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Transgenic and mutant animal models to study mechanisms of protection of red cell genetic defects against malaria

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Abstract. Malaria, caused by members of the genus *Plasmodia*, is still the most prevalent parasitic disease in the world. In an attempt to understand genetic factors conferring resistance to malaria, mouse models of thalassemia, sickle trait, and ankyrin and spectrin deficiency were studied during infection with species of malaria infectious to rodents. Although growth of *P. falciparum* is not inhibited in thalassemic erythrocytes in culture, mice carrying a β-thalassemia mutation were protected from *Plasmodium chabaudi adami*, supporting epidemiologic findings. Transgenic mice expressing β^S hemoglobin were also significantly protected from two species of rodent malaria. Importantly, a significant role for the spleen in protection in the β^S transgenic mice was found. Finally, mice deficient in spectrin and ankyrin were studied with respect to their ability to support the growth of malaria. It was found that spectrin deficient mice were almost completely refractory to *P. chabaudi adami* and *P. berghei*. These models will allow further study of host factors in resistance to malaria.

Key words. Malaria; *Plasmodium falciparum*; *P. chabaudi adami*; *P. berghei*; sickle hemoglobin; thalassemia; spectrin; ankyrin.

Malaria, caused by species of protozoan parasites of the genus *Plasmodia*, is responsible for an estimated 2–4 million deaths per year, mostly among children[19]. Progress in eradicating this disease is problematic because of increasing resistance of the parasites to available antimalarial drugs and resistance of the mosquito vector to DDT. Further, progress in development of a vaccine against malaria has been slower than anticipated due to the complexities of designing a recombinant vaccine against a parasite with several antigenically different stages and diverse parasite strains[15].

The life cycle of the malaria parasite is complex. Infections are initiated by the bite of an Anopheline mosquito with injection of the sporozoite stage. Sporozoites quickly circulate to the liver where they develop further in the hepatocytes giving rise to the exoerythrocytic stages. Each exoerythrocytic schizont releases between 10,000 and 30,000 merozoites which begin the infection in the red blood cells. Within the erythrocytes, the parasites undergo development and division, releasing between 8 and 32 new merozoites every 48–72 hours, depending on the species of malaria. It is the periodic rupture of erythrocytes by the parasite that gives rise to the characteristic fevers and chills which are the symptoms of the infection.

Plasmodium falciparum is the most serious of the human malarias and accounts for most of the deaths due to this disease. Merozoites of *P. falciparum* can invade both young and old erythrocytes; therefore, the parasitemia can rise rapidly. A characteristic of falciparum malaria is that the trophozoites and schizonts are not seen in the peripheral blood. This is due to the fact that the later stages of falciparum-infected erythrocytes develop excrescences on the red cell membrane called knobs[41]. The knobs, which contain several parasite-specific proteins, are at least partially responsible for mediating the cytoadherence of the infected erythrocytes to the venular endothelium of the internal organs including the brain[23,34]. This form, known as cerebral malaria[26], can lead to severe sequelae including coma and death.

Innate resistance to malaria involves species specificity of the parasite in relation to both mosquito and vertebrate hosts (reviewed in Miller and Carter[27]). In addition, areas of endemic *Plasmodium falciparum* are strongly associated with the presence in the population of several abnormalities in hemoglobin synthesis and structure which confer partial resistance to this parasite (reviewed in Nagel and Roth[29]). The protection afforded to the heterozygote carriers has caused these traits to be maintained in the genome despite the fact that the homozygous conditions are often deleterious to the host (balanced polymorphism). In addition, certain erythrocyte membrane defects have been shown to affect the ability of the malaria parasite to either invade or develop within the host erythrocytes.

The studies described below have focused on the use of animal models in studying the protection afforded by various hemoglobin and erythrocyte membrane abnormalities against malaria. Although some aspects of resistance have been studied in *Plasmodium falciparum* grown in culture[13,14,30,31,35,36], the use of animal models allows the study of host factors such as the involvement of the immune system and, in particular, the role of the reticuloendothelial system, in the recognition of infected erythrocytes with various abnormalities. This review will describe several aspects of resistance to malaria which have been studied in rodent models.

Rodents are susceptible to several species of *Plasmodia*. These parasites were discovered in Africa and subsequently their mosquito habitat was recreated in the laboratory so that the parasites could be utilized in immunological, chemotherapeutic and other studies[18]. *Plasmodium chabaudi adami* was discovered in the Central African Republic, in the thicket rat, *Thamnomys rutilans*. In laboratory mice, *P. chabaudi adami* preferentially invades mature erythrocytes and causes a self-limiting infection. *Plasmodium berghei*, in contrast, tends to invade reticulocytes and usually causes a lethal infection in laboratory mice. *P. yoelii* is antigenically related to *P. berghei* and invades reticulocytes but causes a non-lethal infection. *P. vinckei* is another species of rodent malaria which invades mature erythrocytes and is highly virulent[20]. In the studies described below, *P. chabaudi adami* and *P. berghei* were used as representative models of lethal and non-lethal infections and as parasites which preferentially invade host erythrocytes of different stages.

Protective effect of the β-thalassemic mutation

The thalassemias are a group of diseases characterized by imbalanced synthesis of either the α or β globin chains of normal adult hemoglobin. The geographic distribution of the various forms of thalassemia in malarious regions supports the concept that carriers of these traits are protected from malaria[12,41]. However, in several studies, culture of *P. falciparum* in thalassemic trait red cells was found to be normal[31,36] except under oxidant stress[14] or when cultured in minimal essential medium[7]. These results suggest that the protection afforded carriers of these defects may be mediated by other host factors such as the mononuclear phagocyte system. It was, therefore, of great interest to study malaria in vivo, in a mouse model of β-thalassemia.

C57BL/6J mice with a homozygous β-thalassemia syndrome[40] as well as transgenic thalassemic mice which carried copies of the human β^A gene[8] were infected with $1.5–2.0 \times 10^3$ or 10^4 *Plasmodium chabaudi adami*, *P. chabaudi*, strain 1309, or *P. berghei*-infected erythrocytes. The course of infection was followed by taking blood smears from the tail vein approximately every other day. The smears were stained with Giemsa and counted by light microscopy under oil immersion.

In thalassemic mice infected with *P. chabaudi adami*, parasites first appeared in the blood 48 hours later than in control mice. In addition, the level of peak parasitemia was reached 48 hours later than in controls (fig. 1)[37]. Importantly, the level of peak parasitemia in the thalassemic mice was approximately 40% of that in the normal and β^A transgenic mice. The normal and transgenic mice were not significantly different from one another. When mice were infected with a more virulent strain of *P. chabaudi* (strain 1309), a similar delay in patency and peak parasitemia were observed but the levels of parasitemia were similar to controls[37].

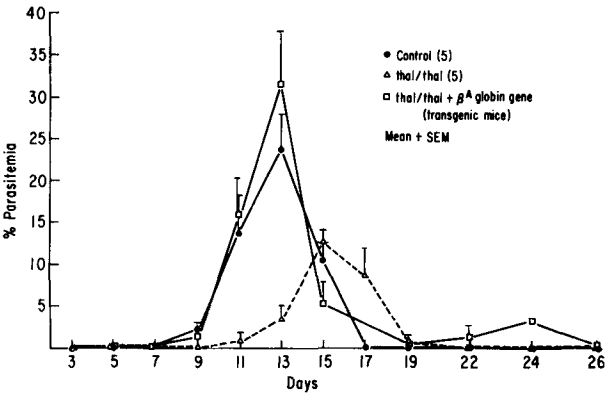

Figure 1. Course of *P. chabaudi adami* infection in C57BL/6J. ●, Normal mice (5); △, β-thalassemic mice (5); □, thalassemic mice with a transgenic human β^A-globin gene (5). Horizontal axis, days; vertical axis, percent parasitized red cells shown as mean + SEM.

These studies indicate that the thalassemic mutation protects mice from one species of rodent malaria, *P. chabaudi adami* and *P. chabaudi*, strain 1309. Correction of the thalassemic condition with the β^A transgene abolished the protective effect, suggesting that the thalassemic condition was indeed responsible for the protection seen.

Interestingly, thalassemic mice were not protected from *Plasmodium berghei* (fig. 2). Indeed, parasitemia was higher in the thalassemic mice than in either normal or β^A transgenic mice. Although the course of parasitemia in the β^A transgenic mice was similar to the control mice, they also had a slightly higher course of parasitemia as compared to the normal mice[37].

The failure of thalassemic mice to be protected from *P. berghei* may be due to the high level of reticulocytes in these mice. The small but significant increase in parasitemia seen in the β^A transgenic mice may, therefore, be due to the slightly elevated reticulocyte count that persists in the transgenic mice. It is unlikely that the protection seen with *P. chabaudi* infection is due to reticulocytosis since the number of mature red cells available for parasite invasion far exceeds the number of infected red blood cells.

The use of in vivo models allows the study of host factors in the protection observed in this model. Recent data have shown that *P. falciparum*-infected α- and β-thalassemic erythrocytes bind greater levels of IgG from endemic sera than do normal infected erythrocytes[25]. Such antibody might mediate enhanced uptake of these cells by the mononuclear phagocyte system. It has been observed that thalassemic erythrocytes are more easily phagocytosed than normal erythrocytes[33] and this effect may be even more marked with malaria-infected red blood cells. Indeed, enhanced phagocytosis of infected erythrocytes carrying several different variants of α chain synthesis has been observed[43]. In our study, however, the spleen had a paradoxical effect on the course of infection in thalassemic mice in that splenectomized thalassemic

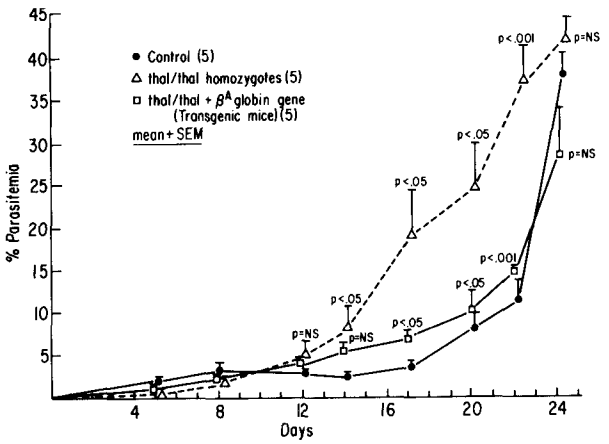

Figure 2. Course of *P. berghei* infection in C57BL/6J mice, ●, Normal mice (5); △, β-thalassemic mice (5); □, β-thalassemic mice with a transgenic human β^A-globin gene (5). P values for thalassemic mice (△) refer to the difference between thalassemic mice and normal mice (●). P values for transgenic mice (□) refer to difference between transgenic mice and thalassemic mice (△). Horizontal axis, days; vertical axis, percent parasitized red cells shown as mean + SEM.

mice had a further delay in the rise of parasitemia[37]. Further study is needed to clarify this effect.

Resistance of transgenic mice expressing human β^S hemoglobin to rodent malaria

Several epidemiologic studies have suggested that persons who are heterozygous carriers of sickle hemoglobin (sickle trait individuals) are protected from falciparum malaria[1,5,11] (and reviewed in Nagel and Fleming[28]). The basis for this protection has been studied extensively in vitro. Early work by Luzzatto[24] demonstrated that the rate of sickling in malaria parasitized cells from an AS individual was 8 times faster than sickling in non-parasitized red blood cells under completely deoxygenated conditions. Later studies, utilizing the in vitro cultivation method for the growth of *P. falciparum*[17], confirmed these results using physiologic venous oxygen tensions[35]. Nevertheless, the precise mechanism of protection in sickle trait individuals in vivo is not known. *P. falciparum*-infected erythrocytes develop knobs on the surface of the infected erythrocyte which mediate binding of these cells to endothelial cells[41,23,34]. One hypothesis is that, under the reduced oxygen tensions of the deep vasculature, and lower intracellular pH which occurs in infected erythrocytes, the hemoglobin sickles lead to the death of the parasites[13]. However, until now, it has not been possible to study these phenomena in vivo.

The recent development of the transgenic mouse model of sickle trait[9] has enabled us to confirm and extend the observation of protection of sickle trait against malaria. More importantly, it has allowed us to investigate the mechanism of this protection in vivo.

In these studies a transgenic mouse line expressing both the human β^S and the human α2 globin genes was used[9]. In order to increase the relative expression of β^S, the transgenic mice, originally produced using the normal inbred strain FVB/N, were bred to homozygosity for a β-thalassemia mutation, $Hbb^{d3(th)40}$ on a C57BL/6J background. The human α gene was used because the mouse α chain inhibits the polymerization of HbS. Although the mice displayed normal hematological features in vivo, their red cells were susceptible to extensive sickling when deoxygenated in vitro. The older animals have evidence of selective organ damage. However, in the transgenic line used in this study, changes in the spleen were minor. Thus, they appeared to represent a model intermediate between sickle cell trait and the disease[9].

β^S transgenic mice as well as the two parental mouse strains, C57BL/6J and FVB/N, were infected with 10^6 *P. chabaudi adami*-infected erythrocytes and the course of infection followed. As shown in table 1, there was a significant delay in appearance and reduced level of parasitemia in the β^S transgenic mice as compared to the controls ($p < 0.05$, days 7–9). In addition, β^S transgenic mice and C57BL/6J mice were infected with 5×10^5 *P. berghei*-infected erythrocytes and the course of infection followed. Again, β^S transgenic mice were significantly protected from this species of malaria, although not to as great a degree as with *P. chabaudi adami* (not shown). Further, sickle polymers were observed in *P. berghei*-infected red cells which had been deoxygenated in vitro[39a]. Importantly, the use of a rodent model of malaria in animals expressing sickle hemoglobin, allowed us to address the mechanism of protection in vivo. We hypothesized that either the parasites cannot grow in red cells expressing sickle hemoglobin or that the host removes the infected RBC early in the erythrocytic cycle, before the parasites have a chance to mature ('suicidal infection'). The first possibility is unlikely since the mice do experience a parasitemia, although diminished compared to controls.

In order to investigate the second possibility, mice were splenectomized prior to infection. Animals were then

Table 1. Course of parasitemia in β^S transgenic, C57BL/6J and FVB/N mice

Day of infection	% of infected RBC[a] β^S	C57BL/6J	FVB/N	
4	0.05 (0.01)[b]	0.3 (0.1)	0.9 (0.4)	
7	1.7 (1.0)	13.5 (1.6)	11.1 (3.6)	$p < 0.01$[c]
9	4.7 (1.8)	18.2 (5.3)	17.3 (6.5)	$p < 0.05$
14	5.5 (3.4)	3.9 (3.7)	2.6 (1.8)	
18	0.1 (0.1)	0.1 (0.2)	0.04 (0.2)	

[a]Percent infected erythrocytes as determined on Giemsa-stained blood smears.
[b]SEM.
[c]Difference between β^S mice and C57BL/6J or FVB/N mice using Student's t-test.

inoculated with 5×10^5 *P. chabaudi adami*-infected erythrocytes and the course of infection followed. Splenectomized C57BL/6J mice had a slightly greater level of parasitemia than non-splenectomized C57BL/6J mice. However, there was a striking difference between splenectomized and intact β^S transgenic mice. Intact β^S mice again had a diminished course of infection whereas in the splenectomized β^S the protective effect of the transgene was completely abolished (table 2). This experiment also demonstrates that the β^S erythrocytes were fully able to support the growth of *P. chabaudi adami* and that the protective effect must be at the level of the host[39a].

These studies confirm and extend previous epidemiological and in vitro studies indicating that the sickle trait phenotype is protective against *P. falciparum* malaria[1,5,11,28]. The spleen plays a significant role in protecting the β^S transgenic mice from malaria. Future studies will determine how the spleen exerts this effect. Questions to be addressed include whether the β^S transgenic red cells are more adherent to endothelium or more easily detected by splenic macrophages. It will be important to determine the nature of the molecular interaction between endothelial cells or splenic macrophages and β^S transgenic erythrocytes. The results of these studies may contribute to an understanding of how *P. falciparum* avoids trapping in the spleen and binds to the endothelium in the internal organs such as the brain. Such studies may lead to possible interventions in the disease.

Role of ankyrin and spectrin in invasion and growth of rodent malaria parasites

Invasion and growth of the malaria parasite within the host erythrocyte depends on specific interactions between the merozoite stage of the parasite and receptors on and within the red cell membrane[3,33]. In addition, recent studies suggest that the growth and development of the erythrocytic stage of the malaria parasite depends on host red blood cell membrane proteins. For example, spectrin plays a role in supporting the growth of the human malaria parasite, *P. falciparum*. Erythrocytes from patients with hereditary spherocytosis, which is characterized by spectrin/ankyrin deficiency, were invaded normally by *P. falciparum* but the growth of the parasite was reduced in proportion to the relative amount of spectrin[38]. In addition, erythrocytes from patients with hereditary pyropoikilocytosis, which have a defect in spectrin dimer association[22], also demonstrated a reduced level of invasibility by *P. falciparum*[10]. Several strains of mice and abnormalities of erythrocyte membrane spectrin[4] were, therefore, utilized in order to further characterize the roles of spectrin and ankyrin in malaria infection. Homozygous mice expressing the *nb* mutation synthesize normal amounts of spectrin but no ankyrin. Because of the ankyrin deficiency, only 50% of the normal amount of spectrin is bound to the erythrocyte membrane[6]. As demonstrated by light and scanning electron microscopy and isopycnic gradients, erythrocytes from these mice show striking morphological abnormalities including exovesiculation and stomatocytes as well as cells resembling acanthocytes and an overall reduction in size. This is illustrated in a scanning electron micrograph in figure 3[39].

Mice with the *sph*/spherocytosis mutation synthesize ankyrin but do not synthesize the α chain of spectrin[2]. Therefore, only about 20% of the normal amount of β spectrin is bound to the red cell membranes of these mice. Erythrocytes from an *sph/sph* mouse also exhibited abnormal shape with disappearance of biconcavity, exovesiculation and reduced size[39].

The rodent malaria parasites, *P. chabaudi* and *P. berghei* provided excellent models with which to characterize the roles of the erythrocytes membrane proteins in invasion and growth of malaria.

Homozygous WBB6F$_1$ (*nb/nb*), heterozygous (*nb/+*), and homozygous (+/+) mice were infected with 1×10^6 *P. chabaudi*-infected erythrocytes and the course of parasitemia followed by taking smears from the tail vein. As shown in figure 4, +/+ mice displayed a typical course of non-lethal parasitemia which was patent on day 3, peaked on day 10 and was cleared by day 14 after infection. Heterozygous animals had a lower peak parasitemia which was cleared by day 17. Strikingly, *nb/nb* mice did not display a patent parasitemia. Similarly, *nb/nb* mice were also refractory to *P. berghei*. In contrast, *nb/+*, +/+ and parental C57BL/6J mice all succumbed to this lethal parasite[39].

In order to determine whether these rodent parasites could invade *nb/nb* erythrocytes, a subinoculation experiment was performed. Twenty-four and forty-eight hours after infection, 100 µl of blood was transferred from infected *nb/nb* and control mice to outbred Swiss mice which are susceptible to both species of malaria. Blood taken from *nb/nb* mice infected with either *P. chabaudi adami* or *P. berghei* was infectious to the Swiss

Table 2. Effect of splenectomy on the course of parasitemia in β^S and C57BL/6J mice

Day of infection	% of infected RBC[a]			
	β^S	βSPLX	C57BL/6J SPLX	C57BL/6J
5	0.1 (0.1)[b]	0.4 (0.3)	0.7 (0.3)	3.1 (1.4)
9	3.9 (1.4)	15.3[c] (0.1)	25.0 (5.0)	29.0 (7.5)
11	1.6 (1.3)	46.9[c] (3.6)	12.2 (6.6)	14.2 (4.8)
16	Negative	Negative	0.02 (0.01)	15.5[c] (3.6)
22	Negative	Negative	Negative	2.9 (1.1)

[a]Percent infected erythrocytes as determined on Giemsa-stained blood smears.
[b]SEM.
[c]$p < 0.001$ (Difference between splenectomized and intact mice using Student's t-test).

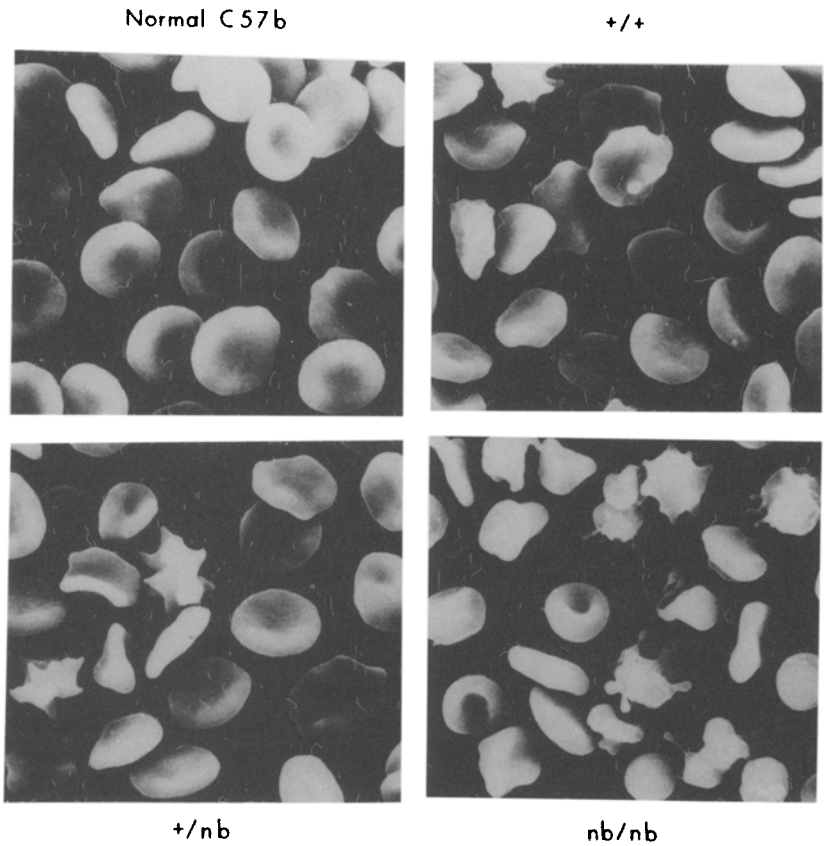

Figure 3. Scanning electron micrographs of heterozygous and homozygous *nb* mouse erythrocytes: (1) normal C57BL/6J mouse; (2) normal +/+ mouse; (3) heterozygote for *nb* mutation (+/*nb*); (4) homozygote for *nb* mutation (*nb*/*nb*). (Magnification for all samples × 3000).

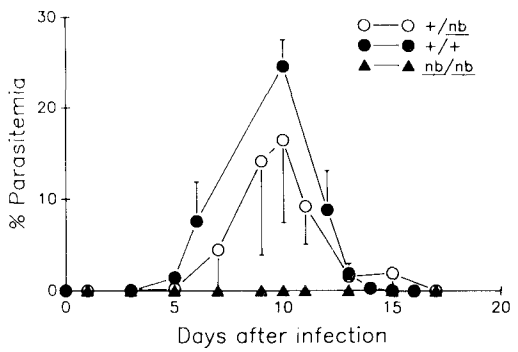

Figure 4. Course of *Plasmodium chabaudi adami* infection in mice carrying the *nb* (normoblastosis) gene. All mice are WBB6F$_1$ (WB/ReJ × C57BL/6J), *nb*/*nb* (triangles, $n = 2$) are homozygous; +*nb* (open circles, $n = 5$). are heterozygous and +/+ (dark circles, $n = 5$) are normal. Data represent the mean parasitaemia ± SD.

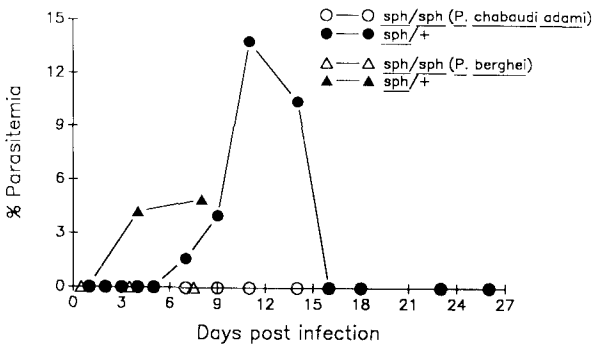

Figure 5. Course of *P. chabaudi adami* (circles) or *P. berghei* (triangles) in WBB6F$_1$ mice carrying the *sph* (spherocytosis) gene, *sph*/*sph* (open symbols) represents one homozygous mouse, *sph*/+ (closed symbols) is one heterozygous mouse.

mice. Therefore, the parasites are able to invade erythrocytes of *nb*/*nb* mice, although probably to a limited extent. However, there was no development of the parasites within the *nb*/*nb* erythrocytes[39].

W/W^V mice, which are anemic due to bone marrow hypoplasia, were also infected with *P. chabaudi adami* to determine whether anemia per se was responsible for the failure of the parasites to develop in mice. However, mice with the W/W^V mutation were completely susceptible to *P. chabaudi adami*[39].

Mice carrying the *sph*/*sph* mutation synthesize ankyrin but only 20% of the normal β chain of spectrin. One *sph*/*sph* mouse was infected with *P. chabaudi adami* and then with *P. berghei*. The mouse was not susceptible to either parasite (fig. 5). It was thus possible to conclude that spectrin is necessary for the growth of these rodent malaria parasites.

The study of rodent malaria in several models of hemoglobinopathies has provided experimental evidence

confirming the protection of the thalassemic and sickle traits against malaria. In addition, these in vivo models offer the possibility of studying host factors in this protection. For example, the spleen was found to play a major role in the protection of the β^S transgenic mice. The use of mutant mice expressing defects in red blood cell membrane proteins has allowed the demonstration that the erythrocyte membrane protein spectrin is necessary for the development of the malaria parasite. Future studies will further characterize host factors in the protection against malaria by red cell genetic defects.

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