Host Defenses in Murine Malaria: Immunological Characteristics of a Protracted State of Immunity to

*Plasmodium yoelii*

JAMES R. MURPHY

Department of Microbiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

Random-bred ICR mice recovered from infection with avirulent *Plasmodium yoelii* were challenged at various later times with virulent *P. yoelii* or with another species of *Plasmodium, P. berghei*, to characterize the immunological nature of the long-term state of immunity generated in response to the avirulent infection. It was found that recovered mice resisted lethal challenge with virulent *P. yoelii* through at least 416 days after primary infection. However, the quality of this immunity changed as the time after avirulent infection increased. Mice challenged early after recovery were able to prevent the development of patent parasitemia. Later, these immune animals lost this capacity and after challenge infections progressed to patency at the same rate as did nonimmune controls. However, after the establishment of parasitemia, those animals which had encountered the homologous parasite a long time before controlled, and then eliminated, blood infection and survived. The "early" state of immunity was expressed by animals which may have harbored small numbers of viable avirulent parasites and possessed a protective humoral factor which could passively transfer anti-*P. yoelii* activity to naive recipients. In contrast, animals with "late" immunity showed evidence of neither persisting avirulent parasites nor serum anti-*P. yoelii* activity. The results support the proposition that immunity to this parasite exists as two distinct but interrelated states of immunological reactivity: an early "active" immunity and a later state which has characteristics suggestive of a state of immunological memory wherewith the animals were capable of anamnestically responding to *P. yoelii* challenge. Little evidence of heterologous immunity to *P. berghei* was observed for animals recovered from *P. yoelii*.

It is established that immunity to erythrocytic murine malaria can be generated in response to attenuated infection (1, 8, 9, 20, 28) and injections of (i) irradiated blood parasites (30, 31), (ii) killed blood parasites (4, 6, 19, 22, 23), or (iii) subcellular blood-form antigens (5, 33). However, these different procedures do not result in states of immunity of uniform quality. Immunization through attenuated infection (20) or injections of irradiated parasites (30) which retain the capacity to synthesize protein (29) can lead to an acquired resistance which prevents or severely restricts the development of patent erythrocytic infection after challenge with virulent erythrocytic parasites. Immunization with dead parasites or parasite products can induce a long-lived state of resistance in which the immunized host responds more rapidly with a successful defense to a virulent challenge than the immunologically naive mouse but lacks the capacity to immediately neutralize the parasites delivered in a challenge (18, 21, 22). This is evidenced by the parallel development of infections in immunized and immunologically naive mice and subsequently more rapid clearance of parasites from previously infected animals (18, 21, 22). It is not clear at this time whether these two patterns of immune response represent qualitatively different states of immunity or whether they are merely quantitatively different.

There is evidence that immune mice with a capacity to immediately restrict *Plasmodium berghei* (18) or *P. yoelii* (20) possess in their serum a factor which can passively transfer to naive recipients a capacity to directly restrict homologous parasites. In contrast, immunized mice which lack the capacity to immediately restrict challenge parasites also lack the protective humoral factor. They do, however, possess for an extended interval after immunization a capacity to rapidly generate such a humoral protective factor subsequent to infection with homologous parasites (18, 22). These observations have been interpreted (18, 21) to support the view that immunity to murine malaria exists as two distinct states of immunological reactiv-
ity: one which resembles active immunity and a second which has characteristics of immunological memory.

This report will show that immunity to *P. yoelii* generated in response to avirulent infection changes with time from a defense consistent with a state of active immunity to a hyperresponsiveness which has characteristics consistent with a state of immunological memory.

**MATERIALS AND METHODS**

**Animals.** Female mice of the random-bred ICR strain were obtained at 5 to 6 weeks of age. The mice were bred at the Trudeau Institute, maintained on a standard diet (4RF, Charles River formula; Agway, Waverly, N.Y.), and provided with water ad libitum.

**Plasmodia.** Virulent and avirulent variants of *P. yoelii* strain 17X were used. The source of these parasites and the procedures employed for their maintenance are described in detail elsewhere (20).

The NYU-2 strain of *P. berghei* was maintained as a frozen (−70°C) seed stock which was passed once in mice before use.

**Infections.** Intravenous inoculation of 10^6 parasitized erythrocytes (PRBC) of the relatively avirulent strain of *P. yoelii* was employed for immunizing infections. Except where noted otherwise, challenge with virulent *P. yoelii* was accomplished by intravenous injection of 10^6 PRBC. *P. berghei* challenges consisted of 10^4 PRBC delivered intravenously.

**Parasitemia.** Thin blood smears were prepared from tail blood and stained by the Giemsa method. If the level of parasitemia was 1% or greater, the number of PRBC in 200 erythrocytes (RBC) was determined. For parasitemia less than 1%, the number of PRBC in a number of microscopic fields containing 2 × 10^4 RBC was determined. Parasitemia is expressed as median PRBC per 10^4 RBC in log_{10} units for each group of mice. Thus, a blood smear showing 10^6 PRBC/10^4 RBC (e.g., 100% parasitemia) is represented as 4 U (e.g., log_{10} 10,000), and a smear showing 1 PRBC/10^4 RBC (e.g., 0.01% parasitemia) is represented as 0 U (e.g., log_{10} 1).

**Assay of sterile immunity.** Mice recovered from infection with avirulent *P. yoelii* were evaluated for latent infection as follows. Splenectomies were performed under ether anesthesia through a flank incision which was closed with Michel clips. Each spleen was placed in 3 ml of 0.9% NaCl and disrupted with a Ten Broeck hand homogenizer, and 0.5 ml of homogenate was injected intraperitoneally into each of five normal ICR mice. Blood smears were prepared from splenectomized mice at 4-day intervals through day 20 after splenectomy.

Recipients of spleen homogenate were challenged with virulent *P. yoelii* 28 days after the intraperitoneal inoculation of the disrupted spleen. Those mice which survived the virulent challenge were considered to have undergone an avirulent immunizing infection as the result of the inoculation of the spleen homogenate. Thus, the donor of a spleen which immunized a recipient was considered to have harbored *P. yoelii* at the time of splenectomy.

Mice which showed no parasitemia after splenectomy and whose disrupted spleens failed to immunize recipients were regarded as sterile with respect to *P. yoelii*.

**Serum transfer.** Blood collected by cardiac puncture of anesthetized mice was allowed to clot, and the serum was removed. The sera were filtered (0.22-μm pore size) and stored at −70°C until assayed.

Recipients were inoculated intraperitoneally with 1 ml of serum and 1 h thereafter were challenged by the intravenous inoculation of 10^6 PRBC of virulent *P. yoelii*. Blood smears were prepared at daily intervals thereafter, and the mean interval in days to 1% infection was determined for each group. An increase in interval to 1% infection for serum recipients as compared to otherwise untreated animals challenged with the same plasmodial inoculum was the measure of anti-*P. yoelii* activity.

**RESULTS**

The course of avirulent *P. yoelii* infection. Figure 1 shows the course of parasitemia after the intravenous introduction of 10^3 PRBC of avirulent *P. yoelii*. It can be seen in Fig. 1 that infections developed uniformly between days 3 and 12 but that the subsequent course of infections varied from animal to animal. Thus, the first mouse to clear patent infection did so by day 18, whereas it took 30 days for the last mouse to reduce blood infection to below measurable levels.

**Characteristics of acquired long-term resistance to reinfection with the homologous parasite.** It was found in a previous study (20) that recovery from infection with avirulent
*P. yoelii* caused the generation of a long-lived capacity to resist lethal challenge with virulent parasites. To determine whether the immunological nature of this long-lived state of resistance changed as the interval from primary infection increased and to estimate the duration of this state of immunity, mice which had recovered from avirulent infection were challenged intravenously with $10^7$ PRBC of virulent *P. yoelii* at intervals between 28 and 396 days after the initiation of the primary avirulent infection.

It was found (Fig. 2) that recovery from avirulent infection resulted in immunity of at least 396 days duration and that the quality of this immunity changed as the interval from primary infection increased. Thus, virulent challenge delivered on day 28 failed to cause patent infections, but similar challenges at day 255 or 396 produced parasitemias which developed through 3 days after the challenge at about the same rate in previously infected mice as in controls. However, mice previously exposed to the live homologous parasite rapidly recovered, whereas the controls progressed to death. These results demonstrate that the host defense which follows an avirulent infection and is accompanied by the capacity to prevent the development of parasitemia wanes as the interval from the immunizing infection increases. However, the capacity to resist a lethal challenge persists for at least 396 days.

Because it is established that a humoral factor, presumably protective antibody, effects in part the elimination of blood forms of *P. yoelii* (10, 20, 22), it was considered probable that the loss of the capacity to immediately neutralize parasites delivered as a challenge would correspond with waning levels of the humoral protective component. This possibility was tested with sera collected 28 or 255 days after avirulent infection.

Figure 3 shows that the passive transfer of 1 ml of day 28 serum protected recipients from *P. yoelii*. Protection was manifested as a statistically significant ($P < 0.01$) analysis of variance, Tukey test) 2-day delay in the onset of 1% par-

**Fig. 2.** Demonstration that the quality of immunity changes as the interval from avirulent "immunizing" infection increases. Ten normal (agematched) mice and 10 mice which had recovered from avirulent *P. yoelii* infection were challenged intravenously with $10^7$ PRBC of virulent *P. yoelii* at the indicated times after avirulent infection.

**Fig. 3.** Demonstration that levels of serum anti-*P. yoelii* activity decline as the interval after recovery from avirulent infection increases. As shown, serum collected at day 28 after donor infection delayed the onset of infection in passively immunized recipients for 2 days, a statistically significant ($P \leq 0.01$) delay denoted by the asterisks. There were five recipients per group.
asitemia for recipients of the day 28 serum as compared to that in controls. The failure of passively transferred day 255 serum (Fig. 3) and normal serum (data not shown) to cause a delay in the onset of infection was evidence of their lack of the protective factor. It was concluded, therefore, that the humoral anti- \textit{P. yoelii} factor generated in response to the avirulent infection fell below detectable levels by day 255 after infection.

To determine whether the host response to the primary avirulent infection caused the elimination of viable parasites, mice were splenectomized on days 28 and 255 after virulent infection. Blood smears were prepared from the splenectomized mice, and the removed spleens were disrupted and inoculated into normal animals in an attempt to identify persisting parasites.

Evidence was obtained (Table 1) which suggested that one of five spleens removed at day 28 contained \textit{P. yoelii}, not a surprising result in that some mice may show low-level (Fig. 1) patent infection at this time. In contrast, no evidence of live parasites was obtained at day 255. It appears, therefore, that the ICR mouse possesses a capacity to generate an immunological defense which in turn is capable of eliminating this avirulent strain of \textit{P. yoelii}.

It is established that immunity to \textit{P. berghei} generated in response to inoculations of Formalin-killed (19) or irradiated (31) blood parasites can be overridden if a sufficient number of PRBC are inoculated. Evidently a threshold is reached above which the growth of the parasite exceeds the capacities of the host defenses even in the immunized animal. Figure 4 shows that late immunity to \textit{P. yoelii} measured here at day 416 after avirulent infection can be overridden by the inoculation of \(10^9\) PRBC. Significantly, it was found (Fig. 4) that infections developed at the same rate in all normal mice and in all mice which had recovered from a previous infection. The coincidence of the growth curves of the parasites in previously infected and normal control mice for each of the doses supports the view that the recovered mice did not possess effective levels of preexisting (at day 416) humoral anti-\textit{P. yoelii} activity. It can also be seen that mice challenged with \(10^7\) PRBC began to clear parasites on day 4 after challenge, whereas those challenged with the smaller \(10^5\) PRBC dose took 2 days longer, until day 6, to begin this process. This inverse relationship suggests that an insuf-

<table>
<thead>
<tr>
<th>TABLE 1. Evidence that long-term resistance to \textit{P. yoelii} persists in the absence of viable parasites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenectomized mice</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Interval to spleenectomy (days)</td>
</tr>
<tr>
<td>28</td>
</tr>
<tr>
<td>255</td>
</tr>
</tbody>
</table>

<sup>a</sup> Day after infection with avirulent \textit{P. yoelii}.

<sup>b</sup> Thin blood smears were prepared immediately before and at 4-day intervals through day 20 after splenectomy.

<sup>c</sup> Spleens were disrupted and inoculated intraperitoneally into normal mice at a ratio of five recipients per spleen. Twenty-eight days thereafter, recipient mice were challenged with \(10^7\) PRBC of virulent \textit{P. yoelii}, a uniformly lethal challenge for nonimmune mice. Survival was interpreted as indicating that an avirulent immunizing infection was caused by the inoculation of the spleen homogenate.

<sup>d</sup> One mouse died at splenectomy.

![Fig. 4. Demonstration that immunity to \textit{P. yoelii} generated in response to avirulent infection can be overridden by increasing the number of PRBC employed for challenge. Five mice recovered from avirulent \textit{P. yoelii} infection and five normal age-matched mice were challenged with the indicated number of PRBC on day 416 after the initiation of the avirulent infection.](image-url)
sufficient number of parasites were delivered with the 10^6 inoculum to cause the immediate triggering of a maximum rate anamnestic response.

Response to heterologous \textit{P. berghei} challenge. It is established (14, 26, 27) that infection of mice with nonlethal \textit{P. yoelii} causes a change in the immunological status of the host which at times allows an animal which has recovered from \textit{P. yoelii} infection to successfully resist otherwise lethal challenge with another species of \textit{Plasmodium}, \textit{P. berghei}. To determine whether this heterologous immunity possessed characteristics similar to those of homologous immunity, mice which had recovered from avirulent \textit{P. yoelii} infection and normal age-matched controls were challenged at intervals with 10^6 PRBC of \textit{P. berghei}.

Figure 5 shows that \textit{P. berghei} infections developed at similar rates in most \textit{P. yoelii}-immune and normal mice and that the interval from \textit{P. yoelii} infection to \textit{P. berghei} challenge did not influence the course of \textit{P. berghei} infections in the majority of mice. Only one previously \textit{P. yoelii}-infected mouse survived subsequent virulent \textit{P. berghei} challenge. This mouse developed patent infection at the same rate as lethally infected mice, but unlike those destined to die, arrested the progress of infection by day 9 and cleared overt infection by day 12.

Notably, the duration of survival after \textit{P. berghei} challenge (Fig. 5) was markedly different for normal mice and for mice infected 255 or 396 days previously with \textit{P. yoelii}. At each interval, the median survival after \textit{P. berghei} infection of the control mice was 18 days, whereas the median survival was 8 days or 10 days after \textit{P. berghei} challenge for mice infected 255 or 396 days previously with \textit{P. yoelii}. It appears, therefore, than an encounter at a distant interval with \textit{P. yoelii} leaves some mice at a disadvantage with respect to the interval over which they can survive \textit{P. berghei} infection.

**DISCUSSION**

In human populations, infection-acquired resistance of high quality to malaria is manifested only after repeated infections (2, 3, 11, 15–17). Furthermore, if the contact between an individual with such infection-acquired immunity and the parasite is broken for an extended period of time and natural elimination of harbored parasites occurs, the quality of resistance to further homologous infection lessens (12, 16). These difficulties in generating and maintaining immunity to plasmodial infections under natural conditions have, in the past, discouraged attempts to develop immunoprophylactic procedures. This view has grown less popular with increasing reports of the successful generation of immunity to malaria in laboratory models. However, recent observations on immunity to blood stage plasmodial infections as generated by the mouse in response to injections of killed blood-inhabiting parasites (18, 19, 21, 22) suggest that the fundamental immunological nature of sterile immunity to blood stage malaria may be such as to provide the vaccinated individual with a level of resistance superior to that of an immunologically naive host but nevertheless a level of immunity which does not afford a capacity to prevent the development of parasitemia.

For example, it is established that mice can develop homologous immunity to \textit{P. berghei} (4, 5, 18, 19, 21), \textit{P. yoelii} (22), and \textit{P. vinckeii} (22) in response to injections of nonliving antigens. Mice immunized in this manner, when chal-
lenged, are unable to immediately restrict the growth of the virulent organisms but develop a much more rapid immune response than the nonvaccinated controls. Thus, after the introduction of live parasites into previously vaccinated mice, infections progress, at times to substantial levels, before being cleared.

Mice that have recently recovered from plasmodial infection (18, 20, 21) or mice that were recently vaccinated with irradiated but metabolically active blood parasites (30) have the capacity to prevent or suppress parasitemia when challenged with virulent homologous plasmodiae. Furthermore, the serum of such animals passively transferred to naive recipients results in significant protection (18, 20, 23). In contrast, animals immunized with killed whole organisms are unable to prevent or suppress parasitemia when challenged any time after immunization, nor can their serum transfer protection (18). But, as mentioned previously, such animals have a brisk immune response when challenged with virulent homologous organisms (18). It would seem, therefore, that immunoprophylaxis should be aimed at stimulating antiplasmodial humoral factors.

However, in a recent (18) study of immunity to P. berghei in mice, evidence was presented that levels of humoral anti-P. berghei activity wane rapidly after recovery from infection. This demonstration that the intense immunological stimulation of infection failed to cause the protracted maintenance of high levels of humoral protective factor brings into question the feasibility of inducing such a sustained state through less severe manipulations. On the other hand, this observation might be interpreted to suggest that the quality of infection-acquired immunity declines rapidly to a state of enhanced responsiveness which persists in the absence of circulating humoral antiplasmodial activity, a state of immunity which might well be similar to that raised directly by injections of killed antigens (21, 22).

The present study addresses these possibilities by determining whether immunity to P. yoelii changes with an increasing interval from immunizing infection. It shows that infection with avirulent P. yoelii causes the generation of a state of immunity to virulent P. yoelii of at least 416 days duration, but shows also that the nature of this immunity changes as the interval from immunizing infection increases. Thus, virulent parasites delivered at day 28 after immunizing infection were rapidly neutralized and failed to cause patent parasitemia, whereas challenges delivered on days 255, 396, and 416 caused transient patent parasitemia before being elminated. These results, together with the demonstration of a passively transferrable protective humoral factor on day 28 but not on day 255, support the view that the capacity to restrict ab initio the growth of a challenge inoculum of parasites is directly linked to the presence of the protective humoral factor. The results of challenges delivered after day 28 demonstrate that infection-acquired immunity to lethal infection may persist for extended intervals in the absence of demonstrable humoral protective activity.

The long persistence of homologous immunity observed in these studies agrees with previous demonstration of a protacted state of immunity to P. yoelii engendered by avirulent infection as described by Barker (1). The results of both studies support the view that long-term immunity to P. yoelii persists in the absence of residual viable parasites, e.g., as a state of immunological memory as opposed to premonition (24, 25).

The pattern of changing immunity observed in these studies is not unique to the P. yoelii-ICR mouse experimental model. Recently Eling (7) presented evidence of a similar change with time in the nature of immunity to P. berghei acquired through drug-attenuated infection. Maier and Coggshall (13) 35 years ago argued for a similar pattern of acquired resistance to P. knowlesi infection of the rhesus monkey, identifying states of immunity which they characterized as (i) complete immunity, (ii) partial immunity, and (iii) no immunity. The similarities in the patterns of acquired resistance to these different species of parasite in diverse species of host give credence to the view that acquired immunity to blood-stage malaria exists as two interrelated states: (i) active immunity and (ii) immunological memory. Active immunity in rodent models appears to be dependent on the presence or recent presence of live parasites and decays precipitously (perhaps within weeks) after the sterilization of the parasites. Immunological memory, which appears to persist in the absence of live parasites, decays only slowly, with an apparent half-life measured in months or years. The former state appears to lead to the latter, from which it can be regenerated anamnestically following a subsequent encounter with live parasites.

This study fails to detect a significant level of heterologous protection to P. berghei in mice recovered from P. yoelii. This result stands opposite to numerous demonstrations (9, 14, 26, 27) of such cross protection and indeed is opposite to observations made in this laboratory where up to 90% of mice recovered from P. yoelii were found to resist a challenge with 10^2 PRBC.
of P. berghei delivered on day 28 after P. yoelii infection (unpublished data). The possibility that the larger P. berghei challenge (10^6 PRBC) employed for the present study caused the reduction in the level of heterologous immunity must be considered, a possibility which is supported by the demonstration (Fig. 4) that even homologous immunity can be overridden if a large enough dose of PRBC is employed for challenge.

ACKNOWLEDGMENTS

This work was supported by the Pangborn Research Fund; by Public Health Service grants AI-07015 from the National Institute of Allergy and Infectious Diseases and 5507 RR 05705 from the Division of Research Resources; and by the Department of Microbiology of the University of Maryland School of Medicine. I thank Pamela S. Logie and George Waterson for excellent technical assistance and Cecilia Queen for typing the manuscript.

LITERATURE CITED