Plasmodium berghei induces apoptotic changes in splenic and peripheral blood cells

Kapoor, G.¹, Bagai, U.² and Banyal, H.S.¹*
¹ Laboratory of Parasitology and Immunology, Department of Biosciences, Himachal Pradesh University, Shimla 171005, India
² Department of Zoology, Panjab University, Chandigarh 160014, India
* Corresponding author email: hsbanyal@yahoo.co.in
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Abstract. Intracellular parasites manipulate host cell apoptosis in different ways either to increase their life span within infected cells or to spread infection. The present data provided information on the cellular changes taking place in spleen and peripheral blood during Plasmodium berghei-infection and indicated apoptosis mediated host immune response during infection. Our results suggested a significant change in cellular composition and absolute number of white blood cells in spleen and peripheral blood of P. berghei-infected Balb/c mice. Plasmodium berghei-infection was associated with marked increase in percentage of apoptotic mononuclear cells compared to polymorphonuclear white blood cells.

INTRODUCTION

Infection with Plasmodium is characterized by both activation and suppression of the immune system during different phases of the disease. Apoptosis or programmed cell death (PCD) is an important mechanism regulating the development, maturation and activation of lymphocytes. Apoptosis is involved in wide range of pathological and physiological events and characterized by chromatin condensation, fragmentation of DNA into oligonucleosome size pieces, swelling and progressive cell degradation. Apoptosis acts as a regulatory mechanism in the development and homeostasis of immune response.

A strong level of apoptosis has been associated with experimental animals whereby apoptosis may act as a protective mechanism that limits the excess inflammatory responses or mechanism to escape the immune response. Apoptosis has been shown to be induced in in vitro-cultured spleen cells from mice infected with Schistosoma mansoni (Fallon et al., 1998), Trypanosoma cruzi (Lopes et al., 1995), and Toxoplasma gondii (Kumararatne et al., 1987) where parasites use apoptosis to escape the immune response. To establish itself inside the host, Plasmodium appears to manipulate the host immune system for its survival by down regulating anti-parasitic inflammatory responses and the host cell apoptosis is influenced by Plasmodium berghei. Apoptosis triggered by malaria has been reported in Plasmodium falciparum (Pino et al., 2003) and Plasmodium chabaudi (Helmby et al., 2000; Sanchez-Toures et al., 2001) infections.

In human malaria, the numbers of circulating lymphocytes in peripheral blood, particularly T cells decrease substantially and frequently (Lisse et al., 1994). The pathophysiology of this lymphopenia became clear when Toure-Bald et al. (1996) reported increase in spontaneous apoptosis of peripheral blood
mononuclear cells in patients with acute *P. falciparum* malaria in comparison to healthy controls. Riccio *et al.* (2003) reported that consistent proportion of T lymphocytes population dies by apoptosis during malaria infection of *P. falciparum* or *Plasmodium vivax*.

The regulation and development of different lymphocyte population in spleen and blood during malaria infection is still poorly understood. An increasing body of evidence suggest that apoptosis is an important factor in host pathogen interaction. The parasite manipulates host cell apoptosis to increase its life span and spread infection. Present study was undertaken to prove the physiological relevance of these results and investigate the changes occurring in cellular composition and absolute number of mononuclear and polymorphonuclear white blood cells in spleen and peripheral blood of *P. berghei*-infected mice.

**MATERIALS AND METHODS**

**Parasite**

*Plasmodium berghei* (NK-65) was maintained in Balb/c mice. A group of 40 mice of either sex 5-6 weeks old weighing 20-25g was injected intraperitoneally (i.p) with 1 x 10⁵ *P. berghei* infected erythrocytes and the course of parasitaemia was monitored till the death of the animal by preparing Giemsa stained thin blood smears from tail blood. A couple of infected animals were sacrificed from the above group having almost the same percent parasitaemia on alternate days post inoculation for tissue and blood collection. All experiments were carried out by procedures authorized by the Institutional Animals Ethics Committee (IAEC) of the university.

**Collection of blood and spleen**

Normal or *P. berghei*-infected mice were anaesthetized with diethyl ether and blood collected in citrate saline, spleen removed on ice weighed, washed with citrate saline and homogenized in appropriate volume of phosphate buffer saline (PBS, 0.01M) pH 7.2 in Potter-Elvehjem homogenizer (Kapoor & Banyal, 2009).

**Separation of WBC using double density gradient**

Normal or *P. berghei*-infected blood was subjected to double density gradient centrifugation for separation of mononuclear and polymorphonuclear (PMN) white blood cells. Double density gradient was formed by layering 3 ml of Histopaque 1077 (Sigma) over 3 ml of Histopaque 1119 (Sigma). Blood was slowly poured over gradient and centrifuged at 700g for 30 min at room temperature. The upper white layer of mononuclear WBC aspirated after removing plasma. Second layer of PMN cells aspirated similarly. Spleen suspension was processed in similar way to obtain mononuclear WBC and PMN cells. WBC obtained were washed twice with 0.01M PBS, pH 7.2 and centrifuged at 800g for 5 min at room temperature.

**Fluorescent staining using acridine orange (AO) and ethidium bromide (EB)**

Morphological assessment of apoptosis was done using acridine orange and ethidium bromide staining by the method of Kasibhatla (1998). The assay method is cheap and can be performed quickly and provides a very useful qualitative evaluation. A suspension of 2 x 10⁶ WBC in 25µl of 0.01M PBS, pH 7.2 was incubated with 10µl each of AO and EB solution (1:1) in 0.01M PBS, pH 7.2. 10µl of above suspension was placed onto microscopic slide and observed under fluorescent microscope (Leica, Germany) for quantification. At least 500 cells were examined under fluorescent light using fluorescein filter before calculating number of apoptotic cells. Live cells stained fluorescent green, whereas, apoptotic cells had orange nucleus showing condensation of chromatin whereas necrotic cells display an orange nucleus with intact structure under UV light.
RESULTS

**Percentage of dead white blood cells in blood**

High level of apoptosis was detected in white blood cells of *P. berghei*-infected mice. In normal blood, the number of dead mononuclear cells was 14.7% and that of dead PMN cells was 9.4% (Table 1).

From day 1 post inoculation the percentage of apoptotic mononuclear cells increased till day 3 (32.8%). The percentage of apoptotic cells decreased on day 5 (30.7%), 9 (19.2%) and 11 (8%) post inoculation respectively. After day 11, the percentage of apoptotic mononuclear cells kept on increasing till day 19 post inoculation having 40% apoptotic cells in peripheral blood (Table 1).

The number of apoptotic PMN cells recorded on day 1 post inoculation was 28.4% which is almost 3 times the number of dead PMN cells in normal mice. Number of apoptotic PMN cells decreased to 18.3% on day 3 to rise again to 38.6 on day 5 (Fig. 1). Thereafter it decreased and increased alternatively.

**Percentage of dead white blood cells in spleen**

On day 1 post inoculation, the percentage of apoptotic mononuclear cells increased from 10% to 35.5% in spleen which increased to 89.5% on day 5 with 10% *P. berghei*-infection. At this time 69.2% of live mononuclear cells were recorded in peripheral blood. From day 7, the percentage of apoptotic mononuclear cells increased regularly till day 11 with maximum 99.8% apoptotic mononuclear cells in spleen as compared to peripheral blood where 91.6% live mononuclear cells were recorded. In contrast to blood mononuclear cells the number of spleen mononuclear apoptotic cells increased on day 13 (45.8%).

8.8% apoptotic PMN cells were recorded on day 3 which increased to 56% on day 5 having 10% infection. An increase on day 11 and 13 with 67.2% and 69.2% apoptotic PMN cells respectively were recorded in spleen.

DISCUSSION

Our results showed significant changes in cellular composition and absolute number of white blood cells in spleen and peripheral blood of *P. berghei*-infected Balb/c mice. The absolute number of mononuclear and PMN cells in spleen and peripheral blood differed significantly (p < 0.05) from each other.

Initial lymphopenia was observed in the present study after inoculating the parasite in the host. The number of live mononuclear cells in blood of normal mice fell from 85% pre-infection to 60% on day 3 post-inoculation. Monocytes have been reported to participate in production, mobilization and regulation of immune effector cells. Mononuclear phagocytic cells have been reported to play an important role in *in vivo* elimination of *P. chabaudi* infection (Stevenson et al., 1989). Lymphopenia in cerebral malaria patients has been attributed to significant increase in spontaneous apoptosis of peripheral blood mononuclear cells (Bald et al., 1995). During viral infections the immune response is often associated with high levels of apoptosis in the spleen through selection of antigen specific cells in early stages of infection as well as reducing the immune response (Welsh & McNally, 1999). In parasite infection, apoptosis have been reported to be induced in fresh spleen cells and *in vitro* cultured cells from mice infected with *Trypanosoma* (Lopes et al., 1995), *Schistosoma* (Fallon et al., 1998) and *Toxoplasma* (Khan et al., 1996). The frequency and number of T cells, B cells and macrophage population in spleen and peripheral blood from *P. chabaudi*-infected Balb/c mice have been reported to be significantly altered during acute infection. Both Fas /FasL expression have been reported to be increased in spleen (Helmby et al., 2000).

Malaria parasite induced apoptosis of white blood cells in the initial stages may be beneficial for the survival of the parasite in the host because it down regulates the anti parasite inflammatory responses of the host during acute infection. The mechanism
of cell death in the subset of white cells might be through the Fas mediated apoptosis of the host T cells (Kern et al., 2000).

Splenomegaly and polyclonal B cell activation are common phenomena associated with malaria in human and experimental murine models. The spleen plays a dual role in both clearing of the parasite from circulation as well as providing a strong haematopoietic response during acute infection (Yap & Stevenson, 1992; Alves et al., 1996). Dkhil (2009) reported splenomegaly and rapid changes
in splenic cell numbers which were influenced by apoptotic events during *P. chabaudi* infection. Marked splenomegaly was also observed with the increase in parasitaemia in the present study.

The percentage of live mononuclear cells in the spleen was almost 90% which fell to 64% just after 24 hours of infection with *P. berghei*. On day 11 when the parasite infected reticulocytes, almost 99% mononuclear cells in the spleen were apoptotic. It corresponded to almost 90% live cells in the peripheral blood at the same time. A similar type of distribution of different cell types in the spleen and peripheral blood with T and B cells appearing in the blood at the time when their absolute number in the spleen were low has also been reported in non-virulent *P. chabaudi* AS (Helmby et al., 2000). Apoptosis has been shown to be increased with increasing parasitaemia in the spleen.

Achtman et al. (2003) described *P. chabaudi*-infection inducing strong B cell response and temporary changes in distribution of lymphocytes in the spleen. Previous observations have supported the role of Fas mediated apoptosis of T cells by *Plasmodium*. Rapid elevation of FasL level in serum of two species of macques infected with *P. coatenii* (Mastumoto et al., 2000) and increased susceptibility to apoptosis and cytokine production among T lymphocyte during cerebral malaria (Kemp et al., 2002) have been reported. A consistent proportion of T lymphocyte population died during *P. vivax* and *P. falciparum*-infection (Riccio et al., 2003).

The present study demonstrated that apoptosis levels were significantly correlated with *P. berghei* infection. We observed that parasite was responsible for an increase in the level of apoptosis of host WBC. The idea that *Plasmodium* can tamper with apoptotic signals to use them for their advantage is interesting as it may explain how the parasite becomes established in their host leading to chronic infection despite the host immune response. The mechanism and the factors responsible for malaria induced apoptosis remains to be fully established. Understanding of parasite immunomodulation can enable us to devise strategies to control the infection.

REFERENCES


