

Identification of Carbohydrates on *Eimeria stiedai* Sporozoites and Their Role in Invasion of Cultured Cells in vitro.

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Invasion specificity of *Eimeria stiedai* sporozoites in cultured cells was examined. Intracellular sporozoites were observed in hepatobiliary epithelial cells, as early as 3 hours post inoculation (p.i.) but the infection rate was monitored for 6 hours. No intracellular parasites were found in rabbit parenchymal hepatocytes and rabbit kidney cells, even on prolonged culturing. In the hepatobiliary epithelial cells inoculated with fixed sporozoites, no intracellular parasite were found. Sporozoites attached on the cell surface of the hepatobiliary epithelial cells fixed with paraformaldehyde but did not penetrate.

The carbohydrates present on *Eimeria stiedai* sporozoites and their functional role in the process of invasion of host cells were also examined. Lectin binding sites on the surface of sporozoites were detected by means of peroxidase-conjugated lectins. Sporozoites showed specific binding with UEA-I and PNA lectins, which bind L-fucose and D-galactose, respectively. Exposure of sporozoites to 100 µg/ml UEA-I significantly reduced their ability to invade primary rabbit hepatobiliary epithelial cells, but similar treatment with PNA had no such effect. Preincubation of these cells in Dulbecco's minimum essential medium containing 10% fetal bovine serum and 1% L-fucose suppressed the invasion activity of the sporozoites, but preincubation of the sporozoites in the same medium without L-fucose had no effect on cell penetration. D-galactose added to the medium had no effect on the invasion activity of sporozoites. These results indicate that L-fucose residues on *E. stiedai* sporozoites and L-fucose binding sites on host cells both are associated with recognition and/or invasion process.

Keywords : Carbohydrates, *Eimeria stiedai*, Hepatobiliary epithelial cell, Invasion, Rabbit

INTRODUCTION

Eimeria stiedai (*E. stiedai*), a coccidian that infects the liver of the domestic rabbit, may cause clinical symptoms related to obstructive jaundice. Lesions occur primarily in the liver, especially the bile ducts. Factors responsible for the specificity of the interaction between the biliary cells and *E. stiedai* have not yet been elucidated. The interaction between host liver tissue and the parasite have not yet been clearly elucidated, although there have been reported the in vitro culture of *E. stiedai* merozoites (Fitzgerald, 1970; Doerr and Hohn, 1972; Coudert and Provot, 1974).

During penetration, *Plasmodium falciparum* merozoites recognize sialic acid on the

plasmalemma of erythrocytes (Hadley et al., 1986). Malaria sporozoites also recognize liver-specific heparan sulfate proteoglycans (Cerami et al., 1992; Frevert et al., 1993; Shakibaei and Frevert, 1996). Sporozoites of *Cryptosporidium parvum* have glycoproteins which play a role in adhesion to and/or penetration of human enterocytes. In addition, enterocytes have glycoproteins with concanavalin-like activity which also play a role in these processes (Kuhls et al., 1991). Thea et al. (1992) reported that lectins occur on the invasive sporozoites of *Cryptosporidium parvum*. Augustine (1985) reported that, among avian *Eimeria* species, penetration of *E. meleagridis* sporozoites into host cells is

inhibited by wheat germ agglutinin lectin (WGA), but enhanced by N-acetylglucosamine, the sugar residue for which WGA has high affinity. She suggested that the inhibition of invasion that occurs as a result of exposing host cells to WGA is due to its binding to negatively-charged moieties on the cell surface rather than specific binding of the lectin to N-acetylglucosamine.

Strout *et al.* (1994) investigated parasite lectins of *E. tenella*, *E. acervulina* and *E. maxima* using hemagglutination inhibition assays. They found that different stages of these parasites have specific surface sugar lectins. They hypothesized that the lectins found on the surface of the sporozoites play a role in determining the site of infection within the intestine of the host. On the other hand, Baba *et al.* (1996) reported that D-galactose residues are present on the surface of *E. tenella* sporozoites and that lectin-like receptors specific for D-galactose are present on primary chicken kidney cells. They concluded that these factors are important for the parasite's invasion.

In the present study, we prepared cultured cells from liver tissue organs of rabbit and compared penetration activity of *E. stiedae* sporozoites in those cultured cells. we also investigated the role of sugar residues and lectin-like components in the process by which *E. stiedae* sporozoites penetrate cultured cells.

MATERIALS AND METHODS

1. cultured cells

Rabbit parenchymal hepatocytes were isolated from male and female rabbits weighing 500-700 g by *in situ* perfusion on the liver with collagenase as described elsewhere (Nakamura, *et al.* 1983). The cell suspension was centrifuged at 600 g for 7 min. After that, the cells were resuspended at 5×10^4 cells/ml in William medium containing 10 % fetal bovine serum, 1mM Dexamethazone and 2nM insulin, then were incubated with collagen-coated petridishes (Falcon Inc.,) at 37°C in 5% CO₂.

Rabbit hepatobiliary epithelial cells were isolated from specific-pathogen-free male and female rabbits weighing 500-800 g. Briefly, approximately 25 g of the bile duct tissue was cut into small pieces, and suspended in 12.5 ml of 0.25 % trypsin in phosphate buffered saline (PBS), and incubated

at 37°C for 30 min with stirring. After centrifugation at 600 g for 7 min, the cells were suspended at 5×10^4 cells/ml in Dulbecco's modified minimum essential medium containing 10% fetal bovine serum (D-MEM10FBS) and incubated at 37°C in loosely capped culture flasks (75 cm²) overnight. After washing thoroughly by pipetting, the adherent cells were further incubated at 37°C, in an atmosphere of 5% CO₂ and 95% air. For use in the experiments, the cells were harvested from the culture flasks by trypsinizing. Approximately 2×10^4 cells in 0.2 ml were cultured on a 15 mm diameter round coverslip at 37°C in 5% CO₂ overnight before inoculation.

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 5×10^4 cells/ml in D-MEM10FBS and incubated as described above. During 1 week, fibroblasts multiplied to become monolayer in the cell culture.

2. Parasites

Eimeria stiedae oocysts were collected from the feces of infected rabbits by sucrose floatation. Sporozoites were released from sporulated oocysts by incubation in the presence of 0.8% sodium taurocholic acid and 0.2% trypsin in PBS at 37°C for 45 min. The released sporozoites were separated from the unbroken oocysts and debris by filtration using a polycarbonate membrane filter (Nucleopore®, pore size; 3 µm, Costar, Cambridge, MA). The sporozoites were washed twice with PBS to remove the sodium taurocholic acid by centrifugation at 1,200 g for 10 min, then suspended in D-MEM10FBS at 1×10^4 parasites/ml. Some of the sporozoites were used to infect cultured cells without further treatment, while others were fixed with 1 % v/v paraformaldehyde in PBS at 4°C for 15 min before being used to infect other cultured cells.

3. Carbohydrates and lectins

D-galactose, α -L-fucose, and α -D-fucose, (Wako, Osaka, Japan) were each dissolved in D-MEM10FBS at a concentration of 1 %. The solutions were sterilized by filtration

using a cellulose acetate membrane filter (Millipore, pore size; $0.2\ \mu\text{m}$, Bedford, MA). Horse radish peroxidase (HRP)-conjugated lectins (*Ulex europaeus*, UEA-I; *Lens culinaris*, LCA; *Arachis hypogaea*, PNA; *Canavalia ensiformis*, Con A; *Triticum vulgaris*, WGA; *Phaseolus vulgaris*, PHA-E4; *Dolichos biflorus*, DBA,) (Honen Co., Tokyo, Japan) were each dissolved in phosphate buffered saline, pH 7.4 (PBS), containing 3% bovine serum albumin (BSA).

4. Detection of lectin binding sites on sporozoites and host cells by HRP-conjugated lectins

Sporozoites and host cells were fixed with 1% paraformaldehyde in PBS at 4°C for 15 min. After washing the sporozoites in chilled PBS three times, the sporozoite and host cell suspensions were dropped onto glass slides, dried at room temperature and the slides were stored at -80°C . HRP-conjugated lectins were serially diluted with PBS, applied to the sporozoite-coated slides and the slides were incubated at 37°C for 1 hour. Thereafter, the slides were washed with chilled (4°C) PBS and lectin binding was detected by peroxidase staining using diaminobenzidine-4 HCl in 0.1 M Tris-HCl buffer pH 7.4 containing 0.01% H_2O_2 .

5. Inoculation of cell cultures with sporozoites.

The culture medium was removed from the cell monolayered coverslips, and 0.2 ml of sporozoites suspension was seeded onto the coverslips. After incubation at 37°C for 5 hours, the specimens were washed in PBS, fixed with methanol and stained with Giemsa. They were then examined under a light microscope, and the number of penetrated sporozoites (stumpy and surrounded by a parasitophorous vacuole) per 400 cells was determined. Results were expressed as mean $\pm$ SD and data from each experiment were evaluated statistically using the Student's *t*-test.

Each experiment was repeated at least three times.

6. Effect of carbohydrates on sporozoite invasion of cultured cells

Sporozoites were suspended at 1×10^5 sporozoites/ml in D-MEM10FBS supplemented with carbohydrate at a concentration

of 1%. Cell monolayered coverslips were inoculated with each suspension, as described above.

7. Effect of UEA-I, PNA and LCA lectins on sporozoite invasion of cultured cells

Ten thousand sporozoites were suspended in D-MEM10FBS containing either UEA-I or PNA at a concentration of $100\ \mu\text{g/ml}$. The same number of sporozoites were preincubated in the medium containing either the lectin or PBS-BSA at 37°C for 1 hr. After washing twice by centrifugation, the sporozoites were suspended in D-MEM10FBS and host cell cultures were inoculated 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 in the cytoplasm. Intracellular sporozoites were found in hepatobiliary epithelial cells at 3 hours post inoculation (p.i.). The number of intracellular sporozoites increased gradually, therefore, the infection rate was monitored for 6 hours in the cell culture. Infection rates for hepatobiliary epithelial cells was approximately 9% at this time. Whereas, no intracellular parasites were found in the parenchymal hepatocytes and rabbit kidney cells. In the hepatobiliary epithelial cells inoculated with fixed sporozoites, no intracellular parasites were found. Interestingly, sporozoites attached on the cell surface of the hepatobiliary epithelial cells fixed with paraformaldehyde. At 4°C , no intracellular sporozoite was found in the cultured cells.

Detection of lectin-binding sites : The entire anterior end of the sporozoites incubated with UEA-I, was stained an intense brown (Fig. 1).

A less intense brown staining region was found between the anterior one third and the middle of the sporozoites incubated with PNA (Fig. 2).

No brown staining region(s) were observed on the cultured cells regardless of the lectin used.

Effect of UEA-I, PNA and LCA lectins on sporozoite invasion : Supplementation of the medium with UEA-I significantly



Fig. 1 Peroxidase staining of *E. stiedai* sporozoites reacted with HRPO-conjugated UEA-I. The deep brown stained part (black region) is located on the anterior end of the sporozoite. The staining on one terminal part of the sporozoites indicates the presence of L-fucose residues.

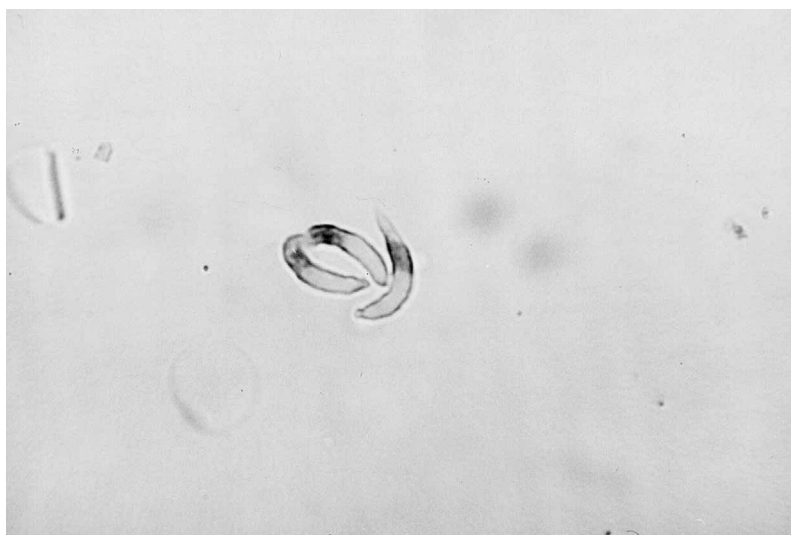


Fig. 2 Peroxidase staining of *E. stiedai* sporozoites reacted with HRPO-conjugated PNA. The light brown stained part (blackish-grey region) is located about one to the middle-half of the sporozoite. The staining indicates the presence of D-galactose residues on one part of the sporozoites.

reduced sporozoite penetration ($P < 0.05$). However no significant reduction of penetration was observed in the medium supplemented with PNA or LCA or in the unsupplemented control (Fig. 3). Preincubation of the host cells with UEA-I resulted in no

inhibitory effect on sporozoite penetration (Fig. 4).

Effect of carbohydrates on sporozoite penetration into cultured cells: Supplementation of the medium with L-fucose significantly reduced sporozoite penetration into cultured

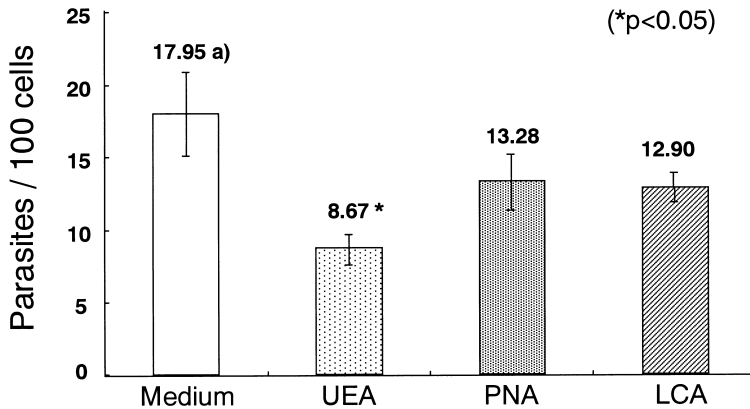


Fig. 3 Effect of lectins on *E. stiedai* penetration of host cells.
a), mean of number of penetrated sporozoites (The bar indicates standard deviation)
UEA: co-cultured with UEA-I. PNA: co-cultured with PNA. LCA: co-cultured with LCA.

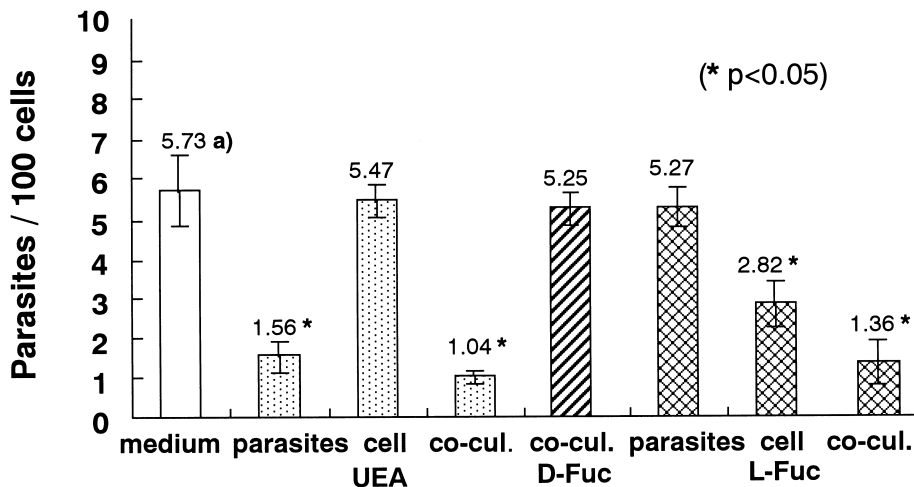


Fig. 4 Effect of carbohydrates and UEA-I on *E. stiedai* penetration of host cells.
a), mean number of penetrated sporozoites (The bar indicates standard deviation).
parasites: sporozoites pretreated with either UEA-I or carbohydrate.
cell: host cells pretreated with either UEA-I or carbohydrate.
co-cul: sporozoites and host cells co-cultured with either UEA-I or carbohydrate.
UEA: treated with UEA-I. D-Fuc: treated with D-fucose. L-Fuc: treated with L-fucose.

cells ($P<0.05$). Preincubation of the cultured cells with L-fucose also significantly reduced sporozoite penetration ($P<0.05$). However, preincubation of the sporozoites with either D- or L-fucose did not cause significant drop in sporozoite penetration (Fig. 4).

DISCUSSION

In the liver tissue, there are some kind of cell types, i.e. parenchymal hepatocytes,

hepatobiliary epithelial cells, blood vessel epithelial cells, kupfer cells and fibroblasts. The results in the present study indicated that *E. stiedai* sporozoites have penetration activity to the hepatobiliary epithelial cells, restrictedly. The sporozoites adhered on the surface of host cells which were fixed with para-formaldehyde. This suggests that the sporozoites have binding sites on the surface of the host cells and they would cause pene-

tration process, after the attachment which is temperature dependent.

In the present study, UEA-I lectin-binding sites were detected as regions of intense brown staining on the anterior part, seemingly the conoid, of the sporozoites. In members of the apicomplexa, this region is known to function in attachment to and penetration of host cells (Chobotar and Scholtyseck, 1982; Sam-Yellowe, 1996). The inhibition of penetration observed when the sporozoites were pretreated with UEA-I specific for L-fucose residues, and also when the cultured cells were pretreated with L-fucose, but not D-fucose, indicates that L-fucose binding sites are present on the surface of this cell type. The reduction of sporozoite penetration observed after their pretreatment with L-fucose may partly be due to specific competition with L-fucose residues on their surface. These findings indicate that *E. stiedai* sporozoites possess L-fucose residues on the outer surface which are associated with the penetration activity.

Although the sporozoites have the capacity to bind PNA, which specifically recognizes D-galactose residues, supplementation of the culture medium with PNA lectin had no effect on penetration activity of the sporozoites. It seems likely that the D-galactose residues may be localized on an inner site, or in the cytoplasm of the sporozoites, and the significance of this observation is not clear.

Our findings are consistent with the report by Baba *et al.* (1996) indicating that attachment of such sugar residues on the sporozoites to the binding sites on the surface of the host cells leads to penetration by the sporozoites. Strout *et al.* (1994) demonstrated by hemagglutination and hemagglutination inhibition assays that *E. tenella*, *E. acervulina* and *E. maxima*, all which are avian *Eimeria* species had a lectin on their sporozoites. To explain the mechanisms of host cell recognition and penetration by *E. stiedai* sporozoites, more detailed research on the interaction between the carbohydrates and the binding sites is necessary. This is especially important because a certain degree of penetration was observed under all situations, indicating that more than one factor is responsible for sporozoite penetration.

In the present study, the biological characteristics of the target cells were unclear. It is

also unclear whether L-fucose binding sites are expressed on a restricted group of cells in rabbit liver, recognized as host cells and target organ specific. Attempts to isolate the lectin-binding substance(s) from the surface of *E. stiedai* sporozoites and investigation of the biochemical characteristics of the lectin binding epitopes are underway.

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