Horizontal Transmission of Toxoplasma gondii in Squirrel Monkeys (Saimiri sciureus)

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Abstract: The possibility of horizontal transmission of T. gondii was examined in squirrel monkeys. After three monkeys were inoculated perorally with 1.1–2.1 × 103 cysts of the T. gondii ME49, the animals were divided into two cages and maintained with one normal monkey for each cage as a cagemate. Two out of the three T. gondii-inoculated monkeys died, and the remaining one monkey was sacrificed in a moribund state one week after infection because of acute toxoplasmosis. Many T. gondii tachyzoites were recovered from broncho-alveolar lavages and were also found histopathologically in the lung, liver, spleen, kidney and lymph nodes and impression smears of tissues from the three T. gondii-inoculated monkeys by Giemsa staining. Anti-T. gondii antibody was examined by immunoblot assay in these animals, and the antibody to T. gondii major surface membrane protein (p30) could be detected after the start of experiment. Furthermore, a specific band of T. gondii NTPase gene was observed by PCR in the liver and lung of infected and cagemate monkeys, and the sequence of the second PCR products obtained from the cagemates, which were clinically normal but gave a positive result in immunoblotting assay, was exactly the same as the sequence of the NTPase gene of T. gondii ME49. These findings suggested that transmission of T. gondii from the infected monkeys to cagemates occurred easily, and since many T. gondii tachyzoites were recovered from the broncho-alveolar lavages of the three T. gondii-inoculated monkeys, we suggest that aerosol infection plays an important role for the enzootic toxoplasmosis in colonies of squirrel monkeys.

Key words: horizontal, transmission, squirrel monkey, toxoplasmosis

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**Introduction**

*Toxoplasma gondii* (T. gondii) is an obligate intracellular protozoan parasite that commonly infects many warm-blooded animals, including human beings [5]. Although chronic infection of *T. gondii* is normally asymptomatic, *T. gondii* has emerged as a major opportunistic pathogen in AIDS patients due to immunodeficiency [12]. Recently, spontaneous cases of fulminating fatal toxoplasmosis were reported in adult squirrel monkeys (*Saimiri sciureus*), which were immunologically normal [8]. Regarding the infection of *T. gondii* in nonhuman primates, New World monkeys are considered to be more susceptible to *Toxoplasma* infection than Old World monkeys, based on reported cases and on experimental infection [17]. Recently, we had the opportunity to study enzootic fatal toxoplasmosis in colonies of squirrel monkeys in Japan, and the results suggested the possibility of horizontal transmission of *T. gondii* among those monkeys. In this study, we tried experimental infection of *T. gondii* in squirrel monkeys to examine the possibility of horizontal transmission of *T. gondii* by maintaining a normal cagemate with *T. gondii*-infected monkeys in the same cage. This is the first report to confirm the possibility of horizontal transmission of *T. gondii* in squirrel monkeys.

**Materials and Methods**

**Animals:** Three male and two female squirrel monkeys (*Saimiri sciureus*) aged one to two years old were obtained from a domestic supplier and were housed in stainless steel cages placed in a room of a P-3 facility. They were fed commercial primate food (Oriental Yeast Co. Ltd., Tokyo, Japan) with water *ad libitum* throughout this experiment. The monkeys were divided into two groups, one group consisted of two *T. gondii*-inoculated and one intact squirrel monkeys (cagemate), and the other group was consisted of one *T. gondii*-inoculated and one intact squirrel monkey (cagemate). Another six normal, healthy squirrel monkeys which had been kept at the Amami Laboratory of Injurious Animals, Institute of Medical Science, the University of Tokyo were used for negative control of the nested PCR test. The present study was approved by the Animal care and Use Committee in Azabu University.

**Parasites and inoculation:** An avirulent strain of *T. gondii* ME49 has been routinely maintained by peroral passage in ddY mice (SLC Inc., Hamamatsu, Japan) in our laboratory, and the cysts of *T. gondii* ME49 were obtained from the brains of chronically infected ddY mice. Briefly, four or five mice were sacrificed under ether anesthesia, and their brains were removed and then triturated in saline with a mortar and pestle. After an aliquot of the brain suspension was placed on a slideglass, and the number of cysts in the brain was counted under microscopy, the suspension containing 20 cysts was injected intraperitoneally into new mice for the next passage. At 3–4 months of the infection, the brain was removed aseptically from the mice and used for the experiment as inoculum. In the present study, brain tissues containing 1106, 1925, or 2090 cysts of *T. gondii* ME49 were fed to three monkeys, Nos.1, 2, and 4, respectively. After inoculation, sera were taken from each monkey every week, and the main organs were collected from the animals for histopathology and detection of *T. gondii* parasites when they were sacrificed.

**Antigen of parasites:** Tachyzoites of *T. gondii* ME49 were grown in a monolayer culture of Vero cells with RPMI 1640 (GIBCO BRL, Life Technologies, Inc., Grand Island, N.Y.) containing 100 U/ml penicillin G (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) and 10 µg/ml streptomycin sulfate (Meiji Seika Kaisha, Ltd., Tokyo, Japan), and 10% heat-inactivated fetal calf serum (Bioserum Ltd., Victoria, Australia) at 37°C in 5% CO₂ atmosphere as previously described [7]. *T. gondii*-infected Vero cells were routinely subcultured at a 3–5 days interval after trypsinization of confluent monolayers. Cultured tachyzoites were purified by filtration through 3.0 µm polycarbonate membrane filters (Millipore Corp., Bedford, MA), washed three times with 50 mM Tris buffered saline (TBS, pH 7.5) and centrifuged at 1500 × g for 5 min. Tachyzoites were sonicated twice at 5A for 30 sec using a sonicator (type MR-52, Fuji Electrical Inst. Co., Ltd., Tokyo, Japan) followed by three cycles of freeze-thawing. The resulting suspension was used as the soluble crude antigen.

**Immunoblotting:** SDS-PAGE was performed according to Laemmli using a 10% polyacrylamide gel [11]. For each well on the gel, an aliquot of 10 µg of soluble
T. gondii tachyzoite antigen was subjected to electrophoresis for approximately 2 hr at 20 mA, and then the separated proteins were transferred onto a polyvinylidene difluoride (PVDF, Millipore Corp.) membrane using semi-dry electroblotting apparatus (AE-6670, Atto Corporation, Tokyo, Japan) [10]. After PVDF membranes were blocked with 5% non-fat dry milk (Difco Laboratories, Detroit, MI) for 1 hr, followed by washing three times with TBS (pH 7.5) containing 0.05% Tween-20 (Wako Pure Chemical Industries, Osaka, Japan), the membranes were incubated with 1:20 diluted sample sera at room temperature overnight. After washing, the membranes were incubated with a 1:500 dilution of horseradish peroxidase-conjugated goat anti-rhesus monkey IgG (H+L) (Bio-Rad Laboratories, Richmond, CA), and positive bands were detected with chromogenic reagents, nitro blue tetrazolium (NBT) (Boehringer Mannheim, Mannheim, Germany) and 5-bromo-4-chromo-3-indolyl phosphate (BCIP) (Boehringer Mannheim).

DNA extraction and nested PCR: DNA was prepared from the organs with phenol-chloroform (Wako Pure Chemical Industries) extraction after Proteinase-K (Sigma Chemical Co. St. Louis, MO) digestion, and was precipitated with ethanol. After the precipitation, DNA was resuspended with 10 mM Tris-HCl-1 mM EDTA 2Na buffer (pH.8.0) and stored at –20°C until use. PCR was done by the nested PCR method based on the NTPase gene of T. gondii [2] for detecting the presence of T. gondii. The outer primers were 5'-GCG GAA TTT GCT CCA ACC CG -3' and 5'-GTC GAC GGA TTC GAA GCC CG-3', and the PCR product was a 378 bp fragment. The internal primer set for the nested reaction was 5'-CGA AGT CGA GGT CCG TTC GAA GCC CG-3', and the PCR product was a 275 bp fragment. Amplification was performed with 2.5 units of Taq DNA polymerase (Sigma Chemical Co.), PCR buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2 mM MgCl₂, 200 µM of each deoxynucleotide (Sigma Chemical Co.), and 5 µM of primers. Denaturation was carried out at 94°C for 30 sec, primer annealing at 50°C for 8 sec and at 60°C for 30 sec, and extension at 72°C for 1 min, and this cycle was repeated for 35 cycles using a GeneAmp PCR system 7500 (Perkin Elmer Cetus, Norwalk, Conn). Final PCR products were resolved by 1.5% agarose gel electrophoresis, and the specific band was observed under UV light.

DNA sequencing: To confirm further whether the PCR products obtained in this study were actually derived from T. gondii DNA, we tried sequencing the second PCR products to examine the specificity of the nested PCR. After the bands had been recovered from the gels, DNA was extracted with phenol-chloroform and cloned using a TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The DNA sequences were determined by an automated DNA sequencer.

Histopathological examination: Organs were fixed in neutralized buffered 10% formalin, paraffin sections were made and then stained with hematoxylin-eosin.

Results

Histopathology

Two of three monkeys died on day 7 after T. gondii infection, and the remaining one monkey was sacrificed because of severe wasting syndrome (Table 1). No notable clinical signs were observed in the T. gondii-infected squirrel monkeys before death, except for hypokinesia which was observed on the day before death. At the necropsy, however, remarkable changes were found in the main organs such as heart, lung, liver, kidney and spleen. The lungs were uniformly red, congested and edematous, with petechiae, and numerous T. gondii tachyzoites were recovered from broncho-alveolar lavages. The spleen was enlarged, and the liver was congested and had an accentuated lobular pattern with a uniform finely granular surface. Mesenteric lymph nodes were enlarged, and their cut surfaces varied from uniformly dark red to mottled with red, gray and tan areas. In the lungs, microfindings common to all three monkeys were necrosis with or without inflammatory cell response. In all the T. gondii-inoculated monkeys, the lungs were edematous, with multifocal hemorrhage and sometimes serofibrinous exudate in the alveolar spaces, and focal interstitial pneumonia. Cellular debris was observed in thickened alveolar walls with minimum inflammatory response. With some difficulty, tachyzoites were identified within alveolar epithelial cells and macrophages (Fig. 1a). In
the liver, numerous necrotic foci were randomly scattered with slight response of macrophages. Many tachyzoites were observed within the necrotic foci and especially in Kupper’s cells and endothelial cells, but were less frequently seen in hepatocytes throughout the liver (Fig. 1b). Propagation of *T. gondii* tachyzoites in hepatic endothelial cells of blood vessels and blood monocytes was thus observed (Fig. 1c). Large numbers of tachyzoites were detected in the macrophages of spleen, and free tachyzoites were also present throughout the red pulp. Cell debris were present throughout the spleen, predominantly in the white pulp. Lymph node lesions were striking, and almost the entire area of enlarged lymph nodes was occupied by necrosis and hemorrhage. Many macrophages and reticuloendothelial cells in the lymph nodes contained large numbers of tachyzoites. In the small intestine of the two monkeys (Nos. 1 and 2) that died naturally, necrosis was seen in villi and submucosal lymphatic nodules, and the lamina propria were edematous. Loss of the mucous membrane and thinning of the intestinal wall were observed in the most severe lesions (Fig. 1d). Multiple tachyzoites were detected in macrophages in the lamina propria and submucosal lymphatic nodules. In the sacrificed monkey (No. 4), the lamina propria of the small intestine was enlarged due to edema, with dilatation of capillary and lymphatic vessels, and cell debris and infiltration by neutrophils were observed. Many tachyzoites were present in epithelial and reticuloendothelial cells and free tachyzoites were widely observed in the lamina propria. In other organs, such as the heart and kidneys, free tachyzoites were observed within necrotic foci, and tachyzoites were also present in both endothelial and parenchymal cells.

Remarkable histopathological changes were not observed in the two cage mates (Nos. 3 and 5) at the end of the experiment.

**Immunoblotting**

The sera obtained from *T. gondii*-infected and cagemate monkeys were analyzed by immunoblotting. The sera obtained from one *T. gondii*-infected monkey sacrificed at 1 week after infection (Fig. 2, lane 7, No. 4) did not show any band, however, the sera from two cagemates (Fig. 2, Nos. 3 and 5) showed a band of 30 kDa from one to three weeks after the start of the experiment (Fig. 2, lanes 10–13). The intensity of the band stained by DAB at 3 weeks is stronger than that of 2 weeks. No bands were observed in the sera taken before the experiment (Day 0) (Fig. 2, lanes 2–6).

**PCR and Sequencing**

To examine whether these monkeys were infected with *T. gondii* or not, we tried a nested PCR assay to detect the genome of *T. gondii*. As shown in Fig. 3, the specific band for the NTPase gene of *T. gondii* was recognized in livers and lungs of *T. gondii*-infected monkeys at one week and cagemate monkeys sacrificed at three weeks by the nested PCR, suggesting that transmission of *T. gondii* had occurred from *T. gondii*-infected monkeys to cagemate monkeys in the same cage. No bands were found in the livers and lungs of normal healthy squirrel monkeys obtained from the Amami Laboratory of Injurious Animals, Institute of Medical Science, the University of Tokyo as negative controls. To confirm further whether the PCR products obtained in this study were actually derived from NTPase gene of *T. gondii*, we tried sequencing of the

### Table 1. Clinical findings and detection of *T. gondii* in Toxoplasma infected and cagemate monkeys

| Inoculum | Detection of *T. gondii* | Anti-*T. gondii* antibody hallmark
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<td>Monkeys (No. of cysts) Survival days Clinical symptoms</td>
<td>Giemsa stain PCR</td>
<td></td>
</tr>
<tr>
<td>No. 1</td>
<td>1106</td>
<td>6‡</td>
</tr>
<tr>
<td>2</td>
<td>1925</td>
<td>6‡</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>21&lt;</td>
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<tr>
<td>4</td>
<td>2090</td>
<td>74</td>
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<tr>
<td>5</td>
<td>–</td>
<td>21&lt;</td>
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* T. gondii tachyzoites were detected by Giemsa stain in stamped smears of brains, lungs, livers, spleens, kidneys, and mesentric lymph nodes. ‡ Nested PCR for detecting *T. gondii* was done using liver and lung DNA as templates. * Anti-*T. gondii* antibody was detected by immunoblot. ‡ moribund. ‡ dead.
Fig. 1.  

a: Propagation of *T. gondii* tachyzoites within an alveolar macrophage. Alveolar exudates and thickened alveolar septa were observed with minimum inflammatory response. The arrow indicates *T. gondii* tachyzoites in a macrophage. No. 1 *T. gondii*-inoculated squirrel monkey.  ×400. 
b: Propagation of *T. gondii* tachyzoites found in a macrophage adjacent to a focus of necrosis in the liver. Many tachyzoites were scattered in a necrotic focus. The arrow indicates *T. gondii* tachyzoites in a macrophage. No. 1 *T. gondii*-inoculated monkey.  ×400. 
c: *T. gondii*-infected monocyte in a blood vessel of the liver. Many tachyzoites were found within a cell. The arrow indicates *T. gondii* tachyzoites in a blood monocyte. No. 1 *T. gondii*-inoculated monkey.  ×400. 
d: Necrosis of the villi, loss of the mucous membrane and thinning of the intestinal wall were observed in the most severe lesions. No. 2 *T. gondii*-inoculated monkey.  ×100.
second PCR product to examine the specificity of the nested PCR. The sequences of the PCR products derived from the lungs and livers which were obtained from Nos. 3 and 5 monkeys, clinically normal organs which gave a positive result in the immunoblotting, were exactly the same as the sequence of *T. gondii* ME49 (Fig. 4). Interestingly, the substitution of two nucleotides as compared with that of *T. gondii* RH published by Bermudes *et al.* [2] was found.

**Discussion**

Toxoplasmosis is a highly fatal disease in squirrel monkeys as previously reported [1, 3, 4, 13, 14]. In the present studies, we used the *T. gondii* ME49 strain, which was originally derived from a diseased sheep and had been serially passaged on mice for a long time. It produces many cysts in the brains and other tissues of mice but is of low virulence to mice.

We used oral infection because it is a more natural route than subcutaneous infection. Oral infection generally induces more severe infection than subcutaneous ones because it produces multiple enteric lesions. Actually severe enteric necrosis with many tachyzoites were observed in the monkeys inoculated with *T. gondii* cysts. Generally, after *T. gondii* cysts are ingested, the cysts rupture in the small intestine, and bradyzoites derived from the cysts invade the tissues of the small intestine, and are then propagated in macrophages and reticuloendothelial cells in the tissues as tachyzoites. After that, they disseminate to extraintestinal tissues through the blood vessels.

After the inoculation of *T. gondii* ME49 cysts orally, three infected monkeys were sacrificed or died of acute toxoplasmosis, and histopathological findings were consistent with the results reported previously [1, 3, 13]. Inflammation, edema, necrosis and congestion were found in the lung, liver, spleen, mesenteric lymph node and small intestine at necropsy. Many tachyzoites could be demonstrated in those tissues histopathologically and were recovered from broncho-alveolar lavages. Massive lymphatic pulmonary edema and acute inflammation with necrosis of the reticuloendothelial system were consistently found histopathologically. Parasitemia and the propagation of parasites in the endothelial cells of blood vessels were confirmed in all of the infected monkeys by histopathological examination, suggesting that early dissemination of this avirulent strain of *T. gondii* from the intestine into the general circulation occurs via blood vessels. On the other hand, not one of the cagemate monkeys showed any histopathological lesions.

In the immunoblotting, *T. gondii* specific antibody was obviously recognized in the two cagemate monkeys (Nos. 3 and 5) at 2 and 3 weeks after the start of the experiment. The results indicate that the horizontal
transmission of *T. gondii* from infected monkeys to cagemates occurred during the experiment. This was supported by the results of the nested PCR, in which the genome of *T. gondii* was detected by the nested PCR in the lungs and livers of the cagemate monkeys at 3 weeks after the start of the experiment. The sequences of the nested PCR products obtained from the lungs and livers which were obtained from Nos. 3 and 5 monkeys, clinically normal but which gave a positive result in the immunoblotting, were exactly the same as the sequence of *T. gondii* ME49, suggesting that the nested PCR used in this study is specific for *T. gondii*.

Interestingly, the substitution of two nucleotides of *T. gondii* ME49 NTPase gene as compared with that of *T. gondii* RH published by Bermudes et al. [2] was found.

Escajadillo et al. [6] reported that contact infection of *T. gondii* may occur between infected *Aotus* monkeys and their cagemates, although the clear route of contact infection was not determined. They showed that *Toxoplasma* tachyzoites were present in milk, saliva, urine or feces and may be ingested by cagemates [6]. Since acute pulmonary toxoplasmosis was found in all *T. gondii*-infected monkeys and many tachyzoites were recovered from the broncho-alveolar lavages in this experiment, the possibility that aerosol infection occurred among the squirrel monkeys in the same cage is suggested. In our preliminary data, normal mice were easily infected with *T. gondii* tachyzoites by intranasal inoculation as well as by intraperitoneal inoculation and died of acute pulmonary toxoplasmosis. In the present study, no *T. gondii* parasites were found by histopathology and Giemsa staining in the tissues of cagemates, although anti-*T. gondii* antibody and *T. gondii* gene were detected by ELISA and PCR after the experiment had started. These findings suggest that subclinical *T. gondii* infection was established in the cagemates by contact with *T. gondii*-infected monkeys as previously reported [6].

Generally, it has been reported that chronic *Toxoplasma* infection is not found in squirrel monkeys and other arboreal New World species and the lemurs, like the night monkey (*Aotus lemurinus*, and *A. zonalis*) [6].
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15], red crested barefaced tamarin (Saguinus oedipus) [3], spider monkey (Ateles geoffroyi) [14], marmoset (Marikina geoffroyi) [15], whiteface monkey (Cebus capucinus) [16] and ring-tailed lemurs (Lemur catta) [9]. All of these animals are extremely susceptible to Toxoplasma infection, and usually succumb to disseminated acute toxoplasmosis. The extremely high susceptibility to T. gondii was circumstantially attributed to absence of exposure and lack of selection by Toxoplasma infection in these arboreal monkeys. Therefore, because they are apparently unselected by Toxoplasma infection, these monkeys provide a sensitive primate model for safety tests of drugs and vaccines against Toxoplasma infection. The fact that T. gondii can easily infect squirrel monkeys and produce fatal diseases, suggests that persons who are working with squirrel monkeys in zoos or keeping them as a pet in the house should be aware of their susceptibility to Toxoplasma infection and take proper precautions to decrease or to prevent their exposure to the reservoir animals or contaminated caging facilities and foods with T. gondii.

References