Simultaneous increases in immune-competent cells and nitric oxide in the spleen during Plasmodium berghei infection in mice

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Background and Purpose: Nitric oxide and other reactive nitrogen intermediates (RNI) are thought to be important mediators of both immunological and pathological responses of the vertebrate host to malaria infection. The role of RNI has been studied most often by assay of stable RNI metabolites (nitrites, nitrates) in blood. This study evaluated the nature of the RNI response of mice to malaria by analyzing the subsets of immune-competent cells within the organ displaying increased RNI in vivo.

Methods: We measured RNI production indirectly, as stable metabolites of nitric oxide activity in tissue homogenates (brain, liver, spleen) from mice infected with Plasmodium berghei. Only spleen exhibited an RNI concentration response during rising parasitemia. Subsets of immune-competent cells (B cells, CD19⁺), macrophages/monocytes (MOMA₂⁺) and T cells (CD4⁺, CD8⁺) in the spleen were assayed by fluorescence activated cell scan flow cytometry.

Results: The spleen was confirmed as a major source of RNI during mid-phase P. berghei infection. Significant increases in CD19⁺ and MOMA₂⁺ spleen cells were evident during the mid-phase of P. berghei infection in MF1 mice when RNI are maximally elevated.

Conclusions: The time courses of the cellular and RNI responses indicate that CD19⁺ and MOMA₂⁺ cells may be responsible for the increase in RNI in the spleen. However, experiments in vitro are needed to make a definitive identification of the cell type(s) responsible for the increase in RNI in the mouse spleen during P. berghei infection.

Keywords: B lymphocytes, macrophages, malaria, nitric oxide, Plasmodium berghei, spleen

Introduction

Potential effectors of immunity against malaria include B cells, cytotoxic T cells, macrophages and a variety of soluble mediators including nitric oxide (NO) and other reactive nitrogen intermediates (RNI) [1]. An essential role for cell-mediated immunity against Plasmodium infections has been established in experimental animals [2], and several types of immune-competent cells produce RNI after stimulation [3,4]. In addition to a putative defence role, RNI are also thought to be important mediators of malaria pathology and hence potential targets for novel drug therapy [3,5]. Exo-antigens released during malaria stimulate macrophages and eosinophils to produce RNI, which cause cell death in several ways [4,6-9].

The mechanisms mediating host defence in malaria are numerous and diverse, and include immunological and non-immunological interactions with parasitized red blood cells (PRBC) [10]. Consequently, the possible importance of RNI in host defence has been studied most often by assay of stable RNI metabolites (nitrites, nitrates) in blood. However, the presence of RNI metabolites in circulating body fluids gives no indication of where the actual RNI were generated, and yet the tissue site of production is critical due to the short life of NO as a reactive entity [11]. Published reports on RNI in host organs during malaria infection include studies from this laboratory, which also document changes in NO per se as well as expression...
of inducible nitric oxide synthase (iNOS) in malarial mice [12,13].

The objective of this study was to evaluate further the nature of the RNI response of mice to malaria by analyzing the subsets of immune competent cells within the organ displaying increased RNI in vivo.

Methods

Animals
Male MF1 mice (mean ± standard error of the mean [SEM] body weight on day of inoculation, 28.1 ± 1.7 g) were supplied by the Biological Services Unit, University of Manchester. Mice were housed in plastic cages at 19-22°C, with unlimited access to CRM feed pellets and water. Experiments were licensed under the Animals (Scientific Procedures) Act 1986. In compliance with the conditions of the licence, mice were humanely killed when humane endpoints were reached.

Malaria
*Plasmodium berghei* N was maintained as described previously [12] by blood passage in mice when infected experimental animals were required, otherwise parasites were stored at −70°C in Alserver’s solution. Experimental malarial mice were injected intravenously with blood containing $2 \times 10^7$ PRBC. Control mice received an equivalent volume (0.2 mL intravenously) of uninfected red blood cells (URBC) diluted with 0.85% saline to the same degree as malarial blood. Parasitemia was measured as Leishman-positive cells as previously described [12].

Preparation of tissue homogenates for RNI assay
Mice were terminally anaesthetised by inhalation of diethyl ether (BDH, UK) 1 h after determination of parasitemia and humanely sacrificed by cervical dislocation. Whole brains, livers and spleens were removed postmortem and weighed. Tissues were placed in separate 1.5 mL microfuge tubes and homogenised in ice-cold deionised water (d.H$_2$O; 0.1 g wet tissue/mL) using an electrical homogeniser (Model RS541-242; RS Components, UK). Homogenates were centrifuged at 5500 g (Model 1-13 Microcentrifuge; Sigma Co., UK) for 15 min.

Griess micro assay
RNI were measured as the concentration of combined nitrates and nitrites using the Griess reaction adapted with modifications from Rockett et al [14] as described fully elsewhere [12]. Nitrite and nitrate standards were prepared in experimental fluids and final values corrected for assay losses [12]. Briefly, 60 µL samples were treated with 10 µL nitrate reductase (NAD[P]H Aspergillus spp.; Sigma Chemical Co., UK) and 30 µL NADPH β-nicotinamide adenine dinucleotide phosphate (Sigma Diagnostics, St. Louis, USA). 200 µL Griess reagent (5% phosphoric acid, 1% sulfanilic acid and 0.1% N [1-naphthyl-1]-ethylenediamine dihydrochloride) [all from Sigma] dissolved in 100 mL d.H$_2$O were added and proteins subsequently precipitated by 200 µL trichloroacetic acid 10% (BDH). Tube contents were mixed then centrifuged at 5500 g, transferred to a 96-well flat-bottomed microplate (Costar, USA), and read at 520 nm using a microplate reader (Dynatech; MRX, USA). Values are presented as mean ± SEM nmol/g wet weight of tissue.

Fluorescence Activated Cell Scanning
Fluorescence activated cell scan (FACScan) technology was applied to count subset populations of immune-competent cells after fluorescent labeling of cell surface markers [15,16]. Whole spleens were removed aseptically postmortem and placed in ice-cold Hank’s Balanced Salt Solution (HBSS) phenol red (Gibco, UK). Cell suspensions were prepared separately for each mouse by gently pressing the tissue through a sterile metal mesh into a Petri dish containing ice-cold HBSS [17]. Cells were washed twice with HBSS, centrifuged at 140 g (Centaur 2; MSE, UK) for 10-15 min and re-suspended for cell counting in diamino-benzidine (DAB)/2/azide: 480 mL DAB solution [2.5 mL DAB mineral salts (50 mg calcium chloride and 50 mg magnesium chloride in 100 mL d.H$_2$O)] in 500 mL autoclaved phosphate-buffered saline (PBS, Sigma-Aldrich, Poole, UK), 10 mL fetal bovine serum (FBS; Gibco) and 10 mL sodium azide 0.1 M (Sigma-Aldrich). Cells were counted using a haemo-cytometer slide after adding 180 µL 1% trypan blue (Sigma-Aldrich) to 20 µL cell suspension. Cell suspensions were diluted to appropriate cell concentrations for flow cytometry, between $10^5$-$10^6$ cells in less than 0.3 mL [17].

Immunofluorescence staining
Spleen cells were suspended in 10 mL DAB/2/azide and pipetted into LP, FACS tubes (Alpha Laboratory Co., UK). Cells were incubated with 10 µL monoclonal antibodies (mAbs) conjugated to fluorescein isothiocyanate at 4°C for 30 min; mAbs (Serotec Co., UK)
used: immunoglobulin G (IgG), b rat anti-CD4 mAb to mark CD4+ T-helper cells; IgG, a rat anti-CD8 mAb for CD8+ T-cytotoxic cells; IgG, a rat anti-CD19 mAb for B cells and IgG, b rat macrophages/monocytes (MOMA2) mAb for macrophages and monocytes. Fluorescent goat anti-rat IgG was used as a negative marker control. Contents of MOMA2+ marker tubes were permeabilised with 4 µL permeabilisation buffer (PB; 1% FBS, 0.1% sodium azide and 0.1% saponin, all from Sigma-Aldrich) before staining with mAb. Two negative controls were used: (a) with PB in PBS for MOMA2+ and (b) without PB for CD4+, CD8+ and CD19+ cells. Cells were washed twice with 0.3 mL DAB/2/azide, centrifuged at 140 g for 15 min and pellets re-suspended. After the second wash, cells were suspended in 0.3 mL PBS and inactivated by 2% paraformaldehyde (Sigma-Aldrich). Cells were then counted on a FACScan Flow Cytometer (Becton Dickinson Laboratory Systems, Belgium) gated for marked cells without PB (IgG, b-PB, CD4+, CD8+, CD19+) and for marked cells with PB (IgG, b+PB, MOMA2+). Values are presented as percentages of total blast cells and as total numbers of cells in the spleen.

Statistical analysis

Values are presented as mean ± SEM for groups of n = 4 or 5 mice. The significance of differences between means was determined by Student’s t test or one-way analysis of variance using GraphPad Prism Software (GraphPad, CA, USA).

Results

The time course of P. berghei parasitemia in mice in this study was 1.5 ± 0.3% (24 h), 6.3 ± 0.5% (48 h), 37.0 ± 1.9% (72 h) and 69.3 ± 3.4% (96 h). Concentrations of RNI in brain, liver and spleen were measured throughout P. berghei infection in mice, but no changes were detected in these organs with the exception of an increase in the spleen during the middle phase of P. berghei infection when parasitemia was rising (Fig. 1).

Spleens were collected for FACScan analysis on days 1-4 following intravenous injection with P. berghei in malarial mice and URBC in time-matched control mice. Subsets of CD4+, CD8+, CD19+ and MOMA2+ cells all altered during P. berghei infection in MF1 mice. When expressed as a percentage of total marked blast cells, CD4+ T cells and CD8+ T cells were smaller on day 3, but unaltered on days 1, 2 and 4 when parasitemia was either low (<7%) or high (about 70%) [Fig. 2]. Significant increases in the percentage population of CD19+ B cells on day 2 and MOMA2+ cells on day 3 were observed in malarial mice when compared with time-matched control animals, which had been injected with URBC on day 0 (Fig. 2).

P. berghei infection in mice causes an increase in spleen size. When subpopulations of immune-competent cells are expressed as the total number of cells in the spleen, increases in the numbers of CD4+ T cells and CD8+ T cells were observed in malarial mice when compared with control animals, but only on day 4 (Fig. 3). Significant increases in the total numbers of CD19+ B cells and MOMA2+ cells in the malarial spleen were observed on day 3, associated with elevated RNI (Fig. 1) during the phase of rising parasitemia. The number of CD19+ B cells remained high in the spleen on day 4, but the number of MOMA2+ cells was not significantly elevated at this later stage.

Discussion

The role of the spleen in the resolution of acute malaria has been established [18-23]. Published data support a key role for this organ in killing malaria parasites [24,25] and/or acting as a filter to remove modified...
Spleen cells and NO in malaria

erythrocytes [26-28]. Hence, splenectomised animals have peak parasitemias greater than those of intact animals [29] or fail to resolve the infection [24].

We have previously reported the detailed time courses (days 1-5) for *P. berghei* parasitemia and the associated changes in concentrations of both NO and RNI in spleen, brain, liver and blood in the MF1 mouse [12]. Day 3 was used in the study reported here to confirm the RNI response documented previously, because it represents the middle phase of this lethal malaria in this mouse strain, when plasma concentrations RNI are maximally elevated [12]. In the experiments reported here, concentrations of RNI were raised in the spleen, but not the brain or liver, during the middle (day 3) and late (day 5) phases of this malaria infection. Hypotheses to explain increased levels of RNI in the spleen include circulating blood depositing stable NO metabolites in the organ, as well as the spleen being the source of NO production. Consistent with the latter hypothesis, expression of iNOS in spleen is increased in this model of malaria [13]. Our earlier reports did not identify the type(s) of cells within the spleen increasing NO production or iNOS gene expression, hence this study was conducted in order to examine a possible correlation between the cell type(s) increasing in number in the spleen at the time of increased RNI in vivo [12,13].

Increased NO production in the spleen would be consistent with it having a defensive role against malaria [30], although the role of the spleen in resistance to malaria may depend on the genotype of the rodent host [31]. The

![Graphs](image_url)
possibility exists that other organs and tissues may also produce RNI during *Plasmodium* infection in other host/parasite combinations. For example, a protective role for the liver has been confirmed during malaria [9,30,32-35]. In this study, however, hepatic RNI was not changed by lethal *P. berghei* infection in MF1 mice, in agreement with our previous report [12]. In the spleen, CD4+ T cells and macrophages are required to resolve *Plasmodium* infections [18,19,21,22,30]. Endogenous mediators, for example macrophage chemotactic factor and migration inhibitory factor, can recruit mononuclear phagocytes into the spleen and activate them to release inhibitors of parasite replication [27]. High numbers of B cells and monocytes have also been observed in the spleen during malaria infection [36-40].

In the study reported here, the percentages of total blast cells marked as CD4+ T cells and CD8+ T cells were lower on day 3 and unaltered on days 1, 2 and 4 (Fig. 2). Nevertheless, the total numbers of both cell types were increased in the spleen, but only on day 4 when

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**Fig. 3.** Comparison of total numbers of immune-competent cells in spleens from control and *Plasmodium berghei*-infected mice. Bars show total numbers of cells marked CD4+, CD8+, CD19+ and macrophages/monocytes (MOMA2+) in spleens collected postmortem from MF1 mice injected intravenously with uninfected red blood cells (control) or *P. berghei* parasitized red blood cells (malaria) at time 0. Values are mean ± standard error (n = 4). * p<0.05, ** p<0.01, time-matched control versus malaria values (Student’s t test).
compared with values for time-matched control animals. Day 4 after inoculation is late in the time course of this lethal malaria model; parasitemia is maximal and associated with increased RNI concentrations in the spleen [12]. Significant increases in the percentage populations of CD19+ B cells and MOMA2+ cells were observed during the middle phase of P. berghei infection (days 2 and 3, respectively) when parasitemia was submaximal and increasing. The total number of CD19+ B cells in the spleen increased progressively throughout days 1-4 of infection, but the number of MOMA2+ cells was only significantly higher on day 3 (Fig. 3). Values for malarial mice were compared with cell populations for time-matched control animals infected with URBC on day 0. Subsets of cells in control mice showed some fluctuation in value presumably in response to URBC and experimental stress. These observations indicate that increases in spleen CD19+ B cells and MOMA2+ cells correlate more closely with the increase in RNI in the spleen in vivo during the middle phase of P. berghei infection in mice. CD4+ and CD8+ T cells may contribute to the late RNI response in the spleen [12].

The changes observed for MOMA2+ and CD4+ T cells in this in vivo model of malaria are consistent with the published literature. Although CD4+ T cells play a crucial role in the protective immune response to the erythrocytic stages of malaria [41], an increase in CD4+ T cell activity can exacerbate malaria pathology in the host [42]. Macrophages appear to be the major phagocytes in the host defence against malaria. They can engulf entire PRBC and generate reactive oxygen intermediates as well as RNI to damage intracellular parasites [6,21]. MOMA2+ cells were not significantly increased either as a percentage of marked blasts or as the spleen content on day 4 of this lethal malaria, when parasitemia was high. During this late phase, plasma concentrations of RNI in vivo decrease and mice approach death on day 5 [12]. However, this study does not establish a direct causal relationship between the subsets of spleen cells and RNI. Experiments with cultured cells are required to elucidate further the relationship between the different cell types and RNI during malaria. Experiments of this nature have been conducted with cells from rats infected with P. berghei [15], but this parasite is not lethal in rats and FACScan analysis showed no change in macrophage populations in either spleen or lymph nodes. Differences such as these in rodent host responses to the same strain of malaria emphasize caution in extrapolating information between different models of malaria.

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References