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Bone Marrow Nitric Oxide Production and Development of Anemia in *Trypanosoma brucei*-Infected Mice

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Mice infected with *Trypanosoma brucei* rapidly develop anemia, with the number of circulating erythrocytes reduced by 50% within a week after infection. The present study investigated the relationship between anemia and bone marrow nitric oxide (NO) production. Bone marrow cell populations from *T. brucei*-infected mice exhibited elevated levels of NO synthase activity which was inhibitable by N^G-nitro-L-arginine methyl ester. NO production was found to coincide with suppressed bone marrow T-cell proliferation in response to stimulation with the mitogen concanavalin A both in vitro and in vivo. As this indicated that NO may inhibit proliferation in other cell types, particularly hemopoietic precursors, we examined the role of NO in anemia during trypanosome infection. NO production correlated directly with the development of anemia, and treatment of infected mice with N^G-nitro-L-arginine methyl ester in vivo to systemically inhibit NO synthesis led to a significant reduction in the anemia. Thus, elevated NO production in the bone marrow of *T. brucei*-infected mice is likely to play a significant role in the anemia resulting from *T. brucei* infection.

African trypanosome (*Trypanosoma brucei*) infections in humans, livestock, and experimental rodent hosts result in the development of parasite-induced anemia. The condition is characterized by a reduction in erythrocyte survival time (2, 5, 19) and significantly decreased packed cell and total erythrocyte volumes (19). Depletion of erythrocytes may occur through intravascular hemolysis or phagocytosis. Although *T. brucei* infection results in a severe suppression of lymphocyte proliferation in response to specific antigens (1), there is also a massive polyclonal B-cell proliferation. This results in the production of large quantities of immunoglobulins, particularly of the immunoglobulin M class (11). Parasite-specific antibody production is only a small fraction of the elevated immunoglobulin M production, with the majority of the antibodies reacting with nonparasite antigens. Some of these antigens have been identified as host antigens (10), including the constituents of erythrocytes (13), and opsonization of erythrocytes by autoantibody is one suggested cause of anemia in trypanosomiasis. However, intravascular hemolysis by complement is unlikely to be a major mechanism of cell destruction because infected C5-deficient mice exhibit a degree of anemia similar to that of normocomplementic mice (12). Erythrocyte destruction may also be a consequence of the phagocytosis of opsonized cells, and macrophages engorged with erythrocytes have been observed in *T. brucei* infections (18, 25). However, experiments involving the artificial induction of immunoglobulin M directed against autologous erythrocytes suggest that this mechanism alone does not account for the development of anemia. Maintenance of a mature cell pool depends on both the availability of immature precursors (mainly from the bone marrow) and their ability to mature fully. Studies have shown that the bone marrow is severely stressed during infections with *T. brucei* (4) and *Trypanosoma musculi* (8). Impairment of bone marrow function and of iron reutilization in cattle infected with *Trypanosoma congolense* has also been reported (5). As we have previously shown that nitric oxide (NO) is involved in splenic immune dysfunction in *T. brucei* infection, in this paper we describe the role of NO in erythropoiesis in the bone marrow.

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MATERIALS AND METHODS

Trypanosomes and mice. Pleomorphic *T. brucei* AnTaT 1.1 (26) was used throughout this study. The bloodstream forms of this parasite were axenically cultivated in vitro in a modification (14) of the medium previously described by Baltz et al. (3). Female C3H/He mice (8 to 12 weeks old) were obtained from Harlan Olac (Bicester, United Kingdom), and infections were initiated with 5×10^5 parasites injected intraperitoneally. Control mice received phosphate-buffered saline (PBS) alone. In some experiments, following inoculation, mice from each experimental group were randomly divided into two groups; one group received untreated drinking water, and the other group received water supplemented with N^G-nitro-L-arginine methyl ester (L-NAME; Sigma, Poole, United Kingdom) at 1 mg/ml. This solution was prepared daily. The parasitemia was monitored at various times by diluting 5 μl of tail blood with 95 μl of 0.85% NH_4Cl solution and counting with a hemocytometer.

Hematological observations. Samples (5 μl) of tail blood were taken at various times and diluted 1:200 in PBS-heparin. Erythrocytes were enumerated by hemocytometer counting. To determine the total blood hemoglobin concentration, tail blood was lysed with distilled water, cell debris was removed by centrifugation ($13,000 \times g$, 5 min), and the hemoglobin concentration in the supernatant was estimated by difference spectroscopy as previously described (9). Bovine hemoglobin (Sigma) was used as a standard.

Bone marrow cell proliferation. Femurs were removed and flushed with 5 ml of ice-cold RPMI 1640 (Gibco, Paisley, United Kingdom) and the resulting cell suspension was washed by centrifugation at $250 \times g$ for 10 min at 4°C. The cell suspension was depleted of erythrocytes by NH_4Cl lysis, washed, and resuspended in RPMI 1640 supplemented with 100 μg of penicillin-streptomycin (Gibco) per ml, 2 mM L-glutamine (Gibco), and 5% heat-inactivated horse serum (Gibco). Quadruplicate cultures were established in 96-well plates with 2×10^5 cells per flat-bottomed well and stimulated with 5 μg of concanavalin A (Boehringer-Mannheim, Lewes, United Kingdom) per ml. In parallel sets of cultures, L-NAME was included in the medium at 0.5 mM. The final volume of each culture was 200 μl. After 48 h of incubation at 37°C in a 5% CO_2-air atmosphere, cultures were pulsed for 16 h with [^{3}H]thymidine (25 Ci/mmol; 0.5 μCi per well; Amersham, Little Chalfont, United Kingdom). Cells were harvested, and thymidine incorporation was determined by liquid scintillation counting.

Nitrite and nitrate assays. For the measurement of NO production in bone marrow cells, cultures were established in 24-well plates with 10^6 cells per well. In parallel sets of cultures, L-NAME was included in the medium at 0.5 mM. The total volume of each culture was 1 ml. Following a 48-h incubation at 37°C in a 5% CO_2–air atmosphere, culture supernatants were sampled for the determination of nitrite concentration by the Griess assay as previously described (6). To measure NO synthesis in vivo, serum nitrate was measured. Tail blood samples (5 μl) were diluted 1:20 in PBS, and the cells were removed by centrifugation at $250 \times g$ for 5 min. The supernatant was retained, and 80-μl volumes were assayed

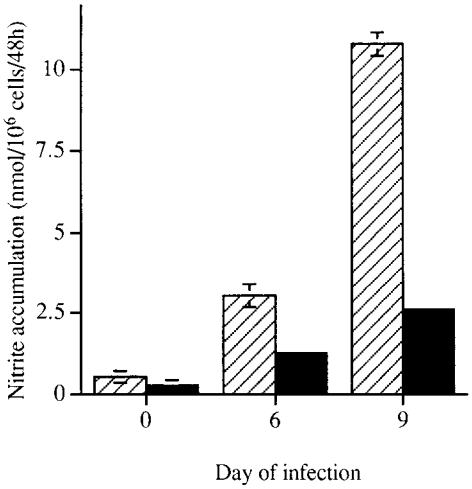

FIG. 1. NO synthesis by bone marrow cells from *T. brucei*-infected mice. Bone marrow cells from control, 6-day-infected, and 9-day-infected mice were cultured for 48 h with (solid bars) or without (hatched bars) L-NAME (0.5 mM) prior to nitrite measurement. Each point is the mean ± standard deviation for quadruplicate cultures (absence of error bar indicates a standard deviation smaller than the resolution of the histogram).

for nitrate with *Aspergillus* nitrate reductase followed by the Griess assay as previously described (14).

RESULTS AND DISCUSSION

Production of nitric oxide by bone marrow cells from *T. brucei*-infected mice. Bone marrow cells from naive and 6-day and 9-day *T. brucei*-infected mice were cultivated for 48 h ex vivo, and the nitrite concentration in the supernatant (NO_2^-, the stable end product of NO synthase activity) was determined. Nitrite accumulation was significantly enhanced in cell cultures derived from infected mice, compared with accumulation in cells from naive mice (Fig. 1). When the NO synthase inhibitor L-NAME was included in the culture medium, nitrite accumulation was significantly reduced, indicating that the cells were producing NO via L-arginine-dependent NO synthase.

Nitric oxide suppresses bone marrow proliferation in vitro and in vivo. Previous work has suggested that production of NO by the bone marrow may limit bone marrow cell proliferation (20). In order to determine whether the production of NO in the bone marrow during *T. brucei* infections inhibits the cell proliferation in this tissue, bone marrow cell cultures were stimulated with concanavalin A. Cultures from naive mice typically showed thymidine incorporations of 10,000 cpm, reflecting the proliferation of the T cells present in the bone marrow. In infected mice, proliferation was significantly reduced compared with that of control mice (Fig. 2a). When L-NAME was included in the culture medium, the suppressed proliferative response was completely abrogated. The proliferative response of bone marrow cells from naive mice was not affected by the inclusion of L-NAME in the culture medium. Background proliferation of unstimulated cells was typically 500 cpm in both naive and infected animals and was not significantly affected by treatment with L-NAME either in vivo or in vitro. This result provides evidence that the inhibition of proliferation in bone marrow cell cultures is mediated by NO. In order to determine if NO is also involved in the inhibition of bone marrow proliferative responses in vivo, we systemically inhibited NO synthesis in experimental mice by the addition of L-NAME to the drinking water (23). Treatment of infected

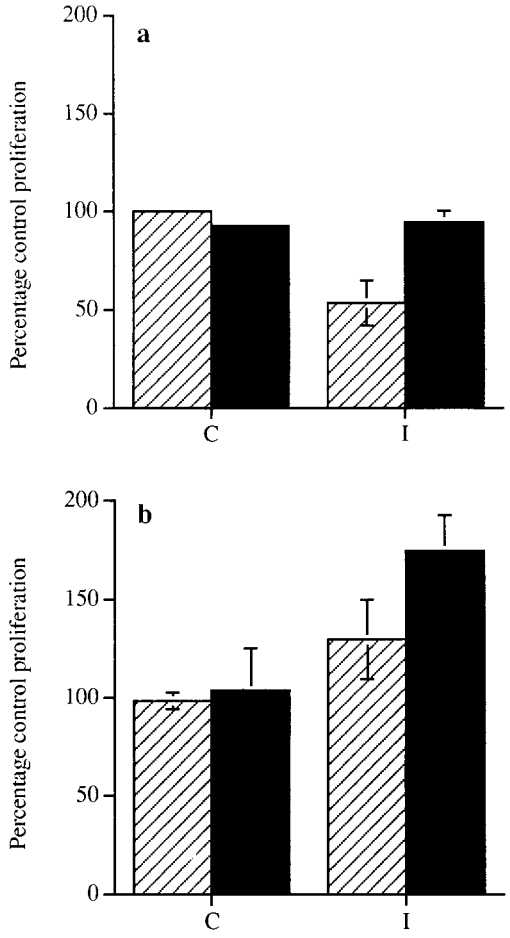

FIG. 2. Suppression of bone marrow cell response to concanavalin A. Mice were given untreated drinking water (a) or drinking water containing 1 mg of L-NAME per ml (b) for the duration of the experiment. Bone marrow cells were cultured at 2×10^5 per microtiter well with 5 μg of concanavalin A per ml (hatched bars). L-NAME was added to a parallel set of cultures at 0.5 mM (solid bars). Each result is the mean ± standard deviation for quadruplicate cultures and is representative of at least two experiments. C, control mice; I, infected mice.

mice with L-NAME from the time of infection completely abolished the suppression of T-cell proliferation (Fig. 2b). L-NAME treatment had no significant effect on the proliferation of bone marrow cells from naive mice. Interestingly, the combination of L-NAME treatment of both infected animals in vivo and the resulting bone marrow cell cultures ex vivo led to a significant increase in proliferative activity over that of controls. This may be related to the role of restricted levels of NO production in the normal control of cell proliferation in this tissue, as suggested elsewhere (20). Thus, during *T. brucei* infection, bone marrow-synthesized NO directly inhibits T-cell proliferation. Other workers have provided evidence for the role of NO in the control of proliferation in the myelomonocytic cell compartment in the bone marrow (20) and lymphocytes in the spleen (16).

Relationship between anemia and NO production in *T. brucei*-infected mice. The result described above indicates that in *T. brucei* infection, NO inhibits bone marrow lymphocytes, and this raises the possibility that hemopoietic cells are affected in a similar manner. In order to test this hypothesis, we examined the relationship between parasitemia, serum nitrate concentra-

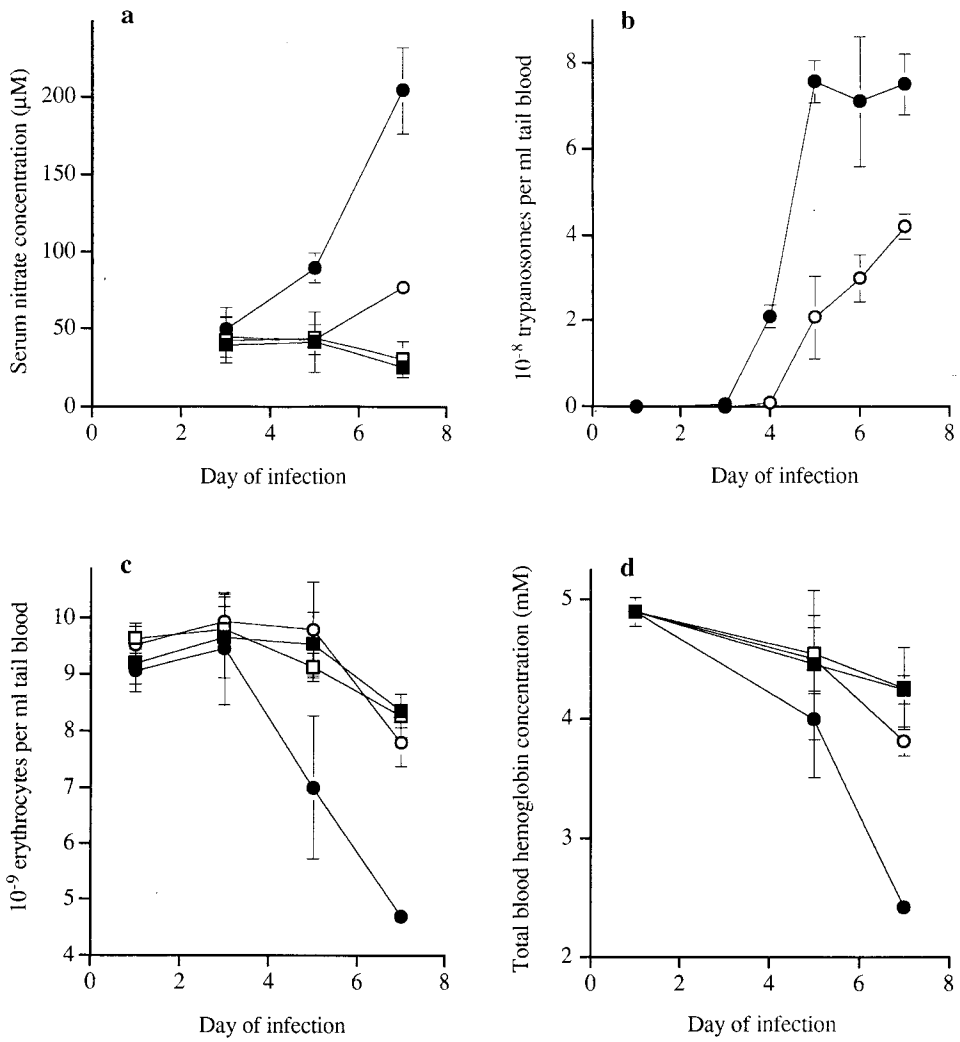

FIG. 3. Changes in hematological parameters in naive (squares) and *T. brucei*-infected (circles) mice. (a) Serum nitrate concentration; (b) parasitemia; (c) erythrocyte density; (d) hemoglobin concentration. Each point is the mean ± standard deviation for five mice and is representative of three similar experiments. Closed and open symbols represent mice given untreated and L-NAME-treated drinking water, respectively.

tion, and erythrocyte and hemoglobin concentration in tail blood of *T. brucei*-infected mice, one group of which was treated in vivo with L-NAME.

Figure 3a and b show that serum nitrate concentration correlated with rising parasitemia in the infected animals. Mice treated with L-NAME showed an effective blockade of NO synthesis, as evidenced by the reduction in serum nitrate concentration to control levels (Fig. 3a). This treatment also resulted in a reduced parasitemia (Fig. 3b), a phenomenon which has been reported previously (23). During infection mice rapidly developed severe anemia, evidenced by a 50% reduction in erythrocyte numbers in tail blood samples, and this reduction was totally reversed in mice which were treated with L-NAME (Fig. 3c). Likewise, when blood hemoglobin concentration was measured, infected mice showed a significant reduction which was also abolished in L-NAME-treated infected mice (Fig. 3d). These results demonstrate a causal relationship between NO production in *T. brucei*-infected mice and the development of anemia. In light of this finding combined with the findings on NO production in the bone marrow of infected animals and its effect on bone marrow cell proliferation, we propose that NO acts directly on the proliferation of immature erythrocytes or hemopoietic stem cells. Interestingly, these findings clarify an earlier observation that corticosteroid treatment, which down-regulates NO synthesis (7, 15), reduces the development of anemia in *T. brucei*-infected mice (2). It is possible that, as in the case of immunosuppression (21), factors other than NO are also involved in causing anemia in trypanosomiasis. One candidate is tumor necrosis factor alpha, which is up-regulated during trypanosomiasis and has been directly implicated in causing anemia in other parasitic infections including malaria (22).

These results provide a new example of the involvement of NO production in pathogenesis during *T. brucei* infection. We have previously shown that NO production is not a significant mechanism against bloodstream trypanosomes in vivo, as it rapidly reacts with hemoglobin in erythrocytes to yield methemoglobin and nitrate (14, 23). Instead, it seems likely that NO causes damage to host cells. In the spleens of *T. brucei*-infected mice, this is apparent in the suppression of lymphocyte responses to both mitogen and specific antigen (21, 24) and is likely to be an important factor in the generalized immuno-

suppression observed in trypanosomiasis. We propose that in the bone marrow, NO has a similar antiproliferative effect, one manifestation of which is anemia resulting from an inhibition of hemopoiesis. There remains the question of why the damaging effects of NO in both spleen and bone marrow are not buffered by the large population of mature erythrocytes. One possibility is that the architecture of these organs leads to a close physical association of activated macrophages and relevant stem cells. Treatment of infected animals with an inhibitor of NO synthase leads to the restoration of erythrocyte and hemoglobin levels as well as the recovery of lymphoproliferative responses. Interestingly, this treatment leads to improved control of acute parasitemia (23). While inhibitors of NO synthesis such as L-NAME have too broad a range of physiological effects (17) to be considered effective therapeutic agents, an understanding of the mechanisms of macrophage activation in trypanosomiasis may enable the development of effective NO inhibitory strategies and hence allow improved control of trypanosome infections.

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