Apoptotic changes induced in mice splenic tissue due to malaria infection

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Background and purpose: Malaria is a life-threatening parasitic disease transmitted by mosquitoes and causing multiple alterations in the infected host. The objective of this study was to investigate the apoptotic changes in murine splenic tissue due to infection with Plasmodium chabaudi.

Methods: Flow cytometric analysis was used to quantify normal and apoptotic spleen cells in P. chabaudi-infected female C57BL/6 mice. Splenic tissue was also examined for histological study.

Results: Challenge of mice with parasitized erythrocytes induced damage to splenic tissue. Splenomegaly and changes in splenic cell numbers were the most common changes linked to P. chabaudi. Also noteworthy was that apoptotic splenic cell numbers correlated with changes in parasitemia.

Conclusions: This study suggested that reversible splenic lesions might develop after persistent low-grade parasitemia. Rapid changes in cellular splenic populations are influenced by apoptotic events, proving the role of the spleen in protective immunity against blood-borne infections due to P. chabaudi malaria.

Key words: Apoptosis; Malaria; Mice; Spleen

Introduction

Malaria remains a major public health problem in the developing world, affecting approximately 500 million people worldwide. The disease is responsible for approximately 2.5 million deaths each year [1]. Despite significant progress in the treatment of malaria, the condition has re-emerged in many areas of the world due to the development of drug-resistant parasites [2].

The spleen is believed to participate in clearing parasites from the circulation and in providing a strong hematopoietic response during acute infections [3].

Programmed cell death, apoptosis, is an important mechanism regulating the development, maturation, and activation of lymphocytes. In addition, apoptosis may also prevent or terminate the lymphocytic response [4]. In mice, both cellular and humoral responses play an important role in immunity against malaria infections [5].

In this study, C57BL/6 mice were infected with Plasmodium chabaudi chabaudi. The resultant apoptotic changes in the spleen were measured and the histological alterations were studied.

Methods

Animals and infection

Forty five female C57BL/6 mice aged 9 to 12 weeks were used. The mice were bred under specific pathogen-free conditions, and fed a standard diet (Norlin, Bad Salzuflen, Germany) and water ad libitum. Blood levels of Plasmodium sp. were maintained in 9- to 14-week-old NMRI (Naval Medical Research Institute, Hanover, Germany) mice (weight, 28-35 g) by weekly passages of infected blood as described previously [6]. The parasitemia was evaluated in Giemsa-stained smears prepared from tail blood [7]. Both NMRI and experimental mice were challenged with 1 × 10⁶ P. chabaudi-infected erythrocytes. Twenty five mice were used for...
the histological and flow cytometric studies at days 0, 4, 8, 12, and 35 postinfection with *P. chabaudi* (5 mice at each time point). Survival of mice (n = 20) was determined in a separate experiment. All experiments were performed in accordance with the German Animal Protection Law and were approved by the Animal Research Ethics (Düsseldorft, Germany).

**Isolation of spleen cells**

Mice were sacrificed by cervical dislocation then weighed. Spleens were aseptically removed and weighed. A small piece from each spleen was used for the histological study and the rest of spleen was gently disassociated through a stainless steel sieve into RPMI (Roswell Park Memorial Institute) medium (GIBCO-BRL, Eggenstein, Germany) supplemented with 5% fetal calf serum (PAA Laboratories, Linz, Austria). Splenic erythrocytes were removed by ammonium chloride lysis [8].

**Determination of apoptotic cells**

Splenic cells were incubated with Annexin V conjugated to Alexa fluor 568 (Molecular Probes, Leiden, The Netherlands). 7-Aminoactinomycin D (BD PharMingen, Heidelberg, Germany) was used to detect apoptotic cells. Spleen cells were analyzed by flow cytometry (FACS Calibur; BD Bioscience, Heidelberg, Germany) as detailed previously [8,9]. FACS (fluorescence-activated cell sorting) analysis was done with a sample size of 10,000 cells gated on the basis of forward and sideward scatter. Data were stored and processed by using Cell Quest Pro software (BD Bioscience).

**Histopathology**

Pieces of spleen were fixed in 10% neutral buffered formalin. Paraffin sections of 5 to 7 μm were cut and stained with hematoxylin and eosin for histological observations [10].

**Statistical analysis**

Statistical analysis was performed by using an unpaired Student’s *t* test. The data were analyzed by using MS Excel 2003 (Microsoft, Rochester, NY, USA) and SigmaPlot 2001 (Systat Software, Inc, Chicago, IL, USA).

**Results**

**Characteristics of *Plasmodium chabaudi* infection**

Infection of C57BL/6 mice with 10^6 parasitized erythrocytes became evident on day 4. Parasitemia increased to reach its peak (parasitemia ~45%) on day 8. Parasitemia then decreased rapidly to approximately 1% on day 12 and stabilized at approximately 0.5% until day 35 (Fig. 1). C57BL/6 mice exhibited a significantly high survival rate of 86% (*p* < 0.5) [Fig. 1].

**Increase in spleen cell number during *Plasmodium chabaudi* infection**

Splenomegaly due to infection with *P. chabaudi* was expressed as a splenic index (ratio of spleen weight in mg/mouse to body weight in g/mouse). On day 8 postinfection, the index in the infected mice (25.5 ± 3.5) was 5 times that in the non-infected mice (3.7 ± 0.74) [*p* < 0.5] (Fig. 2).

Flow cytometric analysis showed that the total number of cells in the spleen increased rapidly during
the early phase of infection, reaching a maximum of \(2.8 \times 10^8\) cells on day 8. By day 12, the number had rapidly decreased to \(0.89 \times 10^8\) cells, whereas on day 35, the number was \(1.5 \times 10^8\) cells (Fig. 3).

**Investigation of the apoptotic cells in the spleen**

The differentiation of the spleen to an antimalaria effector is associated with splenomegaly and reorganizations of the spleen at maximal parasitemia. There is an apparent enlargement of white pulp areas preceding the disappearance of white and red pulp segregation. Total spleen cell number paralleled the increase in the number of apoptotic cells. After maximal parasitemia \((0.3 \times 10^8\) apoptotic cells), the absolute number of apoptotic cells decreased rapidly and almost returned to normal values \((0.09 \times 10^8\) apoptotic cells) by day 35 (Fig. 3).

**Histological changes in the spleen**

The structure of the non-infected spleen was composed of white and red pulps surrounded by a capsule of dense connective tissue (Fig. 4A and Fig. 4B). The white pulp was composed of a central, T-cell rich zone, and a peri-arterial lymphoid sheath surrounded by B-cell-rich primary follicles. The white pulp was separated from the red pulp by the marginal sinus embedded in a layer of marginal zone lymphocytes. When the infection became detectable on day 8, the white pulp enlarged due to cellular proliferation. The limit between white and red pulp started to disappear (Fig. 4C and Fig. 4D), and the spleen increased in size. Vacuolation of some splenic cells was detected. Most of the cells were darkly stained and the sinusoidal spaces were large. On day 12, it was difficult to differentiate between white and red pulp. This disorganization was due to hyperplasia of the lymphoid tissue (Fig. 4E and Fig. 4F). Apoptotic cells were abundant; spleen size increased as evidenced by the splenic index (Fig. 2).

Malaria pigments were more easily detected starting from day 8. On day 35, the parasitemia was less than 1%. The red and white pulps became distinguishable. There was still a lot of malaria pigment, but the spleen had almost regained its normal structure (curative stage) and organization of white and red pulps (Fig. 4G and Fig. 4H).

**Discussion**

In an effort to find new strategies for malaria control, recent research has investigated the role of innate immunity in regulating infections with *Plasmodium* spp. [11]. Clearance of malaria parasites appears to be mediated by both acquired [12,13] and innate [14] immune responses. Female C57BL/6 mice were able to heal infections with *P. chabaudi* and to develop long-lasting immunity against homologous rechallenge. The results of this research support other studies that found an increase in parasitemia during crises [15,16].

The spleen acts as an effector against malaria infection [15]. The total number of spleen cells increased during peak parasitemia and then dropped to lower levels. Shortly after, their numbers increased again until the *P. chabaudi* infection was cleared. This may reflect lymphocyte migration to the peripheral blood as previously reported [16,17]. The flow cytometric analyses revealed that the number of spleen cells decreased during the days shortly before and shortly after peak parasitemia. During this time, there was an enlargement of the spleen with a considerable influx of non-B non-T cells [18,19].
Apoptosis has been described in other parasitic infections and may be involved in down-regulating inflammatory T-helper cell–1 responses [20]. Other mechanisms involved in apoptosis may include tumor necrosis factor-α, nitric oxide, or reactive oxygen [21], all of which are present at high concentrations in the spleens of mice during acute infections with *P. chabaudi*.

The splenomegaly detected in infected C57BL/6 mice was associated with expansion of both white and red pulp due to increased follicle size. This reaction is due to increased hematopoietic support [21] and increased numbers of macrophages [9,22]; the macrophages increase due to erythrophagocytosis [23]. Spleen cellularity and architecture also change dramatically during malarial infection [16]. Depending on the mouse strain and parasite species, these changes have been shown to be associated with either resistance or susceptibility to infection.

Histologic examination of spleen tissue of C57BL/6 mice during the course of *P. chabaudi* infection showed disturbed T-cell areas and changes in splenic architecture [18]. The hemozoin granules generated by intra-erythrocytic parasites to detoxify the lytic host ferrirrotoporphyrin IX [9,24] released during hemoglobin digestion by parasites [25] were localized almost exclusively in the red pulp, thus indicating that the red pulp predominantly harbors the antimalaria effectors of the spleen. The cytoplasmic vacuolation in splenic cells is mainly a consequence of considerable disturbances
in lipid inclusions and fat metabolism occurring under pathological changes [26,27].

The results of this study showed that the change in apoptotic cell number runs parallel to the change in parasitemia, as previously reported [28]. The curative stage in the spleen (day 35 postinfection) after crisis due to destruction of the parasitized erythrocytes indicated a role of the spleen in the defense mechanism against infection.

This study indicates that a reversible splenic lesion (curative stage) had been achieved after the persistent low-grade parasitemia. Rapid changes in cellular splenic populations are influenced by apoptotic events, demonstrating the role of splenic protective immunity against hematologic *P. chabaudi* malaria.

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