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NITRIC OXIDE IS INVOLVED IN EARLY HOST DEFENSE AGAINST A PRIMARY INFECTION WITH *BABESIA MICROTI* IN MICE

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ABSTRACT

The role of nitric oxide in immune defense against primary infection with *Babesia microti* was investigated in the present study. *Babesia microti* infected C57Bl mice reach peak of parasitemia around day 14 PI. with approximately 45% PRBC and a moderate fall in hematocrit . C57Bl mice treated with the iNOS-inhibitor aminoguanidine reached a 55% PRBC peak parasitemia and the hematocrit dropped to approximately 29% PBCV. C57Bl iNOS-/- mice reached a peak parasitemia of more than 80% PRBC within 10 days PI. and their hematocrit dropped to about 20% PBCV. The clearance of parasites from the peripheral blood and recovery of the hematocrit in aminoguanidine-treated C57Bl mice and iNOS-/-mice takes much longer than in the C57Bl wildtype control mice. During the course of infection there were marked differences in cytokine expression patterns of IFN-γ and TNF-α between C57Bl wildtype and C57Bl iNOS-/- mice. C57Bl wildtype mice treated with anti-IFN-γ mAB followed a different course of infection and survived the infection. On the other hand iNOS-/-mice treated with anti-IFN-γ mAB failed to control the rapid rise in parasitemia and died. Surviving animals of the iNOS-/- and anti-IFN-γ mAB-treated iNOS-/- groups subsequently developed sever kidney damage. These results show that NO is involved in early control of primary infection with *Babesia microti* , but also suggest that for the control of the acute stage of infection the effect of IFN-γ is not exclusively mediated through the induction of NO.

INTRODUCTION

Babesiae are tick-transmitted hemoprotozoan parasites which infect wild and domestic animals and cause enormous economical losses in cattle (McCosker 1981). *Babesia microti* is a rodent-pathogen species. In mice *B. microti* causes a transient high parasitemia and moderate drop in PBCV, but generally is not lethal, infected mice recover spontaneously (Ruebush and Hanson 1979). Cell-mediated immunity mediated by CD4+T cells and IFN-γ plays an important role in protecting the mice against primary infection (Igarashi et al. 1994, Shimada et al.1996).

Nitric oxide is a highly reactive compound with numerous physiological properties (Nathan and Xie 1994, Schmidt and Walter 1994). Nitric oxide plays an important role in the immune response against protozoan parasites such as *Plasmodium* species (Nussler et al. 1993, Seguin et al. 1994), *Toxoplasma gondii* (Scharton-Kersten et al. 1997) and *Leishmania major* (Liew et al. 1990). During infection IFN-γ and TNF-α trigger the

inducible isoform of nitric oxide synthase in macrophages to produce NO (Beckerman et al. 1993, Liew et al. 1990). To demonstrate the role played by NO in the immune defense against *B. microti* the selective iNOS-inhibitor Aminoguanidine (Griffiths et al. 1993, Misko et al. 1993) and mice genetically deficient in iNOS (McMicking et al. 1995, Wei et al. 1995) were used. Because parasitemia in iNOS-/- mice shows some similarities to the course of parasitemia in mice depleted of IFN- IFN-γ (Igarashi et al. 1994), and the importance of IFN-γ for the induction of NO production is also well known (Liew et al. 1990, Beckerman et al. 1993) the comparison of mice treated with mAB against IFN-γ and iNOS-/- mice, as well as iNOS-/- mice treated with mAB against IFN-γ should show if the protective effect of IFN-γ is mediated through the induction of NO.

MATERIALS AND METHODS

Mice and parasites: Breeding pairs of mice with a disrupted iNOS gene (iNOS-/-) were generously provided by Dr. J.S. Mudgett (Merck Research Laboratories). BALB/c mice and the C57Bl/6 mice used as controls for this study were purchased from CLEA (Tokyo/Japan). All mice were between 6-9 weeks of age at the beginning of the experiments, all experiment groups were sex and age matched. *B. microti* (Munich strain) was kindly provided by Prof. A.O. Heydorn from the Institute of Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin. The mice were inoculated intraperitoneally (i.p.) with 1x107 parasitized erythrocytes (PRBC).

Treatment with aminoguanidine: Mice were injected with 0.2 ml of a 45 mg/ml solution aminoguanidine hemisulfate (Sigma Chemical Co., St. Louise, USA) 2x daily i.p. from day 1 to day 10 post inoculation with the parasites (PI) (Stevenson et al. 1995).

Parasitemia and hematocrit: Parasitemia was monitored every 2 days by counting the PRBC in Giemsa-stained blood smears. Hematocrit was monitored at 4 day intervals in blood from orbital puncture using the microhematocrit method.

Monoclonal antibodies mAB: Ascites fluid from nude mice injected i.p. with the anti-IFN- γ mAB secerning hybridoma cell clone XMG1.2 was kindly provided by Dr. Igarashi. The mAB were purified by 50% ammonium sulfate precipitation and dialyzed against phosphate buffered saline (PBS). The resulting protein concentration of the mAB solution was determined by the Bradford method (Bradford 1976) using bovine serum albumin as a standard. Mice were injected i.p. with 1 mg of the anti-IFN- γ mAB in 0.2 ml saline on day -1 PI, day 0 PI and there after at 4 day intervals until the end of the experiment. Control mice were injected at the same time with 1 mg normal rat IgG (SEROTEC, UK).

Measurement of NO, IFN-γ and TNF- γ: Blood samples of individual mice were collected by orbital puncture using microhematocrit capillary tubes. The serum was separated and stored in -80°C until use. For measurement the serum samples of the individual mice of each group were pooled and the concentrations of NO, IFN-γ and TNF-α measured by a colorimetrical reaction (NO) and ELISA test kits (IFN-γ and TNF-α) (Cayman Chemical Company, Ann Arbor, USA and ENDOGEN Inc., Cambridge, USA, respectively).

Tissue sections: Tissue samples from selected animals were collected at the end of the experiment and stored in 10% phosphate buffered formalin. For histological examination paraffin sections of kidneys, livers and spleens

were prepared by a commercial company (Sanco Medical Center, Obihiro, Japan) and stained with hematoxylin/eosin (HE) and periodic acid/Schiff's reagent (PAS).

Urine examination: 1,2 ml urine was obtained from the bladder of a just died mouse. The protein concentration of the urine was determined using the Bradford method with bovine serum albumin as a standard. The size of the proteins in the urine was determined by running the urine and normal mouse serum in SDS-PAGE, using a 7,5% gel concentration in a MINI-PROTEAN II cell (BIO-RAD, Richmond, USA). After electrophoresis the proteins were stained with 0.1% Coomasie® Brilliant Blue (FLUKA Chemie AG, Buchs, Switzerland). For examination of the sediment the urine was centrifugated at 15,000 rpm for 15 min, the sediment transferred on a glass slide and examined with a light microscope.

RESULTS

Normal C57Bl mice infected with *B. microti* developed acute infection with a peak parasitemia of 45.5 % PRBC and a hematocrit of 31 % PBCV at day 14 PI, whilst C57Bl mice treated with aminoguanidine had a peak parasitemia of 54.8 % PRBC and hematocrit of 29.3 % PBCV, also around day 14 PI. (Fig. 1). This differences became even clearer in BALB/c mice. Here the normal BALB/c mice and Aminoguanidine-treated BALB/c mice had peak parasitemia of 44.9 % PRBC to 62.8 % PRBC and the hematocrit was 27.8 % PBCV and 20.8 % PBCV, respectively (Fig. 2). Mice treated with aminoguanidine showed more severe clinical signs with forced breathing, piloerection and cold limbs and recovery was later than in their untreated controls.

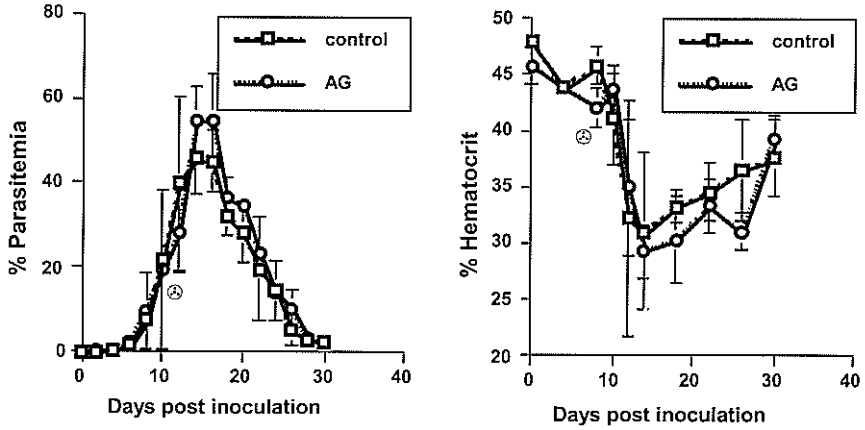

Figure 1. Course of *B. microti* infection and hematocrit in untreated (control) and aminoguanidine-treated (AG) C57Bl mice. Parasitemia and hematocrit as mean of 4 animals in both groups. > = death of 1 animal in the AG-treated group at day 10 PI

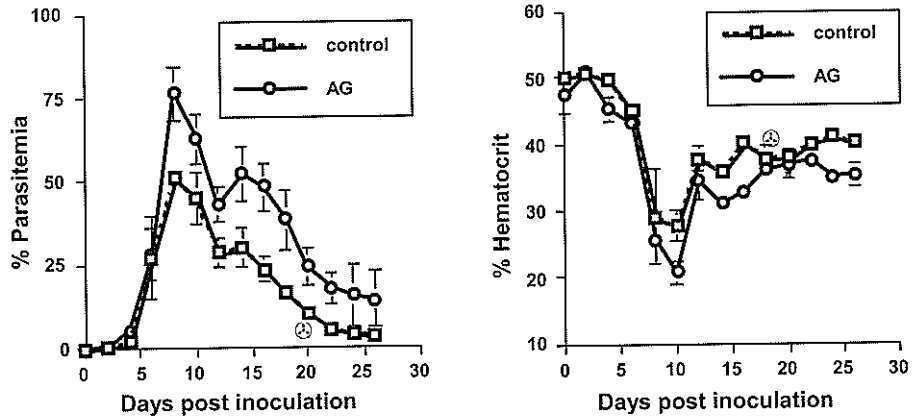

Figure 2. Course of *B. microti* infection and hematocrit in untreated (control) and aminoguanidine-treated (AG) BALB/c mice. Parasitemia and hematocrit as mean of 4 animals in both groups. > = death of 1 animal in the AG-treated group at day 20 PI.

In iNOS-/- mice peak parasitemia occurred much earlier, on day 10 PI as compared to day 14 PI in C57Bl controls. The peak parasitemia in iNOS-/- mice was with 83.3 % PRBC much higher and the hematocrit with 21.3 % PBCV much lower (Fig. 3). However, in contrast to the aminoguanide-treated C57Bl mice the iNOS-/- mice showed comparatively mild clinical signs, indistinguishable from the clinical signs observed in control mice.

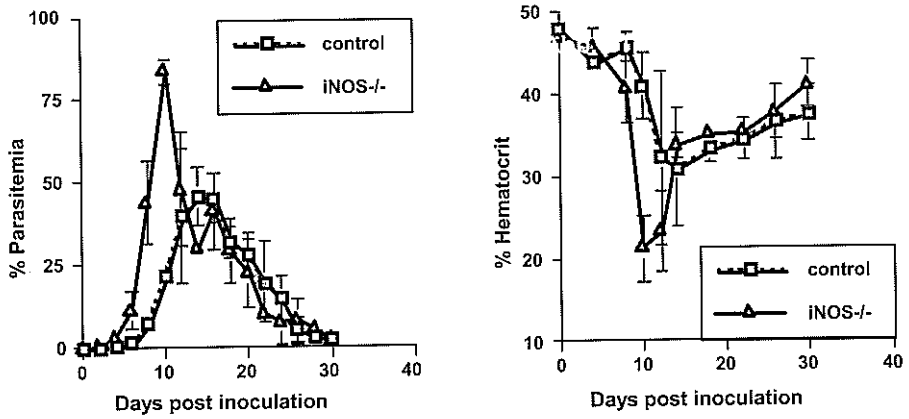

Figure 3. Course of *B. microti* infection and hematocrit in C57Bl (control) and iNOS-/- mice. Parasitemia and hematocrit as mean of 4 animals in the control group and 3 animals in the iNOS-/- group.

In normal C57Bl mice infected with *B. microti* the concentration of NO had two peaks. The first peak concentration was 4 days PI. when the first parasites appeared in the peripheral. During the high parasitemia phase the concentration decreased markedly, only to increase again later reaching a second peak at the end of the parasitemia. In aminoguanidine-treated mice the concentration of NO remained low throughout the duration of parasitemia (Fig. 4). The expression patterns of IFN-γ and TNF-α in control C57Bl mice and aminoguanidine-treated mice were in principal similar. There were two peak concentrations in both groups. The highest, peak concentration occurred at peak parasitemia and a second moderate second peak concentration occurred during the last third of parasitemia. The second peak concentration was much lower in aminoguanidine-treated mice. In contrast, the IFN-γ and TNF-α concentrations in iNOS-/- mice had only a moderate first peak, but later increased to very high levels at the end of parasitemia (Fig. 5 and 6).

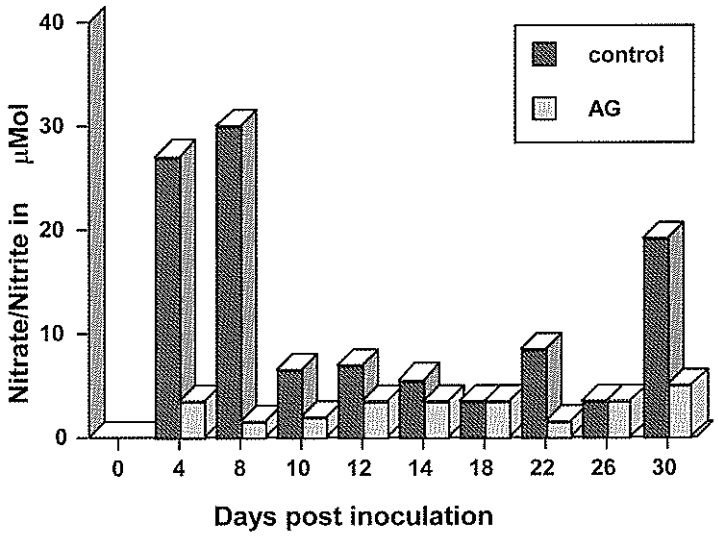

Figure 4. NO levels measured as nitrate/nitrite in the serum of untreated (control) and aminoguanidine-treated (AG) mice during an infection with *B. microti.* Pooled serum of 4 animals in the control group and 5 animals in the aminoguanidine-treated group.

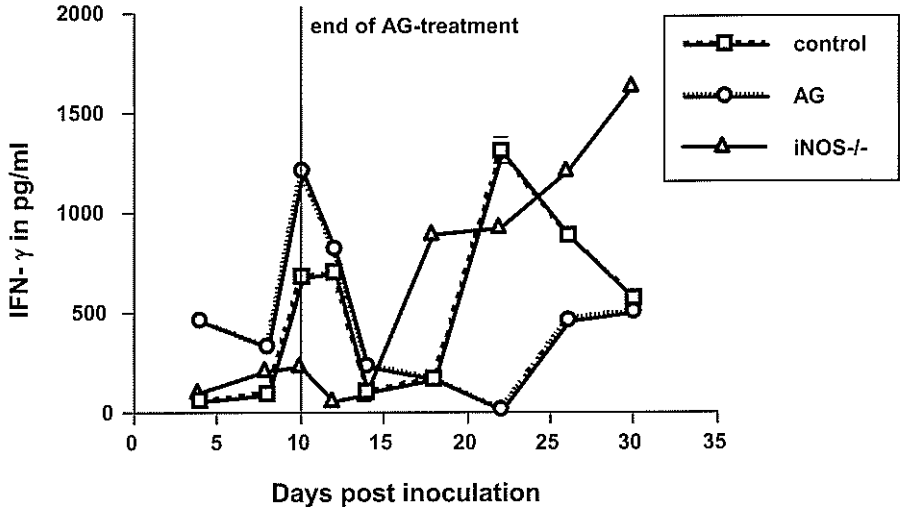

Figure 5. IFN-γ expression in untreated C57Bl mice (control), aminoguanidine-treated C57Bl mice (AG) and iNOS-/- mice during the course of infection with *B. microti*. Pooled serum of 4 mice in the control, 5 mice in the aminoguanide-treated group and 6 mice in the iNOS-/- group.

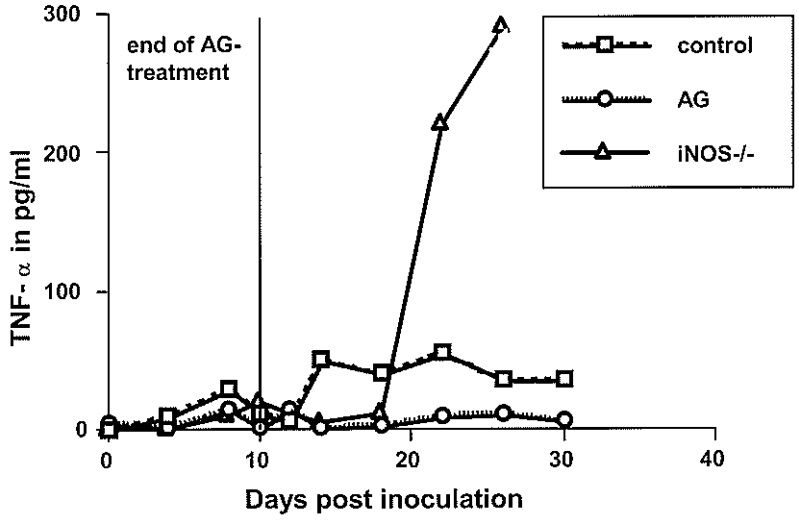

Figure 6. TNF-α expression in untreated C57Bl mice (control), aminoguanidine-treated C57Bl mice (AG) and iNOS-/- mice during the course of infection with *B. microti*. Pooled serum of 4 mice in the control, 5 mice in the aminoguanide-treated group and 6 mice in the iNOS-/- group.

Groups of C57Bl and iNOS-/- mice treated with anti-IFN-γ mAB developed a more severe course of infection when compared to their untreated controls, but the outcome of infection in iNOS-/- mice was still different from

that of control mice with NO (Fig. 7). Hematocrit showed an inverse proportionality to the parasitemia in all four groups of mice (Fig. 8).

Normal C57Bl mice and iNOS-/- mice showed similar mild clinical signs, but anti-IFN-γ mAB-treated C57Bl mice developed more serious clinical signs. Anti-IFN-γ mAB-treated iNOS-/- mice showed severe clinical signs and most mice of this group died at peak parasitemia. Prior to death the mice had a low body temperature, overall weakness of the limbs and facial edema as the major clinical signs. At death these mice had lost about 20 % of their body weight when compared to day 0 of the experiment (data not shown).

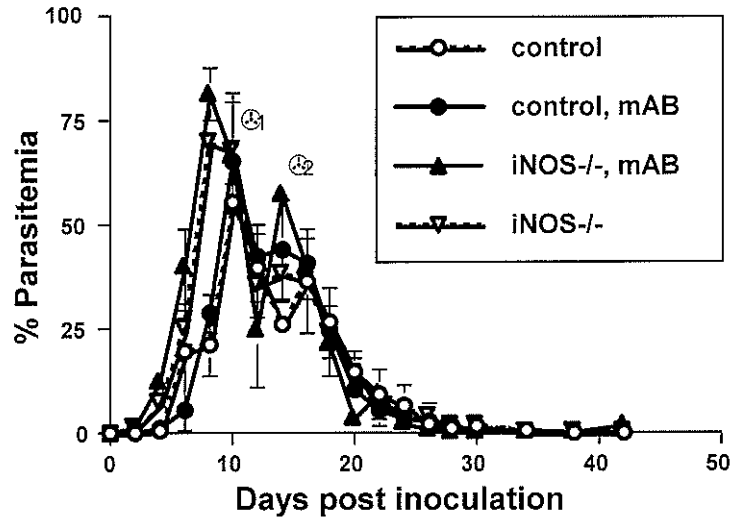

Figure 7. Course of parasitemia in iNOS-/- and C57Bl mice treated with mAB against IFN-γ and their control groups without mAB-treatment. Mean of 3 animals in the untreated C57Bl group (control), 4 animals in the mAB-treated C57Bl group (control, mAB) and 5 animals in the mAB-treated and untreated iNOS-/- groups. >1 = day 10 PI only 3 mice in the mAB-treated iNOS-/- group left, >2 = day 14 PI only 2 mice in the mAB-treated iNOS-/- group left.

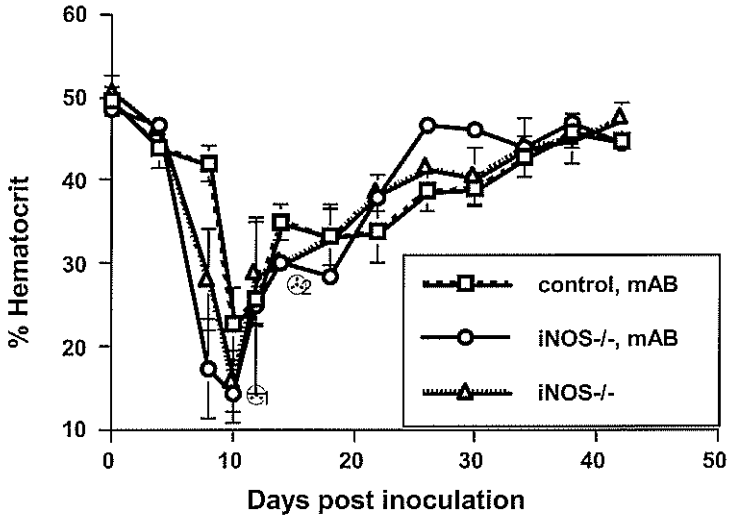

Figure 8. Hematocrit during *B. microti* infection in iNOS-/- and C57Bl mice treated with mAB against IFN-γ and their control groups without mAB-treatment. Mean of 4 animals in the mAB-treated C57Bl group (control, mAB) and 5 animals in the mAB-treated and untreated iNOS-/- groups.>1 = day 10 PI only 3 mice in the mAB-treated iNOS-/- group left, >2 = day 14 PI only 2 mice in the mAB-treated iNOS-/- group left.

Urine from the anti-IFN-γ mAB-treated iNOS-/- mouse showed proteinuria with 2,78 mg/ml protein in the urine and abundant erythrocytes in the sediment. SDS-PAGE analysis of the urine showed a large band of 66 kDa molecular weight that corresponds to the albumin-fraction of the mouse serum (data not shown). Also histologically there was a clear difference between the iNOS-/- mice and the control groups. While in both C57Bl groups only minor mesangial changes could be observed, with slightly higher cell density in glomerula of mAB-treated mice, iNOS-/- mice had broad cellular infiltrations around degenerated glomerula and mAB-treated iNOS-/- mice showed enlargement of the Bowman's capsula with cell proliferation and amorphe masses in the capsulae (Fig. 9). Also in the spleen group-specific differences could be observed: control mice showed a clear pattern of follicles with single mitosis in untreated animals, but "tubes" of dense packed dark cells in the follicle centers of mAB-treated control mice, while in untreated iNOS-/- mice the follicles were somehow confluent and the follicle centers appeared depleted of cells. In mAB-treated iNOS-/- mice a clear follicle pattern was lost, the observed picture "chaotic" (Fig. 10).

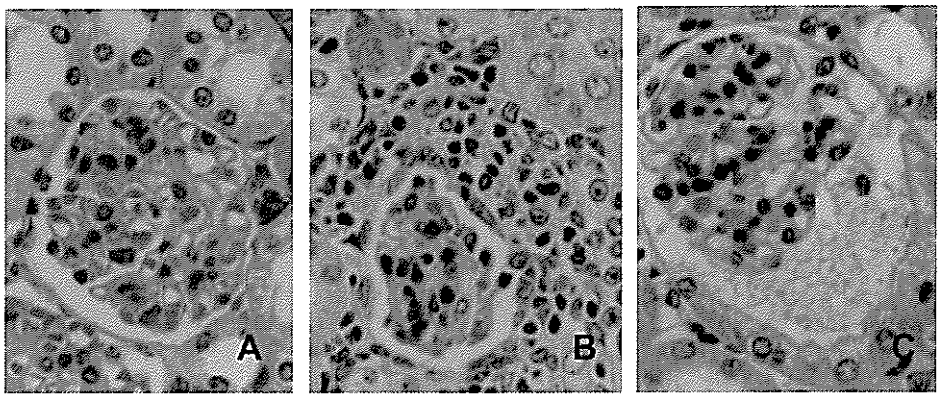

Figure 9. HE stained kidney sections. A, mesangial changes at the glomerulum of a mAB-treated control mouse. B, cellular infiltrations around the degenerated glomerulum of a iNOS-/- mouse. C, enlargement and cell proliferation of the Bowman's capsula of a mAB-treated iNOS-/- mouse.

DISCUSSION

Mice -either genetically or by treatment with aminoguanidine- unable to produce NO have difficulties in controlling the multiplication of *B. microti* in the blood and subsequently suffer from severe anemia. This is more evident in iNOS-/- mice which genetically lack the NO synthesis mechanism.

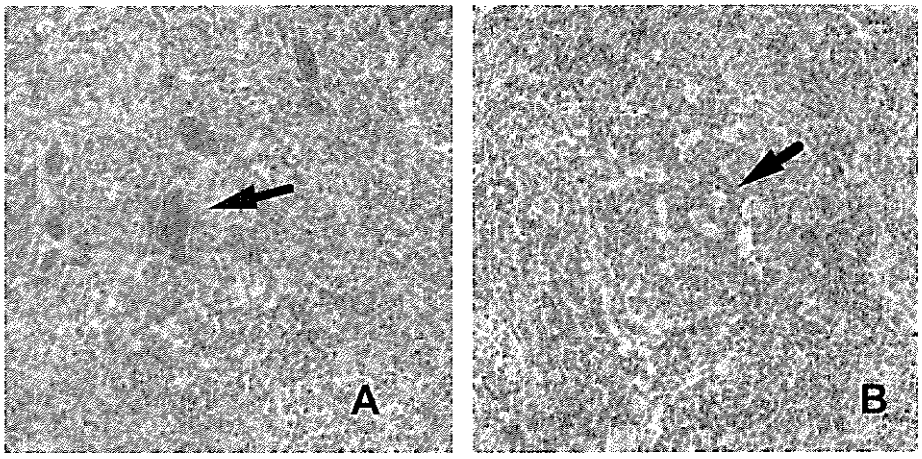

Figure 10. A, tubes of dark staining cells in the spleen of a mAB-treated C57Bl mouse. B, area depleted of cells in the follicle center of a iNOS-/- mouse.

The early peak of NO in the serum (during *B. microti* infection) suggests that this compound plays a major role in non-specific cell-mediated immune response against the parasites, thus determining to some extent the course

of infection. The different patterns of cytokine-expression between iNOS-/- mice and wildtype controls suggest that the two groups of mice employ partly different immune defense mechanisms against the invading parasites than their controls with the principle ability to produce NO. For deeper knowledge of the involved mechanisms more experiments to this point need to be done.

The results of the experiments with mAB against IFN-γ in iNOS-/- mice and wildtype controls show that despite some similarities in the infection-course -higher main peak, marked second peak and later remission of the parasitemia- the course of parasitemia in iNOS-/- mice and anti-IFN-γ mAB-treated mice is still different. This suggests that in immune defense against *B. microti* the protective effect of IFN-γ is not only based on the induction of NO production but acts through other mechanisms independent from the building of NO, too. This conclusion gets supported by the fact that mice without NO additionally treated with mAB against IFN-γ are unable to control the infection and die or suffer severe organ damage.

Taken all our results together we can conclude that NO is also involved in early immune defense against *B. microti* in mice. The effect of IFN-γ herein bases only partwise on the stimulation of macrophages (activation) and involves some other effector mechanisms beside the NO system.

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