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Host Cell Specificity on The Entry of *Eimeria stiedai* Sporozoites in Cultured Cells

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ABSTRACT

Invasion specificity of *Eimeria stiedai* sporozoites to cultured rabbit liver biliary cells, parenchymal hepatocytes and kidney cells was monitored. Intracellular sporozoites were observed in liver biliary epithelial cells, as early as 3 hrs when an infection rate of 5% was recorded. Infection rate for rabbit liver biliary epithelial cells was monitored for 6 hrs and increased to approximately 9% at this time. No intracellular parasites were found in rabbit parenchymal hepatocytes and rabbit kidney cells, even on prolonged culturing. In the liver biliary epithelial cells inoculated with fixed sporozoites, no intracellular parasites were found. Sporozoites attached on the cell surface of the liver biliary epithelial cells fixed with paraformaldehyde, but did not penetrate.

Serum of mice immunized with rabbit liver biliary epithelial cells, showed antibody reactivity to liver biliary epithelial cells, higher than, that of rabbit parenchymal hepatocytes and rabbit kidney cells. After absorption with parenchymal hepatocytes, antibody reactivity to liver biliary epithelial cells in the serum still retained. Sporozoite penetration activity in the liver biliary epithelial cells reduced by the supplement of the serum of mice immunized with rabbit liver biliary epithelial cells in the culture medium. These results suggest that *Eimeria stiedai* sporozoites penetrate the hepatobiliary cells selectively and these epithelial cells have the sporozoite binding site(s) which might be identified by specific antibody.

INTRODUCTION

Eimeria stiedai (*E. stiedai*), a coccidia of liver of the domestic rabbit, causes clinical symptoms related to obstructive jaundice. The pathogenicity is restrictedly located in liver tissue, specially bile ducts. The interaction between host liver tissue and the parasite has not yet been clearly elucidated, although there have been reports on the in vitro culture of *E. stiedai* merozoites (Fitzgerald 1970; Doerr and Hohn 1972; Coudert and Provot 1974).

This study, was conducted to examine the host specificity of *E. stiedai* sporozoites in cultured cells from rabbit liver tissue and compare the penetration activity of *E. stiedai* sporozoites in those cultured cells. To identify the liver origin of the cultured cells, immunofluorescence was performed in liver tissue.

MATERIALS AND METHODS

Cultured cells

Rabbit parenchymal hepatocytes were isolated from male and female rabbits weighting 500-700g by in situ perfusion on the liver with collagenase as described elsewhere (Nakamura et al. 1980). The cell suspension was centrifuged at 600*g* for 7 min. After that, the cells were resuspended at 5 x 10^4 cells/ml in William medium containing 10% fetal bovine serum, 1mM Dexamethazone and 2 nM insulin, then were incubated with collagen-coated petridishes (Falcon Inc.) at 37°C in 5% CO_2. Another cell type of cultured rabbit liver cells were isolated from rabbits similar weighting as described above. Briefly, approximately 20g of the bile duct and liver biliary tissues were cut into small pieces, and suspended in 50 ml of 0.025% collagenase in phosphate buffered saline (PBS) containing $CaCl_2$ and $MgCl_2$, incubated at 37°C for 30 min with stirring. After centrifugation at 600*g* for 7 min, the cells were suspended at 5 x 10^4 cells/ml in Dulbecco's modified minimum essential medium containing 10% fetal bovine serum (D-MEM10%FBS) and incubated as described above. During 2 weeks, epithelial type cells multiplied in the cell culture. This cultured cells were tentatively named liver biliary epithelial cells. Rabbit kidney cells were isolated from the same rabbits as described above. Briefly, the kidney tissues were cut into small pieces, and suspended in 50 ml of 0.25% trypsin in PBS, incubated at 37°C for 15 min with stirring. After centrifugation at 600*g* for 7 min, the cells were suspended at 5 x 10^4 cells/ml in D-MEM10%FBS and incubated as described above. During I week, fibroblasts multiplied to become monolayer in the cell culture. BS-C1 cells, an African Green Monkey kidney cell line, was purchased form Dainippon Pharmaceutical Co. (Osaka, Japan) and cultured in D-MEM 10%FBS. Bovine bile epithelial cells were harvested from bile ducts of cattle by scraping and incubated as described above. Mouse embryonic fibroblasts were obtained from embryo of

ICR mice on the 14th-16th day of the gestation. For experiments, each type cells were removed from cultured petri dishes by trypsinizing, respectively. Zero point two ml, of the cell suspension, at a concentration 5 x 10^4 cells/ml, was cultured on 15 mm round coverslip at 37°C overnight, before inoculation. In the case of hepatocytes, the cells were cultured on collagen-coated coverslips (Iwaki, Tokyo, Japan).

Parasites

Eimeria stiedai and *Eimeria magna* oocysts were collected from feaces of infected rabbits by sucrose floatation. Sporozoites were released from sporulated oocysts by use of 0.4% taulocholic acid and 0.2% trypsin in PBS at 37°C for 2 hrs. The released sporozoites were separated by filtration using polycarbonate membrane filter (Nucleopore, 3 μm pore size, Costar, Cambridge, MA) and centrifuged at 1,200*g* for 10 min. The sporozoites were suspended in D-MEM 10%FBS at a concentration of 10_5 sporozoites/ml. Some of the sporozoites were fixed with PBS containing 1% paraformaldehyde at 4°C for 15 min.

Penetration activity of sporozoites in the cultured cells

Culture medium was removed from the cell monolayered coverslips, and 0.2 ml of sporozoites suspension was mounted onto the coverslips, incubated either at 37°C or at 4°C for a specifying length. Some of coverslips were pretreated by fixation with PBS containing 1% paraformaldehyde at 4°C for 15 min. After incubation, the coverslips were washed in PBS and fixed with methanol and stained with Giemsa. The number of intracellular sporozoites was counted per 400 cells. Each experiment was repeated at least three times.

Immunization in mice

To prepare antisera, against the cultured liver biliary epithelial cells, mice were immunized with the cultured cells. The cultured liver biliary epithelial cells harvested, were washed by centrifugation at 600*g* for 10 min, 3 times, in PBS; fixed with chilled PBS containing 1% paraformaldehyde for 15 min and resuspended in PBS at a concentration of 10^7/ml. The cell suspension was mixed with the same volume of Freund's complete adjuvant. Three BALB/c female adult mice were inoculated intraperitoneally with 0.5 ml of the emulsion. The mice were inoculated intravenously with 0.1 ml of the cell suspension for 2 weeks interval 3 times. On the 10th day post final inoculation, the serum of mice obtained were inactivated at 56°C for 30 min, then pooled and stored at -20°C until use. The serum of mice obtained on the 1st day before immunization was used as control.

Indirect immunofluorescence antibody test (IFAT)

The antibody reactivity, against each cultured cells, was performed by IFAT. Each cultured cells were fixed with PBS containing 1% paraformaldehyde at 4°C for 15 min. After washing in chilled PBS three times, the cultured cell suspensions was dropped onto slide glasses, dried at room temperature and stored at -80°C until use. The serum of mice obtained, four fold serial diluted in PBS were mounted on the cultured cells coated slide glasses at 37°C for 1 hr. After washing with chilled PBS, FITC-conjugated anti-mouse IgG, 200 fold diluted in PBS was added and the incubation continued at 37°C for 1 hr. The presence of specific fluorescence on the cell membranes was examined by fluorescence microscope.

Absorption of cultured cells

To examine the antibody specificity, the serum was done by absorption with cultured cells. Briefly, 100 μl of the tested serum was mixed with 10 mg of either parenchymal hepatocytes or liver biliary epithelial cells at 4°C overnight, under gentle shaking. After that, the suspensions were centrifuged at 12,000*g* for 20 min, the supernatant was stored at -20°C until use.

Effect of immunized serum on sporozoite penetration activity

Cultured cells monolayered on coverslips were incubated in D-MEM 10%FBS containing inactivated serum of either non-infected mice or that of immunized mice, at 37°C for 1 hr. After washed twice with the medium, the sporozoites suspension was mounted onto the cover slips and incubated at 37°C for 6 hrs. The penetration activity was measured as described above.

RESULTS

Penetration activity of sporozoites in the cultured cells

Intracellular sporozoites were easily distinguished under a light microscope, because the penetrated sporozoites became stumpy and surrounded by parasitophorous vacuole (Fig. 1). Intracellular sporozoites were found in biliary epithelial cells (5%) at 3 hrs post inoculation (p.i.). The number of intracellular sporozoites increased gradually , therefore, the infection rate was monitored for 6 hrs in the cell culture. Infection rate for biliary epithelial cells was approximately 9% at this time. However, no intracellular parasites were found in the parenchymal hepatocytes and rabbit kidney cells. At 4°C, penetration activity was not detected. When the liver biliary epithelial cells were fixed, the sporozoites attached on the cell surface but not penetrated (Fig. 2). To compare the penetration activity of *E. stiedai* sporozoites, *E. magna* sporozoites were challenged against the same cultured cells. It was observed that *E. magna*

sporozoites have a predilection for the rabbit bile cells where the penetration was up to 6.64%. In other cell types was 2.36% for BS-C1, 1.8% for rabbit kidney, 0.65% for BFPTC and 0.6% for bovine biliary epithelial cells.

Figure 1 Stumpy intracellular sporozoites surrounded by parasitophorus vacuole.

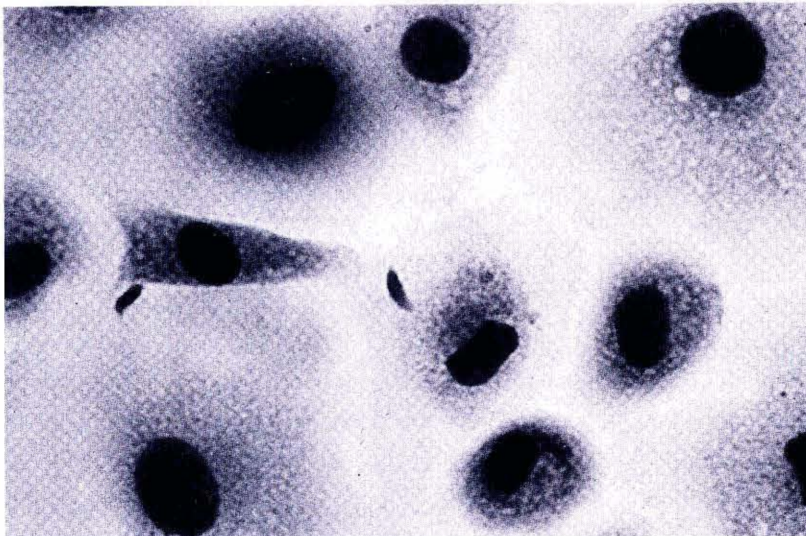

Figure 2 Sporozoites attached on the cell surface but did not penetrate.

Indirect immunofluorescence antibody test

Specific fluorescence was found on the rabbit liver biliary epithelial cells incubated with the immunized serum, up to 1 : 4,096. The serum also showed antibody reactivity to hepatocytes up to 1 : 256. After absorption with the hepatocytes, antibody reactivity to the hepatocytes reduced to <4. While, antibody reactivity to liver biliary duct cells retained to 1 : 256. On the other hand, after absorption with the rabbit liver epithelial cells, the reactivity to liver biliary epithelial cells reduced to 1 : 64. Serum of mice preimmunized with rabbit liver biliary epithelial cells showed no specific fluorescence to any kind of cultured cells.

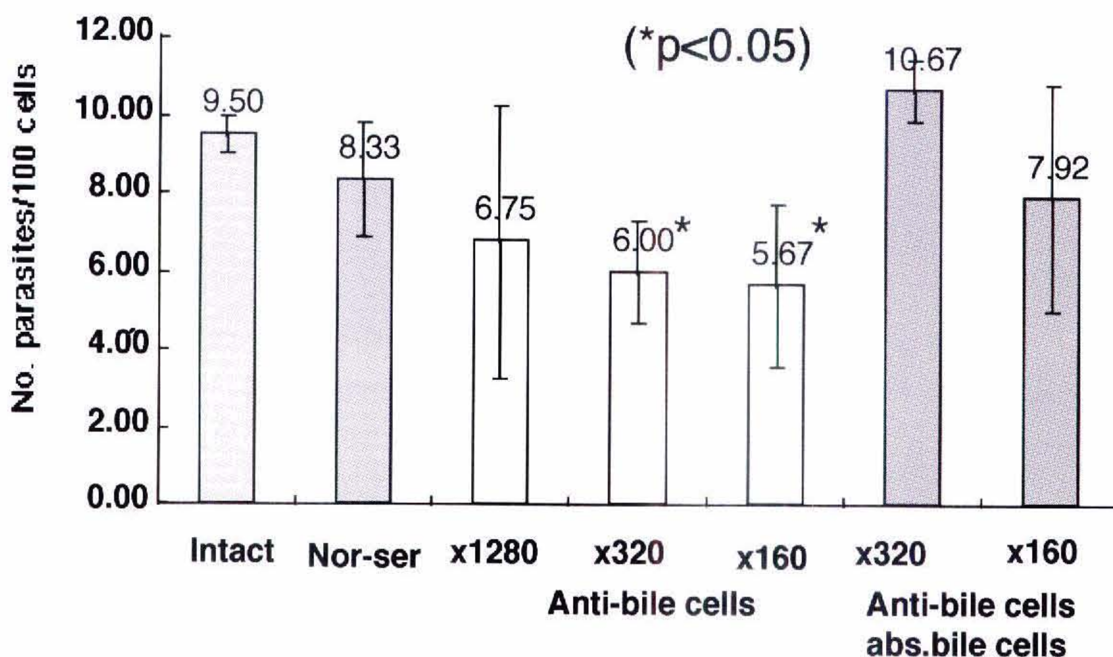

Figure 3 Effects of anti-rabbit biliary epithelial cell antiserum on the *E. stiedai* sporozoites penetration of the rabbit biliary epithelial cells.

Effect of anti-rabbit liver biliary epithelial cell antiserum on sporozoite penetration activity

Supplement of the immunized mice serum diluted 1 : 160 fold in the cell culture showed no cyto-pathogenic effect. Preincubation of the immunized serum diluted 1: 160 and 1: 320 fold in the culture medium markedly reduced the sporozoites penetration activity ($P<0.05$). Preincubation of the immunized serum which was absorbed with the rabbit liver biliary epithelial cells, showed no significant difference on penetration activity, compared to that of unsupplemented

control. Preincubation of the cells with preimmunized serum, diluted 1:40, showed no inhibitory effect to the sporozoites penetration (Fig. 3).

DISCUSSION

Our results showed that *Eimeria stiedai* sporozoites have penetration activity to the liver biliary epithelial cells, but not to hepatocytes and kidney cells. These results were consistent with the observation of Coudert et al. (1994). Anti-cultured rabbit liver biliary epithelial cells serum, reacts the sporozoites binding sites to reduce the sporozoites penetration activity. These suggest that *E. stiedai* sporozoites have binding capacity to rabbit liver biliary epithelial cells, to penetrate the cells and the binding site is antigenic and specific for the liver biliary epithelial cells. In contrast, *E. magna* can penetrate various kind of cell cultures, not only rabbit cells, but also bovine and Green Monkey kidney cells. It is considerably that the binding sites (receptors) of *E. magna* and *E. stiedai* would be different, the receptor of *E. stiedai* is located on rabbit biliary epithelial cells, while the receptor of *E. magna* is distributed on many kind of tissue cells.

We have no data, however to show our hypothesis in this study. We observed that *E. stiedai* sporozoite has L-fucose residues which play important roles for attachment and/or penetration of the host cells, while the host cell has lectin-like substance to bind-L-fucose residues (unpublished data). One possibility would be considered that the antigenic binding site specific for the liver biliary eppithelial cells is the lectin like substance. Further study is necessary to determine whether the antigenic binding site which we observed in this study, is a lectin like substance.

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REFERENCES

Coudert, M. P. & Provot, F. 1974. Parasitologie, -Development interne et schizogonie en culture cellularis d' *Eimeria stiedai* (Lindemann 1865) Kisskalt et Hartmann 1907. CR. Acad. Sc. Paris. t.279, serie D 911-913.

Doerr, U. & Hohn, H. 1972. Zur in vitro-kultur von Eimeria stiedai. *Zeitschrift fur Parasitenkunde. 3:* 56-57.

Fitzgerald, P. R. 1970. Development of *Eimeria stiedai* in avian embryos. *J. Parasitology 56:* 1252-1253.

Nakamura, T., Yoshimoto, K., Nakayama, Y., Tomita, Y. and Ichihara, A. 1983. Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell-cell contact and cell membranes. *Proc. Natl. Acad. Sci. USA. 80:* 7229-7233.