Reduction of the Infectivity of *Toxoplasma gondii* and *Eimeria stiedai* Sporozoites by Treatment with Bovine Lactoferricin

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**ABSTRACT.** Sporozoites of *Toxoplasma gondii* preincubated with lactoferricin showed decreased activity in penetration of mouse embryonal cells. Mice inoculated with 10^5 sporozoites preincubated with lactoferricin showed a higher survival rate than those inoculated with the same number of untreated sporozoites. Likewise, sporozoites of *Eimeria stiedai* preincubated with lactoferricin also showed decreased activity in penetration of rabbit hepatobiliary cells. Rabbits inoculated with 10^5 sporozoites preincubated with lactoferricin shed fewer oocysts than those inoculated with the same number of untreated sporozoites. These results indicate that lactoferricin is effective to reduce the infectivity of sporozoites of *Toxoplasma gondii* and *Eimeria stiedai*.  

**KEY WORDS:** *Eimeria stiedai*, lactoferricin, *Toxoplasma gondii*.

*Toxoplasma gondii* (*T. gondii*), an obligatory intracellular parasitic protozoan is an intestinal coccidium of felids. In intermediate hosts, the parasites multiply as tachyzoites which cause host cell rupture and as bradyzoites which form tissue cysts. In feline intestinal epithelium, the parasites develop through a sexual cycle, resulting in oocyst shedding. The oocysts are resistant to environmental stress and undergo sporogony to form sporozoites which have infectivity in the intermediate and definitive hosts.

Lactoferricin (Lfcin) generated by pepsin digestion of bovine lactoferrin (Lf) is composed of 25 amino acid residues, and is known as an antimicrobial peptide able to damage microbial membranes directly [1, 2]. Previous studies have shown that Lfcin has parasiticidal effects against tachyzoites and bradyzoites/cysts of *T. gondii* [7]. Peroral administration of Lfcin has been shown to have a protective effect against infection by extra-intestinal stage parasites [4]. Whether Lfcin has such an inhibitory effect against sporozoites, however, has not yet been investigated.

In the present study, we examined whether Lfcin is effective to reduce the infectivity of sporozoites of *T. gondii*. In order to examine whether the actions of Lfcin are specific for *T. gondii* or whether it has similar inhibitory effects against other coccidian sporozoites, in similar experiments we tested its effect on sporozoites of *Eimeria stiedai* (*E. stiedai*) which develops in hepatobiliary cells of rabbits and causes extensive obstructive jaundice due to oocyst shedding.

Bovine lactoferricin was prepared from bovine lactoferrin by the method of Bellamy et al. [1]. For experiments, Lfcin was dissolved at a concentration of 2 mg/ml in Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin (D-MEM1%BSA) just before use.

Rabbit hepatobiliary cells (RHC) were harvested from the liver of rabbits as described by Njenga et al. [5]. Mouse embryonal cells (MEC) were prepared as described elsewhere [6]. Both types of cells were cultured in D-MEM containing 10% fetal bovine serum (D-MEM10%FBS). For experiments, the cells were removed by treatment with 0.025% trypsin in phosphate-buffered saline (PBS). A 0.2 ml portion of the cell suspension, at a cell concentration of 2 × 10^4 cells/ml, was applied onto round coverslips (15 × 15 mm diameter; Matsunami, Osaka, Japan). To avoid the influence of lactoferrin present in FBS, the cell culture medium was replaced with D-MEM-1%BSA in each instance for 24 hr before the experiments.

Oocysts of *T. gondii* Beverley strain were isolated from fecal materials of infected cats, and oocysts of *E. stiedai* were isolated from fecal materials of infected rabbits, by the sucrose flotation method described by Dubey and Beattie [3]. To avoid bacterial contamination, oocysts were suspended in 1% sodium hypochlorite on ice for 10 min, then washed by centrifugation at 1,200 g for 10 min in PBS three times. For oocyst sporulation, oocysts were suspended in distilled water, then placed in a 9-cm diameter glass plates at room temperature for 3 days and preserved in 2.5% potassium dichromate at 4°C. Excystation was accomplished by incubation in PBS containing 0.2% trypsin and 0.8% sodium taurocholic acid for 2 hr at 37°C with agitation. Sporozoites of both *T. gondii* and *E. stiedai* were isolated by filtration through a 3-µm membrane filter (Nucleopore, Coster, Acton, MA). After washing by centrifugation, the sporozoites were suspended in D-MEM-1%BSA, at a concentration of 1 × 10^6 parasites/ml.

To examine the inhibitory effect of Lfcin, aliquots of a suspension of the sporozoites in D-MEM1%BSA in a series of tubes were treated with Lfcin at 0 (control), 100 or 1,000 µg/ml at 37°C for a certain period. The parasites in each of
the tubes in the series were then washed thoroughly three times in D-MEM by centrifugation at 1,200 g, for 10 min each time, and resuspended in D-MEM1%BSA at $5 \times 10^5$ sporozoites/ml.

In vitro infection of host cells by sporozoites of *T. gondii* and *E. stiedai*, using RHC and MEC, respectively, was examined as described by Tanaka et al. [7]. Briefly, the host cell monolayers on cover slips were preincubated for 24 hr with D-MEM1%BSA, then challenged with 0.2 ml of either treated or untreated sporozoites and further incubated at 37°C. At 18 hr post-inoculation, the cover slips were washed with PBS thoroughly, fixed with methanol, and stained with Giemsa. The number of intracellular parasites was counted examining a total of 500 cells and the number of intracellular parasites per 100 cells is shown. All experiments were performed in triplicate and repeated at least twice. Data from each experiment were evaluated using Student’s test. The level of significance in all analysis was 95%.

The infectivity of treated sporozoites of *T. gondii* and *E. stiedai* after incubation with 1,000 µg/ml Lfcin for 1 hr was also examined by inoculation test in mice or rabbits, respectively. Five mice in each group were inoculated intraperitoneally with $10^5$ *T. gondii* sporozoites, either untreated or treated with Lfcin. The survival time and mortality of the mice were monitored for up to 30 days post-inoculation. Samples from the mice that died during the monitoring period were examined microscopically to detect the presence of parasites in the peritoneal cavity. The concentration of cysts in the brains of individual mice surviving on the 30th day post-inoculation was examined as described by Isamida et al. [4]. Three rabbits in each group were inoculated intravenously with $10^5$ *E. stiedai* sporozoites, either untreated or treated with Lfcin. The number of oocysts shed by individual rabbits per day was monitored for up to 60 days post-inoculation. The ratio of liver weight to body weight for individual rabbits was determined on the 60th day post-inoculation.

As shown in Fig. 1, after preincubation with Lfcin at 1,000 µg/ml for 0.5 hr, the *T. gondii* sporozoites showed significantly less penetration activity than the untreated parasites. The penetration activity of the parasites treated with Lfcin at 100 µg/ml also showed a tendency to be diminished compared with that of the untreated parasites. Loss of infectivity of the sporozoites was confirmed by inoculation of mice. Four out of five mice inoculated with $10^5$ untreated sporozoites died within 14 days post-challenge. The remaining mouse of this group died on the 24th day post-challenge. In contrast, all five mice inoculated with the same dose of sporozoites treated with Lfcin at 1,000 µg/ml for 1 hr survived for more than 30 days post-challenge without showing any clinical signs of infection, although all mice had cysts in the brain (the number of cysts in the brain of the individual mice was 400, 600, 400, 400, and 300).

The effect of Lfcin on the viability and infectivity of *E. stiedai* sporozoites was also examined in both in vitro and in vivo experiments. Sporozoites retained activity in penetration of hepatobiliary cells after 2 hr incubation in the medium. In contrast, the sporozoites preincubated with Lfcin at 1,000 µg/ml for 0.5 hr showed significantly less activity in penetration of the host cells (Fig. 2). The infectivity of sporozoites after incubation with either Lfcin or the

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**Fig. 1.** Effect of Lfcin on the infectivity of *T. gondii* sporozoites in penetration of mouse embryonal cells. Sporozoites were incubated either in the presence or absence of Lfcin for the time indicated, then the cell culture was inoculated and incubated for 18 hr. The infection rate shown is the number of intracellular parasites per 100 cells, determined by counting the number of parasites in a total of 500 cells. Mean ± SD of triplicate samples.
Fig. 2. Effect of Lfcin on the infectivity of *E. stiedai* sporozoites in penetration of rabbit hepatobiliary cells. Sporozoites were incubated either in the presence or absence of Lfcin for the time indicated, then the cell culture was inoculated and incubated for 18 hr. The infection rate shown is the number of intracellular parasites per 100 cells, determined by counting the number of parasites in a total of 500 cells. Mean ± SD of triplicate samples.

Fig. 3. Infectivity in rabbits of *E. stiedai* sporozoites after treatment with Lfcin or without such treatment. Sporozoites were incubated either in the presence or absence of Lfcin for 1 hr, then rabbits were inoculated intravenously. The number of oocysts in the feces of each rabbit was monitored daily. Mean ± SD of triplicate samples.
medium alone for 1 hr was also examined by an inoculation test in rabbits. All rabbits inoculated with $10^5$ untreated or treated sporozoites shed oocysts between 16 days and 35 days post-challenge. The number of oocysts shed per day in the case of the rabbits inoculated with the untreated sporozoites was higher than that in the case of the rabbits inoculated with the treated sporozoites (Fig. 3). The total number of oocysts shed by the three rabbits inoculated with untreated sporozoites was 103.1, 102.2 and $35.7 \times 10^8$ oocysts. However, the three rabbits inoculated with the same dose of sporozoites treated with Lfcin at 1,000 µg/ml for 1 hr shed less, i.e., 33, 0.9 and $24 \times 10^8$ oocysts. At necropsy, all three rabbits inoculated with $10^5$ untreated sporozoites showed intensive cholestasis. Many abscesses were observed. The ratio of liver weight to body weight in the case of these three rabbits was 19.6, 15.7 and 22.1%. In contrast, the livers of all of the rabbits inoculated with the treated sporozoites had less abscesses and the bile ducts were not swollen. The ratio of liver weight to body weight in the case of these three rabbits was 10.1, 5.7 and 7.4%.

Thus, the present study indicates that Lfcin at a certain concentration has parasiticidal effects against sporozoites of *T. gondii* and *E. stiedai* and the treated sporozoites show reduced infectivity in the host. Hence, considering that sporozoites excyst from the sporulated oocysts in the intestine and invade host enterointestinal cells, and considering that Lfcin is produced by gastric pepsin cleavage of Lf, it is conceivable that a sufficient amount of Lfcin or Lf provided in the diet may be effective to inactivate such food-borne pathogens.

Further studies are necessary to examine whether oral administration of Lf or Lfcin is effective in preventing such coccidian infection.

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REFERENCES