The development of murine cerebral malaria does not require nitric oxide production

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SUMMARY

Nitric oxide (NO) production has been suggested to be required for the development of cerebral malaria. However, the importance of this molecule for the appearance of this pathology is debated. To assess whether murine cerebral malaria is NO dependent, we investigated the course of blood-stage *Plasmodium berghei* ANKA (PbA) infections in inducible nitric oxide synthase (iNOS)-deficient mice. Parasitaemia, haematological alterations, survival and development of cerebral malaria 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 a crucial factor for the development of murine cerebral malaria.

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

INTRODUCTION

For humans, it has been hypothesized that NO could play a role in the appearance of cerebral malaria symptoms as plasma reactive nitrogen intermediates (RNI) concentrations correlate with the severity of the disease (Clark, Rockett & Cowden, 1992; Nüssler, Eling & Kremsher, 1994; Al Yaman *et al*. 1996; Anstey *et al*. 1996). Furthermore, we showed previously that TNF-α/β double-deficient mice infected with PbA were protected against cerebral malaria. The NO production was impaired in these mice, suggesting that TNF induced NO may also have a pathogenic role in mice (Rudin *et al*. 1997).

However, the importance of NO in cerebral malaria is debated. Other experimental data indicate that NO might not be crucial in the development of murine cerebral malaria (Asensio, Oshima & Falanga, 1993; Kremsner *et al*. 1993; Jones *et al*. 1996). Moreover, we have previously shown that IFN-γ receptor (IFN-γR)-deficient mice are protected against cerebral malaria, although in these experiments no significant differences in NO production were found between IFN-γR-deficient and wild-type mice during blood-stage infections (unpublished results).

In the present report, we infected iNOS-deficient mice with PbA, a *Plasmodium* strain that induces cerebral malaria in mice (Taylor-Robinson, 1995*a*), to investigate the role of NO for the development of this complication. 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*.

Parasites and infection

Plasmodium berghei ANKA was a kind gift from Dr D. Walliker (Edinburgh, Scotland). Infections were performed by i.p. injection with 10^5 parasitized red blood cells. Parasitaemia was scored on Giemsa-stained tail-blood smears. Standard haemograms were performed daily with 10 μl of fresh heparinized tail-blood on a Sysmex microcellcounter F-500 (Digitana AG, Zürich, Switzerland).

Aminoguanidine treatment

Aminoguanidine (Sigma, Buchs, Switzerland), a specific nitric oxide synthase inhibitor, was added to

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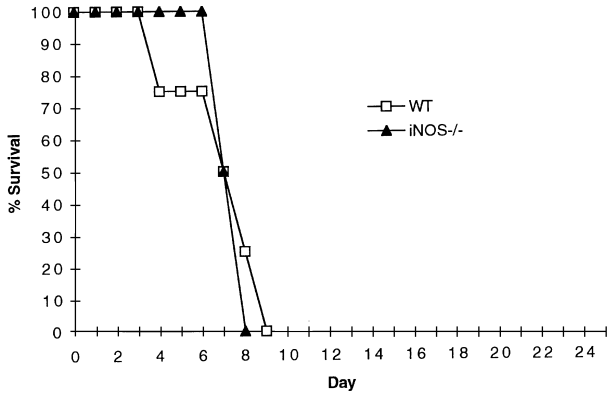

Fig. 1. Survival in wild-type mice (□) and iNOS-deficient mice (▲) after PbA infections. Pooled data from 2 experiments ($n = 10$).

the drinking water of PbA-infected iNOS-deficient and wild-type mice at a concentration of 40 mM, as previously described (Haddad, Duclos & Baines, 1995).

Measurements of nitric oxide (NO)

As no NO_2^- was detectable in serum, NO_3^- was reduced using nitrate reductase (Boehringer Mannheim, Rotkreuz, Switzerland) as described elsewhere (Rockett *et al*. 1992). Briefly, 25 μl of serum were incubated with 10 μl of enzyme per well and 50 μl of Tris–nicotinamide–adenine dinucleotide phosphate for 1 h at 37 °C. Then 10 μl LDH/pyruvate solution were added followed after 15 min by the addition of 100 μl of Griess reagent. The absorbency (A_{540}) was measured using an ELISA reader.

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 assess the role of NO in the development of cerebral malaria, we infected iNOS-deficient mice with 10^5 red blood cells infected with *P. berghei* ANKA (PbA).

Development of cerebral malaria

As shown in Fig. 1, iNOS-deficient and wild-type mice did not survive PbA infections and all mice died between day 5 and day 10. Wild-type and iNOS-deficient mice treated with AG, a specific nitric oxide synthase inhibitor (Haddad *et al*. 1995), displayed the same results as non-treated mice (data not shown).

All the animals showed neurological symptoms before death, such as postural disorders, paralysis, impaired reflexes and loss of grip strength (Rudin *et al*. 1997). This was also observed in all the control groups (wild type, wild type + AG, iNOS + AG). No difference was observed between all 4 groups for the time of the onset and severity of cerebral malaria. Micro-vascular brain lesions, assessed by the leakage of i.v. injected Evans blue (Rudin *et al*. 1997), were observed in all PbA-infected animals (data not shown), in accordance with the appearance of symptoms of cerebral malaria. Parasitaemia at the onset of cerebral malaria (10–15 %) was similar in all groups.

Parasitaemia and haematological values

We investigated whether iNOS deficiency has any influence on parasitaemia and on haematological alterations during PbA infections. Fig. 2 shows the course of parasitaemia in wild-type and iNOS-deficient mice infected with PbA. There was no significant difference in parasitaemia between the different groups of mice at any time of the infections. The results obtained with AG-treated iNOS-deficient and wild-type mice were similar to those obtained with non-treated mice. In PbA infections, no differences were observed in the extent of the anaemia and leukocytosis at any time of the infection (data not shown).

NO levels in serum

NO levels detected shortly before death during PbA infections in wild type mice (45 μM ± 25, $n = 5$) were slightly, but not significantly, increased as compared to naive mice (20 μM ± 8, $n = 3$). NO was not detectable in the sera of iNOS-deficient mice and of the AG-treated animals at any time during PbA infections.

DISCUSSION

Using *Plasmodium berghei* ANKA infections, a murine experimental model for cerebral malaria, we show that iNOS deficiency does not protect mice from fatal cerebral malaria.

We have reported previously that TNF-α/β double-deficient mice and IFN-γR-deficient mice are protected against *P. berghei* ANKA-induced cerebral malaria (Rudin *et al*. 1997). A significant reduction of NO production was found in TNF-α/β double-deficient mice but not in IFN-γR-deficient mice, as compared to wild-type mice. Our present

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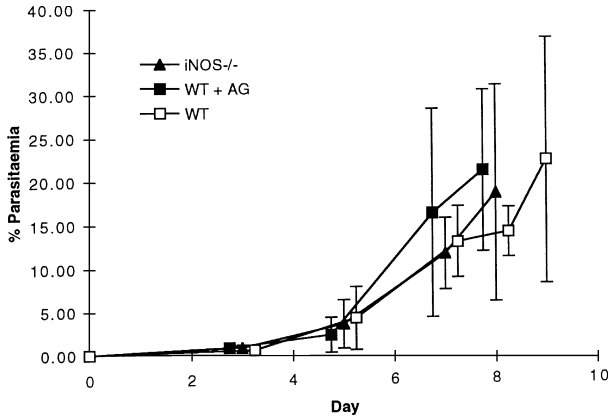

Fig. 2. Parasitaemia in wild-type mice ($n = 5$), wild-type mice treated with AG ($n = 5$) and iNOS-deficient mice ($n = 7$) infected with 10^5 PbA-parasitized RBC i.p. at day 0. Data from 1 experiment. Results are represented as means ± S.D.

observations with iNOS-deficient mice, suggesting that NO is not required for the development of cerebral malaria, confirm these data.

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 murine malaria. In human cerebral malaria, *Plasmodium falciparum* parasitized red blood cells are sequestered in the brain capillaries, whereas murine cerebral malaria models display sequestration of leukocytes (Taylor-Robinson, 1995*a*). Therefore, the pathological mechanisms leading to cerebral malaria might, in part, be different between mice and men. In addition, 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 (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.

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. Similar to iNOS deficiency, this treatment did not affect the course of the infections. Thus, our data demonstrate that NO production is not required for the development of murine cerebral malaria. However, the combination of our results with the ones obtained with C57BL/6 mice (Jacobs, Radzioch & Stevenson, 1995; Favre, Ryffel & Rudin, 1999) 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 where found between different populations, 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., MORKELA, 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 oxide 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.

ASENSIO, V. C., OSHIMA, H. & FALANGA, P. B. (1993). *Plasmodium berghei*: is nitric oxide involved in the pathogenesis of mouse cerebral malaria? *Experimental Parasitology* **77**, 111–117.

CLARK, I. A., ROCKETT, K. A. & COWDEN, W. B. (1992). Possible central role of nitric oxide in conditions clinically similar to cerebral malaria. *Lancet* **340**, 894–896.

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. (1999). Parasite killing in murine malaria does not require nitric oxide production. *Parasitology* **118**, 139–143.

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.

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.

KREMSNER, P. G., NUSSLER, A., NEIGER, S., CHAVES, M. F., BIENZLE, U., SENALDI, G. & GRAU, G. E. (1993). Malaria antigen and cytokine-induced production of reactive nitrogen intermediates by murine macrophages: no relevance to the development of experimental cerebral malaria. *Immunology* **78**, 286–290.

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.

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.

NÜSSLER, A. K., ELING, W. & KREMSHER, P. G. (1994). Patients with *Plasmodium falciparum* malaria and *Plasmodium vivax* malaria show increased nitrite and nitrate plasma levels [letter]. *Journal of Infectious Diseases* **169**, 1418–1419.

ROCKETT, K. A., AWBURN, M. M., AGGARWAL, B. B., COWDEN, W. B. & CLARK, I. A. (1992). In vivo induction of nitrite and nitrate by tumor necrosis factor, lymphotoxin, and interleukin-1: possible roles in malaria. *Infection and Immunity* **60**, 3725–3730.

RUDIN, W., EUGSTER, H. P., BORDMANN, G., BONATO, J., MULLER, M., YAMAGE, M. & RYFFEL, B. (1997). Resistance to cerebral malaria in tumor necrosis factor-alpha/beta-deficient mice is associated with a reduction of intercellular adhesion molecule-1 upregulation and T helper type 1 response. *American Journal of Pathology* **150**, 257–266.

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.

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