Humoral Immune Response to *Isospora felis* and *Toxoplasma gondii* in Cats Experimentally Inoculated with *Isospora felis*

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Feline apicomplexan parasitic protozoans, *Toxoplasma gondii* (T. gondii) and *Isospora felis* (I. felis), are prevalent all over the world. The biological and epidemiological significance of the latter species is well-documented, since *I. felis* infection has been shown to trigger oocyst re-shedding in cats chronically infected with *T. gondii* [2, 3].

We reported in a previous study that cats naturally infected with *I. felis* produced lower anti-*I. felis* antibody and, following *T. gondii* inoculation, showed an increase in anti-*I. felis* antibody titer [10]. This finding suggests either immunoproliferating phenomenon due to *T. gondii* infection or the presence of similar antigen(s) to these two species.

In order to understand more the immune reactions in cats inoculated with *I. felis* in this paper, we examined anti-*I. felis* and anti-*T. gondii* antibody reactivities in cats experimentally inoculated with *I. felis*.

Cysts of S-273 *T. gondii* strain were prepared from brains of mice from the 5th to 7th week post-exposure (p.e.) and density was adjusted to 10⁵ cysts in one ml of phosphate buffered saline (PBS). Trophozoites of RH and S-273 strains of *T. gondii* were obtained from infected mouse embryonal cells (MEC) as described previously [9]. *I. felis* oocysts were collected from feces of infected cats by the flotation method with 

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\text{ZnSO}_4 \text{ solution, followed by succrose density gradient centrifugation [1] and stored at 4°C in PBS supplemented with 100 μg/ml of Kanamycin. Oocysts were examined within two months after collection. Excretion was done by incubating the oocysts in PBS containing 0.5% taurocholic acid and 0.1% trypsin at 37°C for 30 min. Sporozoites of } I. \text{ felis and } T. \text{ gondii were purified by the percoll-sucrose density gradient centrifugation [1].}
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Two female six-week-old cats born to a mother that shed no *I. felis* oocysts were used as *I. felis*-free animals, and were kept in individual cages strictly isolated in the animal care facilities at Shionogi-Aburahi Laboratories, Shiga. The cats were orally inoculated with 5×10⁸ oocysts of *I. felis*. Cat No. 1 was orally inoculated with 5×10⁴ oocysts of *T. gondii* orally on day 28 p.e. and cat No. 2 was also inoculated at the same dose as of *T. gondii* on day 56 p.e.

Feces of each cat were daily collected with the floating method as described previously [4] and were examined microscopically for the presence of coccidian oocysts.

Serum obtained weekly from cats at the jugular vein were used to measure anti-*T. gondii* and anti-*I. felis* antibody IgG titer, and to determine the common antigen(s) to the two species. Anti-*T. gondii* and anti-*I. felis* antibody IgG was titrated by the indirect immunofluorescence assay (IFA) described in a previous report [9].

To define the antigenicity between *T. gondii* and *I. felis*, the extracts of both parasites were examined by the immunoblotting assay. Oocysts of *I. felis* or S-273 or RH strain of *T. gondii* were suspended in a fixed amount of solubilizing buffer [2% 2-mercaptoethanol, 12.5 mM Tris-HCl buffer pH 6.8, 4.6% sodium dodecyl sulfate (SDS), 2 mM phenyl methyl sulfonyl fluoride] for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the suspension was boiled for 5 min, ultrasonicated for 10 sec and centrifuged at 12,000 g for 5 min. The supernatants were applied on a 10% gel of SDS-PAGE.

After electrophoresis, the separated proteins were electrophoretically transferred into nitrocellulose membrane (GVHP, Nihon Millipore, Yonezawa, Japan) as described by Towbin et al. [12]. The membrane sheets were immersed in PBS containing 5% skim milk (Milk-PBS) for blocking at room temperature for 1 hr and then washed in PBS containing 0.05% Tween 20. Cat sera tested were incubated with either MEC fixed with 1% paraformaldehyde in PBS or parasites of *T. gondii* S-273 strain at 4°C overnight to remove non-specific or cross-reactive antibodies, and then were diluted 50-fold in Milk-PBS. Membrane sheets were incubated with the sample sera at 4°C overnight, followed by reaction with peroxidase-conjugated anti-cat IgG (American Qualex, La Mirada, CA) diluted with Milk-PBS. The antigen was visualized by reaction with 0.02% diaminobenzidine-4HCl and 0.1% H₂O₂ in 0.1 M Tris-HCl buffer at pH 7.4.

As shown in Fig. 1a, cat No. 1 showed two peaks of *I. felis* oocyst shedding between days 7 and 10 and between days 14 and 16 p.e. The number of oocysts in the first peak was approximately 1.3×10⁷, which was significantly greater than that in the second peak. Cat No. 2 showed also transient oocyst shedding on days 8 to 21 p.e. (Fig. 1b). The number of oocysts in the first peak on day 10 p.e. was 1.4×10⁷ and that in the second peak on day 18 p.e. was 0.4×10⁷. After challenged with *T. gondii* oocysts, cat No. 1 showed transient shedding of *T. gondii* oocysts on days
24 and 25 p.e. to *T. gondii*. The total number of oocysts was 5.5 × 10^6. Cat No. 2 also showed transient shedding of *T. gondii* oocysts from days 30 to 33 p.e. to *T. gondii*. The number of oocysts per day was below 10^4 and most of the oocysts were not completely sporulated.

By indirect immunofluorescence assay, both *I. felis* sporozoites and *T. gondii* parasites incubated with non-infected cat serum showed fluorescence at the on terminal part of parasites, which was recognized as non-specific reaction and observed up to a serum dilution of 4^4. Specific fluorescence on the whole body of parasites was found in cat No. 1 up to a titer of 4^4 on days 7 and 21 p.e., and then decreased to a titer of 4^2 on day 28 p.e. Likewise, anti-*T. gondii* IgG increased to a titer of 4^3 on day 21 p.e. Anti-*I. felis* IgG antibody in cat No. 2 also appeared on day 7 p.e., and it increased transiently to a titer of 4^3 on day 21 p.e. and subsequently, decreased to 4^2 on day 42 p.e. Anti-*T. gondii* IgG appeared on day 10 p.e. and on day 21 p.e., it increased to 4^3 in titer, and then decreased to 4^1 on day 42 p.e. After cat No. 1 was challenged with *T. gondii*, anti-*I. felis* IgG increased to 4^3 in titer on day 21 p.e. and decreased to 4^2 on day 28 p.e. to *T. gondii*. Anti-*T. gondii* IgG also increased to 4^3 on day 21 p.e. to *T. gondii* and was maintained at 4^6 from day 28 p.e. to the end of experiment. Anti-*I. felis* IgG in cat No. 2 also increased to 4^2 on day 28 and further to 4^4 on day 35 p.e. to *T. gondii*. Anti-*I. felis* IgG also increased to 4^3 on day 14 p.e. and was maintained at 4^6 up to the end of experiment.

To examine the reactivity of cat sera to *T. gondii* and *I. felis* by immunoblotting method, the extracts were prepared by boiling parasites with solubilizing buffer. Whole parasites of *T. gondii* and *I. felis* oocysts containing sporozoites were dissolved with solubilizing buffer, and oocyst walls were removed by centrifugation. Transblotted membrane made to react with cat No. 2 serum collected on day 28 p.e., showed a broad band with a m.w. of 22 kDa in the *I. felis* extract lane. In the lanes of *T. gondii* strains (RH and S-273) extracts, specific bands with a m.w. of 22, 45, 58 and 62 kDa appeared. Membranes made to react with cat No. 1 serum showed similar band in
the lane of I. felis extract, while a faint band with a m.w. of 45 kDa was detected in the lane of RH strain T. gondii. The same serum sample (cat No. 2) preincubated with T. gondii parasites showed only one band of a m.w. of 22 kDa in the lane of I. felis extract, but not in that of T. gondii. The membrane made to react with the serum obtained from the cats pre-inoculated with I. felis showed no specific bands (the data not shown).

Results of the present study demonstrate that I. felis can survive considerably for a long time in the host, but it does not reproduce in enormous numbers just like T. gondii; consequently, the antigenic stimulation by the parasite is likely to be quantitatively weaker than that by other pathogenic microorganisms. In recent years, stage-specific antigenicity T. gondii has been documented [6-8]. It is plausible that I. felis also is antigenically transformed from sporozoites to trophozoites or hypnozoites. This antigenic transformation, if it really occurs, may be associated with the variation of anti-I. felis antibody titer in cats infected with I. felis, as noted in this study. In a previous report, cats naturally infected with I. felis showed low antibody production [10]. One reason for the difference in anti-I. felis antibody titers between experimentally and naturally infected cats noted by us, may be explained by the difference in the inoculum dose of parasites to which the cats were exposed. We surmise that the relatively lower anti-I. felis antibody titer in naturally infected cats may be due to the past exposure to lower inoculum dose.

Furthermore, cats inoculated orally with I. felis oocysts showed a transient appearance and increase in anti-T. gondii antibody titer on day 7 p.e. The prepatent period and the pattern of anti-T. gondii antibody titer in cats in the present study are somewhat different from those of naturally infected cats [9, 10]. Contamination of the materials used in this experiment with T. gondii is probably negligible, because it has been shown that cats infected with T. gondii can sustain a high anti-T. gondii antibody production for a long period of time. Our finding suggests that cats infected with I. felis would very likely show the positive range of anti-T. gondii antibody titer useful to serodiagnosis within the first 21 days after exposure. The titer, however, is relatively lower than that of T. gondii infected cats [9, 10]. It seems reasonable to do serodiagnostic test at least twice at more than three week interval in order to determine whether cats showing relatively lower titer of anti-T. gondii antibody IgG is infected with T. gondii or not.

By immunoblotting assay with I. felis-infected cat sera, we have noted the presence of cross reactive antigens with m.w. of 45, 58 and 62 kDa between T. gondii and I. felis. Absorption test indicated that the band of a m.w. of 22 kDa is specific to I. felis. The increase in anti-T. gondii IgG antibody titer observed in cats infected with I. felis may have resulted from an accelerated production of antibody to the cross-reactive antigen. Recently, cross reactive antigen located in the apical region of sporozoan parasites has been documented [11]. Cat serum is recognized as "positive" by the fluorescence pattern against the whole parasite. This suggests that some of the cross-reactive antigens observed by immunoblotting analysis in this study may be associated with apically-located antigens [11] or antigens with similar antigenicity. The reason why common antigen(s) distributed on the whole body was not detectable by immunoblotting analysis may be explained that the sensitivity of immunoblotting analysis for detection of antigen is lower than that of immunofluorescence method. Further studies are necessary to examine whether antibody production to these cross reactive antigens has protective roles and some biological significance.

REFERENCES