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T and B Cell Population Changes in Young and in Adult Rats Infected with *Plasmodium berghei*[1]

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Malaria infection in young rats is characterized by high parasitemia, severe anemia, and death. Parasitemia is lower in older rats, and the rats usually survive. This study was designed to investigate the immunological basis of this difference. T cell numbers in the thymuses and spleens of young (4 weeks old) and in adult (18 weeks old) infected and control rats were determined by killing with anti-theta serum and complement. The number of complement receptor lymphocytes (B cells) in spleens was determined after these cells had formed rosettes with sensitized, complement-coated sheep erythrocytes. Infection in young rats was characterized by progressive and severe thymic involution and by decreasing numbers of T and B cells in the spleen. In 18-week-old rats, T cell numbers in the spleen were slightly below those of controls early in infection but exceeded normal values by day 15. Progressive thymic involution was not a feature of infection in adult rats. The number of complement receptor lymphocytes in the spleens of adult rats decreased dramatically early in infection but were nearly normal by day 15. Severity of malarial infection in young rats is related to the inability of their lymphocytes to respond to *Plasmodium berghei* antigens early in infection in a way that leads to immunity.

Adult rats are more resistant to malarial infection than are young rats (17). Because *Plasmodium berghei* merozoites preferentially invade reticulocytes, severity of malarial infection in young rats has been attributed to the high percentage of circulating reticulocytes in the blood of younger animals (26). Since adult rats are more resistant to infection with *Plasmodium vinckei* (25), a parasite that invades all available erythrocytes, circulating polychromasia of the young is not an adequate explanation for the severe plasmodial infections observed in young animals. An important factor in age-related immune phenomena in rats is the delay in the development of acquired immunity in young as compared with mature rats (26).

The development of immunity to malarial infection requires functioning T cells. Rats treated with antithymocyte serum were unable to recover from *P. berghei* infection (22). Adult rats that had been thymectomized at birth were also unable to control *P. berghei* infection (4). Whether T cells are required as helpers in the establishment of a humoral response or as effectors of cell-mediated immunity during malarial infection has not been determined.

T and B cells may be identified by using methods based on their surface properties. Thymus-derived cells have the alloantigen θ on their surfaces. This antigen has been identified on thymocytes, on peripheral T lymphocytes, and in neural tissue of mice (21) and in other species, including rats (9, 12, 20). T cells are identifiable on cytotoxicity assays with anti-theta serum and complement. B lymphocytes have a receptor for the third component of complement and may be identified after they have formed rosettes with sensitized, complement-coated sheep erythrocytes (2).

Using the methods described, we studied the changes that occurred in the thymuses and in the T and B cell populations in the spleens of young and adult rats infected with *P. berghei* to determine if the behavior of these cells would give clues about the mechanisms of age-related immunity associated with malarial infections.

MATERIALS AND METHODS

Animals. Four- and 18-week-old Fischer 344 female rats (CD*F, Charles River Breeding Laboratories, Wilmington, Mass.) were used. The animals were housed in the Laboratory Animal Facility of the Department of Microbiology, Ohio State University. They were allowed food and water ad libitum.

Parasites. *P. berghei berghei*-infected mouse blood (Walter Reed Army Institute of Research isolate) was maintained frozen in liquid nitrogen. Inoc-

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ula for rats were obtained by injecting Swiss mice with the stabilate. After a high parasitemia was established in the mice, they were exsanguinated under ether anesthesia by cardiac puncture, and their highly parasitized erythrocytes were injected into 4-week-old CD*F rats. These rats were bled by cardiac puncture on day 5 of the infection, and 2×10^7 infected rat erythrocytes were inoculated intraperitoneally into the rats of each age group.

Five infected rats and three controls from the groups were killed every 5 days for 20 to 25 days. Rats were weighed and exsanguinated by cardiac puncture under ether anesthesia. The spleens of these rats were removed and weighed. Thymuses were removed, rinsed, trimmed of fat and parathymic lymph nodes, drained, and weighed. The entire experiment was repeated once.

Parasitemia. Parasitemias were determined by counting the number of parasitized erythrocytes in 10,000 erythrocytes scanned by light microscopy at ×1,000 in Giemsa-stained thin blood films.

Lymphocyte harvests. Lymphocyte suspensions were obtained by extruding the thymus or the spleen through 60-mesh stainless-steel screens and rinsing the screens with Hanks balanced salt solution (HBSS). Seven milliliters of diluted lymphocyte suspension was layered onto 3 ml of Ficoll-Hypaque solution in a 15-ml screw-cap tube. Lymphocytes were harvested from the interface of the buffer and Ficoll-Hypaque layers after centrifugation at 2,000 rpm for 5 min in an International centrifuge, model CM. This method for harvesting lymphocytes is a modification of the method for isolating leukocytes from the blood described by Boyum (3). Harvested cells were washed twice in HBSS and resuspended in HBSS. Cell counts were made in a hemocytometer. Viable cells were identified by their ability to exclude trypan blue. All suspensions were adjusted to contain 5×10^6 viable cells/ml. Trypan blue for all tests was prepared as a 0.2% stock solution in sterile distilled water. One milliliter of 5× HBSS was added to 4 ml of 0.2% trypan blue to make the standard counting solution.

Cytotoxicity testing. Brain-associated anti-theta serum was prepared as described by Golub (11) except that CD*F rat brains were used. Two CD*F rat brains were homogenized in 2 ml of HBSS in a Virtis blender. The homogenized brains were emulsified in 5 ml of Freund complete adjuvant (Difco, Detroit, Mich.). Approximately 8 ml of emulsified brain was injected subcutaneously into four different areas on a 4.1-kg New Zealand white rabbit. Injections were given weekly for 3 weeks, and at week 4 the animal was bled and injected again. Bleeding and injecting was continued weekly until the rabbit was exsanguinated at week 9. Prior to the scheduled injections, the rabbit was bled by cardiac puncture for normal serum.

Five-milliliter samples of normal rabbit serum and brain-associated anti-theta were absorbed at 4 C for 1 h with 1.25 ml of washed, packed CD*F erythrocytes, 1.25 ml of packed CD*F liver homogenate, and 0.5 g of agarose. Five-milliliter samples of guinea pig complement (Grand Island Biological Supply Co., Grand Island, N.Y.) were absorbed at 4 C for 30 min with 1.25 ml of washed, packed CD*F erythrocytes, 1.25 ml of packed liver homogenate, and 1.25 ml of packed CD*F thymocytes. Serum and complement were clarified by centrifugation at 5,000 rpm in a Sorvall RC-2B ultracentrifuge. Clarified sera were stored in 0.2-ml aliquots at −20 C.

Absorbed sera were titrated to determine the optimum dilutions needed for the cytotoxicity assay (Table 1). On the basis of this titration, the 1:8 dilution of brain-associated theta (BAθ) and of normal rat serum (NRS) was chosen for use in all subsequent tests. The complement used in these studies was diluted 1:4 in HBSS. Some lots of complement were extremely cytotoxic for CD*F thymocytes and could not be used even after exhaustive adsorption with thymocytes and agarose. The cytotoxicity assay was set up as described by Barker (1). Briefly, 0.05 ml of lymphocyte suspension, 0.05 ml of BAθ or NRS, and 0.05 ml of complement were mixed in small tubes. (All dilutions were made in HBSS.) The tubes were incubated at 37 C for 45 min in a humid CO_2 incubator. After incubation, 0.1 ml of trypan blue in HBSS was added to each tube. The number of dead versus living cells was determined by counting 500 cells in a standard hemocytometer. The cytotoxic index was determined as follows (11): cytotoxic index = (percent dead with BAθ − percent dead with NRS)/(100 − percent dead with NRS).

The specificity of rabbit anti-brain-associated theta was determined as follows. One-milliliter aliquots of anti-theta were adsorbed with 1-g quantities of shredded CD*F rat brain tissue for 1 h at 4 C. This adsorbed serum was titrated against thymocytes and splenocytes of 4- to 6-week-old animals. Brain tissue takes out substantial activity, but more than one adsorption with brain tissue is required to remove all activity (Fig. 1).

Assay for complement receptor lymphocytes (CRL). The rosette assay for B cells was set up as described by Bianco et al. (2). Sheep erythrocytes were washed in HBSS, and a 5% suspension in HBSS was prepared. A 1:1,000 dilution of anti-sheep hemolysin (Grand Island Biologicals, Grand Island, N.Y.) was prepared in HBSS. Equal volumes of sheep cells and hemolysin were mixed, and the mixture was incubated at 37 C for 30 min with frequent shaking. After incubation, the cells were washed twice in HBSS and resuspended in enough HBSS to make a 5% solution. The sensitized sheep erythrocytes (EA) were then mixed with an equal volume of fresh mouse complement, which was diluted 1:10 in HBSS. The mixture was incubated at 37 C for 30 min with frequent mixing. After incubation, the cells were washed three times in HBSS and resuspended

TABLE 1. *Determination of the optimum dilution of rabbit anti-brain-associated theta for cytotoxicity testing*

Organ	Cytotoxic indexes						
	2[a]	4	8	16	32	64	128
Thymus	1.00	0.99	0.98	0.97	0.97	0.95	0.94
Spleen	0.33	0.39	0.37	0.38	0.36	0.32	0.17

[a] Dilution of antisera.

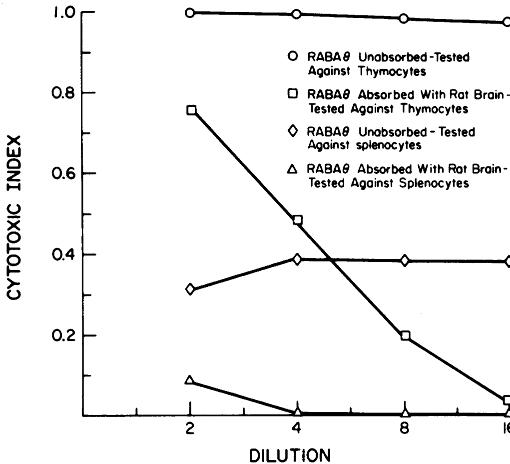

FIG. 1. *Demonstration of the activity of rabbit anti-rat brain-associated theta after adsorption with shredded CD*F rat brain.*

to make a 5% solution in HBSS (EAC). Two-tenths-milliliter volumes of EAC and of lymphocytes (5×10^6 viable cells/ml) were mixed and incubated at 37 C for 30 min with gentle mixing. After incubation, the mixtures were centrifuged at 500 rpm in an International clinical centrifuge for 3 min. A 0.6-ml portion of HBSS was added to each tube to facilitate counting. The contents of each tube were mixed, and the percentage of lymphocytes forming rosettes with EAC (lymphocytes with three or more erythrocytes adhering) was determined by counting the number of rosettes in the total number of lymphocytes in the center square of a hemocytometer. All counts were done in duplicate.

RESULTS

Plasmodial infection in 4-week-old rats was characterized by high parasitemia, emaciation, and death of thymocytes, followed by severe thymic involution. In young infected rats, the thymus was not visible on gross examination by day 20 of the infection. In 18-week-old rats, on the other hand, parasitemia was mild and of short duration, and body weight was maintained. In the older rats, the proportion of dead cells in thymuses never exceeded 10%. By day 25 of the infection, the proportion of dead cells in the thymuses of 18-week-old infected rats was less than that of controls. By day 20 of infection, the thymuses of the older rats had hypertrophied. The behavior of the spleens as well as of thymuses differed in the older and younger infected rats. The older infected rats increased their spleen weight more rapidly than did the younger rats, but they did not increase them as much as the younger rats did and, after 15 days, their spleens began to involute. In the young infected rats, the spleens continued to hypertrophy until death stopped the process (Fig. 2).

The changes in the T cell population in the spleens reflected to some degree the changes in the thymuses. In the young infected animals, the splenic T cell population dropped precipitously. At day 5 of the infection, T cell percentages in the spleen were slightly below normal; by day 10, the percentage of T cells was only 58% of the control levels (Table 2). In the 18-week-old infected animals, T cell populations in the spleen were also slightly below normal on day 5 of infection but returned to near normal levels by day 10 and remained normal or above for the duration of the study period (Table 3).

B cell percentages in the spleens of the young infected rats did not drop as precipitously as did T cell percentages. On day 5, the animals tested had CRL percentages that were 85% of the control values. However, the decrease in detectable B cells was progressive, and by day 20 CRLs were 44% of those of controls for the same day. B cell percentages detectable as CRLs dropped in the early stages of the infection more drastically in the spleens of 18-week-old rats than they did in the 4-week-old rats. On day 5 of the infection, CRLs were only 66% of those of con-

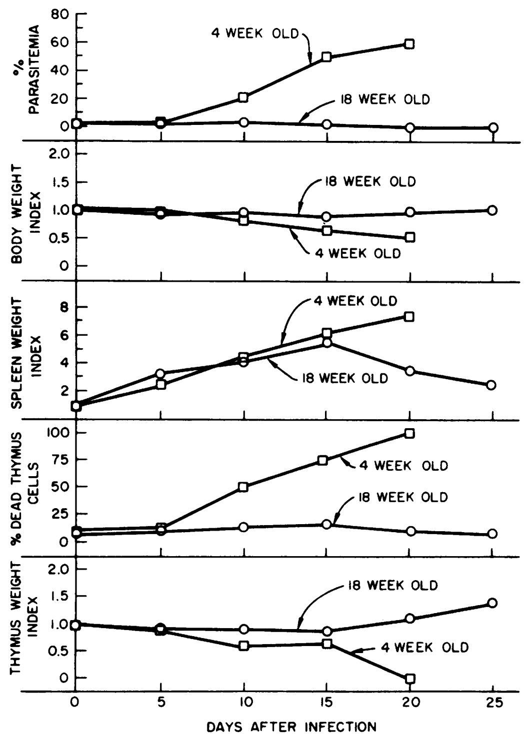

FIG. 2. *Physiological parameters of P. berghei infection in 4- and 18-week-old female CD*F rats.*

TABLE 2. *Behavior of B and T cells in the spleens of P. berghei-infected, 4-week-old female rats*[a]

Day of infection	% Parasitemia	% CRL (B)			% T cells		
		Infected	% of normal	Controls	Infected	% of normal	Controls
5	3.25 (2.10-3.90)	44 (39-47)	85	52 (48-56)	33 (27-38)	87	38 (35-43)
10	21.00 (14.0-33.0)	35 (27-39)	67	52 (48-53)	19 (10-23)	58	33 (28-40)
15	51.00 (38.0-68.0)	31 (25-39)	60	52 (47-56)	19 (14-32)	54	35 (27-42)
20	60.00 (46.0-85.0)	21 (17-32)	44	48 (47-50)	18 (14-32)	47	38 (30-45)

[a] Data presented represent the average percentage of B cells or T cells detectable in spleens as CRLs or by anti-theta serum killing, respectively. Averages were computed from the results of tests on five experimental animals and three controls. Only three test animals were alive on day 20. Data presented in parentheses represent ranges.

TABLE 3. *Behavior of B and T cells in the spleens of P. berghei-infected, 18-week-old female rats*[a]

Day of infection	% Parasitemia	% CRL			% T cells		
		Infected	% of normal	Controls	Infected	% of normal	Controls
5	1.45 (0.96-2.10)	34 (27-40)	66	51 (50-53)	24 (18-26)	89	27 (25-28)
10	2.32 (0.60-5.61)	31 (24-41)	63	49 (45-54)	31 (27-36)	94	33 (28-36)
15	1.40	40 (35-45)	87	46 (43-52)	37 (33-43)	103	36 (32-40)
20	0.00	46 (41-53)	90	51 (50-51)	37 (33-49)	123	30 (25-37)
25	0.00	52 (44-59)	106	49 (49-52)	35 (33-37)	103	34 (28-44)

[a] Data presented represent the average percentage of B cells or T cells detectable in the spleens as CRLs or by anti-theta serum killing, respectively. Averages were computed from the results of tests on five experimental animals and three controls. Data presented in parentheses represent ranges.

trols in adult animals, but the percentage of B cells increased steadily after day 5 until, at day 25, CRL percentages in the spleens of the 18-week-old infected rats were slightly above those of controls for the same day (Tables 2 and 3). The results of the duplicate experiment were essentially the same as those described.

DISCUSSION

Four-week-old rats were unable to mount an immune response capable of bringing about recovery from *P. berghei* infection. This inability to respond was associated with collapses in the lymphocyte populations known to affect both humoral and cell-mediated immune responses. Young rats behaved like mice (17) during *P. berghei* infection. The lymphoid populations of adult rats did not collapse during the infection, but rather, after short initial decreases, the populations hypertrophied.

At day 5 of the infection, the drop in T cell percentages in the spleens of 4- and 18-week-old infected rats was approximately the same, 13 and 12% below control values, respectively. However, decreases in CRL percentages of 18-week-old animals were more dramatic at day 5 than they were in 4-week-old animals. After a B lymphocyte responds to antigen and becomes an antibody-producing cell, that cell is no longer detectable as a CRL (2). The B cells of the adult began to respond immediately to the introduced antigen, but B cells of 4-week-old rats remained unresponsive. It has been suggested that tolerance is more easily induced in neonates than it is in adults (24) and that maturing lymphocytes are more easily made tolerant than are mature ones (19). The initial contact of antigen with the immature lymphocytes of the 4-week-old animal may have produced a toleragenic signal. The lack of response early in the infection may have allowed the parasitemia to increase unchecked. Increasing parasitemia may have increased stress, which may have led to corticosteroid release. Stress-induced corticosteroids may have caused the

death of thymus cells and ultimate thymus collapse.

Severity of malarial infection in young rats has been attributed to circulating polychromasia (26). Polychromasia predisposes the young animal to infection since *P. berghei* merozoites preferentially invade reticulocytes, and compensatory reticulocytosis begins earlier in young rats than in adult rats infected with *P. berghei*.

Acquired immunity develops after about 1 week in adult rats but is delayed until the end of week 2 in immature rats (26). Zuckerman (26) suggested that the delay in the development of acquired immunity in the young rat must be an important factor in age-related immune phenomena, because rats have a clear age immunity to *P. vinckei* and this parasite invades all available erythrocytes. The slow change in the B cell population of young rats reported in this study indicates that young rats have an unresponsive B cell population and supports Zuckerman's suggestion that the delay in the development of acquired immunity, not just polychromasia of the young, is the primary factor responsible for the severe malarial infections observed in young animals.

A possible mechanism for the immunological unresponsiveness of young rats is the presence of large numbers of suppressor T cells in their spleens. Folch and Waksman (10) reported that the spleens of younger rats differ from those of older rats by having larger numbers of suppressor T cells. The presence of suppressor T cells in the spleens of young rats could prevent B cells from responding during malarial infection. The adoptive transfer of 8.7×10^7 normal 18-week-old splenic lymphocytes by intravenous injection to young rats did not enhance the 4-week-old rats' ability to control *P. berghei* infection (Gravely, unpublished data). This may indicate that there is an intrinsic factor in young rats (perhaps a suppressor T cell) that prevents even adoptively transferred, unprimed adult lymphocytes from responding as they would in adults.

It has also been suggested that the severity of malarial infection in young rats is related to the young animal's inability to respond lymphopoietically (8). In the young rat spleen, infection influences the balance between the formation of erythropoietic and immune-oriented cells (8). As infection progressed in rats, Dokow et al. (8) found that the ratio of splenocyte incorporation of ^{59}Fe to [^{3}H]thymidine incorporation reversed from 0.2 to 7.7. Rats made polycytemic by hypoxic conditions did not show the same enhanced ^{59}Fe uptake. It was suggested that a change in the balance between the formation of immunocompetent and erythropoietic cells might be critical in the fate of the infected animal. We suggest that the shift to erythropoiesis is a consequence of the inability of the lymphocytes of young rats to mount an immune response to *P. berghei* antigens. Since no immune response is made early in the infection, parasitemia increases unchecked and leads to anemia and enhanced erythropoiesis.

At day 5, B cells identifiable as CRLs were 66% of those of controls in the 18-week-old infected animals. If it is concluded that antibody-producing cells are not CRLs (2), then the number of cells possibly being transformed to the antibody-producing state upon stimulation by malarial antigen is very large. *P. berghei* is a complex microorganism. Greenwood (13) suggested that plasmodial antigens are mitogenic. It is known that gamma globulin levels, especially immunoglobulin M, increase during malarial infection. Rheumatoid factors, anti-Wasserman globulin (13), and anti-erythrocyte globulins (16) appear during malaria infection. These antibodies are not protective. Histological sections of the spleen have shown that, at the height of *P. berghei* parasitemia in BALB/c mice, the thymus-dependent areas of the spleen are populated by proliferating lymphoid cells of the plasmablast series (18). If a mitogenic capacity of *P. berghei* antigens is an acceptable hypothesis, then large numbers of B cells may have been signaled to transform during infection. This transformation may have resulted in the precipitous drop in CRLs observed in 18-week-old spleens on day 5.

Because antithymocyte serum (22) and neonatal thymectomy (4, 23) abolish the resistance of adult rats to primary infection with *P. berghei*, it may be surmised that T cells are important in the development of immunity to malarial infection. Whereas it has been suggested that T cells are important in cell-mediated immune responses (4, 22), the importance of immune globulin in conferring resistance to malarial infection and in impairing the proliferation of the plasmodia has been shown in passive transfer experiments in humans (7) and in experimental animals (5), in in vitro experiments, and in neutralization tests (14). Since T cells are required as helpers in the humoral response to most antigens (15), T cell depletion by neonatal thymectomy or by antithymocyte serum infections may have abolished helper function during malarial infection. The hypertrophy of the T cell system, followed by the hypertrophy of B cells during the recovery period in adult rats, indicates that both cell types are important in the establishment of the immune response to *Plasmodium*. The behav-

ior of T and B cells in the adults in this study may indicate that T cells are required as helpers in the humoral response.

Our results indicate that it is the inability of the young rat's lymphocytes to respond to *P. berghei* antigens early in the infection that is the principal reason for the severe malaria infections observed in young animals. The inability to respond may be due to lymphocyte immaturity, T suppressor cells, or to tolerance induction.

The collapse of the thymus and accompanying T cell depletion in the spleens of young rats compared with hypertrophy of the T cell system in adults indicate that T cells are important in the establishment of immunity to primary *P. berghei* infection. Because antibody has been shown to be protective (5-7, 14) and because, in these studies, recovery in adult rats was accompanied by hypertrophy first of the T cell system and then of the B cell system, T cells are probably required as helper cells in the establishment of a humoral response to *P. berghei*. The precipitous decrease in CRLs in the spleens of mature rats during infection may also indicate that some of the antigens associated with *P. berghei* are mitogenic for B cells.

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