ON THE SURVIVAL OF HIGH VIRULENT STRAIN OF TOXOPLASMA GONDII INOCULATED INTRAVENOUSLY INTO IMMUNE MICE*

ICHIRO NAKAYAMA

Department of Parasitology, School of Medicine, Keio University, Tokyo, Japan

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As is in other species of pathogenic microorganisms, the virulence of Toxoplasma gondii is different according to the strains and host animals concerned. By the infection of a high virulent strain, a large number of trophozoites appear in many organs and peritoneal exudate of the host. The host usually die of the infection before the cyst is produced. By the infection of a low virulent strain, the host usually survive the infection and the number of parasites which can be recovered from various organs and peritoneal exudate is small, even in which the host animals succumb to the infection. Cysts can be readily detected in the brain after a certain period of infection. Those animals which survived the infection of a low virulent strain usually do not succumb to a challenge infection of high virulent strain.

In this present study, an observation was made on the survival and invasion into tissues of the trophozoites of high virulent strain which were inoculated intravenously in immune mice having survived a previous infection of a low virulent strain.

MATERIALS AND METHODS

Mice weighing about 15g in body weight were intraperitoneally inoculated with 20 to 50 cysts of the low virulent Beverley or S-273 strain and those survived more than 40 days after inoculation were used as experimental animals.

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to be challenged with high virulent toxoplasmas. Although immunological
tests were not performed with these animals, all of them had cysts in the
brain when they were sacrificed later. This means that they had the infection
of a low virulent strain for more than 40 days before they received the
challenge.

The Beverley strain was originally isolated from a rabbit in England. The S-273 strain isolated from a pig in Tokyo by Nobuto et al and has
apparently lower virulence than the Beverley strain. As a high virulent strain, RH strain was used. This strain was isolated from a human being in the
United States and kills all mice inoculated within one week after the inoculation. The RH trophozoites were harvested from peritoneal exudate of mice which
had been inoculated 3 days before.

The challenge inoculation to immune mice was made into tail vein with
1,000 RH-trophozoites with an interval ranging from 40 to 120 days after
they had the primary inoculation of the low virulent strain. They were
sacrificed by ether narcosis from 2 hours to 8 weeks after the challenge. Under deep narcosis, 1.0 ml amount of blood was drawn from heart with a
syringe and immediately diluted in the equal amount of saline. Whole spleen
and brain were removed and emulsified adding with 2.0 ml amount of saline.
Aliquots of blood suspension and organ-emulsions were intraperitoneally subino-
culated into 2 clean mice each. Appearance of a large number of trophozoites
in the peritoneal cavity of subinoculated mice was considered to be the evidence
of the growth of RH-trophozoites, because the low virulent strain does not
produce but a small number of trophozoites in the peritoneal cavity even in
the acute stage of infection. Thus the survival of a high virulent strain in
immune mice can be substantiated, if a large number of trophozoites appear
in the peritoneal cavity of subinoculated mice.

The mode of invasion of trophozoites into tissues was investigated in
sections stained with hematoxylin-eosin or by fluorescent antibody techniques.
Tissues were fixed in formaldehyde for hematoxylin-eosin staining and in alchol
for fluorescent antibody techniques. For the latter techniques, pig antiserum
having the dye test titer 1:1024 was labeled with fluorescein isothiocyanate.
Sections were kept with this labeled antibody in incubater at 37°C for 30 minutes
for conjugation.

RESULTS

Survival of RH-trophozoites in immune mice

Immune mice inoculated with 1,000 RH-trophozoites were sacrificed from
survival of virulent Toxoplasma in immune mice

2 hours to 8 weeks after the challenge. The numbers and percentages of immune mice which still had the RH-trophozoites in blood or organs at the time of examination are shown in the table 1.

The RH-trophozoites were detected from blood of 17 (85%) out of 20 mice which were examined 2 hours after the challenge. At 6 hours period, the percentage of positive mice dropped to 59% and this level was kept until 2 weeks after the challenge, though there were some fluctuations. At the period of 3–8 weeks after the challenge, positive mice decreased in number, the percentage positive ranging from 4 to 13%. The average period of survival of mice subinoculated with materials from immune mice and died of RH-infection was mostly 10 days during 2 weeks after the challenge. In the period from 3 to 8 weeks after the challenge, the survival of subinoculated mice became much longer, indicating that the number of trophozoites in each mouse became markedly smaller at this period when the percentage of positive mice became smaller, too. These data indicated that the number of RH-trophozoites which were inoculated into immune mice decreased markedly 3 weeks after the challenge.

Normal clean mice were inoculated with the same number of RH-trophozoites as controls. They died of the infection within 7 days. The percentages of RH-positive mice as revealed by subinoculation were almost the same as in the immune-mouse group during the first day of inoculation (Table 2). From the second day and later, the percentage increased definitely attaining 100% 4 days after the inoculation. The period of survival of subinoculated mice was almost the same as in immune-mouse group during 2 days after the inoculation, but became much shorter thereafter. This means that the number of trophozoites in blood increased day by day until the death of the host.

By the examination of spleen of immune mice, RH-trophozoites were found in almost all mice during the period from 2 hours to 2 weeks after the challenge. During the period from 3 to 8 weeks after the challenge, RH-trophozoites were found from only about half of the mice examined. The period of survival of mice subinoculated with spleen emulsions were prolonged in the latter period, indicating a decrease in number of trophozoites in spleen 3 weeks after the challenge.

Examination of brain of immune mice were begun 3 weeks after the challenge. The RH-trophozoites were detected from 70–80% of the mice examined. That is to say, parasites were found more frequently in brain than in blood or in spleen at the later period of examination, or 3–8 weeks after the challenge. The period of survival of mice, subinoculated with brain emulsion was prolonged by the examinations in later period (6–8 weeks) as compared with the earlier period (3–4 weeks).
In the control experiments, the RH-trophozoites were always found from spleen from 2 hours to 7 days after the challenge.

**Table 1**

Detection of the challenged toxoplasmas in the organs of immune mice

<table>
<thead>
<tr>
<th>Organs exam.</th>
<th>Periods between challenge and exam.</th>
<th>No. of mice</th>
<th>Average survival-day of dead mice subinoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>exam.</td>
<td>posit. (%)</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>2 hrs</td>
<td>20</td>
<td>17(85%)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>29</td>
<td>17(59%)</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>19</td>
<td>12(63%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25</td>
<td>18(52%)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>29</td>
<td>15(52%)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>22</td>
<td>16(73%)</td>
</tr>
<tr>
<td></td>
<td>1 wk</td>
<td>15</td>
<td>8(53%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15</td>
<td>8(53%)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>24</td>
<td>2(8%)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>25</td>
<td>1(4%)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>15</td>
<td>2(13%)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16</td>
<td>1(6%)</td>
</tr>
<tr>
<td>Spleen</td>
<td>2 hrs</td>
<td>20</td>
<td>20(100%)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>29</td>
<td>29(100%)</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>19</td>
<td>17(90%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25</td>
<td>24(96%)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>29</td>
<td>29(100%)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>22</td>
<td>21(95%)</td>
</tr>
<tr>
<td></td>
<td>1 wk</td>
<td>15</td>
<td>15(100%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15</td>
<td>15(100%)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>24</td>
<td>12(50%)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>25</td>
<td>13(52%)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>15</td>
<td>11(73%)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16</td>
<td>8(50%)</td>
</tr>
<tr>
<td>Brain</td>
<td>3 wks</td>
<td>14</td>
<td>11(79%)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16</td>
<td>12(75%)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>15</td>
<td>10(67%)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16</td>
<td>11(69%)</td>
</tr>
</tbody>
</table>

All mice were challenged with 1,000 RH-trophozoites into the tail vein.

*Mode of invasion of trophozoites from blood vessels into tissues in immune mice*

The RH-trophozoites were inoculated into the tail vein of immune mice and their invasion into tissues was investigated in section preparations stained with hematoxylin-eosin or by fluorescent antibody techniques. As it was very difficult to find out toxoplasma in section preparations at the early stage of infection, a large number of trophozoites (5×10⁶–20×10⁶) were inoculated and examinations were made from 2 minutes to 3 days after the inoculation.

Sections of lung tissues stained by fluorescent antibody techniques revealed
SURVIVAL OF VIRULENT TOXOPLASMA IN IMMUNE MICE

Table 2
Detection of the inoculated toxoplasmas in the organs of non-immune mice (Controls)

<table>
<thead>
<tr>
<th>Organs</th>
<th>Periods between inocul. and exam.</th>
<th>No. of mice posit.(%)</th>
<th>Average survival-day of dead mice subinoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2 hrs</td>
<td>12</td>
<td>9( 75%)</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>18</td>
<td>10( 56%)</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>15</td>
<td>9( 60%)</td>
</tr>
<tr>
<td></td>
<td>2 hrs</td>
<td>25</td>
<td>21( 84%)</td>
</tr>
<tr>
<td></td>
<td>4 hrs</td>
<td>27</td>
<td>27(100%)</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>22</td>
<td>22(100%)</td>
</tr>
<tr>
<td></td>
<td>7 hrs</td>
<td>11</td>
<td>11(100%)</td>
</tr>
<tr>
<td>Spleen</td>
<td>2 hrs</td>
<td>12</td>
<td>12(100%)</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>18</td>
<td>18(100%)</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>15</td>
<td>15(100%)</td>
</tr>
<tr>
<td></td>
<td>2 hrs</td>
<td>25</td>
<td>24( 96%)</td>
</tr>
<tr>
<td></td>
<td>4 hrs</td>
<td>27</td>
<td>27(100%)</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>22</td>
<td>22(100%)</td>
</tr>
<tr>
<td></td>
<td>7 hrs</td>
<td>11</td>
<td>11(100%)</td>
</tr>
</tbody>
</table>

All mice were inoculated with 1,000 RH-trophozoites into the tail vein.

more than 20 trophozoites in one microscopic field (250x) during the period from 2 minutes to 6 hours after the challenge. In section of liver and spleen, however, only a few trophozoites were detected at this stage of infection (table 3). Trophozoites inoculated into control mice gave almost the same pic-

Table 3
Appearance of the challenged toxoplasmas in the organs of immune mice by the examination for the section of tissues stained by fluorescent antibody and hematoxyline-eosin

<table>
<thead>
<tr>
<th>Organs</th>
<th>Detection of toxoplasmas following challenge on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2min 10 30 2hrs 1day 2 3 4 5 1wk 2 3 4 8</td>
</tr>
<tr>
<td>Lung</td>
<td>exp. cont.</td>
</tr>
<tr>
<td></td>
<td>exp. cont.</td>
</tr>
<tr>
<td>Liver</td>
<td>exp. cont.</td>
</tr>
<tr>
<td>Spleen</td>
<td>exp. cont.</td>
</tr>
</tbody>
</table>

No. of toxoplasmas challenged 5x10^6 to 20x10^6 1x10^5 to 2x10^6

D : death ± : 1~2 parasites in several fields unusually + : 2~3 parasites in one field #: several~10 parasites in one field ### : 10~20 parasites in one field #### : 20 parasites or more in one field
tures as in immune mice during this period. These findings indicated that almost all of the RH-trophozoites which were inoculated into the tail vein and attained to lung, penetrated into lung tissues very soon after the inoculation, even within 10 minutes (Plate, Figs. 1 and 2) and only a few of them which could pass through the capillary network of lung would get to liver, spleen and other organs. No difference was seen between control and immune mice in the mode of invasion at this early stage of infection.

One to two days after the challenge, the trophozoites in liver and spleen of immune and control mice increased in number, attaining 10–20 in one microscopic field and multiplication of trophozoites was detected in sections stained with hematoxylineosin. No difference was recognized between immune and control mice as regards the frequency of invasion into host cells at this stage, too. The picture of multiplication was recognized in tissues of immune as well as in control mice (Plate, Figs. 3, 4, 5, 6, 7 and 8). Trophozoites in tissues of immune mice, however, began to decrease in number 3 days after the challenge, followed by sudden decrease on 4th or 5th day, only a few trophozoites being found in one microscopic field of sections of lung, spleen and liver. This state of light infection continued until 2 weeks after the challenge and the number of trophozoite decreased thereafter until the end of examination period or 8 weeks. At this period, only 1–2 trophozoites were found in several microscopic fields, and morphologically, it was impossible to determine whether these trophozoites located sparsely in tissues were of the challenged RH-strain or the low virulent strain of the primary infection. It was demonstrated biologically, however, that they were of RH-strain as has been stated above: the tissue emulsion obtained at this stage of infection caused severe symptoms and subsequent death of all animals having a great lot of trophozoites in peritoneal cavity when inoculated into clean mice.

The RH-trophozoites invaded into alveolar cells of lung except at bronchioles and intralobular bronchus, in lymphatic nodes and venous sinus of spleen, and hepatic and Kupffer's cells of liver (Plate, Figs. 5 and 6). No difference was seen between control and immune mice as to the kinds of cells invaded by toxoplasma. In control mice, however, number of RH-trophozoites in tissues increased rapidly until the host died, usually within 10 days after inoculation. In immune mice, the RH-trophozoites were mostly destroyed within 3–4 days after the challenge, though only a few could survive for a long time in some cases.
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DISCUSSION

It has been recognized by many investigators (Weinman,1) Frenkel,2) Beverley,3) and Nakayama4)) that a host animal which survived an infection of a low virulent strain of Toxoplasma produces an immunity which protects the animal from death when it was subsequently challenged with a high virulent strain. Although these immune animals could survive the challenge infection, that does not always mean that the parasites of high virulent strain used for the challenge were eradicated from the host. According to Nakayama,4) the RH-trophozoites persisted in the brain of immune mice for the examination period of 1–7 weeks after the challenge. Ruchman et al5) examined parasitemia in rats up to 4 weeks after infection and showed that the maximum appearance of parasites was 4 to 5 days after inoculation and the last positive case was observed 16 days later. Jacobs et al6) found parasitemia in 2 of 8 mice during 3 month after infection. The infection was asymptomatic and parasitemia was moderate following inoculation, but the later period parasitites were found in blood at rare intervals and in small number.

Findings obtained in the present study indicates that the RH-trophozoites inoculated into tail vein reach the lung capillary network and most of them penetrate into lung tissues. Only a small percentage of the trophozoites will pass through the capillary network and be distributed in other organs. Their invasion into tissues are very quick, being found in section preparations of lung, liver and spleen already 2 minutes after inoculation. The aspect of this early invasion of parasites into tissues is the case in the chronic infection, and parasites disappear from blood.

Number of parasites in tissues of immune mice decreases remarkably after the 4th day after challenge and it was also difficult to find out the picture of multiplication in section preparation at this period. Using a diffusion chamber inserted into the peritoneal cavity of mice, Nakayama7) recognized that macrophages obtained from immune mice were more resistant to Toxoplasma infection than those obtained from normal mice. Vischer et al8) investigated the multiplication of Toxoplasma in tissue culture of macrophages and reported that combined use of serum and macrophages obtained from immune animals completely prevent the growth of Toxoplasma. The host protection against Toxoplasma infection seems to be involved in the protection of cell-bound and humoral antibody. In the present experiment, however, Toxoplasma inoculated into immune mice persisted for a long period in about half of the animals examined. In these cases, the antibody could suppress the multiplication of Toxoplasma and prevent animals from death, but could not eradicate the parasite.
Observations were made on the survival of high virulent RH strain inoculated intravenously into immune mice which had been infected with the low virulent Beverley or S-273 strain previously. Following results were obtained.

1) Trophozoites penetrated into tissue cells in 85% of immune mice within 2 hours and 59% within 6 hours after the challenge. Even within 2 minutes many trophozoites were already found in tissues of lung. At this stage, however, only a few parasites were found in liver and spleen. The invaded parasites multiplied in tissues and no difference was seen as regards the invasion and subsequent multiplication between immune and control mice in this period.

2) In immune mice, the trophozoites once multiplied in tissues began to decrease in number on the 3rd day from challenge and most of them suddenly disappeared on the 4th and 5th day, only a few being found in lung, spleen and liver. This state of infection continued up to 2 weeks after the challenge. Between 3 and 8 weeks after the challenge, only a few parasites were found in tissue preparations on rare occasions. On the contrary, a large number of parasites were found in tissues of control animals until one week after the challenge and they died of the infection within 10 days.

3) It was demonstrated that organisms inoculated into immune mice were able to multiply in tissues at the early stage of challenge infection, but most of them were soon destroyed. A small number of parasites, however, were able to survive in tissues for a very long time.

REFERENCES

EXPLANATION OF PLATE

Figs. 1 and 2 A single trophozoite is found in endothelial cells in lung of non-immune (Fig. 1) and immune mice (Fig. 2) 10 minutes after intravenous inoculation with RH. (2,000×)

Figs. 3 and 4 Trophozoites begin to multiply in endothelial cells in lung of non-immune (Fig. 3) and immune mice (Fig. 4) 1 day after intravenous inoculation with RH. (2,000×)

Figs. 5 and 6 Trophozoites begin to multiply in Kupffer's (Fig. 5) and hepatic cells (Fig. 6) of non-immune mouse 1 day after intravenous inoculation with RH. (2,000×)

Fig. 7 Trophozoites begin to multiply in splenic cords of immune mouse 1 day after intravenous challenge with RH, (2,000×)

Fig. 8 Multiplied trophozoites are found in liver of immune mouse and the cell invaded is destroyed 3 days after intravenous challenge with RH. (2,000×)