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Murine Trypanosomiasis: Cellular Proliferation and Functional Depletion in the Blood, Peritoneum, and Spleen Related to Changes in Bone Marrow Stem Cells

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Previous reports have described profound effects on the function of the lymphoid system, especially the spleen, in mice infected with *Trypanosoma brucei*. This study provides further evidence of major change in the cell populations of the blood, peritoneum, and bone marrow, but shows that at least some of the stem cells of the bone marrow survive the damage caused by trypanosomes and retain their ability to repopulate the animal. In these infected mice the initial parasitemia was terminated by day 11 and was followed by a subpatent period of approximately 7 days before a final, lethal parasitemia occurred. Lymphopenia preceded the initial and final waves of parasites in the blood, and there was a marked increase in circulating neutrophils and large mononuclear cells for 1 week after the termination of the first wave of bloodstream parasitemia and during the final lethal parasitemia. Dividing macrophages were detected in the peritoneum only briefly during week 1 of infection, but the total number of peritoneal cells was increased from day 8 until the mice died. The bone marrow is severely stressed by the parasite infection. Total cell numbers and spleen colony-forming cells in the bone marrow were profoundly depleted during the resolution of the first parasitemia, but both these parameters largely recovered during the subpatent period before the mice were killed by the disease. Immune function was restored gradually after treatment with Berenil late in infection. We conclude that the progenitors of lymphocytes as well as the mature cells are affected by trypanosomes, but that some of the early bone marrow stem cells escape and rapidly repopulate the peripheral organs upon removal of the parasites.

Trypanosoma brucei infection of mice leads to severe immunosuppression (11, 17, 28). There are many studies of changes in spleen cell function which aim to determine the mechanism underlying the immunosuppression. Splenic B-lymphocytes and null (immunoglobulin −ve, θ −ve) cells, including macrophages, are stimulated to proliferate (26–28); T-lymphocytes also undergo a transient wave of division. Some T-cells become suppressive (7), but many other T-cell functions are rapidly lost (1, 6, 30). Some of the proliferating B-cells differentiate, secreting large amounts of immunoglobulin (6) of diverse specificity (17, 21) but simultaneously becoming refractory to further antigenic or mitogenic stimulation.

One explanation of this splenocyte proliferation and inactivation is that trypanosomes secrete or contain a mitogen (2, 4a, 12, 25). Trypanosomes are numerous in the spleen, but they are also present in many other sites, including bone marrow, peritoneum, and of course peripheral blood. Because the parasites have such a profound effect on mature splenic lymphocytes, we wished to trace their development to see whether lymphocyte precursors are also susceptible. This paper, therefore, describes the impact of the disease on cells in the periphery and their progenitors in the bone marrow.

The immune response is influenced not only by the functional capacity of the mature lymphocytes and their renewal from the bone marrow, but also by lymphoid architecture and recirculation. The last two have long been known to be profoundly deranged by the disease: the tissues are infiltrated by mononuclear cells (14), and edema and circulatory disorders occur (10). We have now quantitated the mononuclear cell infiltration of the peritoneum and have studied the influence of splenic structure and supply of lymphocyte precursors by following recovery of the immune response after drug treatment of the disease.

Although our investigations were by no means

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exhaustive, we conclude that trypanosomes affect lymphocyte progenitors as well as their mature counterparts, but that some of the early stem cells escape. The supply of precursor cells from the bone marrow is decreased but not exhausted, as a combined result of anemia and direct trypanosomal toxicity or mitogenicity.

MATERIALS AND METHODS

Mice. Female (CBA/H × C57BL/6)F_1 mice were bred under specific pathogen-free conditions at the National Institute for Medical Research and used at 4 to 8 months of age.

Trypanosomes. *T. brucei* subsp. *brucei* Lister S42 was kindly donated by K. M. Hudson and R. J. Terry of Brunel University.

Mice were infected with 200 *T. brucei* subsp. *brucei* S42 clone NIM2 intraperitoneally as described previously (6), except in the study of the peripheral blood leukocyte response, in which clone NIM6 was used.

[^{3}H]thymidine uptake by peritoneal macrophages. Cells were aspirated from the peritoneum with 2 ml of medium (RPMI 1640, Gibco Biocult, Scotland) supplemented with 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (0.6 mg/ml), and heparin (10 IU). After centrifugation at 4°C cells were resuspended in 2 ml of medium without heparin, and 0.4-ml portions were incubated for 4 h (37°C, humid atmosphere, 5% CO_2/air) on 12-mm-diameter circular cover slips in leukocyte migration plates with 1 μCi of [*methyl*-^{3}H]thymidine (Radiochemical Centre, Amersham; 1 Ci/mmol). Cover slips were then washed thoroughly with phosphate-buffered saline, dried rapidly, and fixed in absolute ethanol before attachment to glass slides with nail varnish. After subbing with gelatin (0.1%, wt/vol) and chrome alum (0.01%), slides were autoradiographed with Ilford KS nuclear emulsion, developed after 2 days at 4°C, stained with May-Grünewald-Giemsa, and mounted in Depex (Searle).

Cells with 10 or more grains above the nucleus were counted as positive for [^{3}H]thymidine uptake; they were classified as members of the monocyte/macrophage series by morphological criteria (see Results).

Macrophages in the peritoneal cavity were identified by their ability to take up neutral red (5). A 1/10 volume of 0.1% (wt/vol) freshly prepared neutral red in 0.25 M sucrose was added to the cells, which were then incubated for 5 min at 20°C.

Peripheral blood counts. Samples of 5 μl of tail blood were taken at various intervals after infection with NIM6 by using capillaries rinsed with heparin. The samples were diluted 1:40 in 1% acetic acid–0.01 gentian violet for nucleated cell counts, or 1:200 in phosphate-buffered saline for erythrocyte counts. Thin smears of tail blood were also taken, dried in air, fixed in methanol, and then stained for 30 min in 8% Giemsa. These smears were used for differential leukocyte and reticulocyte counts.

Splenic CFU. Splenic colony-forming units (CFU) were assessed by the method of Till and McCullough (33). Femoral bone marrow cells (0.3×10^6 to 1.0×10^6) from normal or infected mice were injected intravenously into syngeneic irradiated (850 R) syngeneic recipients. The spleens were excised 10 days later and fixed in picric acid, and the number of macroscopically visible colonies was counted.

RESULTS

Course of infection. Parasites appeared in the blood 4 days after infection, with a peak of parasitemia on days 7 to 8. After the clearance of this initial wave of parasites, they were undetectable for a few days by the methods used (the subpatent period). After this, trypanosome numbers in the blood increased again until the animals died at about day 25 of the infection. Details are given in Corsini et al. (6).

Development of anemia during infection. Erythrocytes began to decrease coincidentally with the first peak of parasitemia, the anemia being most severe on days 9 to 10 (Fig. 1). In response to the anemia, marked reticulocytosis occurred during the subpatent period—for instance, about 12% of the erythrocytes were reticulocytes on day 12 of the infection. This resulted in a transient resolution of the anemia (day 18), but the erythrocyte count fell again as the parasitemia increased, leading to the death of the infected mice.

Peripheral blood leukocyte count during infection. As shown in Fig. 2A to D, leukopenia was observed during the prepatent period and during the subpatent period after the initial peak in the parasite count. A leukocytosis was observed in association with the initial and final parasitemia. Analysis of the different cell types showed that lymphocytes were significantly decreased in the prepatent period, accompanied initially by a decrease in the number of large mononuclear cells in the circulation, and a second period of lymphopenia was seen at the onset of the terminal wave of parasites. The numbers of large mononuclear cells and neutrophils increased significantly as the parasites were first detected in the blood, and these cells were greatly increased until about a week after the initial parasitemia had been cleared. Both large mononuclear cells and neutrophils then remained at control levels until they increased in number again at the end of the infection. Eosinophil numbers were unchanged by the infection (not shown).

Cellular infiltration of the peritoneum during infection. The yield of macrophages (defined as cells able to phagocytose neutral red [5]) from the peritoneum was stable during week 1 of infection, but rose three- to fourfold after elimination of the first peak of parasites and subsequently remained at this level (Fig. 3). Small mononuclear cells showed a similar increase, and the proportion of macrophages in

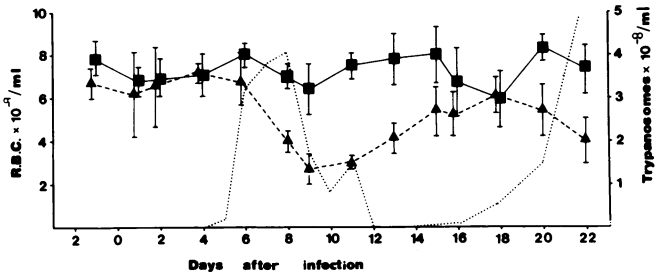

FIG. 1. *Erythrocyte counts and parasite numbers in the blood of mice infected with T. brucei and in a pair of bled control, uninfected mice. Tail blood samples were taken from five mice at each point. Each point shows the mean and standard deviation. Symbols: ■, erythrocytes in control mice; ▲, erythrocytes in infected mice; ·····, parasite numbers in infected mice.*

the cell suspension varied from 25 to 55% (30 to 45% in uninfected animals). From day 10 onwards, the macrophages in the peritoneum were larger than cells from normal mice and were very active in phagocytosis.

The origin of the increase in peritoneal macrophages was investigated by giving the cell a pulse of [^{3}H]thymidine and studying the adherent cells by autoradiography. About 50% of the peritoneal cells adhered to glass cover slips over the 4-h incubation period; 80% of these adherent cells were phagocytic. Macrophages in the fixed and stained preparations were distinguished by their abundant cytoplasm, their spreading habit, and the presence of many pinocytic vacuoles. The autoradiographs revealed a small transient wave of macrophage DNA synthesis on days 4 and 5 of the infection, but this soon declined to less than normal levels (Fig. 4). This result suggests that division in situ does not account for the increase in peritoneal macrophages.

Cell numbers and colony-forming cells in the bone marrow. The yield of nucleated cells from the bone marrow was about half normal on day 7 of the infection, remained at this level until day 19, and then partially recovered during the terminal stage. Splenic CFU showed a similar trend: very few were present at 2 weeks of infection and the colonies formed were small, but later the number of CFU recovered (Fig. 3, 5, and 6).

Recovery of the immune response after drug treatment. The results of these and our earlier studies (1, 6) indicate that most responses in mice infected with *T. brucei* S42 NIM2 are severely depressed at the end of week 2 of the infection. To assess the ability of the immune response of infected mice to recover in the absence of parasites, control mice and mice infected 14 days previously were treated by injecting into each (25 g) mouse 0.75 mg of Berenil in saline intraperitoneally. This dose suppressed the parasitemia to undetectable levels for 3 weeks, after which the infection always relapsed. For complete cure (no parasites for at least 44 days after treatment), it was necessary to give 2 mg of Berenil, but this dose had severe side effects. At 1, 4, or 10 days after drug treatment, mice were primed by injection of sheep erythrocytes and the direct (immunoglobulin M [IgM]) and indirect (IgG) PFC response was assessed (Fig. 7). The drug treatment had no significant effect on the response of uninfected mice. The IgM PFC response of infected, treated animals recovered completely by day 10 after treatment, but the indirect (IgG) response was still suppressed at this time (Fig. 7).

DISCUSSION

Previous studies of the immune response in mice infected with trypanosomes have tested the functional capacity of mature cells. In this study, we have studied the provision of precursor cells from the bone marrow during infection, relating the levels of bone marrow CFU to the parasitemia and changes in mature cells in the periphery.

As expected from the work of previous authors (8, 18, 20, 23), *T. brucei* S42 infection in mice caused anemia with accompanying reticulocytosis. The mechanism of this anemia is still controversial: increases in erythrocyte fragility (4, 18), erythrophagocytosis (20), and various autoimmune phenomena (3, 16, 19, 21, 22, 35) have been invoked. Results from the present study suggested that autoantibodies produced by polyclonal activation by B-lymphocytes (6) were not responsible for the anemia: erythrocyte destruction started before there was any significant rise in the serum immunoglobulin concentration and resolved while serum immunoglobulin levels were maximal.

The peripheral blood leukocyte response to the infection included a reduction in the lymphocyte population just before parasites appeared in the blood and considerable increases

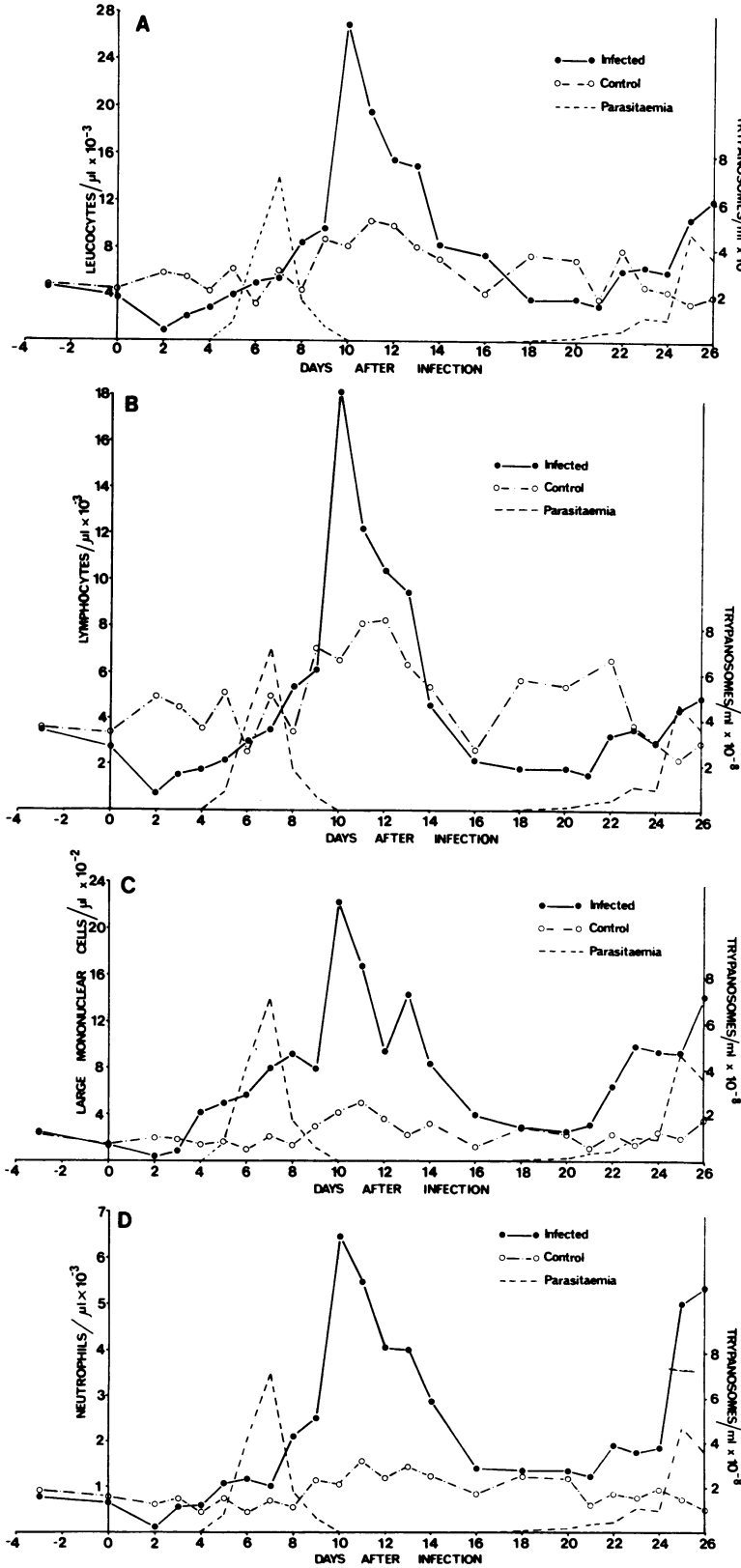

FIG. 2. *Peripheral blood leukocyte counts in infected mice and control, uninfected mice. (A) Total leukocytes, (B) lymphocytes, (C) large mononuclear cells, and (D) neutrophils. Differences between control and infected mice significant ($P < 0.05$, Student's t test) on the following days: (A) 2, 3, 4, 6, 8, 10, 12, 18, 20, 25, and 26; (B) 2 to 5, 8, 10, and 18 to 22; (C) 2 to 14, and 21 to 26; (D) 2, 6 to 14, and 22 to 26.*

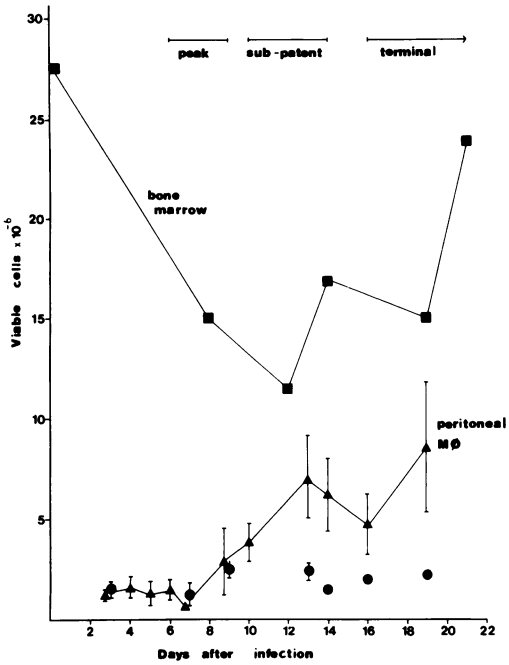

FIG. 3. *Cell numbers in the bone marrow and peritoneum during T. brucei infection in mice. Symbols: ■, bone marrow cells taken from two femurs per mouse, 3–9 mice/point. Macrophages (distinguished by neutral red uptake) taken from five mice per point. ▲, macrophages from infected mice; ●, macrophages from control mice. Arithmetic mean and standard deviation.*

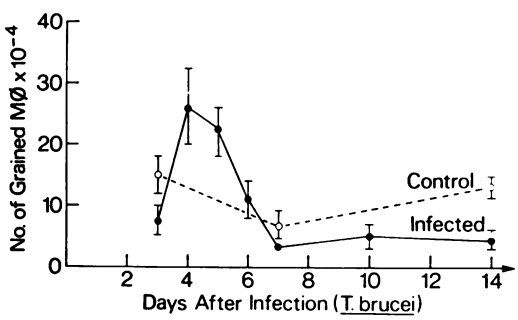

FIG. 4. *Number of peritoneal macrophages taking up [^{3}H]thymidine from (CBA × C57)F$_1$ mice during T. brucei infection. Results expressed as arithmetic mean ± standard error, five mice per group. At least 500 cells were counted per mouse. Symbols: ○, control; ●, infected.*

in the number of large mononuclear cells and neutrophils after the resolution of the initial peak of parasites and in association with the final lethal parasitemia. The increase in large mononuclear cells, which has been seen in a variety of other infected hosts, including humans, (24) could be analogous to the appearance of blast cells in the spleen and bone marrow (26). Alternatively these cells might be transitional and other immature cells related from bone marrow in response to the anemia (15, 36).

There was a dramatic increase in mononuclear cells in the peritoneum associated with the elim-

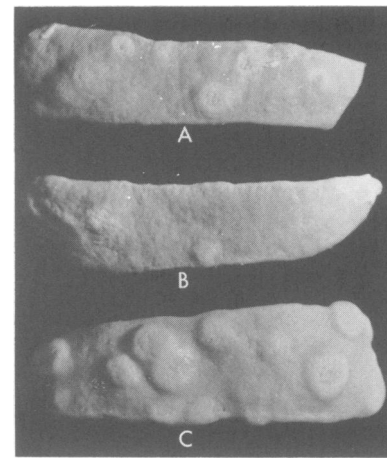

FIG. 5. *Colonies in spleens of irradiated syngeneic mice injected with 0.3 × 10^6 bone marrow cells from normal and infected mice. Spleens after bone marrow cells from mice that were (C) normal (B) infected with T. brucei 14 days previously and (A) infected 21 days previously. Magnification × 4.8.*

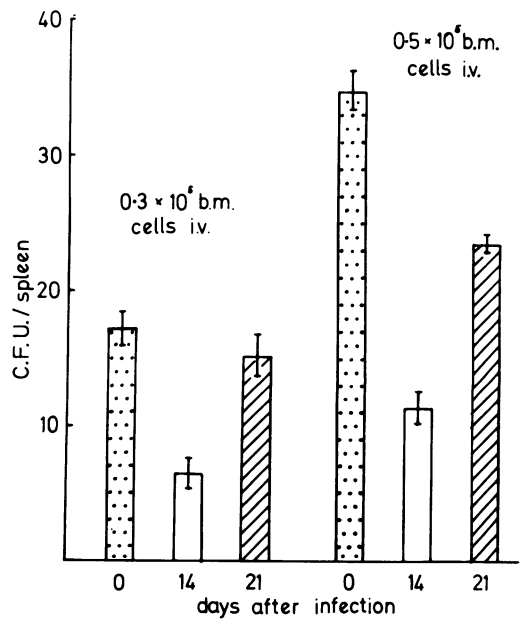

FIG. 6. *The number of splenic colony forming units during infection. Bone marrow from normal or infected mice was injected into irradiated (850 R) recipients and colonies in the spleen counted 10 days later. 5 mice/group; arithmetic mean ± standard error.*

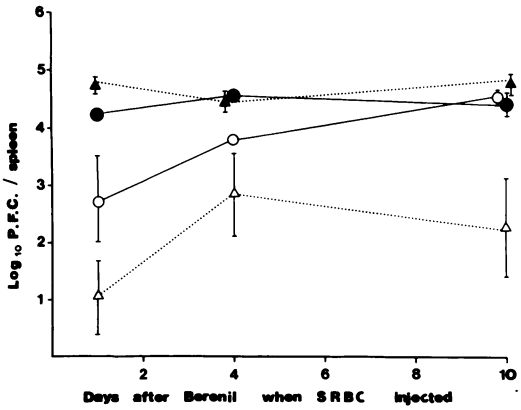

FIG. 7. *Recovery of the immune response after treatment of infection with the drug Berenil. Sheep erythrocytes were injected 1, 4, or 10 days after drug treatment of both infected and control mice, and splenic PFC was assessed 6 days later. Five mice per group. Results are expressed as geometric mean and standard error. Symbols: ▲, control IgG; ●, control IgM; △, infected IgG; ○, infected IgM.*

ination of the first peak of parasites. Trypanosomes are present in the peritoneal cavity and can be seen agglutinating there: they are probably phagocytosed by the macrophages. (Goodwin [10] reviewed similar phenomena seen in ear chambers of rabbits infected with trypanosomes.) We have already shown an increase in splenic macrophages in mice during the disease and demonstrated active suppression of lymphocyte responses by macrophages from infected animals (6). However, the extent of the contribution made by these cells to the overall picture remains to be assessed.

Histological disruption of bone marrow was evident within a few days (White, Corsini, and Askonas, unpublished data), cellularity being halved within 1 week of infection. The splenic CFU of the marrow, which are mostly erythrocyte and granulocyte progenitors (31), were depleted by about 70% at 2 weeks of infection, and the colonies that they formed were smaller than normal. However, after the period of low parasitemia, the cellularity and splenic colony-forming capacity of the bone marrow were partially restored. The very early changes in the marrow are indicative of a direct trypanosome effect. The parasites cause connective tissue disorganization (10), and they or their metabolites have been shown to be either toxic (10, 34) or mitogenic (2, 25, Clayton et al., in press). The qualitative, morphological changes in the bone marrow are analogous to those in the spleen, with an increase in the proportion of large cells (26) and cell destruction (J. White, unpublished data). In addition erythropoiesis in response to anemia is know to decrease CFU in the spleen. Because erythroid, granulocytic, and lymphoid cells have a common stem cell, (31) immunity can be impaired by competition between these three directions of differentiation (9, 13, 29).

The recovery of bone marrow late in infection and of immune responsiveness after drug treatment indicates that some of the pluripotential stem cells escape the effects of the trypanosomes. At day 14 of infection, mice are severely immunosuppressed, and splenic architecture is severely disrupted (1, 6, 17, 28). Nevertheless, the IgM response to sheep erythrocytes recovers within 10 days of drug treatment. Similarly, Berenil leads to recovery of T-cell responses in mice infected with *T. congolensi* and to repopulation of follicular areas (30). If immunosuppression is a direct consequence of trypanosome-induced multiplication and terminal differentiation of B- and T-cells (1, 6, 17), recovery of immune responsiveness will be due to a repopulation of the spleen by lymphocyte precursors. Preliminary cell transfer experiments indicate that both spleen and bone marrow of infected mice contain such precursors. The response to sheep erythrocytes could be restored if spleen or bone marrow cells from infected (14 days) mice were transferred to irradiated recipients and allowed to recover for 3 weeks, further infection being prevented by injection of human plasma (unpublished data).

The IgG response to sheep erythrocytes showed very little recovery within the first 10 days after drug treatment. This delay could be partially due to residual trypanosomes in the animal, but because none was detectable at any stage after the drug cure, we think other explanations more likely. The IgG response is probably more dependent on cooperation between B- and T-cells and, hence, also on restoration of splenic architecture and of T-cell function, than the IgM response. In addition, IgG-producing B-cells take longer to develop from stem cells than IgM producers because they are further along the B-cell differentiation pathway.

In conclusion, we examined the effect of trypanosomiasis on the ability of the mouse host to renew functional lymphoid cells, which is an important factor in the overall immune status of the animal. Maintenance of the mature cell pool depends on both the availability of immature precursors (mainly from bone marrow), and their ability to mature fully. Our investigations revealed that precursors in the bone marrow were drastically depleted in the early stages of the infection, probably due to direct trypanosome toxicity and to the competing demands of

erythropoiesis and lymphopoiesis. However, when the trypanosome numbers were temporarily decreased, the bone marrow was able to regenerate rapidly, indicating that a portion at least of the early stem cells was little affected by the disease.

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