INFECTION AND IMMUNITY, June 1977, p. 821-826
Vol. 16, No. 3

Printed in U.S.A.

Prevention of Recrudescent Malaria in Nude Mice by Thymic Grafting or by Treatment with Hyperimmune Serum

D. W. ROBERTS,* R. G. RANK,[1] W. P. WEIDANZ, AND J. F. FINERTY

Department of Microbiology and Immunology, The Hahnemann Medical College and Hospital, Philadelphia, Pennsylvania 19102, and Laboratory of Microbial Immunity, National Institutes of Health, Bethesda, Maryland 20014*

Received for publication 29 November 1976

Nude mice died when infected with the normally avirulent malarial parasite *Plasmodium berghei yoelii*. Furthermore, malaria recrudesced in Nu/Nu mice after the termination of acute disease by treatment with clindamycin. Recrudescence was not observed in Nu/Nu mice that had been grafted with thymic tissue or treated with hyperimmune serum. Mice made B cell deficient by treatment with anti-μ-chain serum also died when infected with *P. berghei yoelii*. The data suggest that a crucial role of the thymus in preventing recrudescent malaria in this model system is to provide a helper function in the production of protective antibody.

The infection of immunologically intact mice with *Plasmodium berghei yoelii* (Pby) produces a parasitemia of short duration and lifelong immunity to reinfection. In contrast, when athymic "nude" mice are infected with Pby, the number of blood parasites steadily increases until the animal dies (6, 24). These data, and other data obtained by infecting thymectomized rodents (2, 22) or rodents treated with antithymocyte serum (21) or antilymphocyte serum (1, 5), clearly demonstrate that the thymus plays a significant role in the development of immunity to malarial infection. Suggestions that might explain this phenomenon include (i) a T-cell helper function in the production of protective antibodies (3) and/or (ii) the release of nonantibody mediators of immunity by activated T cells (7). Previously, we had observed that B-cell-deficient chickens were immune to subsequent challenge infection with *Plasmodium gallinaceum* provided they had been rescued from acute malaria by means of chloroquine therapy (19). Thus, acute disease constituted an immunizing experience for B-cell-deficient chickens possessing T-cell function. The present investigation was undertaken to determine if acute malarial infection would be an immunizing event in T-cell-deficient "nude" mice. Thus, nude mice were tested for their ability to resist recurrent malarial infection with Pby after the treatment of acute disease with clindamycin. In contrast to the B-cell-deficient chicken, the nude mouse developed recurrent and ultimately fatal malarial infection. However, disease did not recur in successfully thymic-grafted nude mice or in nude mice treated with hyperimmune serum after drug therapy.

[1] Present address: Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR 72201.

MATERIALS AND METHODS

Mice. N/Nu and Nu/+ mice of both sexes were obtained from a closed colony of animals and used at 7 to 12 weeks of age unless noted otherwise. These animals had a BALB/c background and were derived from parental stock kindly supplied by Charles Schaffer, Wittenberg College, Springfield, Ohio.

Thymic reconstitution. Nu/Nu mice, 4 to 6 weeks of age, were grafted under ether anesthesia with thymic tissue derived from neonatal donors according to the procedure described by Rygaard (20). T-cell competence was assessed by sensitizing mice on the flank with 0.25 ml of 10% *p*-amino-*N*-dimethylanaline (NDMA) in acetone and then challenging them 6 days later and measuring increases in ear thickness at 24 h as described by Maguire (16). Nongrafted, immunologically intact Nu/+ mice served as controls.

Experimental infection. Mice were infected via the intraperitoneal route with 10^6 erythrocytes parasitized with the nonlethal 17X strain of Pby. Parasitemias were determined by microscopic examination of blood films prepared from tail blood and stained with Giemsa stain.

Footpad swelling in response to malarial antigen. Footpad swelling was measured in both infected and noninfected mice at various intervals of time after the injection into the left hind footpad of 0.05 ml of frozen and thawed malarial antigen prepared from the 17XL strain of Pby according to the procedure of Finerty and Krehl (10). Measurements were made 3, 6, 12, 24, and 48 h later with a dial micrometer (Schnell-taster) as described previously

(13), and the increase in footpad thickness was recorded for each mouse.

Chemotherapy. Clindamycin HCl hydrate (Cleocin, Upjohn Co., Kalamazoo, Mich.), which has been shown to possess antiplasmodial activity by Lewis (14), was administered in a dose of 74 mg of active drug per kg in water per day for 5 consecutive days by means of oral intubation under light ether anesthesia.

Hyperimmune serum. Nu/+ mice that had survived acute infection with Pby were subsequently hyperimmunized by inoculation with 2×10^6 parasitized erythrocytes at 42 and 70 days after the initiation of acute infection. Mice were bled from the retro-orbital plexus 8 days later under ether anesthesia. The pooled sera were sterilized by filtration through a 0.45-μm grid membrane (Nalge Sybron Corp., Rochester, N.Y.) and stored at −20°C until needed. Normal serum was collected from noninfected Nu/+ mice in the same manner and stored at −20°C.

Treatment with anti-μ-chain serum. Goat antiserum against purified mouse immunoglobulin M (IgM) (MOPC 104E; γ,μ) was obtained through the kindness of Paul Montgomery (University of Pennsylvania, Philadelphia, Pa.). The serum was heat inactivated at 56°C for 30 min and absorbed with 8% washed Nu/$^+$ erythrocytes. The absorbed antiserum was assayed by means of gel diffusion against MOPC 104E; γ,μ myeloma protein (Litton Bionetics Inc., Kensington, Md.) at a concentration of 1 mg/ml and produced a distinct precipitin line at a dilution of 1:256. Serum was sterilized by filtration through 0.45-μm grid membrane and stored at −20°C. Nu/+ mice of both sexes were injected intraperitoneally, using a 30-gauge needle, with 0.05 ml of absorbed goat anti-μ on the day of birth and every other day thereafter during the first week of life (17). The dose was then increased to 0.1 ml and administered at subsequent intervals so that the total volume injected was 2.3 ml/mouse during a period of 11.5 weeks. Both treated mice and untreated control animals were bled 16 days prior to cessation of anti-μ treatment, and the serum was assayed for IgM using commercially prepared radial immunodiffusion plates (Meloy Laboratories, Springfield, Va.) with a working range of 0.06 to 2.1 mg of IgM per ml.

RESULTS

Nu/Nu mice infected with 10^6 Pby failed to resolve their infections, in contrast to Nu/+ littermates (Fig. 1). Although malaria was not fulminant in nude mice, it persisted until the animals died.

To determine if Nu/Nu mice were capable of developing immunity to Pby, Nu/Nu and Nu/+ littermates were infected with 10^6 Pby and then treated with clindamycin after parasitemias became patent. Parasites were not evident in blood films taken 3 days after the termination of drug treatment. Subsequently, malaria recurred in all Nu/Nu mice treated with clindamycin but in none of the four surviving Nu/+ mice that had received the same treatment (Fig. 2). Similarly, when Nu/Nu mice with recrudescent infection were placed on a milk diet therapy shown previously to be efficacious (15), 20 days after drug treatment, their blood films became negative once again, but recrudescence was observed after the mice had been returned to their regular diet.

Since recrudescence did not take place in animals possessing an intact thymus, we next investigated the ability of thymic-grafted Nu/Nu mice to resist recurrent disease after clindamycin rescue. Efforts to demonstrate allergic contact dermatitis to NDMA in thymic-grafted Nu/Nu mice prior to infection with Pby were made. The data revealed that only two of the animals responded with a definitely positive

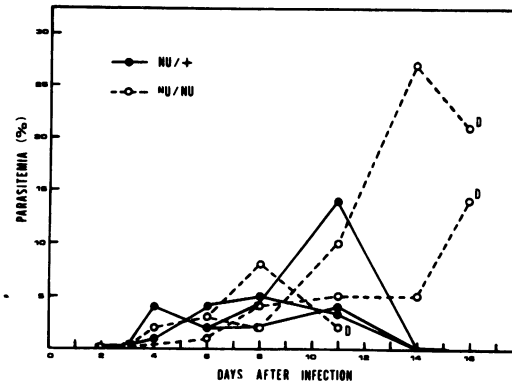

FIG. 1. *Lethal Pby infections in Nu/Nu mice. Parasitemias in individual mice are shown.*

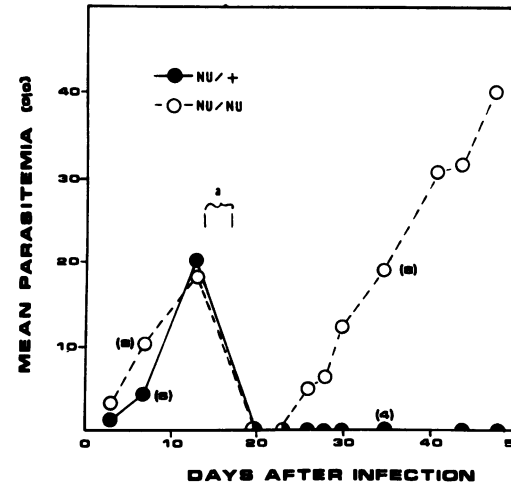

FIG. 2. *Recrudescent malaria in Nu/Nu mice. Parasitemias were averaged for the number of animals shown in brackets. (a) Treatment with clindamycin, 74 mg/kg, during the 5-day period.*

challenge reaction. At 5 months of age, all of the thymic-grafted nude mice as well as non-grafted littermates, both Nu/Nu and Nu/+ mice, were infected with 10^6 Pby and then rescued with clindamycin. Whereas recrudescence was observed in all 7 Nu/Nu mice, malaria did not recur in 12 of the 14 thymic-grafted Nu/Nu mice (Table 1). Similarly, recrudescence was not seen in Nu/+ mice during the observation period of 29 days. Both thymic-grafted Nu/Nu mice and Nu/+ mice were resistant to reinfection with 2×10^6 Pby injected 29 days after rescue. When skin tested with NDMA at 50 days after infection, the thymic-grafted Nu/Nu mice, which resisted challenge infection, showed a mean increase in ear thickness of 17.5×10^{-3} cm. Nu/+ mice receiving identical treatment showed a mean increase of 23.5×10^{-3} cm. Nonsensitized Nu/+ mice developed a mean increase in ear thickness of 2.4×10^{-3} cm upon challenge. Thus, T-cell immunity as measured by this parameter was evident in those thymic-grafted Nu/Nu mice in which recrudescence did not take place.

To determine whether thymic-grafted Nu/Nu mice became sensitized to malarial antigen when infected with Pby, infected mice (Nu/Nu, thymic-grafted Nu/Nu, and Nu/+ animals), as well as uninfected control animals, were tested for footpad swelling in response to the injection of frozen and thawed malarial antigen into the left hind footpad 17 days after drug rescue. The results in Fig. 3 show that footpad swelling was most evident in Nu/+ mice and in thymic-grafted Nu/Nu mice that did not develop recrudescent malaria. Footpad reactions were less marked in infected mice that developed recurrent disease. Noninfected control animals showed a minimal response to the test antigen.

TABLE 1. *Absence of recrudescent malaria in thymic-grafted Nu/Nu mice rescued with clindamycin*

Genotype (no.)	Mean parasitemia 9 days after infection (%)	No. with recrudescent malaria/no. rescued	% Immune to subsequent challenge[b]
Nu/Nu (11)	14	7/7	Not done
Nu/+ (14)	15	0/13	100
Nu/Nu + thymus (14)	11	2/14	100

[a] Parasitemias were arrested by oral treatment with clindamycin (74 mg/kg of body weight) daily on days 9 through 13 after infection.

[b] Mice that had not developed recrudescent infections were challenged with 2×10^6 Pby 29 days after drug rescue. Parasitemias became patent in 13 control mice injected with 10^6 Pby.

Reactions were most pronounced 3 h after injection with antigen and then diminished with the passing of time.

Since the early footpad response to malarial antigen indicated an antibody-mediated reaction (18), it was possible that the role of T cells in preventing recrudescent malaria could be attributed to a helper function in the production of protective antibodies. To test this, Nu/Nu mice were infected with Pby, treated with clindamycin, and then injected daily with 0.2 ml of hyperimmune serum, normal serum, or saline via the intraperitoneal route. Mice given hyperimmune serum were protected from recrudescent malaria, whereas four out of five animals treated with either normal serum or saline showed recurrent disease (Table 2). Although hyperimmune serum did protect athymic mice from recrudescent infection, the mice remained susceptible to challenge infection when the parasites were injected 1 month after the termination of serum treatment. Thus, hyperimmune serum in the absence of a functioning T-cell system prevented recurrent malarial infection but, unlike active immunization in T-cell-bearing animals, it failed to protect the host against subsequent challenge infection.

Whereas these data suggest that antibody may prevent recrudescent malaria, it is difficult to rule out the possibility that T-cell-dependent nonantibody mediators of immunity might account for the absence of recrudescence in Nu/Nu mice treated with hyperimmune serum. To determine if B-cell-deficient mice possessing a functional thymus were capable of developing acquired immunity to malaria, Nu/+ mice were treated from birth with goat anti-μ-chain serum. When assayed by radial immunodiffusion, IgM was not detected in the sera of treated mice. In contrast, the mean concentration of IgM in the sera of untreated age-matched control mice was 28.6 mg/100 ml. At 6 weeks of age the mice were sensitized with NDMA and challenged 6 days later. All animals developed allergic contact dermatitis to NDMA, indicating that T-cell immunity was intact in these B-cell-deficient mice as measured by this parameter. Both B-cell-deficient mice and immunologically intact animals were then infected with 10^6 Pby. The results shown in Table 3 demonstrate that Pby infections were lethal in B-cell-deficient mice, whereas four of five animals possessing B-cell immunity readily survived the infection. These data demonstrate that immunity to acute malarial infection is dependent upon factors other than those usually associated with functioning T cells.

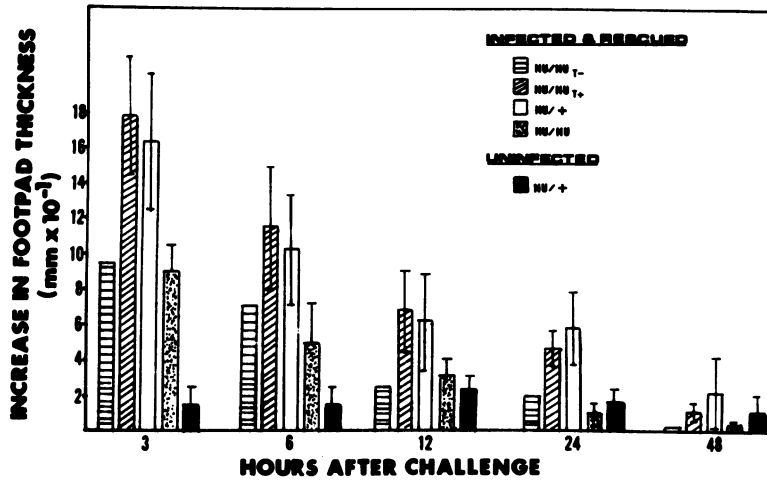

FIG. 3. *Early footpad swelling in response to malarial antigen injected 31 days after initiation of acute infection with Pby. Nu/Nu$_{T+}$ indicates thymic-grafted mice in which malaria did not recur. Nu/Nu$_{T-}$ depicts thymic-grafted mice that developed recurrent disease. Values are means ± standard deviation at hourly intervals noted.*

TABLE 2. *Passive immunization of Nu/Nu mice and subsequent protection against recrudescent malaria*

Genotype (no.)	Treatment after infection and rescue[a]	No. with recrudescent malaria/no. rescued	No. with patent parasitemia/no. challenged[b]
Nu/Nu (5)	Saline	4/5	1/1
Nu/Nu (5)	Normal serum	4/5	Not done
Nu/Nu (5)	Hyperimmune serum	0/5	5/5

[a] Parasitemias were arrested by oral treatment with clindamycin, 74 mg/kg of body weight, daily on days 5 through 9 after infection. Mice were injected intraperitoneally with 0.2 ml of saline, normal serum, or hyperimmune serum on days 9 through 16 after infection.

[b] Surviving mice were challenged with 10^6 parasites 2 months after the initiation of acute infection.

DISCUSSION

The importance of T-cell immunity in malaria is well documented. T-cell-deprived rodents usually suffer more severely from experimental malaria than animals possessing a thymus (1, 2, 5, 6, 21, 22). That T-cell activation does occur in malaria has been demonstrated (12). Further, sensitized T cells have been shown to undergo blast cell transformation in the presence of malarial antigen (23). In fact, it has been suggested that thymus-dependent nonantibody mediators of immunity play a significant role in malaria (7); but much of the evidence available indicates that T cells probably function via a helper role in the production of protective antibodies (3, 4).

In the present study, Nu/Nu mice developed persisting parasitemias and died when infected with the normally avirulent Pby, thus confirming the observations of others (6, 24). Further, only Nu/Nu mice developed recrudescent disease after therapy with clindamycin. It is interesting to note that recurrent malaria in Nu/Nu mice followed an acute course rather than the chronic one which is often observed in hosts that have become partially immune to the parasite. Mice possessing thymic tissue did not demonstrate recrudescent disease, indicating that the thymus played an important role in preventing recurrent infection. Whereas thymus-intact animals demonstrated delayed hypersensitivity to the contactant NDMA, they developed early footpad swelling in response to the injection of malarial antigen. These reactions were consistent with Arthus reactions described by Nelson and Boyden (18) and were probably antibody dependent. Delayed footpad swelling was not observed in any of the mice treated with malarial antigen. The relationship between early footpad swelling and resistance to recrudescent malaria remains uncertain at this time and requires further investigation.

Of greater significance was the observation that recrudescence did not occur in mice that had been treated with hyperimmune serum. In this instance, the parasites in the Nu/Nu mice were destroyed by a component(s), presumably antibody, in hyperimmune serum either acting alone or in conjunction with a non-T-cell effector mechanism. The point is that the role of a functional thymus was fulfilled by injections of hyperimmune serum. This strongly suggests to us that the role of the thymus in preventing recrudescent malaria in the murine model is that of a T-cell helper function in the produc-

TABLE 3. *Lethal Pby infections in mice treated with anti-μ-chain serum*

Group	Treatment[a]	Genotype (no.)	Mean serum IgM levels[b] (mg/100 ml)	Cumulative mortality after infection with Pby (no. died/no. infected)						
				2[c]	5	7	9	13	17	21
A	Anti-μ chain	Nu/+ (5)	<6	0/5	0/5	0/5	1/5	2/5	3/5	5/5
B	None	Nu/+ (5)	28.6	0/5	1/5	1/5	1/5	1/5	1/5	1/5[d]

[a] Mice were treated with goat anti-μ-chain serum.
[b] Serum IgM levels were determined by radial immunodiffusion.
[c] Days after infection.
[d] Blood films were negative for Pby in all surviving mice at this time.

tion of protective antibody. Supporting this was the observation that mice treated with anti-μ-chain serum to suppress B-cell immunity suffered lethal infections with Pby, even though they possessed T-cell function, as demonstrated by their ability to develop allergic contact dermatitis to NDMA. Similar findings have been reported by Weinbaum et al. (24). Furthermore, Brown et al. (4) demonstrated that splenic T cells from rats infected with *P. berghei* conferred immunity when transferred to syngenic recipients. They suggested that T cells exert their protective action mainly through cooperation with B cells. This is consistent with the observation of Gravely and Kreier (11), who demonstrated that the adoptive transfer of immunity to acute *P. berghei* infection in young rats was best achieved with the differentiated B-cell population derived from the spleens of immunized T-cell-intact adult rats.

One of the questions that remains is whether or not T cells may function by mechanisms other than those noted above. This question is prompted by our observation that B-cell-deficient chickens were resistant to an otherwise lethal *P. gallinaceum* infection after the cure of acute disease with chloroquine (19). Thus, in the avian model, nonsterilizing immunity in malaria is antibody independent, but we have not been able to demonstrate the mechanism for this immunity. In this regard the recent findings of Clark et al. (7, 8) prove instructive, since they showed that mice injected with BCG or *Corynebacterium parvum* were protected against certain intraerythrocytic protozoa. They postulate that a nonantibody mediator of immunity, possibly derived from T cells, is responsible (7). These same workers observed that nude mice injected with *C. parvum* resisted infection with *Babesia microti* (8). This may indicate that in the above-cited infections other cells may be the source of nonantibody mediators of immunity. In any event, these data obtained from divergent model systems clearly demonstrate that certain forms of immunity to malaria do not depend upon the action of protective antibodies. The B-cell-deficient mouse maintained on a suppressive regimen of anti-μ serum should provide a useful tool to elucidate the mechanisms involved.

ACKNOWLEDGMENTS

We thank our colleagues B. J. Landau and H. C. Maguire for their helpful suggestions in the preparation of this manuscript. We also acknowledge the expert secretarial assistance of Natalie Retamar.

During the year 1975–1976, W.P.W. was the recipient of an American Cancer Society-Eleanor Roosevelt-International Cancer Fellowship. This work was supported in part by Public Health Service research grant AI-12710 from the National Institute of Allergy and Infectious Diseases (W.P.W.).

LITERATURE CITED

1. **Barker, L. R., and K. G. Powers.** 1971. Impairment of antibody response and recovery in malarial rodents by antilymphocyte serum. Nature (London) 229:429–430.
2. **Brown, I. N., A. C. Allison, and R. B. Taylor.** 1968. *Plasmodium berghei* infection in thymectomized rats. Nature (London) 219:292–293.
3. **Brown, K. N.** 1971. Protective immunity to malaria parasites: a model for the survival of cells in an immunologically hostile environment. Nature (London) 230:163–167.
4. **Brown, K. N., W. Jarra, and L. A. Hills.** 1976. T cells and protective immunity to *Plasmodium berghei* in rats. Infect. Immun. 14:858–871.
5. **Bruce-Chwatt, L. J., J. Darrell, and E. Tipley.** 1972. Antilymphocyte serum and the course of rodent malaria in mice. Trans. R. Soc. Trop. Med. Hyg. 66:522–523.
6. **Clark, I. A., and A. C. Allison.** 1974. *Babesia microti* and *Plasmodium berghei yoelii* infections in nude mice. Nature (London) 252:328–329.
7. **Clark, I. A., A. C. Allison, and F. E. Cox.** 1976. Protection of *Babesia* and *Plasmodium* with BCG. Nature (London) 259:309–311.
8. **Clark, I. A., F. E. Cox, and A. C. Allison.** 1977. Protection of mice against *Babesia spp.* and *Plasmodium spp.* with killed *Corynebacterium parvum*. Parasitology 74:9–18.
9. **Cohen, S., and G. A. Butcher.** 1972. The immunologic response to Plasmodium. Am. J. Trop. Med. Hyg. 21:713–721.
10. **Finerty, J. F., and E. P. Krehl.** 1976. Cyclophosphamide pretreatment and protection against malaria. Infect. Immun. 14:1103–1105.
11. **Gravely, S. M., and J. P. Kreier.** 1976. Adoptive transfer of immunity to *Plasmodium berghei* with immune T and B cells. Infect. Immun. 14:184–190.
12. **Jayawardena. A. N., G. A. T. Targett, E. Leuchars, R.**

L. Carter, M. J. Doenhoff, and A. J. S. Davies. 1975. T cell activation in malaria. Nature (London) 258: 149–152.

13. LaGrange, P. H., G. B. MacKaness, and T. E. Miller. 1974. Potentiation of T-cell immunity by selective suppression of antibody formation with cyclophosphamide. J. Exp. Med. 139:1529–1539.
14. Lewis, C. 1968. Antiplasmodial activity of halogenated lincomycin analogues in *Plasmodium berghei*-infected mice, p. 537–542. Antimicrob. Agents Chemother. 1967.
15. Maegraith, B. G., J. Deegan, and E. S. Jones. 1952. Suppression of malaria (*P. berghei*) by milk. Br. Med. J. 2:1382–1384.
16. Maguire, H. C., Jr. 1976. Specific acquired unresponsiveness to contact allergens with cyclophosphamide in the mouse. Int. Arch. Allergy Immunol. 50:651–658.
17. Manning, D. D., and J. W Jutilia. 1972. Immunosuppression in mice injected with heterologous anti-immunoglobulin antisera. J. Immunol. 108:282–285.
18. Nelson, D. S., and S. V. Boyden. 1964. The cutaneous reactivity of guinea pigs to pure protein antigens. 1. A critical evaluation of methods for the induction of delayed-type hypersensitivity to pure proteins. Int. Arch. Allergy 25:279–303.
19. Rank, R. G., and W. P. Weidanz. 1976. Nonsterilizing immunity in avian malaria: an antibody-independent phenomenon. Proc. Soc. Exp. Biol. Med. 151:257–259.
20. Rygaard, J. 1973. Thymus and self. Immunobiology of the mouse mutant nude. F.A.D.L., Copenhagen.
21. Spira, D. T., P. H. Silverman, and C. Gaines. 1970. Anti-thymocyte serum effects on *Plasmodium berghei* infection in rats. Immunology 19:759–766.
22. Stechshulte, D. J. 1969. Effect of thymectomy on *Plasmodium berghei* infected rats. Proc. Soc. Exp. Biol. Med. 131:748–752.
23. Weinbaum, F. I., C. B. Evans, and R. E. Tigelaar. 1976. An *in vitro* assay for T cell immunity to malaria in mice. J. Immunol. 116:1280–1283.
24. Weinbaum, F. I., C. B. Evans, and R. E. Tigelaar. 1976. Immunity to *Plasmodium berghei yoelii* in mice. I. The course of infection in T cell and B cell deficient mice. J. Immunol. 117:1999–2005.