Preventive Effect of *Toxoplasma* Lysate Antigen (TLA) on Fatal Infection with Mouse Piroplasma

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(Received 7 May 1984/Accepted 7 January 1985)

ABSTRACT. *Toxoplasma* Lysate antigen (TLA), mouse *Babesia* lymphokines (B-LKs) and mouse *Toxoplasma* lymphokines (Tp-LKs), were used to examine the effect of immune adjuvant in reducing clinical symptoms of *Babesia rodhaini* infection in mice. Normal mice were treated twice at an interval of two weeks with an emulsion of TLA in Freund's incomplete adjuvant (TLA emulsion), or a combination of TLA emulsion and B-LKs or Tp-LKs. Two weeks after the second treatment, these mice were infected with *B. rodhaini* (10⁵ parasitized erythrocytes/mouse, i.p.). Thirty five point seven percent (5/14, number of survivals/number examined) of mice treated with TLA emulsion, 53.3% (8/15) of mice treated with a combination of TLA emulsion and B-LKs, and 73.3% (11/15) of mice treated with a combination of TLA emulsion and Tp-LKs, survived for more than 20 days postinfection. None of the mice in the non-treated control group survived for more than 12 days postinfection. The defence mechanisms possibly involved in the group treated with TLA emulsion and combination of TLA emulsion and Tp-LKs, were examined with respect to macrophage activation. Mice treated with TLA emulsion showed a slight increase of leukocyte count, and O₂ release of macrophages. The activity of macrophage migration inhibitory factor (MIF) of spleen cell culture supernatant and serum was not increased in this group. The mice treated with a combination of TLA emulsion and Tp-LKs showed increased MIF activity and greater macrophage phagocytosis. These results suggest that treatment with TLA emulsion and a combination of TLA emulsion and B-LKs or Tp-LKs induces a degree of immunity in the host against *Babesia* infection, and that macrophage activation is part of this defence mechanism.—**Key words: Babesia rodhaini**, *H₂O₂* and O₂ release, Macrophage activation, *Toxoplasma* adjuvant.

When mice were sensitized with *Toxoplasma* antigen, interferon-γ (IFN-γ) appeared in their blood stream and they showed a strong nonspecific resistance [9–11].

Like *plasmodium, Babesia rodhaini,* or mouse *Babesia,* is an intraerythrocytic parasitic protozoa. Basic studies are under way to alleviate clinical infection with it in the host and to detect a substance conferring a preventive effect on the host. The present experiment was carried out as a part of this study with *Toxoplasma* lysate antigen, the emulsion of Freund's incomplete adjuvant, the supernatant of splenic cell culture originated from a group of *Toxoplasma*-immune mice (mouse *Toxoplasma* lymphokines), and the supernatant of splenic cell culture originated from a group of mice suffering from chronic *Babesia* infection (mouse *Babesia* lymphokines). These materials were examined for their effect as immune adjuvant (immune modulator) on *Babesia* infection of mice.

**MATERIALS AND METHODS**

**Experimental mice and organisms:** The Australian strain of *Babesia rodhaini* used in this study was originally supplied by the National Institute of Animal Health, Ministry of Agriculture, Forestry and Fisheries. More than 500 adult male ICR-JCL mice weighing 25–30 g were used in the experiment. An attempt was made to establish *Babesia* infection in mice that the mice might die approxi-
mately 12 days after inoculation (a.i.). In it, each mouse was inoculated intraperitoneally (i.p.) with approximately $1 \times 10^2$ parasitized erythrocytes (PE).

Production of Toxoplasma- and Babesia-immune mice: Toxoplasma-immune mice were prepared in the same manner as described previously [6]. Male mice were inoculated i.p. with approximately $1 \times 10^5$ Toxoplasma protozoa. Then the mouse was treated orally with 2 mg of sulfamethopyrazine (SMPZ, Kyowa Hakko Co., Tokyo) as a therapeutic 7, 9 and 11 days after the initial inoculation. Five weeks after inoculation, the mice were subjected to the first challenge by i.p. with $1 \times 10^5$ trophozoites of the highly virulent S-273 strain. Mice which survived after this challenge were subjected to the second challenge with $1 \times 10^5$ trophozoites of Toxoplasma RH strain four weeks after the first challenge. Mice which survived for 2 weeks after the challenge were used as Toxoplasma-immune mice.

Mice were inoculated i.p. with approximately $1 \times 10^2$ PE each. Then they were treated by intramuscular injection with 0.1 mg of 4, 4-diazoaminodibenzamidine diaceturate (Ganazeg; E. R. Squibb & Sons, Inc.) per head 7, 8, 9, 11, 12 and 13 days a.i. Mice which survived for 4 weeks a.i. were challenged by intraperitoneal inoculation with $1 \times 10^2$ PE. Those which survived for 4 weeks after challenge were used as Babesia-immune mice.

Preparation of Toxoplasma and Babesia lysate antigen: Toxoplasma lysate antigen (TLA) was prepared by the method described by Igarashi et al. [3]. As for Babesia lysate antigen (BLA), essentially the same method as that of Itoh et al. [5] was used for the preparation of freeze-thawed antigen extracted with water from erythrocytes infected with Babesia rodhaini.

Preparation of spleen cells and lymphokines: Spleen cells were separated from the suspension of the spleen from normal, Toxoplasma-immune and Babesia-immune mice by a slight modification of the Conray-Ficoll method [5]. When spleen cells were collected from a mouse sensitized with TLA emulsion or a normal mouse, Con A was added as mitogen to their suspension that the final suspension might contain 5 $\mu$g of Con A per ml. When spleen cells were collected from a Toxoplasma-immune mouse and a Babesia-immune mouse, TLA and BLA, respectively, was added to the suspension of these cells that the final suspension might contain 50 $\mu$g of protein from each antigen per ml. After Con A or antigen was mixed well with the spleen cell suspension, this suspension was incubated in 5% CO$_2$ at 37°C for 48 hours. The supernatants of the cultures of spleen cells collected from the Toxoplasma-immune mouse and the Babesia-immune mouse were used as Toxoplasma lymphokines (Tp-LKs) and Babesia lymphokines (B-LKs), respectively. A macrophage migration inhibitory factor (MIF) assay was performed by the modified agarose droplet method [1, 3]. Babesia-infected erythrocytes were added to the macrophage monolayers by a slight modification of the method of Ishimine et al. [4] and Takahashi et al. [14].

Estimation of amounts of H$_2$O$_2$ and O$_2$ released by macrophages in the peritoneal cavity: Cells exuded in the peritoneal cavity of the mouse were collected by washing this cavity with HBSS. They were washed with HBSS by centrifugation at 270 g at 4°C for 5 minutes. To the resulting precipitate 0.2% saline was added and allowed to stand for 30 seconds for hemolytic treatment. An equal volume of 1.6% saline was added to the resulting cell suspension, which became isotonic again. This suspension was washed three times with HBSS and once with TC-199 by centrifugation at 270 g at 4°C for 5 minutes. To it was added an amount of TC-199 with addition of 10% CS that the resulting suspension might contain $1 \times 10^6$ cells/ml. Then 1 ml of this suspension was placed in
each well of a culture plate. The culture plate was incubated in an atmosphere containing 5% CO₂ in an incubator at 37°C for 3 hours to estimate the amounts of H₂O₂ and O₂ released and the amount of protein. Tissue blocks were minced into cubes about 0.5 mm in size with a pair of scissors and washed three times with 0.9% saline. About 60 tissue cubes were placed in each of three test tubes and treated for estimation in the same manner as the monolayer culture of macrophages. The activities of H₂O₂ and O₂ were estimated by essentially the same method as described by Suzuki et al. [13].

Estimation of enzymes contained in serum: Serum glutamic-oxalacetic transaminase (GOT) and serum glutamic-pyruvic transaminase (GPT) were estimated by the Karnen method, lactic dehydrogenase (LDH) by the Wroblewski and Ladew method, creatine phosphokinase (CPK) by a modified Rosal-sky’s method, blood urea nitrogen (BUN) by the urease indophenol method, total protein by an Atago albumin refractometer, creatine by the alkaline picric acid method, serum bilirubin by the Evelyn-Marron method, and blood glucose by the enzymatic method.

Preventive effect of TLA on clinical infection with mouse Babesia: They were divided into six groups of three each by the dose of TLA; that is 20, 50, 100, 200, 500 and 1,000 µg. In addition, one untreated control group of three was set up.

TLA was emulsified in Freund’s incomplete adjuvant (FIA). Each mouse was injected intraperitoneally with the TLA emulsion twice at a two-week interval. It was inoculated intraperitoneally with 1×10² Babesia-infected erythrocytes 2 weeks after the second injection with the TLA emulsion. Blood smear specimens were prepared from it 5, 8, 10, 12, 14 and 16 days after infection to examine the rate of Babesia infection in erythrocytes and fatality rate. On each specimen five microscopic fields were examined to observe 200 erythrocytes per field, or 1,000 erythrocytes in total. Erythrocytes infected with Babesia were counted and the rate of infection was expressed in percentage.

Preventive effect of simultaneous administration with TLA emulsion and B-LKs or Tp-LKs on clinical infection with mouse Babesia: The dose of administration of TLA used was 500 µg per mouse. Mice were injected simultaneously with the amount of TLA emulsion containing this dose and either B-LKs or Tp-LKs to determine whether the preventive effect of TLA was enhanced on death from mouse Babesia infection or not. Four groups of normal mice each were set up. One of them served as an untreated control. The other three, groups I, II, and III, were administered with the TLA emulsion alone, with the TLA emulsion and B-LKs, and with the TLA emulsion and Tp-LKs, respectively.

In the three groups, each mouse was administered intraperitoneally with the TLA emulsion twice at a two-week interval. The total amount of this emulsion administered to it contained 500 µg of TLA.

In groups II and III, each mouse was administered intraperitoneally with 1 ml of B-LKs and Tp-LKs, respectively, 3 and 25 days after the first administration with the TLA emulsion. In all the groups, each mouse was inoculated intraperitoneally with 1×10² erythrocytes infected with Babesia 28 days after the initial administration with the TLA emulsion. The erythrocyte and leukocyte counts and hematocrit value were examined in the blood, the rise and fall in parasitemia observed in blood smears, and fatality rate were determined in the inoculated mice every five days up to 20 days after inoculation.

Response of mice to administration with TLA emulsion alone: To clarify the defence mechanism to death from mouse Babesia infection after the sensitization with TLA emulsion, mice were examined for response to administration with TLA emulsion alone.

Five adult female mice of the ICR/JCL
strain were injected intraperitoneally with TLA emulsion twice at two-week intervals. They were used as sensitized mice 2 weeks after infection. A control group was set up with five non-sensitized normal mice. In all the mice the erythrocyte, leukocyte, and differential leukocyte counts, hematocrit and hemoglobin values were determined in the blood, GOT and GPT estimated in the blood plasma, MIF activity and physicochemical properties of protein observed in the serum, and MIF activity were estimated in the supernatant of splenic cell culture. The liver, spleen, kidney, and lung were fixed in formalin, embedded in paraffin, and cut into thin sections, which were stained with hematoxylin and eosin and examined histopathologically. In addition, the amounts of H$_2$O$_2$ and O$_3$ produced by peritoneal macrophages were estimated. The amount of H$_2$O$_2$ produced was determined in the histological sections of liver, kidney, and heart.

**Test of ability of peritoneal macrophages to phagocytize Babesia-infected erythrocytes in mice sensitized simultaneously with TLA emulsion and Tp-LKs:** Two groups of 25 normal mice each were used for this test. In one group, the mice were injected intraperitoneally with TLA emulsion twice at two-week intervals and with 1 ml of Tp-LKs 3 and 25 days after the first injection. This group served as a sensitized one. The other group served as the control consisting of non-sensitized mice. In each group blood samples were collected from 20 mice 27 days after the first injection with TLA emulsion, and serum was separated from them. The spleen was harvested from these mice to culture splenic cells for 48 hours. Then, the supernatant was separated from the splenic cell culture. In each group peritoneal macrophages were collected from the remaining five mice 28 days after the first injection with TLA emulsion. They were cultured for 24 hours to obtain a monolayer culture of macrophages. Babesia-infected erythrocytes treated with homologous serum were added to the monolayer culture of macrophages obtained in each group. A well containing culture medium to which the supernatant of splenic cell culture had been added was compared with a well containing the culture medium alone to observe the difference in the ability of macrophages to phagocytize erythrocytes.

**RESULTS**

*Preventive effect of TLA emulsion on clinical mouse Babesia infection:* In the control group, parasitemia began to be observed 5 days after infection and two and one mice died 9 and 10 days a.i., respectively.

In group I and III where each mouse had been administered with TLA emulsion containing 20 and 100 μg of TLA, respectively, one of the three mice of each group survived even 16 days a.i. In group V where each mouse had been administered with TLA emulsion containing 500 μg of TLA, two of the three mice survived even 16 days a.i. In the other groups, parasitemia began to be noticed 5-8 days a.i. and all the mice died 9–13 days a.i. (Fig. 1).

*Preventive effect of simultaneous administration with TLA emulsion and B-LKs or Tp-LKs on clinical mouse Babesia infection:* The results obtained are shown in Figs. 2 and 3. In the untreated control group, the erythrocyte count and hematocrit value began to decrease and the rate of protozoan infection exceeded 40% 10 days a.i. and all the mice died by 12 days a.i.

In group I administered with TLA emulsion alone, the erythrocyte and leukocyte counts and hematocrit value were within an almost normal range, and five of the 14 mice were alive even 20 days a.i., the survival rate being 35.7%. The rate of protozoan infection was about 5% 10 days a.i., but no Babesia protozoa were detected from any mouse 5, 15, or 20 days after that.

In group II administered simultaneously
with TLA emulsion and B-LKs, the erythrocyte and leukocyte counts and hematocrit value were within an almost normal range, and eight of the 15 mice were alive even 20 days a.i., the survival rate being 53.3%, and the rate of protozoan infection about 2% 10 days a.i.

In group III administered simultaneously with TLA emulsion and Tp-LKs, the erythrocyte and leukocyte counts and hematocrit value were within an almost normal range, and eleven of the 15 mice were alive even 20 days a.i., the survival rate being 73.3%, and the rate of protozoan infection was 1.6% 10 days a.i.

Response of mice to administration with TLA emulsion alone: There were no significant differences in the erythrocyte count, hematocrit or hemoglobin value between the sensitized and non-sensitized groups. The leukocyte count increased a little, showing an increase in the proportion of neutrophils and
monocytes, in the sensitized group (Table 1).

There were no differences in the level of GOT, GPT, or any other enzyme in blood plasma between the sensitized and the non-sensitized group.

There was no difference in MIF activity between serum and the supernatant of splenic cell culture (Table 2).

There were no differences in albumin or globulin level in serum determined by electrophoresis between the sensitized and the non-sensitized groups.

The amounts of release of H$_2$O$_2$ and O$_2$ by cultured macrophages were compared between the two groups (Table 3). There was no difference in the amount of release of H$_2$O$_2$ between these groups. The amount of release of O$_2$ was a little larger in the sensitized group than in the non-sensitized group.

Histopathological examination of the group administered with TLA revealed focal cloudy swelling in hepatic cells (Fig. 4), foci of agglomeration of lymphocytes around bile ducts (Fig. 7), and the activation of cells of the reticuloendothelial system in the liver. The hepatic capsule showed thickening accompanied with the appearance of Langhans's cells (Fig. 6) and the infiltration of eosinophils. The renal and splenic capsules showed the same thickening as this. In the
Fig. 3. Preventive effect of pretreatment with TLA emulsion or a combination of TLA emulsion and B-LKs or Tp-LKs on B. rodhaini infection in mice (hematocrit, RBC and WBC counts). These data were average of 6 mice from 3 independent experiments.

* FIA=Freund's incomplete adjuvant.

Table 1. Response of mice to treatment with TLA emulsion in WBC count and Differential WBC count (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>WBC ($\times 10^3$/mm³)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Eosinophil</td>
</tr>
<tr>
<td>Control</td>
<td>4.35</td>
<td>0</td>
</tr>
<tr>
<td>Treated</td>
<td>7.10</td>
<td>3.5</td>
</tr>
</tbody>
</table>

a) Determined by counting 200 white blood cells on smears.
Table 2. Response of mice to treatment with TLA emulsion in MIF activity a)

<table>
<thead>
<tr>
<th>Group</th>
<th>Splenic cell culture supernatant b)</th>
<th>10% Fresh serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40%</td>
<td>51%</td>
</tr>
<tr>
<td>Treated</td>
<td>49%</td>
<td>56%</td>
</tr>
</tbody>
</table>

\[ \text{MIF(\%)} = 100 \times (1.0 - \frac{\text{Average area of migration in the test material}}{\text{Average area of migration in the control}}) \]

b) Diluted to 66% with 10% CS TC-199.

Table 3. Response of mice to treatment with TLA emulsion in H₂O₂ and O₂ release by macrophages

<table>
<thead>
<tr>
<th>Group</th>
<th>H₂O₂ release a)</th>
<th>O₂ release b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Treated</td>
<td>7.1</td>
<td>7.0</td>
</tr>
</tbody>
</table>

(n mol/100 μg protein)

- a) By Nakano method.
- b) By cytochrome C reduction method.

Fig. 4. Pathological findings on the liver of the mouse administered with TLA. Cloudy swelling of hepatic cells. ×530. H. E. staining.

Fig. 5. Pathological findings on the liver of the mouse administered with TLA. Perihepatitis with infiltration of exudative cells. ×132. H. E. staining.

untreated control group, focal cloudy swelling was seen in hepatic cells and cloudy swelling in epithelial cells of uriniferous tubules in the kidney. There were no serious findings in the lungs of the treated and untreated group.

There was no increase in H₂O₂ release when examination was made on histological...
sections prepared from the liver, kidney and heart (Table 4).

Phagocytic activity of peritoneal macrophages derived from mice sensitized simultaneously with TLA emulsion and Tp-LKs on Babesia-infected erythrocytes: As shown in Table 5, the total rate of phagocytosis was 9.4±1.01% in the non-sensitized group and 13.2±3.02% in the sensitized group. It was 27.4±3.26% in the sensitized group or about 1.5 times as high in this group as in the non-sensitized group when the supernatant of splenic cell culture derived from mice of the same group was added.

When the MIF activity of the supernatant of splenic cell culture derived from a non-sensitized or a sensitized mouse was examined, that from the non-sensitized mouse was 50% and that from the sensitized one 75%. Therefore, this activity was increased a little after the sensitization.

DISCUSSION

Groups of mice were administered with 20-1,000 μg of TLA per head. As a result,
the survival rate was the highest in a group administered with 500 μg of TLA per head, because two mice survived in this group consisting of three mice. Therefore, 500 μg was used as a dose of administration of TLA in the present experiment.

A mild increase in count of neutrophils and monocytes was observed in mice administered with TLA emulsion alone. Histopathological examination revealed localized capsulitis in the liver and other organs. This change can be interpreted to be a transitional reaction to intraperitoneal administration with TLA. It is not so serious as to exert a systemic influence.

Makimura and Suzuki [6] reported that there was a parallel relationship between the release of the intermediate substances of active oxygen (H₂O₂ and O₂) by macrophages derived from mice immunized to Malaria infection and an increase in the phagocytic activity of these macrophages. Moreover, Suzuki et al. [13] observed the same parallel relationship as this between the release of these substances by macrophages cultured in medium with addition of Obioactin and an increase in the phagocytic activity of these macrophages. In the present experiment, an increase was noticed in the phagocytic activity of macrophages derived from mice sensitized simultaneously with TLA emulsion and Tp-LKs. Furthermore, there was an increase in the O₂ release by macrophages derived from mice administered with TLA emulsion alone. These results suggest a possibility that administration with TLA may enhance the phagocytic activity of macrophages.

It was suggested [7, 8] that the active oxygen presenting an antibacterial action might be the hydroxyl radical (OH⁻) or singlet oxygen (¹O₂). In the group administered with TLA emulsion, the O₂ release by peritoneal macrophages was increased, but the H₂O₂ release remained unchanged. In the metabolic pathway of active oxygen (Fig. 8), a reaction may take place that O₂ may be transformed directly into H₂O₂. Iron and H₂O₂, however, are required for this reaction to occur (Haber-Weiss reaction [2]). It is impossible to assume that such reaction may have been induced, since there was no increase in H₂O₂ release in the present experiment. The results of this experiment suggest that the sensitization with TLA emulsion may enhance the phagocytic activity of macrophage and confer an antiprotozoan action to these cells, without inducing a sufficient increase in the ability of these cells to produce an intermediate substance of active oxygen.

Ishimine et al. [4] cultured normal monocytes for 24 hours with Babesia-immune lymphokines or the supernatant of normal lymphocyte culture. They found that the rate of phagocytosis of erythrocytes was about three
times as high in the culture with the lymphokines as in that with the supernatant. In the present experiment, macrophages derived from normal mice or mice sensitized simultaneously with TLA emulsion and Tp-LKs were subjected to monolayer culture for 2 hours with addition of Babesia-infected erythrocytes. When the supernatant of the culture of splenic cells derived from a normal and a sensitized mouse was added to the culture medium for macrophages collected from the normal and the sensitized mouse, respectively, the rate of phagocytosis of erythrocytes by macrophages was about twice as high in the culture of these cells with addition of the supernatant as in that with no addition of the supernatant. When macrophages were sensitized with the supernatant of the culture of homologous splenic cells for 2 hours, they showed an increase in phagocytic activity, but the degree of increase was not so high as reported by Ishimine et al. [4]. The difference in results between the present experiment and that of Ishimine et al. [4] may have been derived from the difference in the time of addition of Babesia-immune lymphokines or the supernatant of splenic cell culture, or that between the degree of activation of cultured monocytes by Babesia-immune lymphokines and that of activation of macrophages by the supernatant of the culture of splenic cells derived from mice sensitized simultaneously with TLA emulsion and Tp-LKs.

There was no distinct difference in MIF activity between the supernatant of splenic cell culture derived from mice administered with TLA emulsion alone and that derived from normal mice. On the other hand, there was an increase in MIF activity in the supernatant of splenic cell culture derived from mice administered simultaneously with TLA emulsion and Tp-LKs. These results of the present experiment suggest that the participation of Tp-LKs may be required for the noticeable production of MIF. They agree quite well with the results of an experiment in vitro conducted by Sakurai et al. [12]. In that experiment, normal lymphocytes were sensitized with TLA or Tp-LKs, and MIF production was clearly higher in those sensitized with Tp-LKs than in those sensitized with TLA.

From the results mentioned above it is clear that the sensitization with TLA emulsion confers an ability to resist Babesia infection to mice. This is probably partly because at least macrophages in the mice are activated by the sensitization with TLA which is a heterologous antigen. Furthermore, the preventive effect on death from infection is enhanced by simultaneous administration with TLA and Tp-LKs. Since MIF activity is raised by the sensitization with Tp-LKs, the cause for the enhancement of this effect may be the intensification of the defence mechanism of the mouse by the sensitization of T-lymphocytes by a certain factor contained in Tp-LKs and the augmentation of activation of macrophages.

ACKNOWLEDGEMENTS. The authors thank all staff of the Department of Veterinary Physiology, Ohihiro University, for their technical assistance. This work was supported partially by a Grant-in-Aid for scientific research No. 56480066 from the Ministry of Education, Science an Culture of Japan.

REFERENCES

個々の効果を示す。従って、マウス体内でのマクロファージ遊走阻遏因子（MIF）生成に対する Tp-LKs の関与が示唆された。