The in Vitro Effect of Plasmodium berghei Antigen Lysate on Natural Killer (NK) Cell Cytotoxic Activity

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The in Vitro Effect of *Plasmodium berghei* Antigen Lysate on Natural Killer (NK) Cell Cytotoxic Activity

**Abstract:** *Plasmodium berghei* is the rodent equivalent of *Plasmodium falciparum* which causes cerebral malaria in humans. However, *P. berghei* does not cause cerebral malaria in BALB/c mice, instead it leads to parasitemia, fever and some degree of liver damage. Although different aspects of immunological response have been intensively investigated in malaria; this is the first time the in vitro effect of *P. berghei* antigen lysate on murine NK cytotoxic activity was studied. It was observed that this parasite antigen lysate suppressed the cytolytic activity of NK cells in a dose-dependent manner. Because the suppression of NK cell activity in a dose-dependent manner by *P. berghei* antigen lysate is not in harmony with the fact that BALB/c mice is resistant to cerebral malaria caused by this parasite in other rodents, we concluded that NK cell activity may not play a major role in this resistance.

**Key Words:** Natural killer (NK) cell, cytotoxicity, *Plasmodium berghei*.

### Introduction

*Malaria* is still one of the major public health problems because of its important morbidity and mortality, and cerebral malaria is the most life-threatening complication (1). South-East Asia cerebral malaria (CM) is accompanied by severe pathology in different organs whereas other organ pathology is usually absent in African children with cerebral malaria (2). While CM is often a fatal complication of human *Plasmodium falciparum* infection, its pathophysiology remains poorly understood (3). The lack of suitable animal model has hampered the progress in the understanding of the human disease process. Nevertheless, a mouse model, despite the numerous differences reviewed by White and Ho in 1992 (3), has allowed the recognition of critical factors which help specifying some steps of human CM (4). TNF plays a pivotal role. It is uncertain what mediates protection against malaria in human, but certain MHC molecules (5) and anti-resetting antibodies (6) have both been associated with protection against severe malaria while antibodies reactive to several different antigens (7–10) and T cell reactivity to merozoite surface protein (10) have been associated with protection against clinical disease.

*Plasmodium berghei* is the equivalent of *P. falciparum* of humans in the rodents. *Plasmodium berghei* is malaria strain causing cerebral malaria in different mouse strains, for example CBA/J mice, but not in BALB/c mice. This parasite mainly causes parasitemia, anemia, fever and some degree of liver damage in BALB/c mice (11). Mononuclear phagocytes from CBA/J, the CM–susceptible mouse, have been shown to produce more nitric oxide than those of BALB/c, the CM–resistant mouse, after stimulation by IFN-γ and TNF-α, two critical cytokines in mouse CM (11). In the *P. berghei* ANKA infections, cerebral pathology is the only pathology found and it can be prevented by treatment with antibody against TNF (12) or by suppression of monocytopenia (13). Activation of the immune system in malaria plays an important role in both defense against parasites and pathogenesis. Thus, in murine malaria, T cells mediate protection against infection (14), and yet contribute to the development of CM in *P. berghei*–infected CBA mice (15).

Protective immunity to *P. berghei* can be obtained by vaccination of mice with irradiated sporozoites injected by intravenous route. This immunity has several unique features; it is stage-specific and species-specific but it is
not strain–specific. Above all it is very strong, sterile type of immunity that enables the animals to resist very high challenge doses (16). Cerebral symptoms in a murine malaria infection (P. berghei) can be blocked by antibodies against TNF. However, in another model, using different strains of same parasite and a different mouse strain, this result cannot be reproduced (17).

As a part of immune system, the role of natural killer (NK) cells in plasmodial infections has been investigated by various scientists. Eugui and Allison reported that mouse strain resistant to Plasmodium chabaudi infection showed high NK cell activities whereas other scientists reported that there was no relationship between NK activity and protection against P. chabaudi infection using NK cell deficient mice (18, 19). There are, however, some findings suggesting that NK cells play certain roles in protection against plasmodial infection: Cytotoxicity of human NK cells to schizonts of P. falciparum (20), natural cytotoxicity of rat spleen cells to P. berghei–parasitized erythrocytes (21) and possible relevance of IFN and NK cell system in P. falciparum infection (22).

In this study, we aimed to show the in vitro effects of P. berghei on NK cell activity of splenocytes of BALB/c mice which have been known to be CM–resistant; this could help to explain, at least in part, why this strain of mouse can be infected by this parasite, but dose not develop CM contrary to most of other rodents.

Materials and Methods

Mice: We used 4 female BALB/c mice (purchased from Harlan) for preparation of antigen lysate, 6 male BALB/c mice in the first set of experiments and 5 BALB/c mice (3 males, and 2 females) in the second set of experiments. All mice were six to eight weeks old and healthy.

Parasite: Plasmodium berghei ANKA 6653 strain was provided by Kemal Çeber from the Institute of Parasitology, Baku, Azerbaijan.

Plasmodium berghei antigen lysate: The P. berghei–infected erythrocytes had been frozen–stroed in liquid nitrogen. After these erythrocytes were thawed and washed twice with 0.9% NaCl solution (saline), they were administered to 4 female BALB/c mice intraperitoneally. Nine days later, mice were anesthetized with ketamine at 80 mg/kg. After opening their thoracic cavities, 1 ml of blood was drawn from heart of each mouse into a heparinized syringe (50 µl liquemin). Heparinized blood was diluted with an equal volume of saline. After diluted blood is layered on 3ml Ficoll–Hypaque (Histopaque–1077, Sigma), it was centrifuged at 1500 rpm for 30 minutes. Under these conditions most of the infected erythrocytes suspended in mononuclear cell containing buffy coat. Buffy coats containing infected erythrocytes were collected, pooled and centrifuged. After washing the pellet with saline solution for 3 times, supernatant was discarded, and 8 ml of pyrogen–free distilled water was added onto the pellet. It was resuspended and left at room temperature for 20 minutes, and then ultracentrifuged at 4°C at 39,000 rpm for 30 minutes using Sorvall TST 60.4 rotor (OTD Combi, Sorvall Ultracentrifuge, Du Pont Company, Wilmington Delware 19898, USA). Supernatant was discarded and 6 ml of pyrogen–free distilled water was added onto the pellet. Sonic oscillation (Vibra Cell, VC 100 Ultrasonic processor, Sonics & Material Inc. Connecticut, USA) was applied to completely disperse the parasite antigen lysate. After sonification, antigen lysate was lyophilized (Vitris Benchtop 3L, Vitris Company, Inc. Gardiner, NY 12525, USA). In the experiments, this lyophilized antigen lysate was weighed, and desired concentrations of antigen solution was prepared by adding RPMI–1640 (Sigma Chemical Co., St. Louis, Ma., USA). Antigen solutions were filtered with 0.2 µm filters (Costar) before usage. After making preliminary experiments, final antigen concentrations were adjusted to desired levels between 25–200 µg/ml, and 0.3–30 µg/ml for the first and the second sets of experiments respectively.

Effector cells: Effector cells were isolated from the spleens of BALB/c mice. BALB/c mice were killed by cervical dislocation and splenectomy was performed with a lateral incision. Each spleen was teased in 5 ml 0.9% NaCl in a small plastic petri–dish and homogenized by flushing up and down several times with a syringe. The spleen cell suspension was layered onto 3 ml of Histopaque (Sigma) and then centrifuged at 1500 rpm for 30 minutes. Buffy coat was collected with a Pasteur pipette and washed twice with 0.9% NaCl. The pellets were resuspended in 4 ml of RPMI–1640 medium (Sigma) with 5% inactivated Fetal Calf Serum (FCS, Biowhitech). Then the cell suspension was transferred into 3x10 mm cell–culture treated plastic petri–dish (Greiner) and incubated in a humidified atmosphere of 5% CO2 at 37°C for 30 minutes. This provided the monocytes to adhere the plastic surface. After the incubation, non–adherent cells were harvested and washed once with 0.9% NaCl. The pellet was thoroughly resuspended in RPMI–1640 supplemented with 10% FCS. Viability of the effector cells was >97% by trypan blue dye exclusion. Effector cell concentrations were adjusted to 1x10³ cells/ml in RPMI–1640 with 10% FCS.
Target cells: YAC–1 cell line was maintained in RPMI–1640 supplemented with 0.3 gr/l L–Glutamine and 10% inactivated FCS in a humidified atmosphere of 5% CO2 at 37°C. Target cell viability was always higher than 90% in each of the experiments.

NK Cytotoxicity Assay: In order to determine natural killer (NK) cell cytotoxic activity, Chromium–51 release assay was used with some modifications of that described elsewhere (23). Briefly, YAC–1 cells were labeled by incubating 2–3x106 cells with 200 μCi of sodium chromate (51Cr) (CJ51, Amersham, UK) for 1 hour at 37°C, pelleted, washed twice with 10 ml of RPMI–1640 and resuspended at the concentration of 1x105 cells/ml. Control cytotoxicity at Effector: Target ratios (E/T) used were 100/1, 50/1, and 25/1 in wells of U–bottomed 96–well microtiter plates. The effect of P. berghei antigen lysate on NK cytotoxic activity was compared to control at E/T: 50/1. Each E:T ratios was studied in triplicate. Antigen suspensions were prepared at 1000, 500, 250 and 125 μg/ml for the first set of experiments and 150, 50, 15, 5 and 1.5 μg/ml for the second set of experiments and 50 μl of each antigen suspension was added upon 100 μl of effector cells in appropriate wells 15 min before adding 100 μl of target cells (1x104 cells). Thus, final antigen concentrations during the assay period became 200, 100, 50 and 25 μg/ml for the first set of experiments and 30, 10, 3, 1 and 0.3 μg/ml (in wells) for the second set of experiments. Microtiter plates were incubated at 37°C for 4 hours. After incubation, the plates were centrifuged at 600 rpm for 6 minutes and 125 μl of supernatant from each well was taken and counted by a gamma counter (LKB 1275, Minigamma counter).

In order to determine the spontaneous release (control), target cells were incubated with 150 μl medium only. Maximum release was obtained by incubating target cells with 150 μl of 2% sodium dodecyl sulfate.

Cytotoxicity was calculated from the average counts per minute (cpm) released into the supernatants of triplicate samples by the following formula:

% specific cytolysis= (sample cpm–spontaneous cpm) / (maximum cpm–spontaneous cpm) x 100

Statistical method: The student’s t–test was used in order to make statistical analysis of differences.

Results

P. berghei antigen lysate was found to suppress NK cell cytotoxic activity in vitro. In the preliminary experiments the effect of P. berghei antigen lysate on NK cell activity was investigated at the final concentrations from 25 to 200 μg/ml. These antigen concentrations were found to be too high to make a decision about the breakpoint effect on NK cytotoxic activity (Table 1).

Therefore, we later adjusted the desired final antigen concentrations between 0.3 and 30 μg/ml. That time, as shown in Figure 1, it was observed that P. berghei antigen lysate suppressed the cytolytic activity of NK cells in a dose dependent manner (p= 0.044 for 3 μg/ml vs control, p=0.0102 for 10 ug/ml vs control, p=0.00478 for 30 μg/ml vs control).

Discussion

Malaria is still a very important public health problem because of its dangerous complications like cerebral malaria caused by P. falciparum in humans (1). P. berghei is the equivalent of P. falciparum of humans in the rodents; however, this species does not cause cerebral malaria in BALB/c mice, instead it causes parasitemia, anemia, fever and some liver damage (11). Many immunologic aspects of P. berghei infection has been intensively studied in different types of rodents; however,

Table 1. Effect of Plasmodium berghei antigen lysate on NK cytotoxic activity at the concentrations of 25–200 μg/ml.

<table>
<thead>
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<th>Mouse</th>
<th>0</th>
<th>25</th>
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<th>100</th>
<th>200</th>
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<td>16.27</td>
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<td>22.29</td>
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<td>9.35</td>
<td>10.11</td>
<td>11.90</td>
<td>13.27</td>
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<tr>
<td>e</td>
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<td>12.11</td>
<td>16.89</td>
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<tr>
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<td>6.23</td>
<td>8.40</td>
<td>9.32</td>
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* % Cytotoxicity.
The in Vitro Effect of Plasmodium berghei Antigen Lysate on Natural Killer (NK) Cell Cytotoxic Activity

Natural killer cells comprise 4 to 15% of the mononuclear cells in the peripheral circulation, and have important roles in the immune responses against tumors and viral infections as well as inducing production of different cytokines like IFN–\(\gamma\) (24). The role of NK cells in the protection against plasmodial infections has remained unclear.

It was reported that mouse strains resistant to P. chabaudi infection showed high NK activity by Eugi, et al. (16), but two other groups of workers suggested that there was no relationship between NK cell activity and protection against P. chabaudi infection using NK cell–deficient mice (18, 19).

The effect of this parasite on the NK activity of splenocytes of BALB/c mice has not been investigated.

Augmentation of NK activity by administration of MgCl\(_2\) failed to protect mice against P. yoelii infection (25). In the experiments performed with bg/bg mutated mice, which were shown to have reduced NK activity (26), it was concluded that the level of NK activity was not related to host resistance to malaria because these mice could resolve P. chabaudi infection (19).

NK cells are thought to have non–specific immunity and not to be involved in immunological memory (21). Solomon et al. reported an earlier onset of parasitemia and earlier death in NK cell–deficient mice with P. berghei compared to normal mice (27). Adoptive transfer of NK cells from infected mice seemed to give no protection except for a slight delay in the onset of infected erythrocytes; that is, naive mice should have sufficient amounts of NK cells and they do not need adoptive

Figure 1. Effect of P. berghei antigen lysate on NK cytotoxic activity at various concentrations. Antigen was present with the effector and target cells during the assay period. NK cytotoxic activity was suppressed with antigen in a dose dependent manner (p=0.0201, \(r=-0.8819\)). Asterisks denotes significant differences (P<0.05) when compared to control (0). Data are the mean±SEM of five independent experiments.
transfer of an excess NK cells to protect them against infection. However, when NK cells were depleted in vivo by monoclonal antibody treatment, mortality increased more than twice compared to controls. Therefore, Kitaguchi et al. thought that NK cells might not prevent mice from increasing parasitemia, but these cytotoxic cells might play an important role in recovery from parasitemia, probably by secreting IFN–γ and might finally decrease the mortality in P. chaubaudi infection (28). Stevenson and his coworkers showed that IL–12 protected mice against blood stage P. chaubaudi infection (29); therefore, NK cells may contribute to protection against plasmodial infections in the early phase through their activation by IL–12 and sequential secretion of IFN–γ (28).

In our first set of experiments, we had used 25–200 µg/ml P. berghei ANKA 6653 antigen lysate; however, these concentrations were too high to make any statistically significant comparison between the spontaneous NK activity and stimulation by antigen. Therefore, in our second set of experiments we decided to lower our antigen doses and repeated our study. This time we used 0.3–30 µg/ml P. berghei antigen lysate, and it was observed that cytolytic activity of mice splenocytes was suppressed significantly with increasing doses of parasite antigen (Figure 1). Nevertheless, we have to decrease the antigen doses to much lower values to see where the suppression ceases. With this purpose we performed some prologue studies with antigen doses of 0.01 µg/ml, 0.03 µg/ml and 0.1 µg/ml in 2 BALB/c mice (data not shown) and saw that suppression of NK activity was overcome when dose was decreased to 0.03 µg/ml in one mouse and to 0.01 µg/ml in the other one. Consequently, we need a large number of experiments with lower doses of P. berghei antigen lysate in order to determine the dose limits acting on NK activity.

NK cells are important cytotoxic cells of immune system, especially effective on tumor cells and virally infected cells. Their role in various parasitic diseases are also under investigation. Parasites which act specifically on spleen, like malaria, might be affected by these cells which are rich in spleen. Eugui et al. investigated the differences in susceptibility of various mouse strains to haemoproteozoa infections and though about the possible correlation with NK activity (30). Although we found out that P. berghei antigen lysate suppressed the NK activity in BALB/c mice, we know that this parasite does not cause cerebral malaria in this mouse strain. However, this does not mean that the parasite does not cause any problem; it causes some parasitemia, etc. (11). This may be explained by the parasite burden; the amount of parasite entering the body may be enough to cause an infection together with NK suppression; however, it may still not be enough to challenge with all other compartments of immunological system in order to cause cerebral malaria.

References

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