STUDIES ON TOXOPLASMOSIS
I. ISOLATION OF TOXOPLASMA FROM MUSCLES OF HUMANS, DOGS, AND CATS

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SUMMARY: Diaphragm muscle specimens from 53 humans who died of various diseases unrelated to toxoplasmosis, and from 87 healthy dogs, and mixed specimens of diaphragm and abdominal muscles from 25 healthy cats were examined for the presence of Toxoplasma. The muscle specimens were digested with trypsin, and the digested materials were inoculated into the peritoneal cavity of mice (gpc strain). Toxoplasma was isolated from 2 (3.8 %) out of 53 humans, 11 (12.6 %) out of 87 dogs, and 17 (68.0 %) out of 25 cat muscle specimens. Eight (15.7 %) out of 51 humans, 15 (17.2 %) out of 87 dogs, and 15 (60.0 %) out of 25 cats were HA test (Hanaki et al. 1964) positive at titers of 1 : 64 or higher. The human cases, from which Toxoplasma was isolated, showed negative HA. In the cases of cats, the results of isolation of the parasite and the HA test were roughly correlated, but this was not the case in humans or dogs.

INTRODUCTION

Acute toxoplasmosis in various warm-blooded animals has been reported, since Nicolle and Manceaux isolated Toxoplasma gondii from Ctenodactylus gondii in 1908. On the other hand, many humans and animals have been suspected to be infected subclinically on the basis of serological results.

Recently, the subclinical infections either in the muscle or in the brain of swine (Jacobs, Remington and Melton, 1960 ; Ishii et al., 1962 ; Hanaki et al., 1963 ; Koshimizu et al., 1963; Jacobs, Moyla and Ris, 1963), and sheep (Jacobs et al., 1960 ; Jacobs et al., 1963) were demonstrated. As for humans, dogs and cats, subclinical or chronic infections in the brain or uterus have been reported. Walls, Taraska and Goldman (1963) isolated Toxoplasma gondii from 1 out of 15 pepsin-treated brains of humans who had died of diseases unrelated to toxoplasmosis. The dye test titer of the case was 1 : 4. Remington, Melton and Jacobs (1960) found 4 cases of residual Toxoplasma infections in the human uterus obtained at 9 autopsies and 23 hysterectomies from patients whose serum showed Toxoplasma antibodies. Krause (1955) inoculated mice with tissues from 30 patients, who had died of chronic degenerative and infectious diseases, and of cancer, and brains from 32 dogs. One Toxoplasma strain was isolated
from one adult dog brain, but not from the human tissues. Gibson and Jumper (1960) examined tissues from 137 healthy dogs in the first series with no success in isolation. In the second series, the parasite was isolated from 2 out of 54 adult dog brains examined, one female and one male with dye test titers of 1:256 and 1:1024, respectively. Jones, Eyles and Gibson (1957) reported that Toxoplasma gondii was isolated from 34 (24.3%) out of 140 Memphis cats and 4 out of 35 Columbia cats which showed no sign of illness. There have been no publication concerning latent infection of Toxoplasma in the diaphragm or skeletal muscles of humans, dogs or cats, although Jacobs et al. (1963) reported that the diaphragm was the most consistently parasitized tissue, psoas the next, brain the last in the experiment with dye test positive sheep.

The purpose of this paper is to demonstrate the presence of latent infection among humans, dogs, and cats by isolation of the organisms from the muscles, as a basic study for the epidemiology of toxoplasmosis.

**MATERIALS AND METHODS**

**Specimens:** Diaphragm muscle specimens were obtained from 53 humans sent for autopsy at the Tokyo Medical Examiner Office. They had died of cardio-vascular disturbances (31), suffocation (5), brain contusion (3), soporific intoxication (3), asthma (2), pneumonia (2), septicemia (1), peritonitis (1), pesticide poisoning (1), drowning (1), tuberculosis and alcoholism (1), tuberculosis and gastric ulcer (1), and poliomyelitis (1). Most of them had died suddenly without any medical precautions. Eighty-seven dogs and 25 cats which had no sign of illness were examined. The dogs were caught in the Tokyo area, and the cats were caught in Saitama Prefecture situated north of Tokyo. They were sacrificed at the laboratory by intravenous or intraperitoneal injections with pentobarbital sodium. Within a few hr after their death, post-mortem examinations were carried out, and the diaphragm from the dogs, and the diaphragm and abdominal muscles from the cats were obtained aseptically for the isolation of Toxoplasma. Specimens were also obtained from the lungs, livers, spleens, kidneys, and lymph nodes of these animals and fixed with 10 % formalin for pathological examinations.

**Isolation of Toxoplasma organisms:** Trypsin digestion method described by Hanaki et al. (1963) was used. Approximately 30 g of the muscle specimen was minced by a meat grinder or cut into small pieces by a surgical scissors. The minced or chopped muscle was suspended in 300 ml of saline containing 0.25 % trypsin (Difco 1:250). The suspension was stirred on a magnetic stirrer at room temperature for 1 hr. Then, the digested material was filtered through 2 layers of gauze to eliminate large particles of the muscle, and centrifuged at 2,500 rpm for 15 minutes. The sediment was washed once with saline and recentrifuged at 3,000 rpm for 10 minutes. The sediment was suspended in 5 ml of saline containing penicillin (100 U/ml) and streptomycin (100 γ/ml). Whole volume of the suspension was inoculated into the peritoneal cavity of a group of mice which consisted of 5 or 6 animals, and at the same time, each of the mice was administered with 5.0 mg of cortisone acetate (corton) intramuscularly. The mice used were of the gpc strain free of Toxoplasma infection maintained in this institute.

All the inoculated mice were sacrificed at the 6th week, and examined for the presence of cysts in the brain by means of an unstained wet impressed smear method. To obtain early predictive informations for the isolation of Toxoplasma in the inoculated mice, a Giemsa-stained peritoneal fluid specimen of a mouse from each group was ex-
amined for the presence of proliferative form of the parasite between the 7th and 10th
days, and at the 5th week all the mice were bled from the orbital sinus and the blood
specimens were subjected to the hemagglutination test. The isolated organisms were
identified as Toxoplasma by the morphology and a fluorescein-labeled antibody technique.

Usually, less than 6 specimens were treated for Toxoplasma isolation on the same
day. A control which consisted of 4 to 6 of mice in a cage was prepared and kept in
the same rooms each day, in order to make sure of being free from Toxoplasma con-
tamination in the mice colonies. Each of the control mice was administered with only
5.0 mg of cortisone, and examined in the same way as that of the inoculated mice.

Hemagglutination test: The hemagglutination test method which was originated
by Jacobs and Lund (1957) and modified by Hanaki et al. (1964) was employed in this
experiment.

Preparation of Toxoplasma antigen: Approximately 10⁶ organisms of RH strain were
inoculated into the peritoneal cavity of 100 mice. The peritoneal exudates were obtained 4 days
after the inoculation. The exudates were pooled and centrifuged at 2,500 rpm for 10 minutes
to harvest the organisms. The organisms were suspended in 10 ml of sterilized distilled water,
and freezing and thawing was repeated 5 times to extract antigen. Then, the suspension was
centrifuged at 5,000 rpm for 30 minutes to eliminate cell debris. Ten ml of sterilized 1.8 %
NaCl solution was added to the separated supernatant.

Fixation of sheep erythrocytes: Sheep erythrocytes were fixed by the method reported by
Park (1961). However, the composition of the fixative and buffered sodium bisulfate solution
was slightly modified as follows:
Alcohol-formalin fixative: 37 % formaldehyde, 440 ml; 95 % ethyl alcohol, 150 ml; \( \text{KH}_2\text{PO}_4\),
6 g; \( \text{Na}_2\text{HPO}_4\), 14 g; distilled water, to make 2,000 ml.
Buffered sodium bisulfate: \( \text{NaHSO}_3\), 100 g; \( \text{KH}_2\text{PO}_4\), 6 g; \( \text{Na}_2\text{HPO}_4\), 40 g; distilled water
to make 2,000 ml.

To eliminate the formaldehyde-bisulfate complex, the fixed erythrocytes were dialized against
chlorine-free running well water through a cellophane bag for 4 days.

Sensitization of sheep erythrocytes: The fixed erythrocytes were sensitized with the Toxo-
plasma antigen with the aid of bis-diazo-benzidine following the method of Butler (1963). Three
ml of the antigen and 0.01 ml of a 50 % suspension of the fixed erythrocytes in phosphate buff-
ered saline (PBS) at pH 7.2 were mixed. Then, 1.5 ml of diluted bis-diazo-benzidine (1:20 in
cold pH 7.2 PBS) was added to the mixture. The mixture was kept at 37°C for 15 minutes
for sensitization. The erythrocytes were then centrifuged at 2,000 rpm for 5 minutes at 4°C,
and washed twice with pH 7.2 PBS. Finally, the sensitized erythrocytes were suspended in
3.5 ml of pH 7.2 PBS.

Blood specimens: Heart blood from the dogs and cats, uncoagulated blood retained in the
human heart, or blood from the orbital sinus of the inoculated mice were absorbed on filter
paper strips, Toyokagaku No. 1 (Fig. 1). The blood absorbing filter paper strips were dried at
room temperature for at least 4 days.

Procedure for hemagglutination test: The blood absorption area of the filter paper strip
was cut, and soaked in 0.6 ml of pH 7.2 PBS for 1 hr at room temperature to elute antibody.
It was confirmed in the preliminary experiment that the concentration of the antibody in the
elute was equivalent to that in the serum diluted to 1:16 from the same individual. Inactivated
(10 lbs, for 10 minutes) 1.5 % normal swine serum-PBS (pH 7.2) was prepared for the dilution of the elute. Six-tenths ml of the diluent was dispensed into each of the 3 shallow holes in a plastic tray (Tominaga). To the 1st hole containing 0.6 ml of the swine serum-PBS, 0.2 ml of the elute was transferred and mixed well. Two-tenths ml of the mixture from the 1st hole was transferred to the 2nd hole, and followed by the same way. The dilution was made up to the 3rd hole. Then, 0.05 ml of the sensitized sheep erythrocytes was added to each dilution. Final dilutions thus obtained were 1: 64, 1: 256, and 1: 1,024. The tray was kept in an incubator at 37°C for 3 hours. After the incubation, the tray was shaken again thoroughly, and allowed to stand at room temperature overnight. The results were recorded on the following day.

RESULTS

Toxoplasma was isolated from 2 (3.8 %) out of 53 humans, 11 (12.6 %) out of 87 dogs, and 17 (68.0 %) out of 25 cats (Table 1). Findings of the 2 human cases at autopsy were as follows; one was a 37-year-old woman who had died of an organic phosphorous pesticide poisoning and the other was a 26-year-old man who had died of bronchopneumonia which was caused secondarily by a traffic accident. The former showed erosion of stomach, cloudy swelling of liver and kidney, hypoplasia of Ramus circumflexus, and her right lung had only two lobuli. The latter had brain contusion, sub-pachymeningeal hemorrhage, hypoplasia of viscera, especially of testis, and chronic nephritis. In the 11 dog cases and the 17 cat cases from which the organisms were isolated, some pathological lesions were observed in the liver, spleen, kidneys, and lungs. However, these humans and animals did not show any symptoms or lesions characteristic of toxoplasmosis at ante- or post-mortem examinations or histopathological investigations.

Table 1. Isolation of Toxoplasma organisms from humans, dogs and cats

<table>
<thead>
<tr>
<th></th>
<th>Number examined</th>
<th>Number isolated</th>
<th>Rate of isolation</th>
</tr>
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<tbody>
<tr>
<td>Human</td>
<td>53</td>
<td>2</td>
<td>3.8%</td>
</tr>
<tr>
<td>Dog</td>
<td>87</td>
<td>11</td>
<td>12.6%</td>
</tr>
<tr>
<td>Cat</td>
<td>25</td>
<td>17</td>
<td>68.0%</td>
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All the strains isolated showed typical morphology of Toxoplasma, and they were stained well by the fluorescein-labeled antibody. Twenty-seven out of 30 strains isolated (2 human, 11 dog, and 14 cat strains) formed cysts in the brain of mice which were sacrificed at the 6th week in the 1st passage. The other 3 strains, which were isolated from cats, killed mice in between 7 and 15 days in the 1st passage without formation of cysts in the brain. At the 2nd passage, 2 out of the 3 cat strains formed cysts in the brain without cortisone administration, but killed mice within 7 days with cortisone treatment. The remaining one cat strain killed mice within 10 days at the 2nd passage without cortisone treatment. Two dog strains and 4 cat strains, which formed cysts in the brain of mice at the 1st passage, have increased their virulence to mice from the 2nd or 3rd passage, killing mice in between 7 and 9 days after inoculation.

HA test results on all the human and animal cases are shown in Table 2. Eight (15.7 %) out of 51 humans, 15 (17.2 %) out of 87 dogs, and 15 (60.0 %) out of 25 cats were positive at titers of 1: 64 or higher. The HA titers of the hosts from which the organisms were isolated are indicated in Table 3. Four (36.4 %) out of the 11 dogs
Table 2. Hemagglutination tests on human, dog and cat specimens

<table>
<thead>
<tr>
<th>Number examined</th>
<th>Hemagglutination titer</th>
<th>% positive ≥1:64</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:64</td>
<td>1:256</td>
</tr>
<tr>
<td>Human*</td>
<td>51</td>
<td>43</td>
</tr>
<tr>
<td>Dog</td>
<td>87</td>
<td>72</td>
</tr>
<tr>
<td>Cat</td>
<td>25</td>
<td>10</td>
</tr>
</tbody>
</table>

* Blood specimens from two cases were not available.

Table 3. Hemagglutination tests on human, dog and cat specimens, from which Toxoplasma were isolated

<table>
<thead>
<tr>
<th>Number of cases</th>
<th>Hemagglutination titer</th>
<th>% positive ≥1:64</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>1:64</td>
<td>1:256</td>
</tr>
<tr>
<td>Human</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Dog</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Cat</td>
<td>17</td>
<td>4</td>
</tr>
</tbody>
</table>

and 13 (76.5%) out of the 17 cats from which Toxoplasma were isolated were HA positive at titers of 1:64 or higher. All the cats with HA titers of 1:256 or higher were positive in the isolation of the organisms. Two human cases from which the organisms were isolated gave negative HA tests.

DISCUSSION

It is very important for elucidation of the epidemiology of toxoplasmosis in the warm-blooded animals to confirm the existence of subclinical infection parasitologically, and to know the mode of transmission. Subclinical infection of the organisms in the muscle of swine and sheep was already reported. However, the infection in the human, dog and cat diaphragm or skeletal muscles has not yet been confirmed parasitologically.

The existence of subclinical infection in the muscles of the host was confirmed in this experiment. The incidence of infection was rather low in humans, but quite high in cats. The infection rate in dogs lay between them. Recently many studies on swine toxoplasmosis have been published in this country. However, epidemiology of toxoplasmosis in warm-blooded animals still remains unknown. From the epidemiological point of view, much attention should be given to the subclinical infection in dogs and cats. The dogs and cats which are infected subclinically may play some important role in the transmission of Toxoplasma in the warm-blooded animals, and there may be some important infective sources in the environment surrounding dogs and cats.

It is considered that the isolated Toxoplasma organisms persisted in the muscles of the host as a cyst form, because the hosts showed no sign of toxoplasmosis at the ante- or post-mortem examinations or at the histopathological investigations, and the isolated
strains were proved to be of weak virulence, except one. Most of the strains were less virulent to mice but formed cysts in the brain of mice without killing them even after several passages. On the other hand, one strain was very virulent and killed mice in the first passage, and some other strains increased their virulence to mice with subsequent passages and acquired the activity of killing mice.

In the cases of cats, the results of isolation and the HA test were roughly correlated with each other, but this was not the case in humans or dogs. The method of the HA test employed in this experiment was developed for the diagnosis of swine toxoplasmosis by Hanaki et al. (1964). Some device will be needed for this modified HA test to raise its specificity, when applied to other host species.

ACKNOWLEDGEMENT

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REFERENCES


