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Genetic Control of Resistance to Murine Malaria

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Strain variation in the level of resistance to malaria was investigated in inbred mice after infection with Plasmodium chabaudi. Following intraperitoneal infection with the typing dose of parasitized erythrocytes, mice of 11 inbred strains could be separated using survival time as the criterium into resistant and susceptible groups. Genetic analysis of F_1 hybrid and backcross progeny derived from one of the most resistant (B10.A) and from the most susceptible (A/J) strains as parents suggested that host resistance in this strain combination was genetically controlled by a dominant, non-H-2-linked, autosomal gene or closely linked genes. Analysis of the mechanisms of resistance to P chabaudi showed (1) phenotypic expression of the resistance gene was apparent within 6 days of infection as a significant difference between resistant and susceptible mice in the level of parasitemia; (2) the level of host NK cell activity was *not* related to the level of host resistance to malaria; (3) compared with susceptible A/J mice, resistant B10.A hosts had an augmented erythropoietic response during the course of malaria as well as during phenylhydrazine-induced anemia and (4) treatment with BCG or P acnes resulted in an equal degree of protection, measured by parasitemia and survival, in both resistant and susceptible mice.

Key words: malaria, inbred mice, genetic control of resistance

It has been well established among human populations that certain genetically determined traits impart to the host a high level of resistance against infection with malaria. These traits, which include absence of the Duffy blood group antigen, sickle cell anemia, thalassemia, and glucose-6-phosphate dehydrogenase deficiency, are phenotypically expressed as biochemical abnormalities in host erythrocytes [1].

Variations in the outcome of malarial infection are also apparent among inbred strains of mice [2–6]. As in the human system, these differences in susceptibility have been shown to be genetically determined in experimental models following infection with either Plasmodium berghei or P chabaudi. In the murine models, however, the mechanism(s) of resistance is unknown. It has been shown recently by our laboratory that the phenotypic expression of the superior resistance to infection with P chabaudi

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that is controlled by a non-H-2-linked, dominant, autosomal gene or closely linked genes is apparent early in the course of the infection and results in survival of the resistant host [6]. In the present study, we have evaluated several parameters of host response to malarial parasites in resistant and susceptible mice to determine the mechanism of genetically determined resistance.

MATERIALS AND METHODS

Mice

Age- and sex-matched mice 8–12 wk old were used in all experiments. B10.A, A/J(A), F_1 hybrid (B10.A × A, A × DBA/2J, and A × BALB/c), F_2, and backcross mice were bred in our laboratory. The following inbred strains were purchased from the Jackson Laboratory, Bar Harbor, Maine: C3H/HeJ, SJL/J, AKR/J, DBA/1J, CBA/J, C57BL/6J, C57L/J, and DBA/2J. BALB/c mice were purchased from Canadian Breeders, St Constant, Quebec, Canada. Beige mice were obtained from Dr John Roder, Queens University, Kingston, Ontario.

Parasite

P chabaudi was a kind gift from Dr Pierre Viens, Université de Montréal, Montreal, Quebec, Canada. This parasite was maintained by weekly passage in (B10.A × A)F_1 male mice. After 12 passages, the P chabaudi preparation was discarded, and a fresh inoculum was prepared from frozen stock cultures stored at −40°C until it was used. For passage or infection of experimental mice, heparinized blood was collected from groups of infected F_1 animals and pooled. Total erythrocyte counts and parasitemia (percentage of 200 Wright-stained erythrocytes infected) were determined for duplicate samples. Erythrocytes diluted in sterile phosphate-buffered saline were adjusted to the desired concentration of parasitized erythrocytes and injected intraperitoneally (IP) into passage or experimental mice. For passage, infection was initiated with a dose of 10^6 to 10^7 parasitized erythrocytes (P-RBC).

Determination of Parasitemia

The course of experimental infections was monitored every third day by examining Wright-stained thin blood smears. Parasitemias of individual mice were determined by counting a minimum of 200 erythrocytes per blood sample. Parasitemia is expressed as the mean percentage of erythrocytes infected from groups of 3 to 10 mice.

Determination of Erythrocyte Characteristics

The number of erythrocytes, hematocrit, and amount of hemoglobin were determined by standard procedures [7] for individual samples of blood from normal or infected mice. Erythrocyte characteristics are presented as the mean values for blood samples from groups of four to eight mice. Reticulocyte counts were determined by counting a minimum of 500 erythrocytes on blood smears stained with new methylene blue (A.J.P. Scientific, Clifton, NJ). Reticulocytosis is expressed as the mean percentage of reticulocytes in blood samples from groups of four to six normal or treated mice. Anemia was induced chemically in mice by IP injection of 3.2 mg of phenylhydrazine hydrochloride (Fisher Scientific, Montreal, Canada) diluted in phosphate-buffered saline and adjusted to pH 7.0. The 50% lethal dose was calculated by

TABLE I. Strain Survey of Resistance to P chabaudi*

Mouse strain	Level of resistance[a]	Mean survival time (days)[b]
C57BL/6	Resistant	>14
C57L	Resistant	>14
DBA/2	Resistant	>14
CBA	Resistant	>14
B10.A	Resistant	>14
A/J	Susceptible	8.3 ± 0.3
BALB/c	Susceptible	8.6 ± 0.5
C3H/HeJ	Susceptible	9.7 ± 0.6
AKR	Susceptible	9.8 ± 0.5
SJL	Susceptible	9.5 ± 0.8
DBA/1	Susceptible	9.2 ± 0.6
(B10.A × A)F_1	Resistant	>14
(A × DBA/2)F_1	Resistant	>14
(A × BALB/c)F_1	Susceptible	8.3 ± 0.3

*Groups of ten mice in two experiments were injected IP with 10^6 erythrocytes parasitized with P chabaudi.

[a]Susceptibility was defined as a mouse strain in which <50% of animals survived the typing dose at day 10 of infection.

[b]Infected mice died at times indicated or survived for more than 14 days. Results are expressed as means ± standard error of the mean.

the method of Reed and Muench [8] and was determined after injection of doses of phenylhydrazine-hydrochloride ranging from 0.8% to 8.0 mg into groups of four mice. Mice were observed daily for 10 days, and mortality was recorded.

BCG and P acnes

Mice were injected intravenously (IV) with 5×10^6 colony-forming units (cfu) of living Mycobacterium bovis, strain BCG (TMC 1029, Phipps substrain, Trudeau Mycobacterial Collection) available from the National Jewish Hospital and Research Center (Denver, CO) or with killed Propionibacterium acnes (50 mg/kg, Wellcome Research Laboratory, Beckenham, England). Seven days later, groups of BCG-treated, P acnes-treated, or normal, control mice were infected IP with 1×10^6 erythrocytes parasitized with P chabaudi.

Results

We recently examined the level of resistance in mice to infection with the murine malarial species P chabaudi [6]. After intraperitoneal infection with a typing dose of 10^6 P-RBC, 11 strains could be characterized as resistant or susceptible. A given mouse strain was considered susceptible if less than 50% survived 10 days after infection with this dose (Table I). Strains C57BL/6, C57L, DBA/2, CBA, and B10.A mice were characterized as resistant and continued to survive beyond day 14. Infection with P chabaudi, in contrast, was lethal to 100% of strain A mice and > 50% of BALB/c, C3H/HeJ, AKR, SJL, and DBA/1 mice with a mean survival time of less

than 10 days. These results suggested to us that the level of resistance to infection with P chabaudi was dependent upon the genetic background of the host. The H-2 complex did not appear to be important in determining the level of resistance, since strain A mice (H-2^a) were highly susceptible, whereas strain B10.A mice which share the *a*- haplotype were among the most resistant.

Next we studied the level of resistance of F_1 hybrids to determine if resistance to malaria is inherited as a dominant trait. Resistance was examined in two hybrid combinations resulting from crosses between susceptible strain A mice and resistant B10.A or DBA/2 mice and an F_1 hybrid derived from two susceptible parents (Table I). In the former crosses, 100% of the (B10.A × A) F_1 or (A × DBA/2) F_1 mice were resistant to the typing dose of 10^6 parasitized erythrocytes and survived beyond day 14. The level of resistance of (A × BALB/c) F_1 mice was low with a mean survival time (8.3 $\pm$ 0.3 days), which was similar to the times of the susceptible parents. Thus, the level of resistance to infection with P chabaudi appears to be inherited as a dominant trait.

Using 10^6 P-RBC as a standard typing dose, susceptible animals succumbed within 10 days of infection, while resistant hosts survived indefinitely. However, when susceptible A mice were infected with a range of doses of parasitized erythrocytes (10^4 - 10^9), it was apparent that the infective dose correlated with the time until death. That is, the length of the mean survival time of susceptible A mice decreased from 13 days after infection with 10^4 parasitized cells to less than 6 days after infection with 10^9 P-RBC. Furthermore, the infection was lethal to 100% of A mice at all doses. Except at a dose of 10^9 P-RBC, 100% of resistant B10.A mice survived. Thus, the 50% lethal dose for susceptible A hosts is apparently $< 10^4$, while for resistant B10.A hosts, it is $> 10^9$ P chabaudi-parasitized erythrocytes.

To further analyze genetic control of resistance to malaria, segregation analysis using backcross and F_2 animals derived from highly susceptible A and very resistant B10.A parental mice was performed using a typing dose of approximately 10^6–10^7 erythrocytes parasitized with P chabaudi. During this investigation, it became apparent that the sex of the host influenced the outcome of the infection. This influence is apparent in Figure 1, where we have presented the results of genetic analysis on separate groups of more than 500 male and female mice. For example, 100% of female B10.A and F_1 mice survived the infection, whereas only 83% of male B10.A and 86% of male F_1 animals survived. Using the criterium of survival at day 14 as a measure of resistance, the following results were obtained: in groups of male mice (expressed as number of animals surviving/total number), 94% (32/35) of F_1 × B10.A, 46% (29/62) of F_1 × A, and 70% (43/57) of F_2 animals were resistant. In groups of female mice, 100% (10/10) of F_1 × B10.A, 75% (24/32) of F_1 × A, and 87% (36/41) of F_2 animals were resistant. These results are consistent with the hypothesis that the major determinant of resistance to infection with P chabaudi in B10.A mice is a single, dominant gene (or group of closely linked genes) that is not expressed in A mice but whose expression is influenced by the sex of the host.

To determine the mechanism of resistance to infection with P chabaudi, we analyzed several aspects of host defense apparent early (during the first 2 wk after infection), in the course of infection, specifically, control of parasitemia levels, NK cell activity, and the erythropoietic response in genetically resistant and susceptible hosts. This approach was chosen because death of susceptible mice occurred by 10

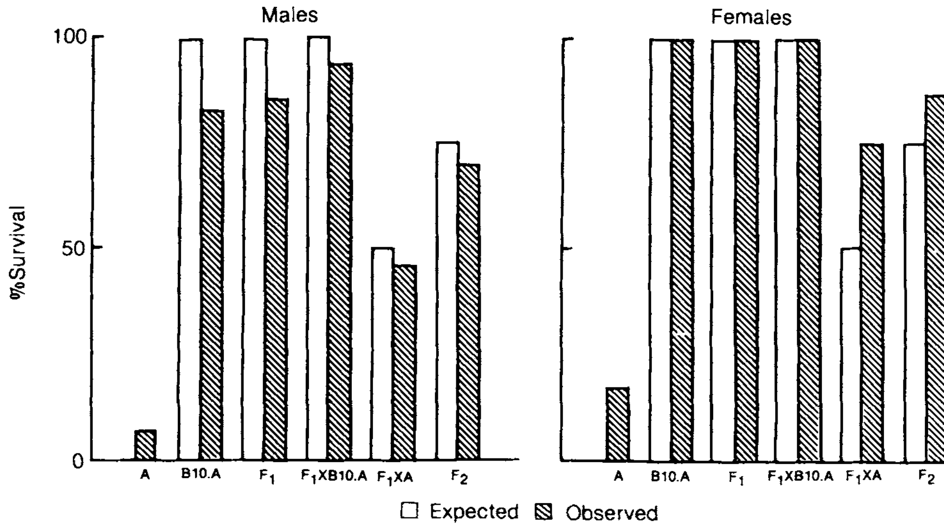

Fig. 1. Segregation analysis of resistance to P chabaudi in male and female mice. Groups of 10 to 100 mice of strains A and B10.A and their F_1, F_2, and backcross progeny were injected IP with 10^6 to 10^7 P-RBC. The outcome of infection (either death or recovery) was followed in individual animals (expected or observed percentage of survival for genetic control by a single, dominant gene[s]).

days, suggesting that the mechanism of genetically determined resistance is phenotypically expressed in the early stages of infection before the development of specific immunity.

First, we measured the parasitemia levels in resistant and susceptible mice after infection with a low dose (10^6 cells) and a high dose (10^8 cells) of P-RBC (Fig. 2). A significant difference was evident between resistant B10.A and susceptible A mice at days 6 and 10 after infection with 10^6 P-RBC. The peak level of parasitemia in A mice was approximately 50% after infection with either dose. When this critical level of parasitemia was reached, the infection terminated in death of 100% of susceptible A hosts. In contrast, the peak parasitemia level in resistant B10.A mice was dependent upon the infective dose. At the low dose, the parasitemia level peaked on day 10 at approximately 35% in B10.A mice. At the high dose, parasitemia approached the 50% level characteristic of susceptible A mice. Despite such a high parasite load, B10.A mice survived the infection, and the level of parasitemia was decreasing by 14 days after infection. These results observed with B10.A mice were confirmed when DBA/2 mice were used as the resistant strain.

Since a low level of NK cell activity has recently been shown to be associated with susceptibility of both humans and rodents to malaria [4, 9–11], we also examined the relationship between these two genetically determined host responses in two separate experimental models of NK-deficient animals [12]. First, the level of resistance to infection with P chabaudi of NK-deficient bg/bg mice on the C57BL/6 background was found to be identical with that of normal bg/+ littermates (Table II). In addition, examination of the two traits in backcross [F_1 (B10.A × A) × A]

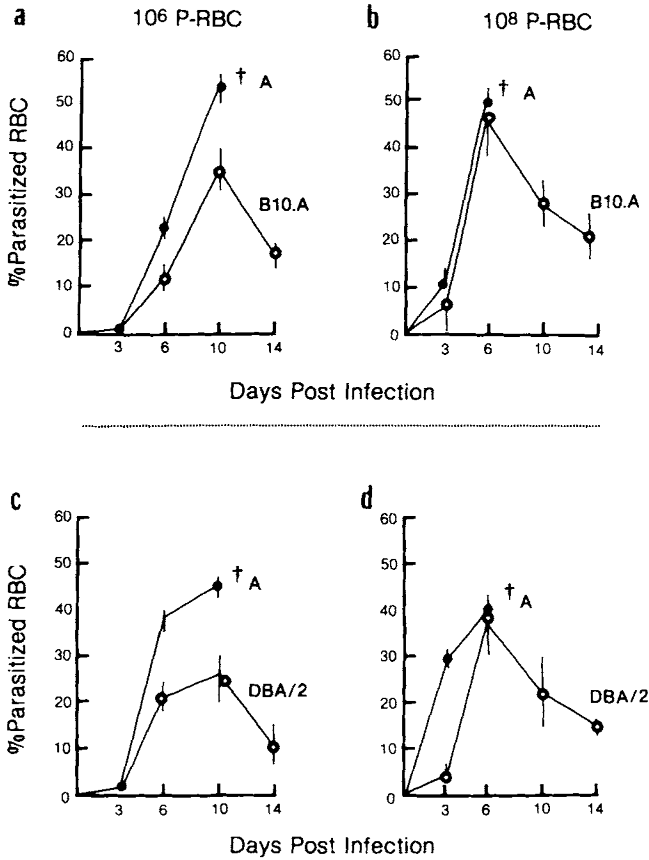

Fig. 2 Levels of early parasitemia in resistant and susceptible mouse strains. Groups of five male mice of strain A (●) or B10.A (○[a and b]) or DBA/2J (○[c and d]) were infected IP with 10^6 (a, c) or 10^8 (b, d) P-RBC. At different times after infection, the percentages of P-RBC were determined as described. The data are presented as mean ± standard error of the mean. The daggers represent death. Similar results were obtained with female mice.

progeny derived from B10.A (malaria-resistant, high NK cell activity) and A (malaria-susceptible, low NK cell activity) parental mice showed that the levels of NK cell activity and resistance to malaria segregated independently (Fig. 3).

The response of host erythropoietic system to the stress of malaria-induced anemia may also be related to superior resistance to infection with P chabaudi. There was no difference between normal, uninfected A and B10.A mice in several erythrocyte characteristics, including numbers of circulating cells, percent hematocrit, and hemoglobin volume [6,13]. However, there were significant differences between the two strains following infection with the typing dose of P chabaudi (Table III). In general, there was an inverse relationship between the level of parasitemia and the erythrocyte traits: a high level of parasitemia was associated with a low hematocrit and low numbers of erythrocytes. A comparison of the absolute numbers of infected

TABLE II. Resistance to Malaria of Beige Mice

	Strain			
	A/J	C57BL/6	Bg/Bg	Bg/+
Resistance to P chabaudi[a]	0/10	9/10	10/10	9/10
NK cell activity[b,c]	4.4 ± .64	19.94 ± .98	4.7 ± .3	22.0 ± 5.2

[a]Number of survivors/total number of mice infected with 5×10^6 P-RBC.
[b]E:T = Effector:Target Cell Ratio 100:1, % lysis ± SEM.
[c]NK cell activity of normal, uninfected mice (5–10 mice per group).

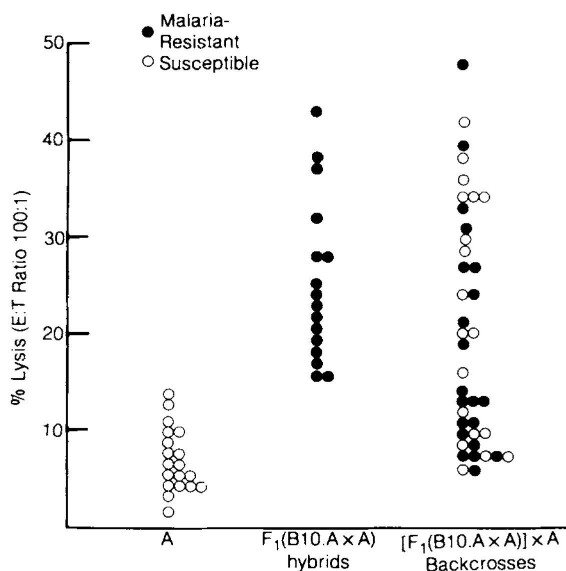

Fig. 3. Comparison of NK cell activity (% lysis) and malaria resistance in individual animals of $[F_1(B10.A \times A)] \times A$ backcross progeny. Mice were hemisplenectomized, and the level of NK cell activity in spleen fragments was determined as previously described [12]. Animals were allowed to recover from surgery for 10–14 days and were infected IP with 5×10^6 P-RBC in three separate experiments. Susceptible animals succumbed to infection within 9–11 days after inoculation.

erythrocytes in resistant B10.A and susceptible A mice showed that, although on a cell-per-cell basis there were more circulating erythrocytes in infected B10.A hosts, there were approximately 3×10^6 parasitized cells in either mouse strain.

Differences in responsiveness to anemia of the erythropoietic systems of malaria-resistant B10.A and susceptible A mice were further characterized by comparing the course of reticulocytosis following injection of the anemia-inducing chemical, phenylhydrazine. As shown in Table III, reticulocyte production in phenylhydrazine-treated B10.A mice was greater than in similarly treated A mice. Although the response peaked on day 6 in both strains, there were almost three times as many reticulocytes in the blood of B10.A hosts. In addition, there was a significant

TABLE III. Erythropoietic Responses of A and B10.A Mice to Anemia

Mouse strain[a]	Malaria-induced anemia %P-RBC	Erythrocytes (10^6 cells/mm^3)	Hematocrit (%)	No. of P-RBC (10^6 cells/mm^3)	Phenylhydrazine-induced anemia LD_{50}[b]	% Reticulo-cytes
A	56.6 ± 2.1*	4.6 ± 0.03*	20.7 ± 7.2**	2.61 ± 0.02***	4.0 mg	18.1 ± 4.5*
B10.A	35.8 ± 2.7*	9.3 ± 0.27*	43.7 ± 1.7**	3.33 ± 0.24***	5.0 mg	46 ± 0.8*

[a]Groups of 5–8 male mice injected IP 7 days previously with 10^6 P-RBC or 6 days previously with 3.2 mg phenylhydrazine. Values are means ± SEM.
[b]Determined by method of Reed and Muench [8].
*Significant ($P < 0.005$).
**Significant ($P < 0.01$).
***Not significant.

difference between the strains in the dose of phenylhydrazine that killed 50% of the animals.

In spite of genetically determined differences in the level of resistance early (within 10 days) in the course of infection with P chabaudi, BCG or P acnes pretreatment resulted in nonspecific immunity against malaria, in both genetically resistant and susceptible hosts (Fig. 4).

The increased protection was evident by a 50% or greater decrease in parasitemia levels in both B10.A and A mice. Moreover, more than 90% of treated A mice survived the usually 100% lethal infection with 10^6 P-RBC.

DISCUSSION

During malaria, host response leading to decrease of the peak parasitemia level apparent at the crisis stage of infection has been shown to be dependent upon the development of specific immune responses. However, the existence of certain genetic traits among human populations that inherently protect an individual against infection is well known. These traits, for example, sickle-cell anemia, are phenotypically expressed as biochemical abnormalities in host erythrocytes and are unrelated to immune mechanisms. In spite of being deleterious, however, the genes have survived with high frequency in malarious areas of the world because of their protective effects [1].

We have described another example of innate resistance against malaria, that is, the high level of natural resistance apparent among certain inbred strains of mice following infection with P chabaudi. While more than 50% of the animals of susceptible strains (A, SJL, C3H/HeJ) succumbed within 10 days of infection with 10^6 parasitized erythrocytes, resistant strains, such as CBA mice or mice with a C57 background (C57BL/6, C57L or B10.A), survived beyond 14 days. Segregation analysis of the level of resistance of hybrid, backcross, and F_2 progeny derived from the parental combination of one of the most resistant strains, B10.A and highly susceptible A mice showed that resistance to infection with P chabaudi is genetically controlled by a dominant, autosomal gene or closely linked genes [6]. The trait of resistance to this malarial species is not H-2 linked, because A mice that are $H\text{-}2^a$

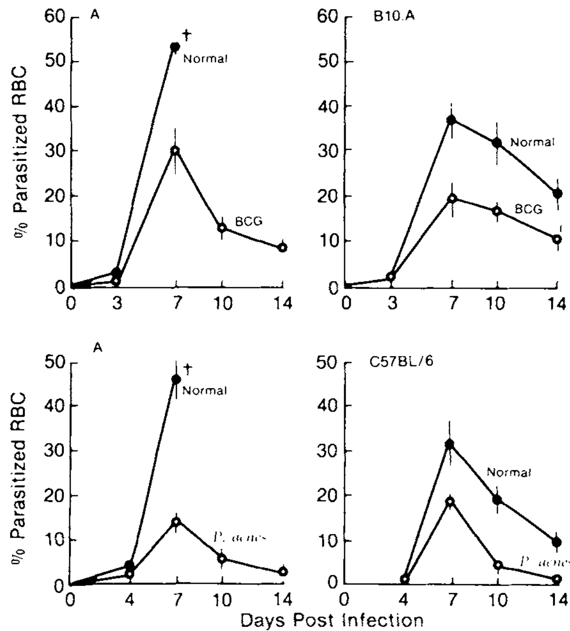

Fig. 4. Level of early parasitemia in BCG or P acnes-treated mice. Groups of five mice were treated with BCG or P acnes as described. Normal and treated animals were infected 7 days later with 10^6 P-RBC. At different times after infection, the percentages of P-RBC were determined. The daggers represent death. The data are presented as mean ± standard error of the mean.

were very susceptible, while B10.A mice, also H-2^a, were very resistant. Differences in the level of resistance between male and female parental and progeny mice suggested that the expression of the trait is influenced by the sex of the host [6]. In addition to the major gene regulating differences in resistance between A and B10.A mice, other genes, probably of lesser influence, contribute to the overall level of host resistance to P chabaudi. The existence of other genes is suggested by several lines of evidence: (1) Mice of several strains classified as susceptible by our criteria actually exhibited a spectrum of mean survival times [6]; and (2) a spectrum of levels of resistance rather than a clear-cut distinction into a resistant and a susceptible group was apparent when resistance to P chabaudi was analyzed in AXB/BXA recombinant inbred mice [Stevenson and Skamene, unpublished observations]. However, since A mice seem to possess no resistance alleles at the other putative loci (that is, A strain is 100% susceptible), analysis of the extreme ends of the spectrum (strains A and B10.A or C57BL/6) should be advantageous for determining a single, major mechanism of resistance without the interference of the other minor gene products.

The phenotypic expression of superior resistance was apparent early in the course of infection. Death of susceptible mice, such as A strain mice, occurred by 10 days after infection with the typing dose of 10^6 P-RBC. Therefore, we examined several early defense mechanisms in resistant and susceptible hosts in order to determine the mechanism of natural resistance to infection with P chabaudi.

First, there was a significant difference at day 6 following infection with a low dose (10^6 P-RBC) between genetically resistant and susceptible mice in the magnitude of parasitemia levels. It is during this early patent period that the number of parasitized erythrocytes is increasing as a result of parasite multiplication. The early rise occurred in both genetically resistant and susceptible mice. Grun and Weidanz [14] have shown that there is no difference in the early rise or parasitemia level between P chabaudi adami-infected immunologically intact mice and B-cell-deficient or athymic nude mice. In our model, there was a difference in parasitemia levels between genetically resistant and susceptible hosts infected with 10^6 P-RBC even though the early parasitemia curves were superimposable. These results support our hypothesis that an innate resistance mechanism apparent before the development of specific immunity is responsible for the early differences in parasitemia levels and leads to superior resistance of some strains of mice, such as B10.A.

The response of the erythropoietic system to the stress of both malaria-induced and phenylhydrazine-induced anemia was also found to be superior in malaria-resistant B10.A hosts. It has been observed that there is diminished erythrocyte production due to depression of erythropoiesis during malaria [1]. Such a depression in susceptible A mice, which are inferior to resistant B10.A mice in the quantity of erythrocytes produced in response to the reticulocytosis-stimulating agent phenylhydrazine, could result in an almost total diminution of erythropoiesis. Thus, unable to replace destroyed erythrocytes, A mice may succumb to malaria because of severe and lethal anemia.

Since the NK cell has been implicated as an effector cell against Plasmodia [4,9–11], we also examined the relationship between the level of NK cell activity and the level of resistance to malaria. Determination of the levels of the two traits in backcross F_1XA progeny derived from B10.A (malaria-resistant, high NK cell activity) and A (malaria-sensitive, low NK cell activity) parental mice and in NK-deficient beige mice on the resistant C57BL background showed that the level of NK cell activity did not correlate with level of host resistance to malaria [12]. The same conclusion was reached by Wood and Clark [15] in their studies using ^{89}strontium-treated mice and beige mice infected with another murine malaria species, P vinckei petteri.

Recent evidence from several laboratories strongly implicates the macrophage as the Plasmodia-effector cell. A factor indistinguishable from tumor-necrotizing factor [16–19] and products of oxygen metabolism, hydrogen peroxide [20], and reactive oxygen metabolites [21], which are produced by macrophages, have been found to be cytotoxic in vivo and in vitro to human and murine species of the parasite. Moreover, macrophages have been shown to be cytotoxic to P yoelii in vitro [22]. Differences between mouse strains in the level of genetically determined resistance to P chabaudi could be due to differences in macrophage response early in the infection. P chabaudi-susceptible A mice are susceptible to infection with a variety of pathogens, for example, Listeria monocytogenes [23] and Rickettsia akari [24]. Susceptibility of A mice in these examples is due to one of several defects in mononuclear phagocytes, including production [25], mobilization [26], and activation [24].

In addition to defects in macrophage inflammatory responses [26], in macrophage activation for rickettsicidal [24] and tumoricidal [27] activities, and in hemolysis-stimulated erythropoiesis [6], strain A mice are known to be deficient in a variety of other traits including polymorphonuclear inflammatory responses [28],

production of eosinophils [29], complement activation [30], interferon induction [31], as well as synthesis of C-reactive protein and other acute-phase reactants [32]. Any of these traits may be considered candidate mechanisms of innate resistance to malaria.

It is of interest to point out that recovery from infection with P chabaudi adami, unlike recovery from other species, such as P yoelii, appears to be due to an antibody-independent mechanism of immunity [14,33]. Additionally, P chabaudi is more susceptible than P berghei or P yoelii to inhibition by nonspecific immunostimulants, such as BCG [34].

We found that treatment of P chabaudi-susceptible A mice with BCG or P acnes resulted in protection against the infection, as measured by both decreased parasitemia levels and survival. This result suggests that genetically susceptible A mice are capable of developing nonspecific immune effector mechanisms necessary for the phase of parasite elimination and recovery in spite of lacking the resistance mechanism necessary during the early phase of parasite multiplication. It has been hypothesized that the final effector mechanism during P chabaudi infection is the activation of macrophages by T cells, either specifically in response to parasite antigens during infection or nonspecifically by agents such as BGG [14,34,35]. An understanding both of the precise mechanism(s) of genetically determined resistance apparent early in the course of infection with P chabaudi and of the final effector mechanisms should provide new strategies for manipulation of the host-Plasmodia relationship in favour of the host.

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