be zero. (**A**) BALB/c ISCs eliminated parasites from BALB/c hepatocytes in two different experiments. In another experiment 1×10^6 BALB/c ISCs were added to eight hepatocyte cultures and 1×10^6 NSCs to eight other cultures. There were 22.6 ± 4.71 (mean ± SE) liver-stage parasites in the ISC cultures and 92.5 ± 4.54 parasites in NSC cultures ($P < 0.0005$, Student's t test, two-tailed), an inhibition of 75.5%. The variation in this experiment was representative of that found in subsequent experiments (B to D). (**B**) BALB/c ISCs specifically eliminated parasites from BALB/c (●), but not C57BL/6 (○) hepatocytes. (**C**) C57BL/6 ISCs (●) eliminated parasites from C57BL/6 hepatocytes, but BALB/c ISCs (○) did not. (**D**) BALB/c ISCs (●) eliminated parasites from BALB/c hepatocytes, but C57BL/6 ISCs (○) did not.

To determine whether other factors secreted by the ISCs had activity against parasites, we collected supernatants at 3, 17, and 24 hours after the addition of cells to hepatocyte cultures (27, 41, and 48 hours after infection), and added the supernatants at dilutions of 1:2, 1:4, and 1:8 to cultures 24, 27, and 41 hours after infection. The supernatants had no effect on the parasites.

Because these data suggested that the elimination of parasites requires the direct interaction of immune T cells with hepatocytes, we conducted a series of experiments with BALB/c ($H\text{-}2D^d$, $H\text{-}2K^d$) and C57BL/6 ($H\text{-}2D^b$, $H\text{-}2K^b$) mice. Results of three different experiments (Fig. 2, B to D) showed that the activity against liver-stage parasites is genetically restricted, indicating that it is mediated by T cells that presumably recognize malaria antigens on the hepatocyte surface. Genetically restricted killing of target cells has been linked only to the

Table 1. Malaria-specific infiltrates in the livers of immune mice after challenge. Groups of three BALB/c mice were immunized with 7.5×10^4 irr-spz (1.5×10^4 rads) or irradiated salivary glands (irr-glands) dissected from the same number of uninfected mosquitoes and processed in the same way as the sporozoites (*8*). The mice were challenged 14 days later by intravenous injection of 7.5×10^4 normal sporozoites (spz) or mosquito salivary glands (glands) (*8*). The mice were killed 43 hours after challenge and the livers were processed for standard light microscopy. The mean number of infiltrates per 20 low-power fields (×125) in mice immunized with irr-spz and challenged with live sporozoites was significantly higher than among the two groups of control mice ($P < 0.05$, Student's t test, two-tailed). Values are means ± SD.

Experiment	Immunogen	Challenge	No. of infiltrates	Diameter of infiltrates (μm)
1	irr-spz	spz	17.7 ± 2.05	69.3 ± 12.10
	irr-spz	glands	3.1 ± 0.64	51.7 ± 11.59
2	irr-spz	spz	17.3 ± 11.0	52.9 ± 28.49
	irr-glands	spz	2.3 ± 2.08	64.6 ± 48.91

MHC. Since hepatocytes express only class I MHC proteins on their surface, these findings suggest that the elimination of infected hepatocytes is class I MHC–restricted. However, since the mice studied were not MHC congenics, we have not formally mapped the restriction to the MHC, and it is possible that non–H-2 loci are involved in regulation of this phenomenon.

After immunization with 7.5×10^4 irradiation-attenuated *P. berghei* sporozoites, BALB/c mice have low serum levels of antibodies to sporozoites, yet they are consistently protected against challenge with 5×10^5 sporozoites (*12*). High concentrations of monoclonal and polyclonal antibodies to the circumsporozoite (CS) protein and to sporozoites can protect against sporozoite challenge (*13, 14*). These antibodies have no effect on the development of liver-stage parasites if added to in vitro cultures after sporozoites have invaded hepatocytes (*5*) and almost certainly protect by interacting with all potentially infective sporozoites during the short period between inoculation and invasion of hepatocytes. Therefore, it is not surprising that such antibodies completely protect against moderate, but not against large, sporozoite challenge. There is also evidence that T effector cells (*14, 15*), specifically cells of the suppressor-cytotoxic phenotype ($CD8^+$) (*3, 4*), are involved in the potent protective immunity induced by immunization with irr-spz. Our data provide an indication that malaria antigens expressed on hepatocytes represent at least one target of a cytotoxic T cell (CTL)–mediated response. Such antigens have not been detected on the surface of hepatocytes with antibody probes, but CTLs can recognize antigens not detectable by antibodies (*16*). The CS protein is present in hepatocytes throughout the liver stage (*7, 17*) and may be a target for this response. However, only a single CTL epitope has been identified on the *P. falciparum* and *P. yoelii* CS proteins (*18*), and the response to CS protein T cell

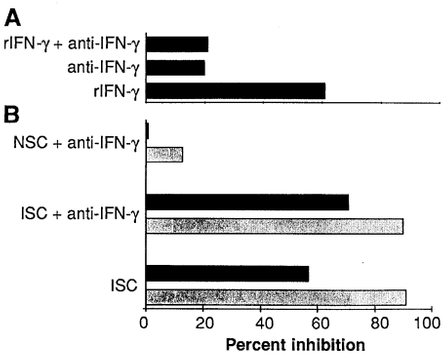

Fig. 3. The effect of anti–IFN-γ on ISC-mediated inhibition. (**A**) Anti–IFN-γ inhibited the effect of rIFN-γ on liver-stage parasites. Recombinant IFN-γ (10^3 units) and anti–IFN-γ adequate to inhibit 10^4 units of rIFN-γ were added to hepatocyte cultures 24 hours after infection with 4×10^4 *P. berghei* sporozoites. The medium was changed, then fresh rIFN-γ and anti–IFN-γ were added to the cultures at 27 and 41 hours, and the parasites per two cultures were counted at 48 hours. (**B**) Anti–IFN-γ does not inhibit the effect of ISC on liver stage parasites. Anti–IFN-γ and (black bar) 0.5 or (gray bar) 1.0×10^6 ISCs were added to the hepatocyte cultures at 24 hours, the medium was changed; fresh anti–IFN-γ was added at 27 and 41 hours, and the number of schizonts per culture was counted at 48 hours. Percent inhibition was calculated as in Fig. 2.

epitopes is severely genetically restricted (*19*). Yet all nine strains of congenic mice that we immunized with irradiated *P. berghei* sporozoites were protected against challenge by 10^4 uncloned NK-65 *P. berghei* sporozoites (*20*), a challenge dose expected to overcome antibody-mediated protective immunity. If effector T cells are important in protecting most of these different strains of mice, it is probable that a number of epitopes on multiple antigens, either on the sporozoite or first expressed in hepatocytes, were the target of these T cells. Further development of preerythrocyte-stage malaria vaccines will require the identification of such antigens and the production of subunit vaccines that induce protective cell-mediated immunity against these antigens.

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8. Six- to 12-week-old BALB/c mice (Jackson Laboratory) were used for all experiments. NK-65 sporozoites were isolated from salivary glands as previously described (*14*). In addition, an equal number of salivary glands from age-matched uninfected mosquitoes that had been identically reared and fed were processed in the same way as infected salivary glands. This material was adjusted to the same concentration as the infected material and used for control immunizations and challenges. After 43 hours the mice were killed, and the livers were removed and processed by standard methodology for histological examination and by immunofluorescence. For immunofluorescence studies, cryosections that had been fixed with acetone were incubated with rat monoclonal antibodies including M1/70 (macrophages, polymorphonuclear leukocytes, and natural killer cells), P 7/7 (nonpolymorphic determinants on mouse class II), YTS 169.4 ($CD8^+$ lymphocytes), YTS 191.1 ($CD4^+$ lymphocytes), and SER-4 (stromal macrophages, including Kuppfer cells). Slides were washed in phosphate-buffered saline and then incubated with a fluorescein-conjugated rabbit antibody to rat immunoglobulin G and examined with a fluorescent microscope.
9. Spleen cells were isolated by standard methods, depleted of $CD4^+$ or $CD8^+$ lymphocytes by incubation with monoclonal antibodies and complement, and transferred into naïve mice that had been exposed to 500 rads (cesium) 2 hours earlier. After 24 hours, the recipients received 1.0×10^4 irr-spz intravenously and 7 days later were challenged with 5×10^4 live sporozoites. The animals were killed 43 hours later and the livers were removed and processed for pathologic examination as in (*8*).
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High-Resolution Epitope Mapping of hGH-Receptor Interactions by Alanine-Scanning Mutagenesis

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A strategy, called alanine-scanning mutagenesis, was used to identify specific side chains in human growth hormone (hGH) that strongly modulate binding to the hGH receptor cloned from human liver. Single alanine mutations (62 in total) were introduced at every residue contained within the three discontinuous segments of hGH (residues 2 to 19, 54 to 74, and 167 to 191) that have been implicated in receptor recognition. The alanine scan revealed a cluster of a dozen large side chains that when mutated to alanine each showed more than a four times lower binding affinity to the hGH receptor. Many of these residues that promote binding to the hGH receptor are altered in homologs of hGH (such as placental lactogens and prolactins) that do not bind tightly to the hGH receptor. The overall folding of these mutant proteins was indistinguishable from that of the wild-type hGH, as determined by strong cross-reactivities with seven different conformationally sensitive monoclonal antibodies. The alanine scan also identified at least one side chain, Glu^{174}, that hindered binding because when it was mutated to alanine the receptor affinity increased by more than a factor of four.

SYSTEMATIC REPLACEMENT OF SEGments (7 to 30 residues in length) of human growth hormone (hGH) with sequences derived from nonbinding growth hormone homologs (homolog-scanning mutagenesis) defined a binding patch on a structural model of hGH that included the NH_2-terminal portion of helix 1, a loop between residues 54 and 74 and the COOH-terminal portion of helix 4 (*1*). This analysis provided a general outline of the receptor binding site, but did not identify the specific residues involved in receptor binding. Here, side chains that are important for modulating binding are located by sequential replacement of residues encompassed in the binding patch with alanine. Alanine was chosen as the replacement residue because it eliminates the side chain beyond the β carbon yet does not alter the main-chain conformation (as can glycine or proline) nor does it impose extreme electrostatic or steric effects. Furthermore, alanine is the most abundant amino acid and is found frequently in both buried and exposed positions and all variety of secondary structures (*2*). Alanine-scanning mutagenesis generates a small and systematic set of mutant proteins that can be readily assayed by quantitative binding analysis and avoids the necessity of sorting a library of random mutants by a genetic screen or selection.

A total of 62 single alanine mutants were produced (Table 1) by restriction-selection (*3*) to efficiently enrich for the mutant sequence after primer-directed mutagenesis on a synthetic hGH gene template (*1*). The mutant hormones were expressed in a secreted form from *Escherichia coli* (*4*), and their binding constants were determined for the extracellular portion of the cloned hGH liver receptor by Scatchard analysis (*1, 5*). This receptor fragment is highly soluble and retains high affinity and specificity for hGH (*5*). The use of the purified truncated receptor in binding assays avoided artifacts associated with binding to receptors on membranes or whole cells.

Overall, the results of the alanine scan (Fig. 1) are consistent with those from the homolog scan (*1*) in showing that the middle and COOH-terminal segments are more important in binding than the NH_2-terminal segment. The largest reductions in binding (~20 times lower) occurred for specific alanine substitutions within the 54 to 74 loop and the COOH-terminal sequence 167 to 191. We extended the alanine scan to include residues 2 to 19 because of uncertainties in the positions of the NH_2-terminal residues in the porcine GH (pGH) structure (*6*). In this segment, alanine substitutions caused more modest reductions in binding; the largest reduction (~6 times lower) was for F10A. For one mutant (E174A) that is located near a number of disruptive alanine mutations (Fig. 1), the affinity for the receptor was actually increased (4.5 times higher).

The most disruptive alanine substitutions form a patch that extends from F10 to R64 and from D171 to V185 (Fig. 2). These side chains appear to be facing in the same direction in the model of hGH. For example, all of the alanine mutants tested in helix 4 that most affect binding (D171A, K172A, E174A, F176A, I179A, C182A, and R183A) are confined to three and one-half turns of this helix, and their side chains

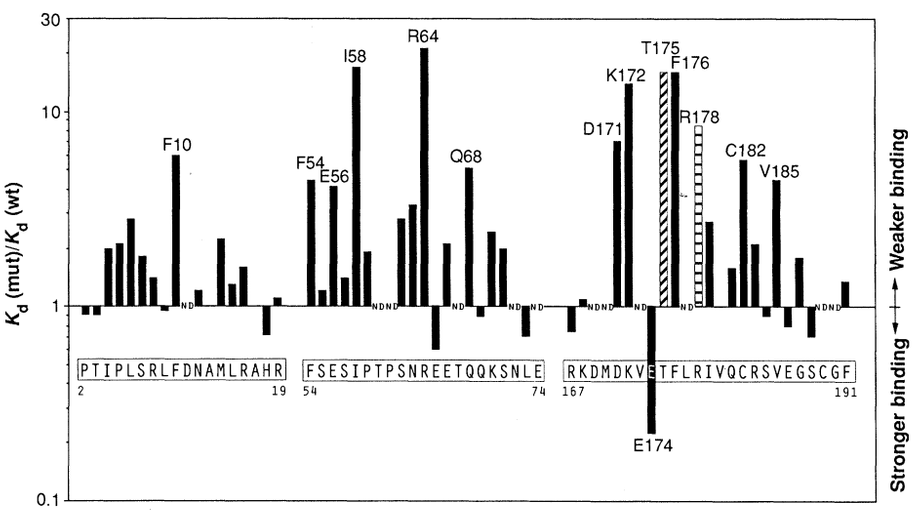

Fig. 1. The change in the dissociation constant relative to wild-type hGH K_d (mut)/K_d (wt) for mutating residues to alanine (black bars), serine (crosshatched bar), or asparagine (hatched bar) within the three binding site segments (Table 1). ND indicates that the K_d (mut) was not determined because of poor expression of the corresponding alanine mutant protein. Residues in which mutations cause a fourfold or greater change in the dissociation constant are labeled. The values are all at or below ±25% (SD).

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