Parasite killing in murine malaria does not require nitric oxide production

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SUMMARY

Nitric oxide (NO) production has been suggested to play a role as effector molecule in the control of the malarial infections. However, the roles of this molecule are debated. To assess whether blood-stage parasite killing is NO dependent, we investigated the course of blood-stage *Plasmodium chabaudi chabaudi* (Pcc) infections in inducible nitric oxide synthase (iNOS)-deficient mice. Parasitaemia, haematological alterations, and survival were not affected by the lack of iNOS. To exclude a role of NO produced by other NOS, controls included NO suppression by oral administration of aminoguanidine (AG), a NOS inhibitor. As in iNOS-deficient mice, no difference in the parasitaemia course, survival and haematological values was observed after AG treatment. Our results indicate that NO production is not required for protection against malaria in our murine experimental model. However, C57BL/6 mice treated with AG lost their resistance to Pcc infections, suggesting that the requirement for NO production for parasite killing in murine blood-stage malaria might be strain dependent.

Key words: malaria, nitric oxide, *Plasmodium chabaudi*, iNOS-deficient mice.

INTRODUCTION

In the case of malaria, several reports suggested that NO might play an important role as an effector molecule in the development of resistance in mice (Taylor-Robinson *et al*. 1993; Mellouk *et al*. 1994; Rockett *et al*. 1994; Jacobs, Radzioch & Stevenson, 1996). NO has been shown *in vitro* to be toxic for exoerythrocytic stages of *P. berghei* (Mellouk *et al*. 1991) and for asexual erythrocytic stages of *P. falciparum* (Rockett *et al*. 1991). Moreover, it has been shown that resistance to blood-stage malaria in mice correlates with nitric oxide synthase expression in the liver (Jacobs, Radzioch & Stevenson, 1995).

However, the importance of NO in malaria is debated. Other experimental data indicate that NO might not be involved in parasite killing (Cavacini *et al*. 1989; Ghigo *et al*. 1995; Jacobs *et al*. 1995; Jones *et al*. 1996). Thus, it has been hypothesized that although NO is an important contributor to the control of malaria, it might not be essential (von der Weid & Langhorne, 1993; Taylor-Robinson, 1995*a*, *b*). Moreover, we have previously shown that in IFN-γ receptor (IFN-γR)-deficient mice the development of anti-malarial protection is impaired, as compared to wild-type mice. However, in these experiments no significant differences in NO production were found between IFN-γR-deficient and wild-type mice during blood-stage infections, indicating that NO production might not be a critical factor for protection (Favre, Ryffel & Rudin, 1997).

In the present report, we analysed the role of NO production in the resolution of murine malaria by infecting iNOS-deficient mice with non-lethal Pcc. Administration of aminoguanidine, a nitric oxide synthase inhibitor, was included to exclude other possible defects due to the iNOS deficiency in these mice and to inhibit NO production by other nitric oxide synthases (Nathan & Xie, 1994).

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MATERIALS AND METHODS

Mice

Wild-type and iNOS-deficient (MacMiking *et al*. 1995) mice (C57BL/6 × 129Sv/Ev) were kindly provided by Professor M. Aguet (ISREC, Epalinges, Switzerland). They were bred and housed under specific pathogen-free conditions with standard chow and water *ad libitum*. C57BL/6 mice were purchased from BRL (Füllinsdorf, Switzerland) and maintained under similar conditions.

Parasites and infection

Plasmodium chabaudi chabaudi AS was a kind gift from Dr D. Walliker (Edinburgh, Scotland). Infections and monitoring were performed as described (Favre, Ryffel & Rudin, 1999).

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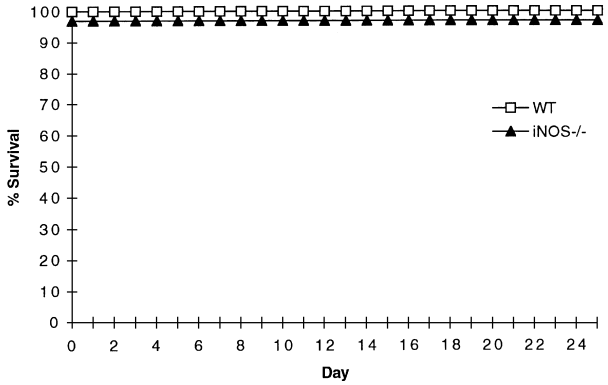

Fig. 1. Survival in wild-type mice (□) and iNOS-deficient mice (▲) after Pcc infection. Pooled data from 2 experiments ($n = 15$).

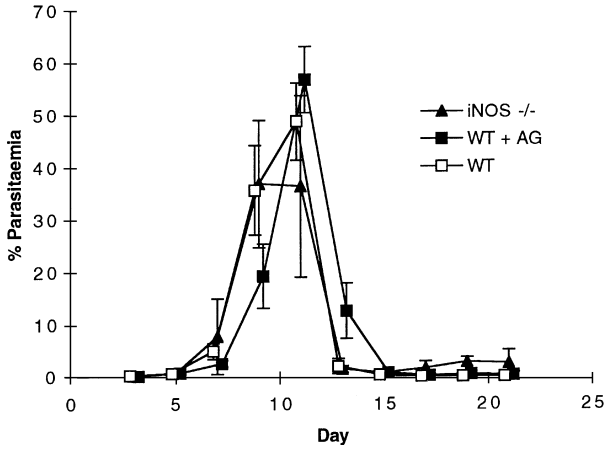

Fig. 2. Parasitaemia in wild-type mice ($n = 5$), wild-type mice treated with AG ($n = 5$) and iNOS-deficient mice ($n = 7$) infected with 10^5 Pcc parasitized RBP i.p. at day 0. Data from 1 experiment. Results are represented as means ± S.D.

Aminoguanidine treatment

Aminoguanidine (Sigma, Buchs, Switzerland), a specific nitric oxide synthase inhibitor, was added to the drinking water as described (Favre *et al*. 1999).

Measurements of nitric oxide (NO)

NO concentration in serum was assessed after reduction of NO_3^- as described (Favre *et al*. 1999).

Statistics

Results are given as means of at least 5 animals ± standard deviation. Differences between wild-type and iNOS-deficient mice were analysed by Student's *t*-test. $P \leqslant 0{\cdot}005$ was considered as being significant. All experiments were repeated at least twice.

RESULTS

To determine the importance of NO production in the acquisition of protective immunity in murine malaria, we infected iNOS-deficient mice with 10^5 red blood cells infected with non-lethal *Plasmodium chabaudi chabaudi* (Pcc).

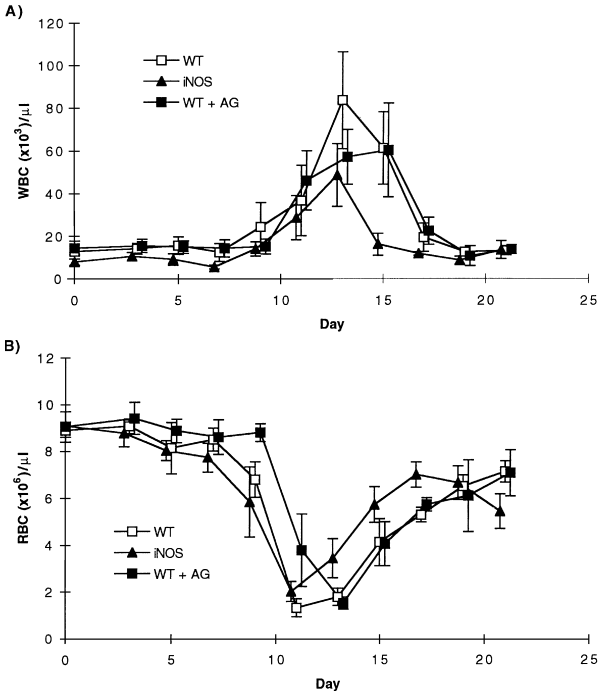

Fig. 3. Time-course of haematological values during Pcc infection. (A) Leukocytes ($\times 10^3/\mu$l) and (B) erythrocytes ($\times 10^6/\mu$l) numbers in tail blood of wild-type mice ($n = 5$), wild-type mice treated with AG ($n = 5$) and iNOS-deficient mice ($n = 7$) infected with 10^5 Pcc-parasitized RBC i.p. at day 0. Values were measured every second day in 10 μl of blood. Data from 1 experiment. Results are represented as means ± S.D.

Survival

We asked whether iNOS deficiency has an influence on the resistance to mouse malaria infections. iNOS deficiency did not alter the course of non-lethal Pcc infection, as 100% of the iNOS-deficient mice survived ($n = 10$), as did wild-type mice (Fig. 1). Wild-type and iNOS-deficient mice treated with AG, a specific nitric oxide synthase inhibitor (Haddad, Duclos & Baines, 1995), displayed the same results as non-treated mice (data not shown).

Parasitaemia, anaemia and leukocytosis

We investigated whether iNOS deficiency has any influence on parasitaemia and on haematological alterations during Pcc infection.

Fig. 2 shows the course of parasitaemia in wild-type and iNOS-deficient mice infected with Pcc. There was no significant difference in parasitaemia between the different groups of mice at any time of the infections. After a second, small parasitaemia peak around day 21, no parasites were observed in blood up to day 60 after infection for all groups. The results obtained with AG-treated iNOS-deficient and wild-type mice were similar to those obtained

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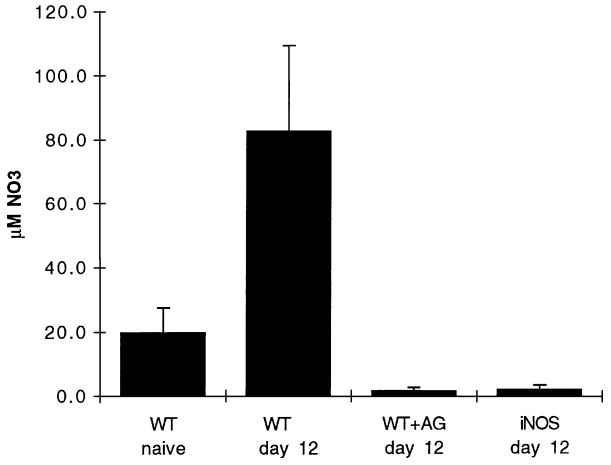

Fig. 4. Serum nitrate (NO_3^-) levels during Pcc infection. Sera were obtained by heart puncture from wild-type mice at day 0 (WT Day 0; $n = 3$), and at the peak of the parasitaemia (WT Peak; $n = 5$), AG-treated wild-type mice (WT + AG Peak; $n = 5$) and iNOS-deficient mice (iNOS Peak; $n = 5$). Serum NO_3^- levels were determined, after enzymatic reduction, by Griess reaction. Results are represented as means ± S.D.

with non-treated mice. All groups of mice were similarly protected against subsequent infections (data not shown).

Fig. 3 summarizes the haematological values in Pcc-infected wild-type mice, iNOS-deficient mice and AG-treated wild-type mice. The extent of anaemia was similar in all types of mice during Pcc infections.

Leukocytosis peaked 2 days after the parasitaemia peak. Interestingly, in iNOS-deficient mice, the peak leukocytosis was lower than in wild-type mice ($48{\cdot}6 \pm 14{\cdot}7 \times 10^3$ WBC/μl, $83{\cdot}7 \pm 22{\cdot}8 \times 10^3$ WBC/μl, respectively; $P < 0{\cdot}005$). The peak leukocytosis observed in wild-type mice treated with AG was intermediate ($57{\cdot}1 \pm 12{\cdot}9 \times 10^3$ WBC/μl).

NO levels in serum

We assessed NO production by measuring the levels of nitrate (NO_3^-) in sera at the time of the peak of parasitaemia. As shown in Fig. 4, high levels of nitrate were only detectable in the sera from wild-type mice infected with Pcc at the time of the parasitaemia peak at day 12.

No NO was detectable in the sera of any of the AG-treated animals during Pcc infection.

DISCUSSION

Here we report that iNOS-deficient mice are equally protected against *Pasmodium chabaudi chabaudi* malaria as wild-type mice are, and that these mice develop protective immunity, as wild-type mice do.

During Pcc infections significant production of NO is usually confined to a brief period, corresponding to the peak of the primary parasitaemia (Taylor-Robinson *et al*. 1993). This happens early in the infection, when a Th1-type immune response predominates (Langhorne *et al*. 1989). It has been suggested that Th1 cells play a role in protective immunity, either by directly producing NO as an effector molecule (Taylor-Robinson *et al*. 1994) or by activating the NO production by macrophages through the secretion of IFN-γ (Taylor-Robinson *et al*. 1993). NO has been shown to have direct microbicidal and parasiticidal activities by interacting with enzymes, sulphydryl groups, or superoxides (Crawford *et al*. 1994; Clark & Rockett, 1996). Moreover, it has been shown that NO regulates IL-2 and IFN-γ production by Th1 cells, and might be important in the regulation of the switching between Th1- and Th2-type immune responses (Taylor-Robinson *et al*. 1994).

We have previously demonstrated for IFN-γR-deficient mice that their susceptibility to *P. c. chabaudi* infection is increased (Favre *et al*. 1997). However, we did not find significant differences of NO serum levels at the time of the parasitaemia peak in IFN-γR-deficient mice, as compared to wild-type mice. These results combined with the present ones obtained with iNOS-deficient mice suggest that parasite control and killing do not require NO production but do, however, depend on IFN-γR signalling. Moreover, IFN-γ itself could be toxic for the blood-stage parasites *in vivo*, as reported for exoerythrocytic stages (Mellouk *et al*. 1991). Alternatively, NO could be more important in anti-sporozoite protection (Tsuji *et al*. 1995), than against the blood-stage forms of the parasite.

Although murine experimental malaria models have proved to be appropriate to understand human malaria (Taylor-Robinson, 1995*a*, *b*), there are discrepancies between human malaria and mouse malaria. The role of macrophage activation and release of nitric oxide might also differ between mice and men (Crawford *et al*. 1994). It has been suggested that, in some cases, human macrophages may preferentially use oxygen-independent pathways for their anti-microbial activity, e.g. degradation of extracellular tryptophan, or modulation of iron metabolism (Moncada, Palmer & Higgs, 1989; Murray *et al*. 1989; Murray & Teitelbaum, 1992; Weinberg, 1992). Therefore, the importance of NO in *Plasmodium* killing might be different between mice and men, as men might preferentially use different pathways in parallel.

Interestingly, in accordance with the observation of Jacobs *et al*. (1995), we observed that treatment with AG depletes the resistance of inbred C57BL/6 mice to Pcc infections (data not shown). However, we did not observe this phenomenon with our C57BL/6 × 129Sv/Ev wild-type mice, although they have a close genetic background. This discrepancy could be due to an increased genetic susceptibility of

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C57BL/6 mice, or to the lack, or dysregulation, of other protective pathways. This observation reinforces the importance of the genetic background for the susceptibility to malaria in mice.

It is possible that knock-out mice develop alternative pathways to overcome in-born deficiencies. Moreover, in iNOS-deficient mice there might be an up-regulation of the constitutive NOS (eNOS, nNOS) to compensate the deficiency of the inducible NOS (Granger & Hibbs, 1996). To avoid such artefacts, we treated mice with aminoguanidine, that completely suppresses NO production, as controls. Similarly to iNOS deficiency, this treatment did not affect the course of the infections. Thus, our data demonstrate that NO production is not required for protection in malaria. However, the combination of our results with the ones obtained with C57BL/6 mice suggest that the importance of NO production in the resistance to malaria might be strain specific. This might explain some of the contradictory data obtained with different human populations, where important differences were found, for example NO correlated with cerebral malaria in Papua New Guinea (Al Yaman *et al*. 1996), but an inverse correlation was observed in Tanzania (Anstey *et al*. 1996).

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REFERENCES

AL YAMAN, F. M., MOKELA, D., GENTON, B., ROCKETT, K. A., ALPERS, M. P. & CLARK, I. A. (1996). Association between serum levels of reactive nitrogen intermediates and coma in children with cerebral malaria in Papua New Guinea. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90**, 270–273.

ANSTEY, N. M., WEINBERG, J. B., HASSANALI, M. Y., MWAIKAMBO, E. D., MANYENGA, D., MISUKONIS, M. A., ARNELLE, D. R., HOLLIS, D., McDONALD, M. I. & GRANGER, D. L. (1996). Nitric oxyde in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *Journal of Experimental Medicine* **184**, 557–567.

CAVACINI, L. A., GUIDOTI, M., PARKE, L. A., MELANCON-KAPLAN, J. & WEIDANZ, W. P. (1989). Reassessment of the role of splenic leukocyte oxidative activity and macrophage activation in expression immunity to malaria. *Infection and Immunity* **57**, 3677–3682.

CLARK, I. A. & ROCKETT, K. A. (1996). Nitric oxide and parasitic disease. *Advances in Parasitology* **37**, 1–56.

CRAWFORD, R. M., LEIBY, D. A., GREEN, S. J., NACY, C. A., FORTIER, A. H. & MELTZER, M. S. (1994). Macrophage activation: a riddle of immunological resistance. *Immunology Series* **60**, 29–46.

FAVRE, N., RYFFEL, B. & RUDIN, W. (1997). The course of *Plasmodium chabaudi chabaudi* infection in interferon-gamma receptor deficient mice. *Parasite Immunology* **19**, 375–383.

FAVRE, N., RYFFEL, B. & RUDIN, W. (1999). The development of murine cerebral malaria does not require nitric oxyde production. *Parasitology* **188**, 135–138.

GHIGO, D., TODDE, R., GINSBURG, H., COSTAMAGNA, C., GAUTRET, P., BUSSOLINO, F., ULLIERS, D., GIRIBALDI, G., DEHARO, E., GABRIELLI, G. & ET, A. L. (1995). Erythrocyte stages of *Plasmodium falciparum* exhibit a high nitric oxide synthase (NOS) activity and release an NOS-inducing soluble factor. *Journal of Experimental Medicine* **182**, 677–688.

GRANGER, D. L. & HIBBS, J. B. JR (1996). High-output nitric oxide: weapon against infection? *Trends in Microbiology* **4**, 46–49.

HADDAD, E. K., DUCLOS, A. J. & BAINES, M. G. (1995). Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. *Journal of Experimental Medicine* **182**, 1143–1151.

JACOBS, P., RADZIOCH, D. & STEVENSON, M. M. (1995). Nitric oxide expression in the spleen, but not in the liver, correlates with resistance to blood-stage malaria in mice. *Journal of Immunology* **155**, 5306–5313.

JACOBS, P., RADZIOCH, D. & STEVENSON, M. M. (1996). In vivo regulation of nitric oxide production by tumor necrosis factor alpha and gamma interferon, but not by interleukin-4, during blood stage malaria in mice. *Infection and Immunity* **64**, 44–49.

JONES, I. W., THOMSEN, L. L., KNOWLES, R., GUTTERIDGE, W. E., BUTCHER, G. A. & SINDEN, R. E. (1996). Nitric oxide synthase activity in malaria-infected mice. *Parasite Immunology* **18**, 535–538.

LANGHORNE, J., GILLARD, S., SIMON, B., SLADE, S. & EICHMANN, K. (1989). Frequencies of CD4+ T cells reactive with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with Th1 and Th2 characteristics during infection. *International Immunology* **1**, 416–424.

MacMIKING, J. D., NATHAN, C., HOM, G., CHARTRAIN, N., FLETCHER, D. S., TRUMBAUER, M., STEVENS, K., XIE, Q. W., SOKOL, K. & HUTCHINSON, N. (1995). Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81**, 641–650.

MELLOUK, S., GREEN, S. J., NACY, C. A. & HOFFMAN, S. L. (1991). IFN-gamma inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. *Journal of Immunology* **146**, 3971–3976.

MELLOUK, S., HOFFMAN, S. L., LIU, Z. Z., DE, L. V. P., BILLIAR, T. R. & NUSSLER, A. K. (1994). Nitric oxide-mediated antiplasmodial activity in human and murine hepatocytes induced by gamma interferon and the parasite itself: enhancement by exogenous tetrahydrobiopterin. *Infection and Immunity* **62**, 4043–4046.

MONCADA, S., PALMER, R. M. J. & HIGGS, E. A. (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochemical Pharmacology* **38**, 1709–1715.

MURRAY, H. W., SZURO-SUDOL, A., WELLNER, D., OCA, M. J., GRANGER, A. M., LIBBY, D. M., ROTHERMEL, C. D. & RUBIN, B. Y. (1989). Role of tryptophan degradation in respiratory burst-independent anti-microbial activity of gamma-interferon-stimulated human macrophages. *Infection and Immunity* **57**, 845–849.

MURRAY, H. W. & TEITELBAUM, R. F. (1992). L-arginine-dependent reactive nitrogen intermediates and the anti-microbial effect of activated human mononuclear phagocytes. *Journal of Infectious Diseases* **165**, 513–517.

NATHAN, C. & XIE, Q.-W. (1994). Nitric oxide synthases: roles, tolls and controls. *Cell* **78**, 915–918.

ROCKETT, K. A., AWBURN, M. M., COWDEN, W. B. & CLARK, I. A. (1991). Killing of *Plasmodium falciparum in vitro* by nitric oxide derivatives. *Infection and Immunity* **59**, 3280–3283.

ROCKETT, K. A., AWBURN, M. M., ROCKETT, E. J., COWDEN, W. B. & CLARK, I. A. (1994). Possible role of nitric oxide in malarial immunosuppression. *Parasite Immunology* **16**, 243–249.

TAYLOR-ROBINSON, A. W. (1995*a*). Murine models of cerebral malaria: a qualified defence. *Parasitology Today* **11**, 407–409.

TAYLOR-ROBINSON, A. W. (1995*b*). Regulation of immunity to malaria: valuable lessons learned from murine models. *Parasitology Today* **11**, 334–342.

TAYLOR-ROBINSON, A. W., LIEW, F. Y., SEVERN, A., XU, D., MCSORLEY, S. J., GARSIDE, P., PADRON, J. & PHILLIPS, R. S. (1994). Regulation of the immune response by nitric oxide differentially produced by Th1 and Th2 cells. *European Journal of Immunology* **24**, 980–984.

TAYLOR-ROBINSON, A. W., PHILLIPS, R. S., SEVERN, A., MONCADA, S. & LIEW, F. Y. (1993). The role of TH1 and TH2 cells in a rodent malaria infection. *Science* **260**, 1931–1934.

TSUJI, M., MIYAHIRA, Y., NUSSENZWEIG, R. S., AGUET, M., REICHEL, M. & ZAVALA, F. (1995). Development of antimalaria immunity in mice lacking IFN-gamma receptor. *Journal of Immunology* **154**, 5338–5344.

VON DER WEID, T. & LANGHORNE, J. (1993). The roles of cytokines produced in the immune response to the eythrocytic stages of mouse malarias. *Immunobiology* **189**, 397–418.

WEINBERG, E. D. (1992). Iron depletion: a defense against intracellular infection and neoplasia. *Life Sciences* **50**, 1289–1297.