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Malaria antigen and cytokine-induced production of reactive nitrogen intermediates by murine macrophages: no relevance to the development of experimental cerebral malaria

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SUMMARY

The *in vitro* production of reactive nitrogen intermediates (RNI) by murine macrophages was evaluated in response to heat-stable malaria antigen and cytokines. Malaria antigen, interferon-γ (IFN-γ) and tumour necrosis factor (TNF) induced RNI production in macrophages in a dose-dependent way. RNI production steadily increased over a 2-day period and was enhanced when the malaria antigen was co-incubated with IFN-γ and/or TNF. RNI production induced by either IFN-γ or malaria antigen or a combination of the two was suppressed by pentoxifylline in a dose-dependent manner. Pentoxifylline did not significantly influence TNF-induced RNI production. L-*N*-monomethyl arginine reduced malaria antigen, IFN-γ and TNF-induced RNI production when these reagents were used in combination or alone. An anti-TNF monoclonal antibody (mAb) reduced IFN-γ-induced RNI production, but did not significantly alter the malaria antigen-induced RNI synthesis by macrophages. The influence of inhibitors of nitric oxide synthase, L-*N*-monomethyl arginine and *N* ω-nitro-L-arginine, was studied in experimental cerebral malaria. They did not exert any significant effect on the development of cerebral malaria in *Plasmodium berghei* ANKA-infected CBA/J mice.

INTRODUCTION

Reactive nitrogen intermediates (RNI) are nitric oxide, nitrite and nitrate, derived from L-arginine along a metabolic pathway including nitric oxide synthase as the key enzyme. They can be produced by many mammalian cells. RNI are bioactive molecules which exhibit *in vitro* cytotoxicity against protozoal parasites such as *Toxoplasma gondii*[1] and *Leishmania major*.[2,3] The toxic effects of RNI are probably due to their interactions with iron-dependent enzymes such as aconitase in the citric acid cycle or enzymes in the mitochondrial respiratory chain containing iron–sulphur prosthetic groups.[4]

Recently it was demonstrated that nitrite and nitrate ions are toxic *in vitro* to asexual blood stages of *Plasmodium falciparum*.[5] Nüssler *et al.*[6] showed RNI-dependent destruction of intrahepatic *P. yoelii* rodent parasites which was incited by tumour necrosis factor (TNF) and interleukin-6 (IL-6). In a similar study Mellouk *et al.*[7] demonstrated an anti-parasitic effect of interferon-γ (IFN-γ) upon pre-erythrocytic stages of *P. berghei*, which could be neutralized by L-*N*-monomethyl arginine (L-NMMA), an inhibitor of nitric oxide synthase. Interestingly, Clark *et al.*[8] hypothesized intriguing links between RNI production and the development of cerebral malaria.

In the present study the *in vitro* effects of malaria antigen and cytokines were examined upon RNI production by macrophages, as well as the effects of anti-TNF monoclonal antibody (mAb) and pentoxifylline both known to inhibit the development of murine cerebral malaria.[9,10] The effects of L-NMMA were also studied *in vitro* and, additionally, *in vivo* in the development of experimental cerebral malaria.

MATERIALS AND METHODS

Malaria antigen and other modulators of RNI production

Malaria antigen was prepared as follows: *P. vinckei*-infected mice with 60% parasitized erythrocytes were bled, erythrocytes were washed in sterile phosphate-buffered saline (PBS), suspended to a concentration of 10^8 parasitized cells/ml, and incubated in PBS for 24 hr at 37° in 5% CO_2. Supernatant was then removed, centrifuged, boiled for 5 min, passed through a 0·2 μm pore filter (Millipore, Eschborn, Germany), and stored at 4° before use.[10] To eliminate any lipopolysaccharide (LPS) contamination in the malaria antigen preparation, 5 μg/ml polymyxin B (Sigma, Deisenhofen, Germany) was added to all

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culture media.[11] LPS (Sigma) was used to check the neutralizing capacity of polymyxin B. In addition malaria-immune serum was generated by the i.p. injection of 200 μl malaria antigen 9 days before bleeding of mice. The inhibitory capacity of this immune serum was investigated on malaria antigen and LPS-induced RNI production by macrophages *in vitro*. Recombinant murine TNF and IFN-γ (Dr G. R. Adolf, Boehringer-Ingelheim, Vienna, Austria) were tested as stimulants in the concentrations given below. A rat anti-mouse TNF mAb (V1q, Dr P. H. Krammer, DKFZ Heidelberg, Germany) was used in a dilution that neutralized 2000 U/ml TNF. Pentoxifylline (Rentschler, Laupheim, Germany) and L-NMMA (Dr S. Moncada and Dr. F. Y. Liew, Wellcome Research Laboratories, Beckenham, U.K.) were tested as inhibitors of RNI production in a dose range as indicated in Results.

Modulation of RNI production by macrophages

Ten-week-old female BALB/c mice (Bundesgesundheitsamt Berlin, Germany) and CBA/J mice (Shaw's Farm, Blackthorn, U.K.) were injected with 3 ml 2% amylose intraperitoneally 3–5 days prior to macrophage harvesting. Peritoneal cells were removed in 5 ml cold RPMI-1640 (Gibco, Gaithersburg, MD) containing polymyxin B, washed and resuspended in CRPMI (RPMI-1640 with 10% heat-inactivated foetal calf serum) to $2{\cdot}5 \times 10^6$ cells/ml. The cell suspension was aliquoted in 100-μl portions onto a 96-well plate (Nunc, Wiesbaden, Germany) and allowed to adhere at 37° in the presence of 5% CO_2 for approximately 3 hr, after which cells were washed twice with medium. Remaining adherent macrophages covered approximately one-third of the well surface area.

Macrophage cultures were stimulated with 100 μl aliquots containing malaria antigen and other stimulants in concentrations as indicated in Results. Experiments were performed three to five times in quadruplicates. After different incubation periods, supernatants were removed to measure RNI.

Nitrite determination

Cell-free culture media were assayed for nitrite, the stable end product of the nitrogen oxidation of L-arginine, with use of the Griess reagent. Briefly, 100 μl aliquots were added to 200 μl of a 1 : 1 mixture combined immediately beforehand of 1% sulphanilamide and 0·1% *N*-1-naphthylethylenediamine in 45% acetic acid. Absorbance was read immediately at 540 nm, cross filtered at 690 nm. A concentration gradient for $NaNO_2$ in medium demonstrated a linear relationship to absorbance, and was used as a standard curve.

Statistical analysis

Statistical analysis was performed using the Wilcoxon rank sum test and the Fisher's exact test.

Treatment of experimental cerebral malaria by L-NMMA

Eight-week-old female CBA/J mice (Bomholtgard, Ry, Denmark) were infected by i.p. inoculation of 10^6 *P. berghei* ANKA infected erythrocytes. Approximately 80% of mice develop acute cerebral malaria within the second week with a parasitaemia of only 5–10%.[12] Infected mice were injected i.p. on day 6 with 5 mg L-NMMA ($n = 10$) or 5 mg *N* ω-nitro-L-arginine (NNA; Sigma, Buchs, Switzerland; $n = 14$) dissolved in normal saline. The dose of 5 mg was chosen because it demonstrated *in vivo* effects in a LPS toxicity model. Control mice received normal saline ($n = 14$).

In a second experiment infected mice received two i.p. injections daily starting on day 1 until day 9 of either L-NMMA (2 mg solved in 200 μl normal saline; $n = 10$) or D-NMMA (Drs Moncada and Liew, Wellcome; 2 mg in normal saline; $n = 10$) or L-arginine (Drs Moncada and Liew, Wellcome; 2 mg in normal saline; $n = 10$). D-NMMA does not inhibit the L-arginine-dependent production of RNI.[3]

RESULTS

RNI production by macrophages stimulated with malaria antigen and cytokines

Spontaneous RNI production by peritoneal macrophages cultured in CRPMI medium served as a control. In several experiments ($n = 5$) malaria antigen induced the RNI synthesis to detectable levels after only 1 day. Optimal induction of RNI synthesis was achieved after 2 days, therefore data are given for this time-point unless otherwise indicated. Polymyxin B only slightly reduced the capacity of malaria antigen to induce RNI production (25%), whereas the effect of 0·1 μg/ml LPS was completely abolished by 5 μg/ml polymyxin B. RNI production was decreased by 50% after 30 min preincubation of malaria antigen with immune serum, diluted 1:50. However, the LPS-induced RNI production by macrophages was not reduced using the same serum (data not shown). Malaria antigen induced RNI synthesis by macrophages of BALB/c mice in a dose-dependent manner (Fig. 1). Both cytokines, IFN-γ and TNF also induced dose-dependent RNI production. RNI were significantly increased in an additive way by co-incubation of cytokines with malaria antigen ($P < 0{\cdot}05$, Fig. 1). The highest level of RNI was observed after stimulation with malaria antigen plus 100 U IFN-γ/ml ($61{\cdot}2 \pm 0{\cdot}4$ μM NO_2^-). These findings were confirmed by identical experiments in BALB/c and CBA/J mice without differences between mouse strains.

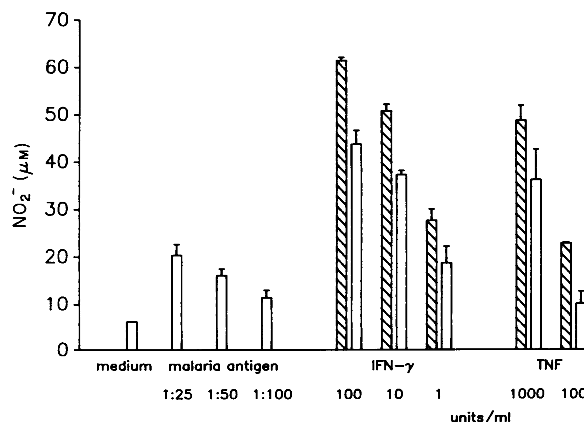

Figure 1. Nitrite (NO_2^-) production by peritoneal macrophages of BALB/c mice cultured for 48 hr with different agents: CRPMI medium with polymyxin B served as a reference for the spontaneous NO_2^- production. Malaria antigen, IFN-γ and TNF were either used alone (□) or in combination (▨). The values shown are means with the upper range of quadruplicates of a representative experiment.

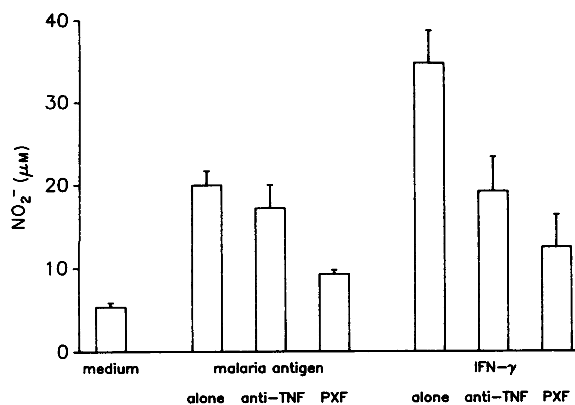

Figure 2. Nitrite (NO_2^-) production by peritoneal macrophages of BALB/c mice cultured for 24 hr in the presence of malaria antigen (1:20) and anti-TNF or pentoxifylline (1 mg/ml) and in the presence of IFN-γ (100 U/ml) and anti-TNF or pentoxifylline (1 mg/ml). The values shown are means with the upper range of a representative experiment. PXF, pentoxifylline.

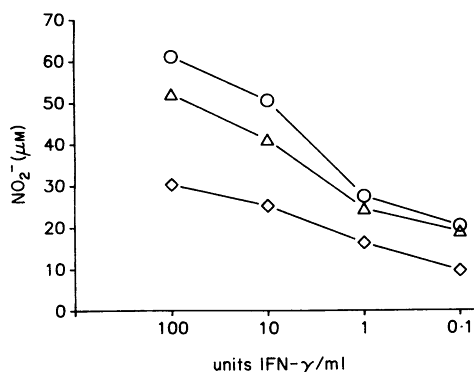

Figure 3. Nitrite (NO_2^-) production by peritoneal macrophages of BALB/c mice cultured for 48 hr in the presence of malaria antigen and different doses of IFN-γ with and without pentoxifylline: IFN-γ + malaria antigen (○); IFN-γ + malaria antigen + pentoxifylline (0·1 mg/ml) (△); IFN-γ + malaria antigen + pentoxifylline (1 mg/ml) (◇). The values depicted are means of quadruplicates of a representative experiment.

Table 1. The influence of L-NMMA, D-NMMA and L-arginine on the development of experimental cerebral malaria ($n = 10$/group). The drugs were injected i.p. 2 mg twice a day starting on day 1 until day 9. The numbers indicate surviving mice/group

	Days after infection						
Treatment	5	6	7	8	9	10	11
L-NMMA	10	9	6	0	0	0	0
D-NMMA	10	7	3	1	1	1	0
L-arginine	10	9	6	2	2	1	0
None	10	10	5	4	1	1	1

RNI production by macrophages inhibited by anti-TNF mAb, pentoxifylline and L-NMMA

Experiments were performed with macrophages of BALB/c mice. Anti-TNF mAb alone did not affect the spontaneous release of RNI by macrophages, but it significantly inhibited the IFN-γ induced RNI production ($P < 0·05$, Fig. 2).

Pentoxifylline (1 mg/ml) significantly reduced both IFN-γ and malaria antigen-induced RNI production of macrophages ($P < 0·05$, Fig. 2), but it did not reduce the TNF-induced RNI synthesis (data not shown). In further experiments ($n = 5$), concentrations of up to 1 mg/ml pentoxifylline showed no inhibitory effect on spontaneous RNI production of macrophages. However, pentoxifylline exhibited a dose-dependent significant suppression of RNI production of macrophages stimulated with malaria antigen (1:100) plus various concentrations of IFN-γ (Fig. 3).

In macrophage cultures using IFN-γ (10 U/ml) and malaria antigen (1:100) for stimulation (100% value) a concentration gradient of L-NMMA (500–2 μM) was used for the inhibition of RNI production. A reduction of 90% and 80% was obtained at 500 and 100 μM L-NMMA, an intermediate inhibition of 40% at 20 μM, and no inhibition at 2 μM. Likewise L-NMMA reduced IFN-γ, TNF and malaria antigen-induced RNI production when these reagents were used alone (data not shown). In macrophages cultured for 1 or 2 days without stimulants a reduction of spontaneous RNI production to background levels of medium was found with 500 μM L-NMMA.

The effect of the treatment of experimental cerebral malaria by L-NMMA

Single injections of either 5 mg L-NMMA or 5 mg NNA on day 6 after infection did not influence the development of murine cerebral malaria. Seventy-six per cent of all mice died with the syndrome of cerebral malaria between days 7 and 9. There was no difference between treated mice and control mice (data not shown). Moreover, using a different protocol of L-NMMA administration (2 mg twice daily from day 1 until death) mice were no more protected against death from cerebral malaria than animals receiving D-NMMA or L-arginine or normal saline, respectively (Table 1). On the contrary, although not reaching the level of statistical significance ($P > 0·05$), L-NMMA-treated mice died of cerebral malaria earlier.

DISCUSSION

In the present study an additive stimulatory effect of heat-stable malaria antigen and cytokines on RNI production of murine macrophages was demonstrated. This parallels findings in one study in which macrophages were stimulated with LPS and IFN-γ[4] and in another, in which *Leishmania major* amastigotes and IFN-γ were used as stimulants.[3] However, in these studies a potentiating synergistic effect of IFN-γ and LPS or amastigotes on RNI production was shown. Results of our *in vitro* investigations support evidence for the hypothesis of Mellouk *et al.*[7] that sequestered malaria parasites, like *Leishmania*, might provide their own second activation signal for maximum RNI production by various cells. The malaria antigen used in the present study is a heat-stable antigen mixture of culture supernatant from *P. vinckei*. This antigen preparation induced macrophages to secrete comparable amounts of TNF as did a

similar antigen preparation from *P. berghei* (P. G. Kremsner, unpublished results). Moreover, the components of exoantigen-activating macrophages are phospholipids. They seem to be closely related between different species of *Plasmodium*.[13] In contrast to the L-arginine analogue L-NMMA, anti-TNF mAb and the phosphodiesterase inhibitor pentoxifylline did not reduce the baseline RNI production by macrophages. It is therefore assumed that the latter agents exert an inhibitory influence on stimulation of RNI production rather than on spontaneous production. However, differences exist between pentoxifylline and anti-TNF as to the position where they exert their inhibitory action on RNI production of macrophages. Pentoxifylline reduced malaria antigen and IFN-γ induced RNI production, but not TNF-induced RNI production. In contrast anti-TNF mAb significantly reduced IFN-γ-induced but not malaria antigen-induced RNI production of macrophages.

Evidence is accumulating that RNI are major antiplasmodial effector molecules in asexual blood[5] and hepatic stages of malaria.[6,7] Thus, it is conceivable that part of the anti-parasitic effect of IFN-γ and TNF observed in different murine malaria infections[14–16] may well be mediated by RNI. However, IFN-γ and TNF are also involved in the development of cerebral malaria. This became obvious when neutralizing antibodies to either IFN-γ or TNF were found to prevent cerebral malaria in *P. berghei* ANKA-infected CBA/Ca mice.[9,17] In addition malaria-immune serum as well as pentoxifylline also prevented murine cerebral malaria.[10,18] All these regimens substantially decrease RNI production possibly via different pathways, as may be inferred directly or indirectly from our *in vitro* studies. Clark *et al.*[8] hypothesized that cerebral malaria may be induced by RNI originating from endothelial cells lining cerebral vessels and from mononuclear cells concentrating in small cerebral vessels during malaria. In view of our results, which show no significant influence of substances like L-NMMA and NNA, which specifically inhibit RNI synthesis, on the course of murine cerebral malaria, it is assumed that RNI do not play a major causative role in this syndrome. This is supported by the observation that inhibitors of RNI production, used in doses similar to those in the present study, exerted biological effects in a LPS mouse toxicity model with a single injection regimen (A. Nüssler, unpublished results) and in murine *P. vinckei* malaria daily injections of L-NMMA led to an aggravation of malaria organ pathology and death despite effective chemotherapy.[19] Enhanced organ pathology after L-NMMA injection has also been reported in mice previously injected with LPS.[20] In rats with endotoxic shock similar doses of L-NMMA accelerated the decline in blood pressure with all animals dying earlier than controls.[21] Our concept that RNI protect against malaria-associated complications such as cerebral malaria rather than induce it, is also supported by other data. The inhibition of RNI production *in vivo* increased leucocyte adherence to endothelium via CD11/CD18 integrins more than 15-fold.[22] Experimental cerebral malaria was abrogated by antibodies to CD11a, the α-chain of the integrin leucocyte function-antigen 1, indicating that CD11a/CD18-mediated adhesion of leucocytes to endothelium is critical in the pathogenesis of cerebral malaria.[23,24] IL-1 can induce RNI production.[25] Murine cerebral malaria was prevented by a low-dose therapy of IL-1,[26] which could have been mediated by the stimulation of RNI production. RNI were first described as endothelium derived relaxing factor leading to vasodilation of blood vessels.[27] Pathological spastic constriction of intracerebral arterioles was identified as being a major cause leading to clinical (*P. falciparum*) and experimental (*P. berghei*) cerebral malaria.[28] Prostacyclin and RNI have similar functions such as the induction of endothelium-dependent relaxation and the inhibition of platelet aggregation.[29] Experimental cerebral malaria was prevented by administration of a stable prostacyclin analogue.[30]

Taken together, these data suggest that RNI do not play a critical role in the pathogenesis of neurovascular lesions characteristic of murine cerebral malaria. However, in this experimental model, only end-stage vascular problems can be assessed: early stages of neurological disturbances go unnoticed. One cannot exclude, therefore, that RNI might explain metabolic changes taking place at very early stages of brain complications, i.e. those observed in patients with cerebral malaria who fully recover from their acute episode. Studies of RNI production in these patients are required to clarify this point.

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