Complete development of Cryptosporidium parvum in MDBK cells

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Abstract

Sporozoites of Cryptosporidium parvum excysted in vitro from bovine oocysts were incubated with monolayers of Madin-Darby bovine kidney cells. The extent of parasite colonisation was monitored by light microscopy and immunofluorescence. Electron microscopy confirmed the complete development and replication of C. parvum within Madin-Darby bovine kidney cells.

Keywords: Cryptosporidium parvum; In vitro cell culture

1. Introduction

Cryptosporidium parvum is an intracellular coccidian parasite that infects the gastrointestinal tracts of a wide variety of mammals, including humans. In immunocompromised individuals, especially in patients with acquired immunodeficiency syndrome, the infection often provokes a severe and persistent life-threatening diarrhoea [1]. No effective therapy for cryptosporidiosis is available. The screening of anticryptosporidial agents is hampered by the lack of a convenient small-animal model system for infection. Moreover, compared with an in vivo model, an in vitro system of infection is less expensive and more suitable to facilitate studies on the basic biology of the parasite and even for the screening of chemotherapeutics and immunological agents. More than 20 papers described various methods of infecting cell monolayers with C. parvum, but with variable results. In addition, the parasite develops poorly, and the quantification of parasite infected cells based on staining techniques or on Nomarski Interference Contrast microscopy is difficult [2]. This report describes a simple and, in our hands, reproducible method of producing infections of C. parvum in vitro using Madin-Darby bovine kidney (MDBK) cells.

2. Materials and methods

2.1. Isolation and preparation of C. parvum

Oocysts of C. parvum isolated from faeces of naturally infected calves were purified with phosphate-buffered saline (PBS) pH 7.2/diethyl ether, and...
were subsequently passed through a discontinuous Percoll gradient, as described previously [3]. To kill microbial contaminants purified oocysts were placed in an antibiotic-antimycotic solution (2000 U of penicillin, 200 U of gentamicin, and 3.12 μg of amphotericin B per ml) at 4°C for 16 h and then exposed to a 5.25% (w/v) sodium hypochlorite solution in an ice-water bath for 8 min. After washing in cold PBS pH 7.2 the pellet was resuspended in 6 ml 1.05% sodium thiosulfate, centrifuged at 1500×g for 10 min, and washed in PBS pH 7.2 until all hypochlorite was removed. Release of sporozoites was achieved by incubating oocysts at 37°C with 0.22% sodium taurocholate and 0.04% (w/v) bovine trypsin in PBS for 45 min. Sporozoites were completely separated from oocyst walls and intact oocysts by gentle filtration through 3-μm pore-size cellulose nitrate filters (Sartorius). Sporozoites which passed through the membrane were washed by centrifugation at 1500×g for 20 min in Alsever solution and twice in s-MEM (see below). Sporozoites were counted in hematocytometer and resuspended in s-MEM to obtain a final concentration of 4×10⁶ sporozoites cm⁻² of confluent monolayers.

2.2. Cell culture

Madin-Darby bovine kidney (MDBK) cells were maintained at 37°C in a 5% CO₂/95% air humidified incubator in 80-cm² tissue culture flasks in minimum essential medium (MEM, GIBCO) supplemented with 2.2 g sodium bicarbonate per l, and 200 U penicillin, 40 U gentamicin, and 0.25 μg amphotericin B per ml (s-MEM). For routine cell passage, 8% foetal calf serum (FCS) was used, whereas 4% FCS was added for infection studies (maintenance medium), to prevent overgrowth of the cells. Monolayers of cells were prepared on 13-mm diameter round glass coverslips in 24-well tissue culture plates or in 25-cm² tissue culture flasks at a concentration of 1.5×10⁵ cells per well or 2.5×10⁶ cells per flask.

Sporozoite and cell viability were determined with a double-staining procedure using fluorescein diacetate and propidium iodide [4].

2.3. Inoculation of monolayers with parasites

24 h after preparing the monolayers the medium was removed. Confluent monolayers were inoculated with 1×10⁶ oocysts or 5×10⁶ sporozoites in 400 μl of maintenance medium per well, or with 100×10⁶ sporozoites in 3.5 ml of maintenance medium per 25-cm² flask. Plates and flasks were incubated at 37°C in a 5% CO₂/95% air humidified incubator.

2.5 h after infection of the cultures cell monolayers were washed 3 times with s-MEM. New maintenance medium was added and monolayers were reincubated at 37°C. The maintenance medium was subsequently changed every 48 h. Coverslips from uninfected and infected wells, and flasks were removed daily for 7 days post inoculation (p.i.).

The development of C. parvum in MDBK cells was monitored by light microscopy, and by indirect immunofluorescence, and was confirmed by electron microscopy (E.M.).

2.4. Light microscopy of infected cells

Coverslips were washed with s-MEM, fixed with absolute methanol, immersed in Giemsa’s Blood Stain (1:20 in distilled water) for 30 min, and washed with distilled water 3 times. The preparations were mounted with DePex and examined with a Leitz Laborlux 12 light microscope under oil immersion (×500 and ×1000 magnification). Approx. 30 fields were examined per coverslips to count the percentage of infected cells.

2.5. Indirect immunofluorescence detection of C. parvum parasites

Methanol fixed monolayers were rehydrated in PBS pH 7.2 containing 1% horse serum for 30 min at room temperature to block non-specific binding. Fixed monolayers were then incubated in a polyclonal anti-C. parvum rabbit antiserum (raised against bovine sonicated oocysts) at a 1:100 dilution in PBS pH 7.2 with 1% horse serum. After 60 min of incubation at room temperature, cell monolayers were washed in PBS pH 7.2 and further incubated for 60 min in a 1:100 dilution of fluorescein isothiocyanate-labelled goat anti-rabbit antibody in Evans blue (1:10000). Coverslips were washed in PBS pH 7.2, and mounted with buffered glycerol. Monolayers were examined with a Leitz Dialux 20 GMBI fluorescence microscope (×400 magnification).
2.6. Electron microscopy of infected cells

Infected MDBK cells in 25-cm² flasks were examined by E.M. from 1 to 5 days p.i. Cells were fixed at 4°C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 for 1 h and washed 3 times with double-distilled water. After scraping of the cell monolayer, infected cells were centrifuged and the pellet was embedded in 3% agar. Blocks of 1 mm³ were cut, postfixed for 1 h with 1% osmium tetroxide in water, and rinsed with double-distilled water. Cells were block stained with 2% uranyl acetate in water overnight, and washed 3 times with distilled water. Samples were dehydrated through a series of graded ethanol solutions, incubated in propylene oxide 3 times for 30 min, and embedded in Epon/Spurr (50/50). Ultrathin sections were stained with lead citrate and examined in a Philips 201C transmission electron microscope.

3. Results

The isolation procedure used in this study yielded sporozoites of which the viability was consistently greater than 90% in all assays.

*C. parvum* infected MDBK cells were detectable 1–4 days p.i. with Giemsa staining. The parasites appeared as basophilic, round-to-oval bodies with 1, 2, 4, 6, or 8 nuclei. However, further differentiation of the various parasitic stages was not possible in these preparations. With this staining parasitic forms were observed in approx. 25% of the cells after 24 h p.i.

Using the immunofluorescence the infection of MDBK cells by *C. parvum* was detected 1–6 days p.i. The developmental stages of the parasite stained bright green and cell monolayers appeared red as a result of the Evans blue counterstain. However, counting of infected cells was hardly possible.

Transmission electron microscopy permitted the identification of developmental stages of *C. parvum* (Fig. 1). The ultrastructures of the parasite infected MDBK cells were comparable to those described during experimental mammalian infection with *C. parvum* [5]. Type I schizonts and type II schizonts were clearly recognised from 24 to 120 h p.i., and macrogametes were observed at 48 and 72 h p.i. *C. parvum* oocysts were observed as early as 48 h p.i.

4. Discussion

A rapid and technically simple system is reported that can be routinely used to infect MDBK cell monolayers with *C. parvum*. In this study we describe the complete development of *C. parvum* in MDBK cells. The oocysts observed in the cell cultures must have resulted from sexual reproduction of the parasite, since the inocula consisted only of sporozoites.

Cells were infected with sporozoites that were earlier liberated in vitro from oocysts. We have chosen for sporozoites as inoculum because during the excystation of *C. parvum* oocysts, as of other coccidial
oocysts, toxic substances are liberated which may have a negative impact on development of this parasite in cultured cells [6]. Although the isolation and purification of sporozoites significantly prolong the inoculum preparation, our results demonstrate that these procedures only minimally interfere with the sporozoite viability, which is essential for the infection of the MDBK host cells.

As determined by Giemsa staining, a parasite/host cell ratio of about 1:4 was obtained, which is higher than the percentages (3–20%) of cell infections that were reported by others [7,8]. Recent reports suggest that infection of Caco-2 cells [4] and HCT-8 cells [9] may produce higher infection rates. Perhaps the parasite development in MDBK cells may be enhanced by addition of glucose, insulin, or vitamins in the medium as was reported for the HCT-8 cells [9].

It should be noted that it is difficult to determine the infection rate of infected host cells. Recently, Woods et al. [10] described a rapid in situ ELISA to quantitate the development of *C. parvum* in cultured cells. In preliminary studies we used Giemsa staining which is time consuming to recognise infected cells. In contrast, the immunofluorescence method permits easy detection of the parasitic forms, and is more sensitive. However, it is hard to distinguish individual host cells, and the *C. parvum* antigens seem to be clustered in certain areas. Therefore, in our experience, it is not easy to determine the parasite/host cell ratio with immunofluorescence as Favennec et al. [2] suggested.

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**References**


