T lymphocytes from mice immunized with irradiated sporozoites eliminate malaria from hepatocytes.
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When mice are immunized with radiation-attenuated sporozoites they are solidly protected against sporozoite challenge by an immune response that has been shown to require CD8+ lymphocytes in several strains of mice. The target of this CD8+ T-cell-dependent immunity 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. Further subunit pre-erythrocytic stage malaria vaccine development will require identification of the antigens recognized by these T cells and a method of immunization that induces such immunity.

Introduction

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 radiation-attenuated malaria sporozoites (irr-spz) protects animals (1) and humans (2-5) against challenge with normal sporozoites. In mice the effector arm of this protective immunity has been shown to require CD8+ lymphocytes and interferon-γ (6, 7). Such immunity does not protect against challenge with erythrocyte-stage parasites (1) or liver-stage merozoites (8), 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 naive animals after infection (9, 10) 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.

Materials and methods

Mice

Six to 12-week-old BALB/c and C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used.

Immunization

Sporozoites. P. berghei sporozoites (uncloned NK-65) were isolated from salivary glands as previously described (11). The sporozoites were attenuated by exposure to 1.5 x 10⁴ rad (150 Gy) (cobalt). The initial immunizing dose was always 7.5 x 10⁴ sporozoites (spz) and booster doses were 2.0 x 10⁴ spz by the intravenous route.

Salivary glands. 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, irradiated when appropriate, and used for control immunizations and challenges.

Challenge

Mice were challenged with sporozoites or salivary gland material by intravenous injection.
Histopathology studies

Groups of three BALB/c mice were immunized with $7.5 \times 10^4$ irr-spz or the equivalent material from uninfected salivary glands. Two weeks later they were challenged with $7.5 \times 10^4$ spz or the equivalent material from uninfected salivary glands. Forty-three hours later, the mice were killed and the livers removed and processed for standard histological examination (haematoxylin and eosin), and for immunofluorescence examination. For immunofluorescence studies, cryosections previously fixed with acetone were incubated with rat monoclonal antibodies including Mac-1 (macrophages, polymorphonuclear leukocytes, and natural killer cells), P 7/7 (nonpolymorphic determinants on mouse class II), Lyt-2 (CD8⁺ lymphocytes), L3T4 (CD4⁺ lymphocytes), and SER-4 (stromal macrophages including Kupffer cells). Slides were washed in phosphate-buffered saline and then incubated with a fluorescein-conjugated rabbit antibody to rat IgG, and examined with a fluorescent microscope.

Adoptive transfer

Spleen cells were isolated by standard methods, depleted of CD4⁺ or CD8⁺ lymphocytes by incubation with monoclonal antibodies and complement, and transferred into naive mice that had been exposed to 500 rad (5 Gy) (caesium) 2 hours earlier. After 24 hours, the recipients received $1.0 \times 10^4$ irr-spz intravenously and 7 days later were challenged with $5 \times 10^4$ live sporozoites. The animals were killed 43 hours later and the livers were removed and processed for pathologic examination.

Liver-stage parasite killing assay

Mice were immunized with three doses of irr-spz at 2-week intervals, and given a final booster dose of $2.5 \times 10^3$ irr-spz. Five days later one to three mice were killed, and immune spleen cells (ISCs) were isolated and added to primary hepatocyte cultures (12) that had been infected with an average of $5 \times 10^4$ normal P. berghei sporozoites 24 hours previously. The media was changed and processed for standard histological examination (haematoxylin and eosin), and for immunofluorescence examination. For immunofluorescence studies, cryosections previously fixed with acetone were incubated with rat monoclonal antibodies including Mac-1 (macrophages, polymorphonuclear leukocytes, and natural killer cells), P 7/7 (nonpolymorphic determinants on mouse class II), Lyt-2 (CD8⁺ lymphocytes), L3T4 (CD4⁺ lymphocytes), and SER-4 (stromal macrophages including Kupffer cells). Slides were washed in phosphate-buffered saline and then incubated with a fluorescein-conjugated rabbit antibody to rat IgG, and examined with a fluorescent microscope.

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Effect of anti-interferon γ on liver-stage killing

Twenty-four, 27, and 41 hours after infection of hepatocyte cultures with $4 \times 10^4$ P. berghei sporozoites, the medium was changed and recombinant IFN-γ (10³ units), ammonium sulfate precipitated rabbit antibodies against rIFN-γ (adequate to neutralize $10^4$ units of rIFN-γ), or both rIFN-γ and anti-IFN-γ antibodies were added to the cultures. The numbers of parasites per two cultures were counted at 48 hours (Fig. 2a). Anti-IFN-γ and $0.5$ or $1.0 \times 10^6$ ISC were added to the hepatocyte cultures at 24 hours, and 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 (Fig. 2b). Percent inhibition was calculated as in Fig. 1.

Results

Hepatic infiltrates

Multiple inflammatory infiltrates were found in livers of mice immunized with irr-spz and challenged with normal sporozoites, but few infiltrates were found in controls. The response is specific for malaria (Table 1).

Table 1: Malaria-specific infiltrates in the livers of immune mice after challenge

<table>
<thead>
<tr>
<th>Experiment and immunogen</th>
<th>Challenge</th>
<th>No of infiltrates per 20 low-power fields (× 125)</th>
<th>Diameter of infiltrates (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. irr-spz</td>
<td>spz</td>
<td>$17.7 \pm 2.05$</td>
<td>$69.3 \pm 12.10$</td>
</tr>
<tr>
<td>irr-spz</td>
<td>glands</td>
<td>$3.1 \pm 0.64$</td>
<td>$51.7 \pm 11.59$</td>
</tr>
<tr>
<td>2. irr-spz</td>
<td>spz</td>
<td>$17.3 \pm 1.0$</td>
<td>$52.9 \pm 28.49$</td>
</tr>
<tr>
<td>irr-glands</td>
<td>spz</td>
<td>$2.3 \pm 2.08$</td>
<td>$64.8 \pm 48.91$</td>
</tr>
</tbody>
</table>

*The mean number of infiltrates per 20 low-power fields (× 125) in mice immunized with irr-spz and challenged with liver sporozoites (spz) was significantly higher than among mice immunized with irradiated salivary glands (irr-glands) and challenged with liver sporozoites, and among mice immunized with irr-spz and challenged with salivary glands (glands) (P<0.05, Student’s t-test, 2-tailed). Values are means ± SD.*
The infiltrates contain numerous macrophages, polymorphonuclear leukocytes, eosinophils, and CD8⁺ lymphocytes. CD4⁺ lymphocytes are present in lower concentration, and the number of Kupffer cells recognized by monoclonal antibody SER-4 is similar in infiltrates and normal hepatic tissue. To further define the infiltrates, we adoptively transferred spleen cells from immune or naive donors into naive recipients, challenged the recipients, and examined their livers 43 hours later. Infiltrates were present in the livers of mice that received unfractionated ISCs (5.2 ± 4.19 infiltrates per 20 low-power fields, mean ± S.D.) and CD4⁺ ISCs (5.5 ± 2.12), but not in those that received CD8⁺ ISCs (0) or normal spleen cells (0.3 ± 0.57).

Elimination of malaria from hepatocytes

When ISCs are added to cultures infected with sporozoites 24 hours previously, they eliminate parasites from the cultures in a dose-dependent manner (Fig. 1A). In another experiment 1 × 10⁶ BALB/c ISCs were added to eight hepatocyte cultures and

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**Fig. 1:** Spleen cells from immune mice eliminate the liver stages of *P. berghei* from hepatocyte culture, and the effect on parasites is genetically restricted.*

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*A. BALB/c ISCs eliminated parasites from BALB/c hepatocytes in two different experiments. B. BALB/c ISCs specifically eliminated parasites from BALB/c (0), but not C57L/6 (0) hepatocytes. C. C57L/6 ISCs (0) eliminated parasites from C57L/6 hepatocytes, but BALB/c ISCs (0) did not. D. BALB/c ISCs (0) eliminated parasites from BALB/c hepatocytes, but C57L/6 ISCs (0) did not.
1 × 10⁶ NSCs to eight other cultures. There were 22.6 ± 4.71 liver-stage parasites in the ISC cultures, and 96.5 ± 15.57 parasites in NSC cultures (P < 0.05, Student's t-test, 2-tailed); an inhibition of 75.5%.

Interferon-γ (IFN-γ) inhibits the development of liver-stage parasites in vitro (13–15). We therefore used an antiserum directed against IFN-γ (anti-IFN-γ) to determine whether IFN-γ mediated the elimination of parasites. Mouse rIFN-γ (1000 units) inhibited parasite development by 60%, and this effect could be blocked by addition to cultures of rabbit anti-mouse rIFN-γ (Fig. 2a). When the antibody to mouse rIFN-γ was added to the ISCs it had no effect on the elimination of parasites (Fig. 2b). 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. To determine whether other factors secreted by the ISCs had activity against parasites, we collected supernatants 3, 8, and 24 hours after addition of cells to hepatocyte cultures (27, unpublished observations). High concentrations of IFN-γ similar to those in cultures to which normal attenuated sporozoites can protect BALB/c mice, elimination of parasites was specific for BALB/c hepatocytes (data not shown). Genetically restricted killing of target cells has been linked only to the 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.

Discussion

After immunization with 7.5 × 10⁴ 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 × 10⁵ sporozoites (S.L. Hoffman & W.R. Ballou, unpublished observations). High concentrations of monoclonal and polyclonal antibodies to the circumsporozoite (CS) protein and to sporozoites can protect against sporozoite challenge (11, 17). These antibodies have no effect on development of liver-stage parasites if added to in vitro cultures after sporozoites have invaded the hepatocytes (8) 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 and that there is evidence that T-effector cells (11, 18), specifically cells of the suppressor/cytotoxic phenotype (CD8⁺) (6, 7), are involved in the potent protective immunity induced by immunization with irradiated sporozoites. Since it was likely that infected hepatocytes were the target of this immunity, we looked at livers of immune mice after challenge with sporozoites and found malaria-specific infiltrates. The numbers of infiltrates in these experiments exceeded the numbers of mature schizonts expected after such a sporozoite challenge. 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, could 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. The data from the studies using the in vitro assay 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 (19, 20). The CS protein is present in hepatocytes throughout the liver stage (10, 21, 22) 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 (23; W. Weiss et al., unpublished observations), and the response to CS protein T-cell epitopes is generally genetically restricted (24–29). Yet all nine strains of congenic mice that we immunized with irradiated P. berghei sporozoites were protected against challenge by 10⁴ uncloned NK-65 P. berghei sporozoites (30), a challenge dose expected to overcome antibody-mediated protective immunity. If effector T cells played a major role in protecting most of these different strains of mice, it is probable that a number of epitopes on multiple antigens, either on sporozoites or first expressed in hepatocytes, were the target of these T cells. Further development of pre-erythrocytic-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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