Upregulation of reactive oxygen and nitrogen intermediates in *Plasmodium berghei* infected mice after rescue therapy with chloroquine or artemether

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*Plasmodium berghei* ANKA infected C57Bl/6 mice develop cerebral malaria at a parasitaemia of 15–25%. When parasitaemia reached 10%, *P. berghei* infected mice were treated with artemether, chloroquine or clindamycin in order to prevent the occurrence of cerebral malaria. Artemether and chloroquine were highly efficient. Functional tests revealed that zymosan stimulated spleen cells from untreated mice with cerebral malaria showed a slight decrease in their capacity to produce reactive oxygen intermediates (ROI) when compared with naive mice. After artemether or chloroquine treatment, the ROI production was significantly enhanced. The interferon-gamma induced production of reactive nitrogen intermediates (RNI) was slightly elevated in mice with cerebral malaria, but markedly elevated in artemether or chloroquine treated mice when compared with naive mice. Moreover, high levels of inducible nitric oxide synthase gene expression could be detected by in-situ hybridization in spleen sections of mice which had been treated with artemether or chloroquine. These findings suggest that increased production of ROI and RNI after chemotherapy may play a protective role for the host during malaria.

Introduction

Reactive oxygen intermediates (ROI) may cause intra-erythrocytic death of malaria parasites as well as tissue damage in the host (Allison & Eugui, 1983; Ockenhouse & Shear, 1984; Wozenkraft *et al*., 1984). ROI have also been shown to be responsible for the development of cerebral malaria (Thumwood *et al*., 1989). Increased ROI production has been described during *Plasmodium chabaudi*, *Plasmodium vinckei* and *Plasmodium berghei* malaria (Stocker *et al*., 1984; Clark *et al*., 1987; Prasad *et al*., 1990), but ROI scavengers were found to enhance the development of murine malaria (Clark *et al*., 1987). However, antimalarials like chloroquine seem to exert their beneficial action by modulation of ROI production (Prasad *et al*., 1990). Other antimalarials, like artemisinin, are known as ROI generators and exert their antimalarial action as promoters of ROI production in an iron dependent manner (Meshnick *et al*., 1993).

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Reactive nitrogen intermediates (RNI), the products of the inducible nitric oxide synthase (iNOS), are considered as important regulator molecules in inflammatory immune responses and antimicrobial defense mechanisms (Nüssler & Billiar, 1993). The production of RNI has also been found to be essential in the defense against malaria parasites like Plasmodium falciparum, Plasmodium yoelii and P. berghei (Mellouk et al., 1991; Nüssler et al., 1991; Rockett et al., 1991). Although possible links between RNI and cerebral malaria were proposed (Clark et al., 1991), it has been demonstrated that RNI are not directly involved in the pathogenesis of cerebral malaria (Senaldi et al., 1992; Asensio et al., 1993; Kremsner et al., 1993b; Grau & De Kossodo, 1994). In contrast, the production of high levels of RNI seems to be associated with protective effects for the host in human and murine malaria (Kremsner et al., 1992; Cot et al., 1994; Kremsner et al., 1996). Chloroquine and artelinate, a derivative of artemisinin, have been found to influence the RNI production in vitro (Kremsner et al., 1993a). Artemether, a methylether derivative of dihydroartemisinin, is highly effective in the treatment of severe P. falciparum malaria, including cerebral malaria (Myint & Shwe, 1987; White et al., 1992; Taylor et al., 1993).

In the present study, we have investigated the capacity of spleen cells to produce ROI and RNI in untreated P. berghei infected mice with cerebral malaria and in mice treated with artemether or chloroquine. The production of RNI during cerebral malaria and after artemether or chloroquine treatment was further examined by in-situ hybridization using a specific DNA probe for the gene encoding iNOS.

Materials and methods

Mice were maintained under specific pathogen free conditions. Female, six week old C57BL/6 mice were infected intraperitoneally (ip) with $2 \times 10^4$ P. berghei ANKA parasitized erythrocytes, and the course of infection was followed by daily determination of parasitaemia on Giemsa-stained blood smears. When the parasitaemia reached approximately 10% (at the onset of the cerebral malaria syndrome), four groups of mice were examined in repetitive experiments. The first group ($n = 22$) was treated with clindamycin 5 mg/kg (Upjohn, Heppenheim, Germany) every 12 h for four days, the second group ($n = 24$) received chloroquine 12 mg/kg (Bayer, Leverkusen, Germany) daily for three days, the third group ($n = 40$) was given artemether 5 mg/kg (dissolved in 100 μL oil) (Sapce S.A. Feinchemicals, Barbengo, Switzerland) daily for three days, and the fourth group ($n = 39$) received placebo treatment corresponding to the mice treated with artemether (three doses of 100 μL oil). All injections were given ip. Parasitaemia, cerebral symptoms and survival times were recorded separately for each mouse. Neurological signs such as paralysis, ataxia and convulsions served as parameters for cerebral malaria. Death due to cerebral malaria occurred within 24–36 h following the onset of neurological signs.

Because optimal results were obtained with artemether and chloroquine, mice from the placebo group and mice treated with artemether or chloroquine were sacrificed when the placebo mice showed full blown cerebral malaria, and the mice under treatment appeared healthy and had already very low parasitaemia. Spleens were immediately removed aseptically, and either teased through stainless steel screens for preparation of spleen cells, or fixed in formaldehyde for paraffin section. Spleen cells were isolated and maintained in culture in order to investigate the in-vitro production of ROI or RNI in supernatants after stimulation with zymosan or murine recombinant interferon-gamma.
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The concentration of hydrogen peroxide in supernatants, which was used as representative molecule of ROI, was determined by the horseradish peroxidase catalyzed oxidation of fluorescent scopoletin to a non-fluorescent product (De la Harpe et al., 1985), following the procedure described previously (Kremsner et al., 1993a). Cell-free supernatants were also assayed for nitrite, the stable end-product of RNI synthesis, by the Griess reaction as described previously (Kremsner et al., 1993a). Statistical analysis was performed using the Mann Whitney U-test for differences in the capacity of ROI and RNI production.

In-situ hybridization was performed on paraffin sections (4 μm) of murine spleens, using a specific cDNA probe for human hepatocyte iNOS (hep-NOS). The gene probe was the 2.1 kb fragment (bp 1057 to 3119) of hep-NOS (Geller et al., 1993), as cloned between the EcoRI and BamHI sites of the plasmid pBluescript SK (Stratagene, Pennsylvania, USA). After digestion of plasmid DNA with the restriction endonucleases EcoRI and BamHI (Boehringer Mannheim, Germany), the corresponding 2.1 kb fragment was isolated by electroelution from 1% agarose gels. The isolated DNA gene probe was further labelled with digoxigenin-dUTP (Boehringer Mannheim). The in-situ hybridization procedure was performed as described previously for interleukin 1 (IL-1), interleukin 6 (IL-6), and tumour necrosis factor (TNF) specific gene probes, including the corresponding control hybridizations (Pringle et al., 1989; Fleming et al., 1992; Prada et al., 1994, 1995).

Results

In the placebo group, the majority of mice (95%) developed neurological signs and died of cerebral malaria between days 8 and 12 after infection with a parasitaemia of approximately 20%. Only two of 39 mice (5%) survived this phase of the infection, and these died two weeks later of hyperparasitaemia and severe anaemia. Clindamycin was not effective in preventing death due to cerebral malaria during treatment. Seventeen mice (77%) died 2-4 days after the start of therapy, the five survivors (23%) showing increasing parasitaemia even 2 days after the initiation of treatment. The subsequent decrease in parasitaemia never reached undetectable levels, and a second rise followed 5 days later. In contrast, the treatment with chloroquine was much more effective in preventing cerebral malaria. Only one mouse died with cerebral signs during treatment. The treated mice had rapid parasite clearance with undetectable parasitaemia during the week following treatment. The mice treated with artemether also showed a high rate of survival (38/40, 95%) and parasite clearance was similar to that of chloroquine treated mice.

Isolated spleen cells from mice with cerebral malaria stimulated for 90 min with opsonized zymosan showed a slightly decreased capacity to produce ROI in each experiment, compared with spleen cells from naive mice. Spleen cells from mice with cerebral malaria stimulated for 60 h with IFN-gamma exhibited an increased capacity to produce RNI in each experiment, when compared with controls. Spleen cells from mice treated with artemether or chloroquine, compared with cells from mice presenting cerebral malaria, had significantly increased capacities to produce ROI and RNI (P < 0.05 for each comparison, Table). These results were further corroborated by the results of in-situ hybridization experiments. The highest levels of iNOS gene expression were observed in the spleens of mice treated with artemether or chloroquine (Figure). The iNOS-mRNA positive cells were mainly located in the red pulp or in the
Table. ROI and RNI production in spleen cells of mice with cerebral malaria (CM) and after chemotherapy with artemether or chloroquine

<table>
<thead>
<tr>
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<th>Mice groups</th>
<th>Naive</th>
<th>CM</th>
<th>Artemether</th>
<th>Chloroquine</th>
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<tr>
<td>Stimulants of ROI</td>
<td>Stimulants of ROI</td>
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<tr>
<td>medium (control)</td>
<td>zymosan</td>
<td>834 (±104)</td>
<td>720 (±153)</td>
<td>1118* (±123)</td>
<td>1505* (±97)</td>
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<td></td>
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<td>834 (±104)</td>
<td>720 (±153)</td>
<td>1118* (±123)</td>
<td>1505* (±97)</td>
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<tr>
<td>Stimulants of RNI</td>
<td>Stimulants of RNI</td>
<td></td>
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<td></td>
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<tr>
<td>medium (control)</td>
<td>IFN-γ</td>
<td>1.9 (±0.3)</td>
<td>7.4 (±2.0)</td>
<td>9.2 (±1.4)</td>
<td>13.3 (±1.8)</td>
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<td>1.9 (±0.3)</td>
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<td>9.2 (±1.4)</td>
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*Values are expressed as means (±S.E.M.), for ROI in pmol H₂O₂/well/90 min/750,000 cells after stimulation with 2 mg/mL zymosan, and for RNI in nmol NO₂⁻/mL supernatant after 60 h stimulation of 5 x 10⁶ cells/mL with 0.1 mg/L IFN-gamma. Four consecutive experiments were performed for ROI production and five for RNI production, each one in double series of triplicates.

**Statistically significant (P < 0.05).**

Discussion

Clindamycin is a slow acting antimalarial, effective in treating experimental and human malaria (Kremsner & Graninger, 1992), but in this study it did not prevent death due to cerebral malaria. In contrast, treatment with artemether was efficacious and comparable to chloroquine. This finding corroborated the value of artemether in severe human malaria (Myint & Shwe, 1987; White et al., 1992; Taylor et al., 1993), and led us to study relevant immune mechanisms in the course of chemotherapy. It has previously been reported that the antimalarial activity of artemisinin and its derivatives is due to free oxygen radical generation (Meshnick et al., 1993). The observed increase in the capacity of spleen cells to produce ROI after artemether treatment further indicates that ROI may play an important role in the antimalarial activity of this drug. It is more likely that it is not the drug itself but instead drug damaged parasites releasing material which stimulate the spleen cells to upregulate ROI production. This possibility is further supported by the similar effect found after chloroquine treatment. Surprisingly, chloroquine has previously been reported to inhibit increased ROI production in Swiss albino mice infected with P. berghei NK 65 (Prasad et al., 1993). However, in this previous study, the infection with P. berghei did not induce cerebral malaria, and all the immunologic changes due to the neurological syndrome were consequently missing. Moreover, the dosage of chloroquine used in the study of Prasad et al. (1993) was higher than in our study, and the ROI production was measured by chemiluminiscence in Kupffer cells.

In the present study, we also found an elevated capacity of spleen cells to produce RNI after treatment with artemether or chloroquine. Since it has previously been described that artemisinin derivatives and chloroquine in high dosage inhibit the in-vitro...
Figure. Detection of iNOS mRNA by in-situ hybridization in spleen sections of *P. berghei* ANKA infected mice during cerebral malaria and after chemotherapy with artemether or chloroquine. (A) naive mouse (control), (B) untreated mouse with 20% parasitaemia presenting cerebral malaria; (C) mouse with 2% parasitaemia after treatment with artemether, and (D) mouse with 2% parasitaemia after treatment with chloroquine. A positive reaction is indicated by dark staining (purple colour in the original). WP, white pulp; RP, red pulp.

production of RNI in murine peritoneal macrophages (Kremsner *et al.*, 1993a), it was of interest to study whether only the spleen macrophages are the cells responsible for the enhancement of RNI production after chemotherapy. The in-situ hybridization
experiments in spleen sections of artemether or chloroquine treated mice showed extensive iNOS activity in the red pulp and in the marginal zone of the follicles where numerous macrophages are present. In addition, other splenic cells may also be responsible for the observed high levels of iNOS expression. It has previously been reported that the generation of free radicals by artemisinin and its derivatives is iron-dependent (Meshnick et al., 1993), and that such generation occurs after binding with the haem groups of haemozoin (Hong et al., 1994; Asawamahasakda et al., 1994). Since it is known that haemozoin inhibits the oxidative burst and the phagocytic activity in macrophages (Turrini et al., 1993), our results suggest that the treatment of *P. berghei* infected mice with artemether may compensate this defect by enhancing the capacity of macrophages to produce ROI. In addition, the production of other important immunomodulators like IL-1 or TNF has been found to be increased by the presence of haemozoin (Pichyangkul, Saengkrai & Webster, 1994; Prada et al., 1995), and this may activate the iNOS gene expression with subsequent increases in the production of RNI. Since enhanced RNI production is associated with rapid parasite clearance (Kremsner et al., 1996) and a reduction in coma duration in patients with *P. falciparum* malaria (Cot et al., 1994), the present finding of an association of increased iNOS gene expression and rescue therapy of cerebral malaria further supports the protective role of RNI in the host during malaria.

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**References**


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