Intraerythrocytic Killing of Malaria Parasites

Annual Report

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The findings in the report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.
The purpose of these studies is to determine the role of activated macrophages in immunity to the blood stages of malaria. This is being accomplished by comparing the activity of macrophages during lethal and non-lethal malaria infections and in malaria-resistant and non-resistant mice. The results indicate that in non-lethal P. yoelii infection of mice, macrophage activity, as determined by $H_2O_2$ release, gamma-interferon production and lymphoproliferation, is high early in infection, declines during the time of peak parasitemia and recovers as the infection is cleared. Although some macrophage functions are enhanced during the early phase of lethal P. yoelii infection, this enhancement is not sustained. Recombinant gamma-interferon, infected into mice, had a protective effect in susceptible mice with lethal infections but not in non-lethal infections.
Foreword

Citations of commercial organizations and trade names in the report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978)."
Title: Intraerythrocytic Killing of Malaria Parasites

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Scientific Report

Research Problem

Evidence from this laboratory indicates that lymphokines (LK), which are produced by the spleen during rodent malaria infections, stimulate normal, resident macrophages to bind and ingest and to kill intraerythrocytic malaria parasites in vitro (1, 2).

Since the killing of the intraerythrocytic parasites in vitro appeared to be mediated, at least in part, by \( \text{H}_2\text{O}_2 \) secreted by macrophages (2, 3), studies were undertaken to determine whether levels of \( \text{H}_2\text{O}_2 \) production varied between lethal and non-lethal infections and between susceptible and resistant mice.

Further, since human gamma-interferon (Genentech, Inc.) was able to activate human monocyte-derived macrophages to kill \( P. \) falciparum (3) and preliminary evidence suggested that LK obtained from malaria-infected mice also contains gamma-interferon, studies were undertaken to determine the levels of gamma-interferon produced during lethal and non-lethal infections and in susceptible and resistant mice. In addition, the effect of recombinant mouse gamma-interferon (Genentech, Inc.) on the course of lethal and non-lethal rodent malaria was studied.

Background

Much evidence is accumulating to indicate that cell-mediated immune responses are very important in the response to some species of malaria (4) and may act as an adjuvant in vaccinated mice (5). However, the actual mechanisms whereby cell-mediated responses protect against malaria are not known.

The overwhelming evidence supports the concept that activated macrophages are involved in controlling several infections such as \textit{Leishmania tropica} (6), \textit{Rickettsia akari} (7), and \textit{Trypanosoma cruzi} (8); that they are cytotoxic to tumor cells (9), and finally that they may be involved in vaccine-induced immunity (5).

We have been studying macrophage activation during rodent malaria (10) and the effects of macrophages activated with other stimulants on malaria-infected erythrocytes (1). Briefly, we have found that spleen cells of mice infected with BCG or malaria, produced factors or lymphokines (LK) which stimulated normal mouse peritoneal macrophages for enhanced phagocytosis of parasitized erythrocytes (1) and for killing of parasitized erythrocytes (2). We have also found that fresh monocytes or LK-stimulated, monocyte-derived macrophages were active in inhibiting the multiplication of \( P. \) falciparum (3). In both systems \( \text{H}_2\text{O}_2 \) seemed to be the active molecule. Killing was observed after parasitized erythrocytes bound to monocytes and was associated with an oxidative
burst in the monocytes. After the interaction, the parasitized erythrocytes appeared to be degenerating and looked like the previously described "crisis" forms (11).

Our results confirm and extend several other findings. Taliaferro and Cannon (11) observed that upon acquisition of immunity in monkeys infected with malaria, some intraerythrocytic parasites appear to degenerate within the erythrocytes. This finding implied that soluble mediators might affect malarial parasites. That such mediators might be secreted by macrophages was first suggested by Allison and Clark (12). Mice treated with BCG are protected against malaria and the mechanism suggested was that parasites are killed by products of activated macrophages. Since then, other parasiticidal factors have also been shown to have an effect on intraerythrocytic malaria parasites, namely, tumor necrosis factor (13), interferon (13) and a lipopolysaccharide-induced serum factor (14).

The concept that oxygen radicals might affect malaria parasites was suggested by the observations that injections of alloxan (15) and t-butyl hydroperoxide (16,17) into mice with P. vinckeii (15, 17) or P. yoelii (16) markedly reduces parasitemia. These compounds generate reactive oxygen intermediates and their activity can be inhibited by iron-chelating agents such as desferrioxamine and diethylthiocarbamate. It is well known that malaria parasites are sensitive to oxidant stress. Cultures of P. falciparum grown in G-6-PD deficient erythrocytes are inhibited under high oxygen tension (18). In addition, dilutions of H2O2 as low as 10^-8M are toxic to P. yoelii and P. berghei in vitro and in vivo (19).

Early studies of Langhorne et al. (20) indicated that incubation of spleen cells from infected monkeys with parasitized erythrocytes reduced their ability to multiply. Later, Taverne et al. (21) demonstrated the killing of P. yoelii by cells of the monocyte-macrophage series. Data from this study also suggested that fresh blood monocytes or peritoneal cells activated by incubation with lymph node cells of immunized mice were more effective than normal peritoneal cells. Our studies show that H2O2 produced upon an oxidative burst in activated macrophages, is lethally damaging to P. yoelii and P. falciparum. Because these studies suggest an important protective mechanism in malaria, studies were undertaken to determine the roles of IFN and H2O2 production in lethal and non-lethal P. yoelii infections and in susceptible and resistant mice.

Approach

We have taken the following approach in these studies:

1. In lethal and non-lethal P. yoelii infections in susceptible Balb/C ByJ mice we have determined:
a) the levels of \( \text{H}_2\text{O}_2 \) produced by peritoneal and splenic macrophages,

b) the levels of gamma-interferon produced by spleen cell cultures,

c) the ability of spleen cell cultures to proliferate in response to a mitogen (Con A) and to \( P. \text{yoelii} \) erythrocyte antigens.

2. Similar studies as in 1. above were carried out in more resistant CBA/J mice.

a) \( \text{H}_2\text{O}_2 \) production

b) gamma-interferon production

c) responses to Con A and \( P. \text{yoelii} \) antigen.

3. We have determined the effect of mouse gamma-interferon on the course of lethal and non-lethal \( P. \text{yoelii} \) infections in outbred mice and inbred susceptible (Balb/C ByJ) and resistant (CBA/J) mice.

**Results**

1. a) The levels of \( \text{H}_2\text{O}_2 \) produced by peritoneal and spleen macrophages measured as in (21), over the course of either \( P. \text{yoelii}17\times\text{X} \) (lethal) or \( P. \text{yoelii}17\times\text{NL} \) (non-lethal) in Balb/C ByJ mice is shown in Fig. 1. In the non-lethal infection, \( \text{H}_2\text{O}_2 \) levels are increased above normal by day 5, decline during the second week of infection (when parasitemia peaks) and then begin to increase after week two. \( \text{H}_2\text{O}_2 \) production by peritoneal macrophages from mice infected with \( P. \text{yoelii}17\times\text{X} \) were not above control levels and declined to below control levels until the mice died. Spleen cells did have increased \( \text{H}_2\text{O}_2 \) production early in the lethal infection, however, this declined and did not recover.

b) Gamma-interferon levels produced by spleen cell cultures pulsed with mitogen or \( P. \text{yoelii} \) antigen were measured by RIA as in (22). In the non-lethal infection, levels of gamma-interferon were greatly increased 4-5 days after infection, declined during the second week and then began to recover to control levels as the infection was cleared (Fig. 2). In the lethal infection, however, gamma-interferon production was at about control levels (gamma-interferon produced by spleen cells from uninfected mice pulsed in the same way) throughout the infection.

c) The proliferative response of spleen cells to the T cell mitogen, Con A, and to a preparation of erythrocytic stage \( P. \text{yoelii} \) antigens (prepared as in 2) followed the same general pattern as the production of \( \text{H}_2\text{O}_2 \) and gamma-interferon (Fig. 3). The response to both Con A and \( P. \text{yoelii} \) was high on day 3, dropped to below control levels by day 12 and then recovered in the non-lethal infection. In the lethal infection the responses were high on day 3, dropped to below control levels and then the animals died.
2. The same three assays were performed on peritoneal and spleen cells from the more resistant strain of mice, CBA/J. These mice generally survive infection with $10^6$ parasitized erythrocytes of *P. yoelii*17XL. The results are shown in Figs. 4-6. $H_2O_2$ release from peritoneal and spleen cells was well above control levels on day 3 of infection, declined to control (peritoneal cells) or below control (spleen cells) levels from days 5-20, and then recovered. During the lethal infection the $H_2O_2$ response was initially high, decreased dramatically on day 5 (peritoneal) or day 10 (spleen) and then recovered temporarily. The gamma-interferon response of cells triggered with Con A or antigen was also high initially, declined during the second week of infection and recovered in both non-lethal and lethal infections. Interestingly, the proliferative responses to *P. yoelii* antigen was high initially, declined and recovered temporarily in the non-lethal infection. However, in the lethal infection there was a different pattern, the response was low initially, peaked during the second week and then declined to below control levels.

3. Effect of gamma-interferon on *P. yoelii* infections in vivo.

SW female mice, 4-6 weeks old were injected with doses of gamma-interferon ranging from 1,000 to 100,000 units/day, intraperitoneally. Controls received either diluent, gamma-interferon plus anti-gamma-interferon or thioglycollate. Fig. 7 shows that mice infected with *P. yoelii*17XL were protected by gamma-interferon in a dose-dependent fashion. Controls were not protected. However, mice infected with the non-lethal *P. yoelii* did not show the same degree of protection (Fig. 8).

Further experiments were undertaken in inbred mice. In susceptible, Balb/C ByJ, gamma-interferon had a protective effect against *P. yoelii*17XL but no effect on the non-lethal infection (Fig. 9, 10). Interestingly, in the more resistant CBA/J mice there was only a slight protective effect against *P. yoelii*17XL (which is not lethal in these mice) and no effect on the non-lethal parasite (Fig. 11, 12).

Discussion and Conclusions.

Our initial observations, that there are indeed differences in the macrophage response to lethal and non-lethal *P. yoelii* infections made during the first year of this project, have now been confirmed and extended with more information on the time course of these changes and the differences between susceptible and resistant strains of mice. In addition, the responses of peritoneal cells compared with spleen cells have been analyzed.

The data indicates that the mice make an early macrophage response to both lethal and non-lethal *P. yoelii* as indicated by $H_2O_2$ production by both peritoneal and spleen cells. In the non-lethal infection, the decline in $H_2O_2$ production is followed
by a recovery whereas in the lethal infection, it is not. These results are in parallel with the cellular response to infection. Both production of gamma-interferon and lymphoproliferation in response to P. yoelii antigen and a T cell mitogen, Con A, show a similar pattern. In non-lethal infections there is a good early response of gamma-interferon, a decline, and a slight recovery. In lethal infections there is hardly any gamma-interferon response at all. Even more striking is the lymphoproliferative response in which spleen cells from mice infected with non-lethal malaria show a striking recovery as the infection is cleared while mice infected with lethal malaria do not.

In more resistant mice (CBA/J), P. yoelii17x is not lethal. Thus, the responses to both lethal and non-lethal P. yoelii more closely resemble the response of Balb/C mice to P. yoelii17xNL, i.e., a good early response, a decline as parasitemia increases and then a recovery. The only exception to this pattern was the lymphoproliferative response of mice infected with P. yoelii17xL. The reason for this is under investigation.

The results of injection of exogenous interferon are interesting in view of the results discussed above. Gamma-interferon appeared to have an effect primarily in the lethal P. yoelii infection. Thus, in animals with poor macrophage responses, gamma-interferon helps. If the animal is making a sufficient response, gamma-interferon has no effect.

Recommendations

During the coming year it will be important to determine how gamma-interferon is mediating its effect on the erythrocytic cycle of malaria. Preliminary experiments indicate that macrophages from mice injected with gamma-interferon show high levels of $H_2O_2$ production and enhanced phagocytosis. However, controls injected with gamma-interferon plus a monoclonal antibody against gamma-interferon also show the same effect. Since this might be due to the injection of immune complexes, studies are being planned in which (Fab)$_2$ fragments of the monoclonal will be injected with gamma-interferon and a polyclonal antiserum against gamma-interferon will be raised in rabbits and (Fab)$_2$ fragments of this antiserum will be utilized. Further, these reagents will be useful in experiments to see the effect of anti-gamma interferon on lethal and non-lethal P. yoelii infection. These experiments are planned for the coming year.

Literature Cited


Fig. 1. H$_2$O$_2$ Response in C3H/10B6J Mice

PyNL Spleen Macrophages  PyL Spleen Macrophages

PyNL Peritoneal Macrophages  PyL Peritoneal Macrophages

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Fig. 2

O-trans response in LEC/CD8 T cells

PyNL ConA

PyL ConA

PyNL Ag

PyL Ag

Time (hours)

0.1
0.2
0.3
0.4
0.5

CONTROL

0.1
0.2
0.3
0.4
0.5

CONTROL

0.1
0.2
0.3
0.4
0.5

CONTROL
Fig 3. Lymphoproliferative response in BALB/cByJ

PyNL Antigen

PyL Antigen

PyNL ConA

PyL ConA

CONTROL
Fig 4: H2O2 response in COHJV mice

PyNL Spleen Resident Macrophages

PyL Spleen Macrophages

PyNL Peritoneal Macrophages

PyL Peritoneal Macrophages

-CONTROL-

5 10 15 20 25 30

-CONTROL-

5 10 15 20 25 30

-CONTROL-

5 10 15 20 25 30

-CONTROL-
Data from "IFN Dose Response to Py Lethal" in SWJ
Data from "IFN Dose Response of P.y (Non)" in S.

Days after Infection

Parasitemia

- Diluent
- Group II
- Group III
- Group IV
- Group V
- Group VI
- Group VII
Data from "PY LETHAL response in CBA/J"
IFN EFFECT ON PY NON LETHAL IN CBA/J

![Graph showing IFN effect on parasite levels over days after infection.](image)

Fig. 12

Days after infection

% Parasitemia
Appendix

Legends to Figures

Fig. 1. \(H_2O_2\) response of spleen and peritoneal macrophages in Balb/C ByJ mice infected with \(P. yoelii\)17xL or \(P. yoelii\)17xNL. Mice were infected I.P. with \(10^5\) parasitized erythrocytes. On the days indicated, animals were sacrificed, peritoneal and spleen cells harvested, plated and \(H_2O_2\) measured. Data represents the mean of two mice/point.

Fig. 2. Gamma-interferon response of spleen cells from Balb/C ByJ mice infected with \(P. yoelii\)17xL or \(P. yoelii\)17xNL. Mice were infected as for Fig. 1. On the days indicated, animals were sacrificed, spleen cells obtained and cultured for 3 days at \(5 \times 10^5\) cells/ml. On day 3, 50 ul of the supernatant was assayed for gamma-interferon by RIA. Data represents the mean of triplicate assays from 2 mice/point.

Fig. 3. Lymphoproliferative response of spleen cells in Balb/C ByJ mice infected with \(P. yoelii\)17xL or \(P. yoelii\)17xNL. Animals were infected as in Fig. 1. On the days indicated, mice were sacrificed, spleen cells obtained and cultured at \(5 \times 10^5\) cells/ml for 4 days. On the 3rd day, cells were pulsed with thymidine and harvested on day 4. Data represents the mean of triplicate cultures from 2 animals/point.

Fig. 4. \(H_2O_2\) response of spleen and peritoneal macrophages in CBA/J mice infected with \(P. yoelii\)17xL or \(P. yoelii\)17xNL. As for Fig. 1.

Fig. 5. Gamma-interferon response of spleen cells from CBA/J mice infected with \(P. yoelii\)17xL or \(P. yoelii\)17xNL. As for Fig. 2.

Fig. 6. Lymphoproliferative response of spleen cells from CBA/J mice infected with \(P. yoelii\)17xL or \(P. yoelii\)17xNL. As for Fig. 3.

Fig. 7. Effects of recombinant gamma-interferon on the course of \(P. yoelii\)17xL in SW mice. Mice were pretreated I.P. for 3 days with the materials shown, in 0.2 ml. On day 0 they were infected with \(10^4\) infected erythrocytes I.P. Injections were continued daily. Data represents the mean of 5 mice/group.

Fig. 8. Effect of recombinant gamma-interferon on the course of \(P. yoelii\)17xNL in SW mice. As for Fig. 7.

Fig. 9. Effect of recombinant gamma-interferon on the course of \(P. yoelii\)17xL in Balb/C ByJ mice. Mice were pretreated I.P. for 3 days with the materials shown, in 0.2 ml. On day 0 they were infected with \(10^4\) parasitized erythrocytes I.P. Injections were continued daily to day 28 except for days 8, 9, 15, 16, 22, 23. Data represents the mean of 4 mice/group.
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