DEVELOPMENT OF *ISOSPORA BELLI* IN HCT-8, HEP-2, HUMAN FIBROBLAST, BEK AND VERO CULTURE CELLS

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Abstract. The development of *Isospora belli*, a human coccidian parasite, was studied in different cell lines. Merozoites were observed in all kinds of cells, whereas sporogony was demonstrated only in Hct-8. This implied that not only the human cell line can be infected, but also some animal cell lines. Unizoites could be found in Vero cells. The merozoites were transferred to a new culture cell for three passages and maintained for two weeks, but no oocyst production was observed in any culture cells during cultivation.

INTRODUCTION

Human Isosporiasis is a coccidian disease caused by *Isospora belli*. The disease occurs in both immunocompromized and immunocompetent hosts (Lindsay *et al*, 1997b). These protozoal parasites have asexual and sexual stages within the intestinal cells of their hosts and produce oocysts. Infection occurs in the small intestine by the ingestion of food contaminated by sporulated oocysts. Symptoms of *I. belli* infection in immunocompromized patients are more severe, with diarrhea being more watery (Lindsay *et al*, 1997b). Extraintestinal stages of *I. belli* and unizoite tissue cysts have been observed in AIDS patients (Lindsay *et al*, 1997a,b; Velasquez *et al*, 2001) but is was unclear whether development occured in those organs. There have also been isosporiasis cases in Thailand since 1987 (Morakote *et al*, 1987), but a study of the life cycle has never been done before. We report here an *in vitro* study of *I. belli* cultivation in various cell types to compare their development. Only a few drugs have been used for the treatment of isosporiasis and the combination of sulfamethoxazole (SMZ) and trimethoprim (TMP), an effective treatment against isosporiasis, is commonly used as prophylaxis in AIDS patients against *Pneumocystis carinii* in Australia (Dehovitz *et al*,

1986) but the mode of action is unknown. However, relapse and resistance have been reported (Lindsay *et al*, 1997a; Bialek *et al*, 2001). Information from this study may be useful for further studies of *in vitro* drug testing in tissue culture models.

MATERIALS AND METHODS

Specimen collection, sporulation and purification of oocysts

A stool specimen was collected from an AIDS patient, immediately mixed in 2.5% potassium dichromate solution and stored for a week. The sporulated oocyst suspension was filtered through five layers of gauze and separated from fecal materials by sugar flotation without phenol (Jensen, 1983). The oocysts were collected, washed 3 times in distilled water and concentrated by centrifugation (10 minutes at 1,000*g*). The sediment, which contained oocysts, was cleaned with 2% sodium hypochlorite solution (V/V) in an ice bath for 10 minutes to kill microorganisms.

Excystation

Sporulated oocysts in 3% sodium hypochlorite solution were washed 3 times in phosphate buffer saline (PBS) and finally suspended in Eagle's MEM tissue culture medium (Nissui, Tokyo, Japan) supplemented with 80 µg/ml gentamicin and kept at 4ºC until used.

Oocysts were broken to release sporocysts using a Teflon-coated tissue grinder. After the

sporocysts were liberated, they were suspended in 3% (W/V) sodium taurocholate (Fluka Chemie, Buchs, Switzerland) and 1% (W/V) trypsin (1:250, Sigma Chemical, St Louis, Missouri) in MEM medium, which was sterilized by filtration and incubated at 37ºC in a CO_2 incubator for 2 hours.

Cell culture and sporozoite inoculation

The harvested sporozoites were centrifuged for 10 minutes at 1,000*g*, and then washed twice with PBS. They were tested on a blood agar plate to check for contamination, counted and varied in inoculum between 10^5 to 10^6/ml before infection. One hundred microliters of sporozoite mixture was then inoculated into Hct-8 (ATCC CCL-244, human ileocecal adeno carcinoma), Hep-2 (human larynx carcinoma), human fibroblast (sourced locally), BEK (Bovine Epithelial Kidney cells) and Vero cell (African green monkey kidney), grown in a monolayer line on coverslips, fitted in 24-well plastic tissue culture plates. RPMI 1640 (Gibco Laboratory, Grand Island, New York) with 2% horse serum was added to the culture. The culture plates were incubated at 37ºC in a CO_2 incubator for 2 hours to allow the sporozoites to penetrate the cells. Any unexcysted oocysts and extracellular sporozoites were removed by washing 3 times with PBS in the absence of calcium and magnesium, and then one ml of MEM medium was added to each well. The culture plates were incubated at 37ºC in a CO_2 incubator for observation.

The effect of serum on the penetration of sporozoites was studied by withdrawing the serum from culture medium one day before and during inoculation.

The coverslips were removed from the culture plates and stained either by hematoxylin and eosin (H&E) or Giemsa's stain at 24-hour intervals post-inoculation. The culture fluids were aspirated from these wells, centrifuged and examined fresh under a light microscope before being inoculated into the new set of tissue culture plates. The coverslips containing infected cells from the second inoculation were fixed and stained to observe the progress of their development for a week.

Host-parasite interaction was studied by inoculation of sporozoites into both young and old cell lines (monolayer and 48 hour after monolayer). Two kinds of bacteria accidentally contaminated culture cells before the inoculation of sporozoites. One was a gram-positive coccus, the other was a non-fermented bacillus. The bacterial association in culture was studied.

Some excysted sporozoites were kept at 4ºC for a week and some were incubated at 37ºC in a CO_2 incubator for 24 hours before infecting the culture cells

RESULTS

The oocysts showed the typical characteristics of the sporulated oocysts of *I. belli*; 4 sporozoites were noted inside the sporocysts (Fig 1). Some oocysts had only one sporocyst with more than 4 sporozoites (Fig 2). The merozoites were observed in culture fluid. The sporozoites and merozoites showed a gliding movement, as described by Lindsey and Current (1983) (Fig 3). The merozoites were rather short and stout while the sporozoites were long and slender. However, neither of them could be differentiated by light microscopic observation alone. After excystation, some sporozoites were liberated from intact sporocysts and the sporozoites were stained more clearly by H&E than Giemsa (Fig 4).

From observation, the development of *I. belli* in culture can be classified into 3 categories. The first group was in a cell line of a specific host and specific site (Hct-8); the parasites developed from early to complete schizogony and the schizonts reached maturity in 5 days (Figs 5, 6). The sporogony stage and microgametocytes were observed within 5 days in the second passage of the culture (Figs 7, 8). The second group was in a cell line of a specific host but an unspecific site (Hep-2); the parasites developed to an early stage of merogony; only a few merozoites were present in each meront. However, those merozoites could be infected in a new passage, but the sexual stage was not observed. The third group was in a cell line that was in neither a specific host nor a specific site (Vero and BEK); only a few dividing stages were observed, whereas most of them developed unizoites in parasitophorous vacuoles (Fig 9). The asexual cycle was not complete and there was no sexual stage formation. For the hu-

Fig 1–Sporulated oocyst of *I. belli* (arrow), four sporozoites in each sporocyst were demonstrated. Bar=10 µm.

Fig 2–Sporulated oocyst without sporocysts of *I. belli* (arrow), containing more than four sporozoites. Bar=10 µm.

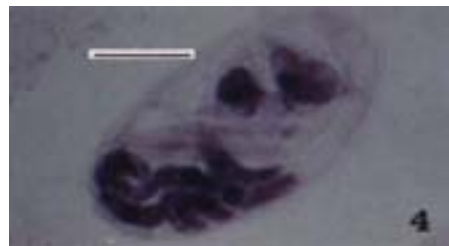Fig 3– A developing schizont in culture medium (arrow), the gliding movement of merozoites was observed. Bar=10 µm.

Fig 4–Staining oocyst and sporozoites; the sporozoites were released from the sporocysts and the oocyst was still intact. Bar=10 µm.
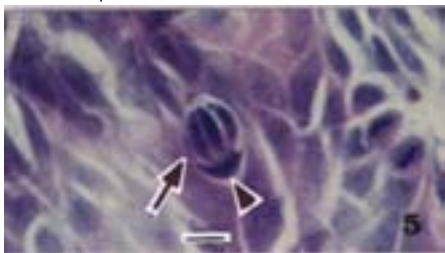

Fig 5–Immature schizont in Hct-8 cell line (arrow). Note the nucleus of the host cell (arrowhead) and merozoites forming the "T sign" (H&E). Bar=10 µm.
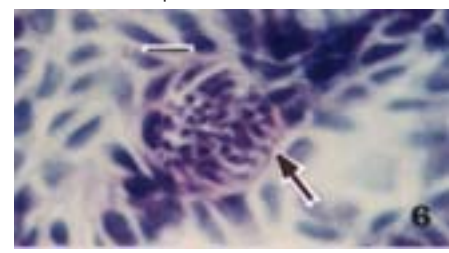

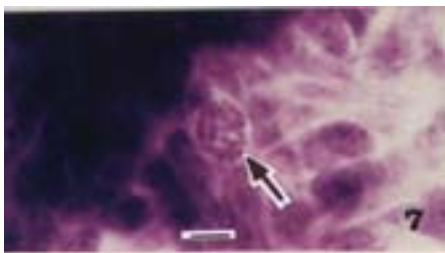Fig 6–Mature schizont in the Hct-8 cell line (arrow), numerous merozoites were present (H&E). Bar=10 µm.

Fig 7–Suspected developing microgametocyte (arrow), the parasites were different from the schizont in Fig 6 (H&E). Bar=10 µm.
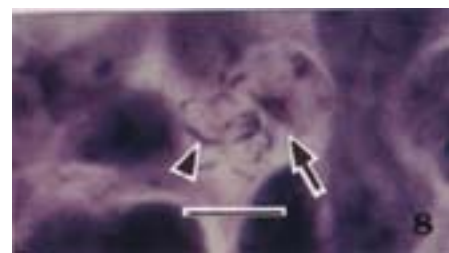

Fig 8–Microgametocyte (arrow), spindle-shaped microgametes were noted (arrowhead) (H&E). Bar=10 µm.

Fig 9–A sporozoite in a vacuole of the Vero cell line (arrowhead); the parasite was present in the culture medium at 48 hours post-infection. Bar=10 µm.
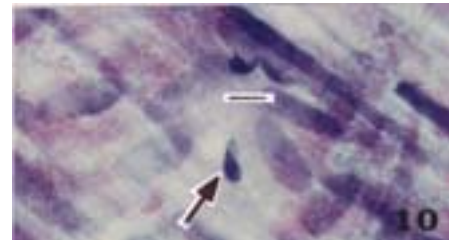

Fig 10–Sporozoites in human fibroblast culture cell (arrow), no development of parasites was observed (H&E). Bar=10 µm.

man fibroblast, the development of the parasite could not be seen, and the sporozoites could not enter the cells (Fig 10).

Host-parasite interaction and culture conditions played an important role in the invasion of sporozoites. We observed first that parasites preferred to penetrate into young culture cells more than old cells. Second, 2 kinds of bacteria accidentally contaminated culture cells before the inoculation of sporozoites. One was a gram-positive coccus which did not interfere with the growth of the cells. We found that it seemed to promote the invasion of sporozoites compared with uncontaminated cells. Another was a nonfermented bacillus that caused severe damage and necrosis to cells. This bacterium destroyed both the culture cells and the developed parasites within a few days. Unfortunately, we did not type the bacterium. Moreover, when the serum was withdrawn from the medium one day before inoculation it also provided sporozoite invasion.

The asexual stage was observed in fewer than 10^4 sporozoites, while merozoites, which can infect new culture cells were observed in 10^6 sporozoites.

The excysted sporozoites, which were kept at 4ºC for a week before infecting the culture cells, could penetrate and develop in the culture cells. However, in the sporozoites, which were incubated at 37ºC in a CO_2 incubator, infection did not occur, and damage to the culture cells was observed.

DISCUSSION

Extraintestinal stages of *I. belli* have been reported in AIDS patients (Lindsey *et al*, 1997a). This observation corresponded to our experiment, where we could infect *I. belli* sporozoites into Hep-2, which are cells from the human larynx, and merozoites developed. The presence of unizoite tissue cysts in patients with acquired immunodeficiency syndrome has also been reported, but it was not proved whether the unizoite tissue cysts were a part of the cycle of *I. belli* or of other species of *Isospora*, because *I. belli* oocysts could not be detected in the stool of the patients (Velasquez *et al*, 2001). Our information supports this parasitic infection in different hosts,

the Vero cell that derived from the kidney cells of monkeys and BEK, which is derived from the kidney cells of bovines. Both were infected in different patterns, for example, unizoite formation.

The formation of merozoites in Hct-8, which is a specific host and specific site, accured within 5 days. Banana-shaped merozoites, subnuclear organisms that created a "T sign", as described by Field (2002), were demonstrated, and the infectivity of the merozoites could be noted. In other cell lines it was quite difficult to harvest the merozoites and transfer them to a new passage because only a few merozoites were obtained from those cells.

The host-parasite interaction was a distinct phenomenon. In healthy cell lines, uncontaminated culture cells, and in culture cells which were supplemented with enriched medium, the penetration of sporozoites was very poor. They invaded the host cell better when the host cells were weak due to bacterial contamination or with insufficient nutrients from the serum. This experiment suggests one explanation as to why this infection is more severe in immunocompromised hosts. There is a diminished immune status and impairment of the epithelial cells of the intestinal lumen due to bacterial or fungal infections. Although some kinds of bacteria in our experiment enhanced infection with *I. belli*, not all of them promoted the penetration of sporozoites. If the bacteria caused severe damage and detachment of cells, the sporozoites also could not survive. Young culture cells grown in a monolayer were more susceptible to penetration than the older culture cells. This was supported by the observation that the merozoites from the first generation refused to enter old culture cells, and were diminished within one week. Our observations correspond to the occurrence of *Cryptosporidium* infection in culture cells (Meloni, 1996). In some instances they could infect a new cell, but the reason for this is still unclear.

The sporozoites, which were incubated at 37ºC in a CO_2 incubator, were found to have the ability to penetrate culture cells, but they caused severe damage to most of the cells and the development of parasites did not occur due to the impairment of the culture cells. It may be hypothesized that of optimum temperature, the sporo-

zoites or intact oocysts excret some chemical agents toxic to cell lines, as described *Eimeria* spp (Jensen, 1983), however excretion of these agents was inhibited at 4ºC. This observation needs further study.

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