

African Journal of Biotechnology Vol. 9(45), pp. 7611-7616, 8 November, 2010
Available online at <http://www.academicjournals.org/AJB>
DOI: 10.5897/AJB10.1231
ISSN 1684-5315 ©2010 Academic Journals

Full Length Research Paper

Different apoptotic responses to *Plasmodium chabaudi* malaria in spleen and liver

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Accepted 27 September, 2010

The purpose of this study is to determine whether the apoptotic responses to *Plasmodium chabaudi* malaria in spleen and liver via mRNA expression of three genes involved in apoptosis (Bax, Bcl-2 and Caspase-3) are similar or not and to detect if these genes could be a good marker for apoptosis due to infection with *P. chabaudi* of female C57BL/6 mice. Mice were injected intraperitoneally (ip) with 106 *P. chabaudi*-infected erythrocytes and then scarified at days (0, 1, 4 and 8, respectively). Quantitative real time-polymerase chain reaction (PCR) was used to quantify apoptotic genes. The levels of Bax and caspase-3 were significantly increased only at days 1 and 8 in the liver cells and at day 8 in the spleen when compared with day 0. The level of B-cell lymphoma 2 (Bcl-2) was significantly increased at all three days after infection in the liver cells, but significantly decreased at all three days after infection in the spleen. In conclusion, present data has shown that infection with *P. chabaudi* stimulated apoptotic genes in the liver and spleen cells in different ways. Based on the obtained data, it can be hypothesized that the out come of malaria triggered different apoptotic pathways in the liver and spleen. Also, these apoptotic genes can be used as a reliable apoptosis detection method.

Key word: Apoptosis, malaria, mice, liver, spleen.

INTRODUCTION

Malaria is one of the most devastating diseases in the world, particularly in tropical countries. Annually, about 400 million of people get affected with malaria around the world and around 2 millions of them die (Guha et al., 2006a). The few available anti-malaria drugs are becoming increasingly ineffective due to the increasing resistance of the parasites (Carlton et al., 2001). All efforts to develop an effective anti-malaria vaccine have failed to date (Hviid and Barfold, 2008). Basic research is therefore more than ever, urgently, required to understand both protective host mechanisms and pathological complications induced by malaria (Callaway, 2007). A rather convenient malaria model system is the *Plasmodium*

chabaudi blood stage infection in mice, since *P. chabaudi* shares several characteristics with *Plasmodium falciparum*, the most dangerous human malaria species (Hernandez-Valladares et al., 2005). Mice are either resistant or susceptible to blood stages with *P. chabaudi* malaria, which is controlled by both genes of the mouse major histocompatibility complex (MHC), the *H2*-complex and genes of the non-*H2* background as well as diverse endogenous soluble factors (Wunderlich et al., 1988; Roberts et al., 2001).

The liver plays a central role in malaria, it is not only the site of preerythrocytic development of *Plasmodium* parasites, but also acts as an effector against malarial blood stages as recently shown (Krücken et al., 2005b; Wunderlich et al., 2005). In particular, the Kupffer cells, which represent about 90% of all host macrophages, are able to eliminate parasite-derived hemozoin and even *Plasmodium*-infected erythrocytes. During this process, Kupffer cells become increasingly activated, which manifests itself as a production of NO (nitrogen oxide) and diverse radical oxygen species (ROS). The NO and

Abbreviations: MHC, Major histocompatibility complex; ROS, radical oxygen species; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; Bcl-2, B-cell lymphoma 2; i.p, intraperitoneally; qRT-PCR, quantitative real-time polymerase chain reaction; Apaf-1, apoptotic protease activating factor-1.

ROS in turn induce an oxidative stress which results in an increase in apoptosis of hepatocytes (Krücken et al., 2005a; Guha et al., 2006a). On the other hand, spleen is believed to participate in cleaning parasites from the circulation and providing a strong hematopoietic response during acute infections (Dkhil, 2009).

Apoptosis is linked intimately with both physiology as well as pathology in variety of cellular systems (Gourley et al., 2002; Alkahtani et al., 2009). The dysregulation of liver apoptosis during malaria is a critical event in liver pathology. Apoptosis does not only play an essential role in development and tissue homeostasis but is also involved in a wide range of pathological conditions (Oh et al., 2004). ROS have been involved in the apoptosis induced by different stimuli as well as the pathologic cell death that occurs in many diseases (Oh et al., 2004).

Apoptosis may occur via a death receptor-dependent (extrinsic) or independent (intrinsic or mitochondrial) pathway. The death receptor pathway comprises of Fas (CD95/Apo-1) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (Apo-2) and this pathway is activated when ligands specific for either Fas or TRAIL bind to their respective receptors, resulting to activation of caspase-8, which finally activates caspase-3 (Lyke et al., 2004). On the other hand, mitochondrial pathway of apoptosis is initiated by the down-regulation of anti-apoptotic proteins (such as B-cell lymphoma 2 (Bcl-2) and Bcl-xl) and/or up-regulation of pro-apoptotic proteins (such as Bax, Bad and Bid), resulting in opening of mitochondrial permeability transition pores and release of apoptosis inducing proteins (cytochrome c, apoptosis inducing factor, etc.) from mitochondria (Akanmori et al., 1996; Barton, 1996; Gourley et al., 2002; Alkahtani et al., 2009).

The present study was undertaken to investigate whether the apoptotic responses to *P. chabaudi* malaria in spleen and liver via mRNA expression of three genes involved in apoptosis (Bax, Bcl-2 and caspase-3) are similar or not and also, to detect if these genes could be a good marker for apoptosis during infection with *P. chabaudi* in the liver and spleen cells of female C57BL/6 mice.

MATERIALS AND METHODS

Animals and infection

Female C57BL/6 mice 10 - 14 weeks old were obtained from the central animal facilities of Heinrich Heine University, Düsseldorf, Germany and housed in plastic cages. Mice were bred and maintained under specified pathogen free conditions. Mice were fed with standard diet (Wohrlin, Bad Salzuffen, Germany) and water *ad libitum*. The experiments were approved by the State authorities and followed German law on animal protection.

Blood stage malaria

A non-clonal line of *P. chabaudi* (Wunderlich et al., 1988) exhibiting a very similar, but not identical restriction length polymorphism

pattern to *P. chabaudi* was used (Kruecken et al., 2005b). Erythrocytic stages of *P. chabaudi* were passaged weekly in NMRI mice. From these mice, blood was taken and 10^6 *P. chabaudi*-infected erythrocytes were injected intraperitoneally (i.p.) in the mice. Parasitemia was evaluated in Giemsa-stained blood smears. The total number of erythrocytes was determined in a Neubauer chamber. A total of five mice were sacrificed at each day (0, 1, 4 and 8, respectively) by cervical dislocation. Livers were removed and cut into smaller pieces and kept at -80°C .

RNA-isolation

Approximately 250 mg frozen liver and spleen were homogenized with an ultraturrax in 5 ml Trizol (Peqlab Biotechnology, Erlangen, Germany) for one minute. After mixing with 1 ml chloroform for 15 s, the suspension was incubated for 15 min at room temperature and centrifuged at 3.000 g for 45 min. After isopropanol precipitation of the supernatant, the pellet was washed twice with 80% ethanol, air-dried and dissolved in 200 μl RNase-free water. RNA concentrations were determined at 260 nm, and the purity of RNA was checked in 1% agarose gel.

Quantitative real-time polymerase chain reaction (qRT-PCR)

All RNA samples were treated with DNase (Applied Biosystems, Darmstadt, Germany) for at least 1 h and then converted into cDNA following the manufacturer's protocol using the Reverse Transcription Kit (Qiagen, Hilden, Germany). qRT-PCR was performed using the ABI Prism[®] 7500HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) with SYBR Green PCR Mastermix from Qiagen (Hilden, Germany). We investigated the genes encoding the mRNA for the following proteins: 18S, Bax, Bcl-2 and Caspase-3. All primers used for qRT-PCR were commercially obtained from Qiagen. PCR reactions and were conducted as follows: 2 min at 50°C to activate uracil-N-glycosylase, 95°C for 10 min to deactivate UNG, 40 cycles at 94°C for 15 s, at 60°C for 35 s and at 72°C for 30 s. Reaction specificity was checked by performing dissociation curves after PCR. For quantification, mRNA was normalized to 18S rRNA. The threshold Ct value is the cycle number selected from the logarithmic phase of the PCR curve in which an increase in fluorescence can be detected above background. The ΔCt was determined by subtracting the Ct of 18S rRNA from the Ct of the target ($\Delta\text{Ct} = \text{Ct-target} - \text{Ct-18S rRNA}$). The relative mRNA levels of non-infected mice are described as a ratio of target mRNA copy to 18S rRNA copy = $2^{-\Delta\text{Ct}}$. The fold induction of mRNA expression on days (0, 1, 4 and 8 *p.i.*) was determined using the $2^{-\Delta\Delta\text{Ct}}$ -method

$$-\Delta\Delta\text{Ct} = \Delta\text{Ct day 0 } p.i. - \Delta\text{Ct day 8 } p.i.$$

Statistical analysis

Two-tailed student's t-test and Fisher's exact test were used for statistical analysis.

RESULTS AND DISCUSSION

Characteristics of *P. chabaudi* infection

The parasitemia of mice with 10^6 *P. chabaudi*-infected erythrocytes became evident on day 4 and rocketed to reach its peak (48%) on day 8 and then reduced rapidly

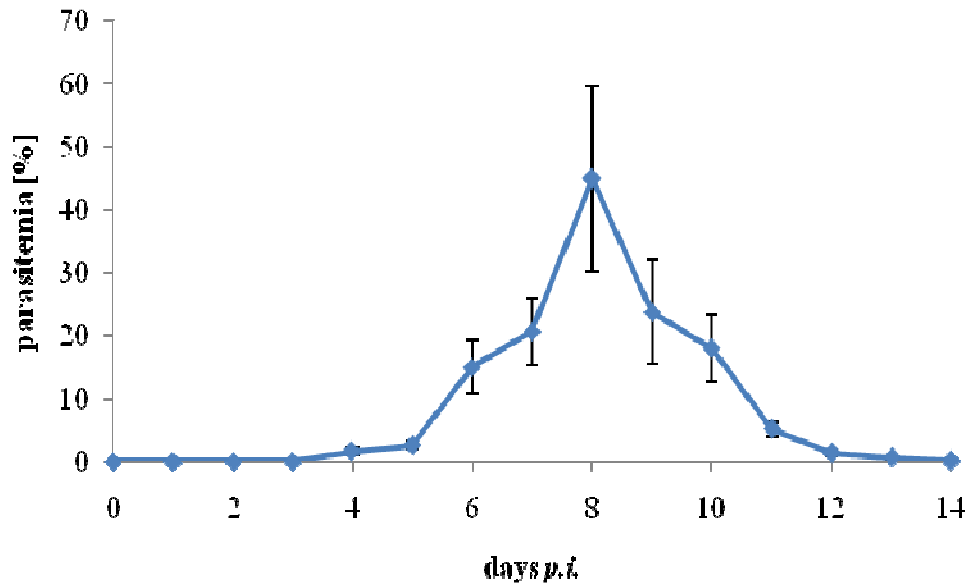


Figure 1. Parasitemia (48%) of female C57BL/6 mice (n = 20) infected with 10^6 *P. chabaudi*-infected erythrocytes.

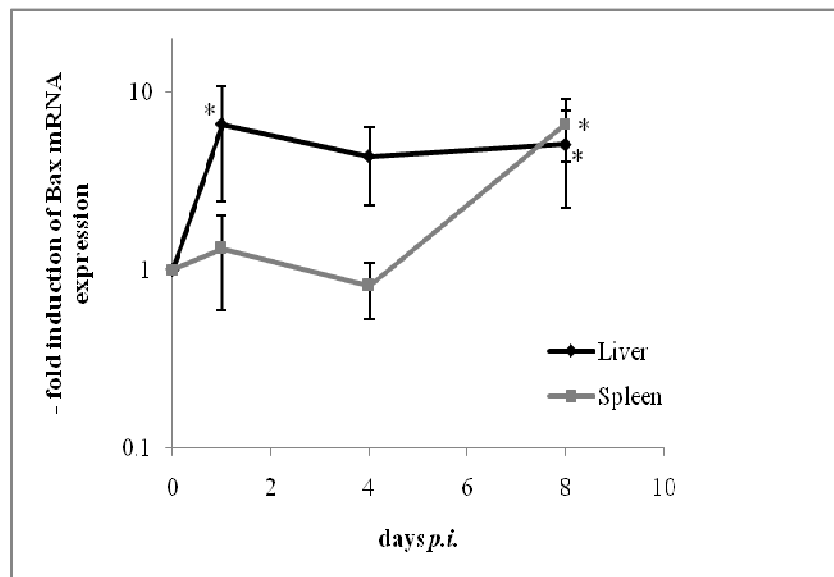


Figure 2. Quantitative real time-PCR of Bax gene expression in the liver and spleen of *P. chabaudi* infected mice. The expression of Bax was measured at different times. The data present are the mean \pm SE (n = 5). *Significant value at (P < 0.05).

to about 0.4% on day 12 (Figure 1).

Apoptotic genes expression

qRT-PCR was used to detect changes in mRNA levels of different apoptotic genes. The level of mRNA expression

for Bax as a pro-apoptotic gene was significantly (P < 0.05) increased at days 1 and 8 after infection in the liver and at day 8 in the spleen when compared with day 0 (Figure 2). The level of Bcl-2 as an anti-apoptotic gene was significantly (P < 0.05) increased at all three days after infection in the liver cells and in contrast, significantly (P < 0.05) decreased at all three days after

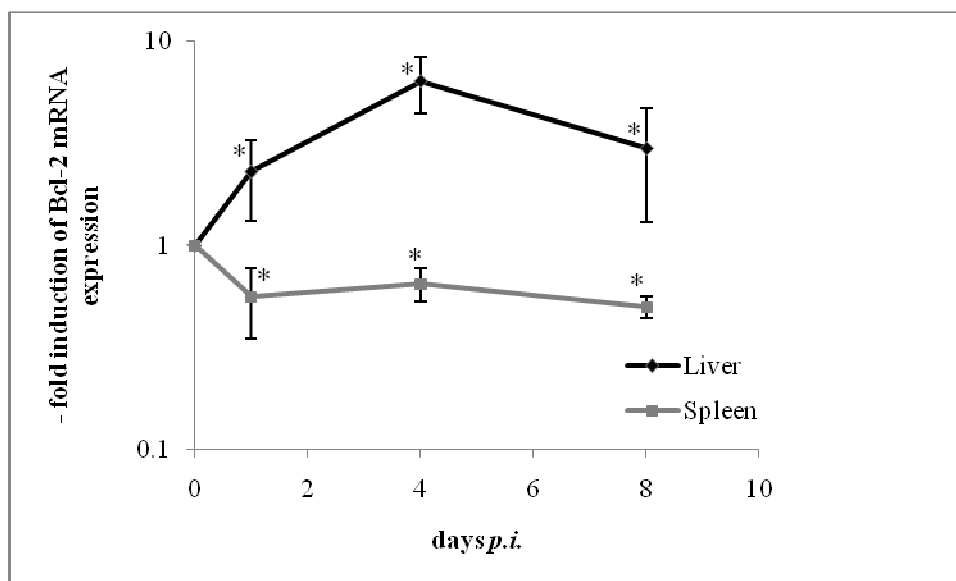


Figure 3. Quantitative real time-PCR of Bcl-2 gene expression in the liver and spleen of *P. chabaudi* infected mice. The expression of Bcl-2 was measured at different times. The data present are the mean \pm SE (n = 5). *Significant value at (P < 0.05).

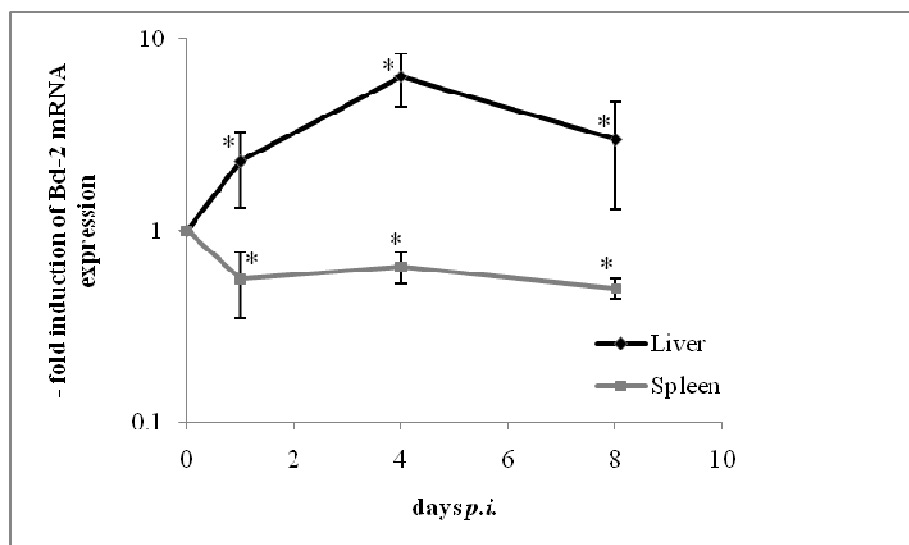


Figure 4. Quantitative real time- PCR of caspase-3 gene expression in the liver and spleen of *P. chabaudi* infected mice. The expression of caspase-3 was measured at different times. The data are the mean \pm SE (n = 5). *Significant value at (P < 0.05).

infection in the spleen cells when compared with day 0 (Figure 3). The level of mRNA expression for caspase-3 as executioner gene was significantly (P < 0.05) increased at days 1 and 8 after infection in the liver and at day 8 in the spleen when compared with day 0 (Figure 4). The present data shows that the infection with *P. chabaudi* induced different apoptotic responses at different time points during malaria infection. These data

are similar with previous results in our laboratory focused on the apoptotic effect (Alkahtani, 2010). Apoptosis is a complex cellular process triggered as a response to a wide variety of stimuli (Bergmann-Leitner et al., 2009). Apoptotic processes are regulated by many of the same biochemical intermediates, including death factors and ROS (Sanchez-Alonso et al., 2004). Presented data agreed with previous results that malaria infection

significantly induces liver apoptosis mediated by oxidative stress mechanisms (Guha et al., 2007). The pathogen of malaria, *Plasmodium*, enters erythrocytes and thus escapes recognition by the immune system and then induces oxidative stress to the host erythrocytes and triggers erythrotosis (Alkahtani, 2009; Föller et al., 2009).

Cell apoptosis is regulated via two major pathways: The intrinsic or mitochondrial pathway and extrinsic or death receptor pathway. Both pathways converge at the level of active effector caspases. Caspase-independent mechanisms may also contribute to cell death. Effector caspases cleave various cellular target proteins finally leading to apoptosis (Alkahtani et al., 2009). Apoptosis could result from a combination of both pathways: the intrinsic apoptosis pathway by generation of oxidative stress and the extrinsic apoptosis pathway by activation of Kupffer cells which can secrete TNF (Alkahtani, 2009). In the spleen, splenomegaly and changes in splenic cell numbers were the most common changes linked to the changes in parasitemia of *P. chabaudi* and this may reflect lymphocyte migration to the peripheral blood. On the other hand, rapid change in cellularenic population proves the role of the spleen in protective immunity against blood-borne infection due to *P. chabaudi* malaria (Dkhil, 2009).

Intrinsic apoptotic pathway is initiated when Bcl-2 lead to the release of cytochrome *c* and other pro-apoptotic cofactors from the inter-membrane space of mitochondria which is consider as an essential cofactor required for the activation of caspases and is regulated by the Bcl-2 family of proteins upstream of caspase activation (Baliga and Kumar, 2002). Once in the cytosol, cytochrome *c* interacts with the apoptotic protease activating factor-1 (Apaf-1) and the procaspase-9 forming the apoptosome complex (Yuan and Yankner, 2000). The result is the cleavage and activation of procaspase-9 and other procaspases that are responsible for the executive stages of apoptotic cell death. The Bcl-2 family of proteins localizes at membrane compartments during apoptosis and can either promote or inhibit apoptosis. Substantial evidence suggests that the primary role of Bcl-2 family proteins is to regulate the release of cytochrome *c* from mitochondria. Briefly, Bcl-2 is an apoptosis suppressing factor that heterodimerizes with Bax and neutralizes the effects of the latter. When Bcl-2 is present in excess, cells are protected against apoptosis. In contrast, when Bax is in excess and the homodimers of Bax dominate, cells are susceptible to programmed death. Therefore, it is the ratio of Bax to Bcl-2 which determines the fate of a cell (Sanchez-Alonso et al., 2004). Infections with intracellular pathogens may provide an appropriate stress-related signal which would normally trigger the intrinsic pathway of apoptosis and, thereby, disturb or even prevent the further development of the affected host cells as a response to intracellular pathogens (Graumann et al., 2009). Recently, it has been reported that the p18/Bax fragment cleaved from full-length Bax (21-kDa) is as efficient as full-length Bax in

promoting cytochrome *c* release (Oh et al., 2004).

Gene expression analysis using reverse transcriptase (RT)-PCR indicates the significant up-regulation of Bax expression in liver of malaria infected mice, suggesting the involvement of mitochondrial pathway of apoptosis (Guha et al., 2006b). However, besides the fact that different apoptotic markers cannot always be detected at the same time, very little is known on the exact timing of the successive steps in the apoptotic pathways and for this reason, it is suitable to confirm this at the protein level. This is in agreement with the opinion of many researchers arguing that the detection of mRNA differences can only indicate a biological significance if protein expression and activity can confirm the results; this is certainly true for caspases because they are secreted as inactive procaspases, which are only active after further modification (Huerta et al., 2007; Vandaele et al., 2008).

As observed in this study, overexpression of apoptotic genes leads to apoptosis. Up-regulation of Bax and/or down-regulation of Bcl-2 mRNA have been observed in several experimental models and indicate that the expression of Bax and Bcl-2 genes may be regulated by p53 (Mishra et al., 2006). Under our experimental conditions, malaria infection with *P. chabaudi* induces apoptosis in the liver and spleen cells via Bax, Bcl-2 and caspase-3 tests in different ways. So this study suggests that more investigations are needed to confirm apoptosis by using other tests such as studying morphological changes, apoptosis mechanism in liver and spleen cells, ultrastructure of cells that may undergo apoptosis and measurement of the protein levels as well.

ACKNOWLEDGEMENT

This study was supported by a grant from Research Center, Teachers College, King Saud University, Riyadh, Saudi Arabia. Project No. (BIO/2010/2) I gratefully acknowledge this support.

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