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Cultivation of *Cryptosporidium parvum* in a non-adherent human monocytic cell line

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Abstract

Cryptosporidium parvum is a coccidian parasite responsible for severe diarrhea in immunocompromised patients. At present, only therapies of limited efficacies are available and despite recent improvements, an easy to perform and reproducible in vitro model is still lacking. The present study shows that the human monocyte cell line THP-1 supports the growth of *C. parvum*. After inoculation with oocysts, all the asexual and sexual developmental stages were found. Immunofluorescence controls showed that few oocysts remained after infection, disappeared within the first 24 h and that sexual stages reappeared after the second day postinoculation. A continuous asexual life-cycle proceeded throughout the experiments, with at least 15-day cultures. Ultrastructural studies evidenced that the parasites were usually localized at the cell surface and also in the cytoplasm, a feature found in vivo with M cells and macrophages. This culture system is easy to initiate and to maintain with adjustable parasitemias and duration which will allow time-dependent drug-testing and also facilitate the study of the biology of this parasite.

Keywords: *Cryptosporidium parvum*; In vitro culture; THP-1 cells

1. Introduction

Cryptosporidium parvum is an enteric protozoan which causes a diarrhea that can be life-threatening to immunocompromised individuals, especially those with AIDS. Although its in vitro cultivation was described a decade ago [1], and despite significant improvements in the supporting cell lines and the number of parasites obtained in culture [2–5], there is still a need for a simple and efficient system that could provide correct amounts of parasites and long-term maintenance. In particular, the overgrowth and peeling of cell monolayers is always a limiting parameter to long-term cultivation. To overcome some of these problems, we decided to test a non-adherent cell line for its ability to support the growth of this parasite.

2. Materials and methods

2.1. Parasites

Oocysts of a *C. parvum* strain originally obtained from an immunocompromised child [6] were pas-

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saged in neonatal calves (INRA, Nouzilly, France). The feces were homogenized, filtered on metal sieves of decreasing pore sizes, stored in 2.5% potassium bichromate at 4°C and were used within 6 months.

After removing the fat by ether treatment (35 ml fecal suspension, 15 ml ether; centrifugation 5 min, 800×*g*), the suspension was concentrated by centrifugation for 15 min at 1500×*g*.

The oocysts were purified using a modification of the method of Arrowood and Sterling [7]. Briefly, 5 ml of the concentrated fecal suspension was carefully layered on the top of a discontinuous sucrose gradient obtained with 10 ml Sheather sucrose solution diluted 1/4 with distilled water overlaid on 10 ml of the same solution diluted 1/2 and the tubes were centrifuged 30 min at 1500×*g*. The white layer at the interface between the two solutions was carefully removed and washed 3 times (15 min, 1500×*g*) in cold saline. The concentrated oocysts were resuspended (v/v) in 5% $K_2Cr_2O_7$ and stored at 4°C until use.

2.2. Culture media

RPMI-1640 (DAP, Vogelgrun, France) containing 25 mM HEPES (Sigma, L'Isle d'Abeau, France) was used as incomplete medium. Culture medium consisted of incomplete medium supplemented with 2 mM glutamine, 10% fetal calf serum (DAP, Vogelgrun, France) and antibiotics (200 U/ml of penicillin/200 μg/ml of streptomycin; Sigma, L'Isle d'Abeau, France).

2.3. Cells

The cells used in this study were the monocyte line THP-1 (ECACC 88081201, Sophia-Antipolis, France). They were maintained in complete medium at 37°C in 150 cm^2 plastic flasks (TPP, Trasadingen, Swizerland) and in a humidified CO_2 incubator. The cells were routinely passaged when they reached a density of about 1×10^6/ml. They were washed once in incomplete medium before inoculation with *C. parvum* oocysts.

2.4. Infection of cells with C. parvum

Prior to infection, the oocysts were washed four times in cold (4°C) saline to remove the potassium bichromate and were suspended in 1 ml saline. They were aseptized with a 1.25% sodium hypochlorite solution on ice for 5 min and centrifuged for 7 min at 1500×*g*. After one wash in cold incomplete medium, they were spun 7 min at 1500×*g* and resuspended in complete medium at 37°C under appropriate volume and concentration, usually a 1:1 oocyst/cell ratio. This suspension was mixed with the previously washed THP-1 cell pellet and distributed in 6-well microplates (Nunc, Denmark) under reduced oxygen in a candle jar, at a cell density of approximatively $3–6\times10^6$/ml. The oocysts were allowed to excyst for 4 to 16 h according to the experiments. The cells were then mixed with a Pasteur pipette, distributed into 15 ml conical tubes and spun for 4 min at 160×*g*. They were washed twice in the same manner with incomplete medium in order to remove the residual free oocysts and cell debris, and reincubated as above. The medium was replaced twice daily for the first 3 days and then once a day.

2.5. Evaluation of parasite growth

When needed, the cultures were resuspended and a small sample was centrifuged in a microtube at 15 000 revs./min for 1 min in a Beckman Avanti centrifuge. The supernatant was discarded; thin smears were made and fixed in 100% methanol. They were stained with alcian blue and Giemsa modified from Rosales et al. [8]. Briefly, the slides were stained for 25 min with 20% Giemsa in water, washed with tap water, briefly exposed to 1% (w/v) alcian blue in ethanol and washed as above. At least 1000 cells were counted with an oil immersion ×100 objective and the parasitemia, i.e. the percentage of infected cells, was assessed.

2.6. Oocyst and sexual stages determination

Propidium iodide was used as counter stain to visualize the cells and parasite nuclei. To monitor the presence of residual oocysts after infection and the appearance of sexual stages in culture, immuno-

fluorescence was performed. After incubation of the methanol-fixed smears with propidium iodide at 100 μg/ml in PBS [9], the slides were washed with PBS, a FITC-labelled monoclonal antibody specific for oocyst antigens (Cellabs PTY, Bradsure Biologicals, UK) was deposited and incubated for 30 min at 37°C in an humidified chamber. After thorough washes with PBS and mounting, the slides were viewed with a Leitz Laborlux microscope equipped with epi-fluorescence.

2.7. Electron microscopy

The cultures were processed as for light microscopy, washed once in incomplete medium and fixed for 2 h at 4°C with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5. After three washes in buffer, they were post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in EPON. Thin sections were cut with a Reichert ultramicrotome, mounted on mesh 300 copper grids and viewed with a Jeol 1200 EX microscope.

3. Results

The THP-1 cell line is fully susceptible to infection. Fig. 1 shows the different development stages observed. The first stages to appear were undifferentiated ('empty') forms and trophozoites. Type I meronts were seen as early as 6 h postinoculation (p.i.) and disappeared after 40 h p.i., whereas 4 nuclei type II meronts began to appear at 15–16 h and were visible throughout the experiments (type II meronts were seen at day 25 p.i.). Gamonts began to appear at day 2 p.i. and oocyst-like bodies were found intracellular at day 4. Low speed centrifugation of the cultures at this time resulted in oocyst-like organisms being concentrated in the supernatant.

After the initial infection, residual free oocysts were scarce and disappeared at 24 h p.i. Immunofluorescence staining performed from day 2 p.i. onward showed a positive staining of the gamonts, the asexual stages remaining unstained (Fig. 2).

As shown in Fig. 3, the percentage of infected cells slowly decreased during the experiments but was still about 20% of the starting parasitemia at day 15 p.i., after a 1:1 dilution of the cultures. Numerous multiparasitized cells were seen, often bearing more than eight parasites, sometimes up to 24, evidencing a net increase in the number of parasites in the first days of cultivation.

Samples were taken at various time intervals and processed for electron microscopy (Fig. 4). Besides classically extracytoplasmic parasites, parasites developing deep into the host cell cytoplasm were also seen.

4. Discussion

Cryptosporidium parvum has been cultivated since 1984 [1] with various adherent cell lines, usually intestinal enterocytes [10–14] and also the Caco2 cell line [2,15]. Recently, a more efficient system has been developed by Upton et al. using HCT-8 cells [3,16,17]. Most of these systems support the growth of *C. parvum* for a limited time, since the plated cells must be infected at sub-confluency and tend to peel off after 72 h. Other non-intestinal cell types have also been used such as MDCK cells [3,18,19], endometrial carcinoma cells [20], bovine fallopian tube epithelial cells [5] and mouse peritoneal macrophages as well [21]. This latter type is important since macrophages are thought to transport the parasites to other non-intestinal locations [22,23] in immunocompromised patients.

Several reasons prompted us to test the present system; (1) accessibility: in monolayers, infective parasites are released into the medium and must reach the cell monolayer in order to continue the cycle. The use of non-adherent packed cells increases the probability for the invading forms to gain access to a receptive cell. The high number of multiparasitized cells bearing more than eight parasites, often many more, evidenced this fact. The probability for microgametes to fertilize macrogametes, a crucial event [4] is also greatly increased; (2) another rationale for using THP-1 cells was the possibility to dilute, expand and regulate the starting parasitemia, something impossible with adherent monolayers; and (3) monolayers are usually monitored with Nomarski interference contrast, an easy

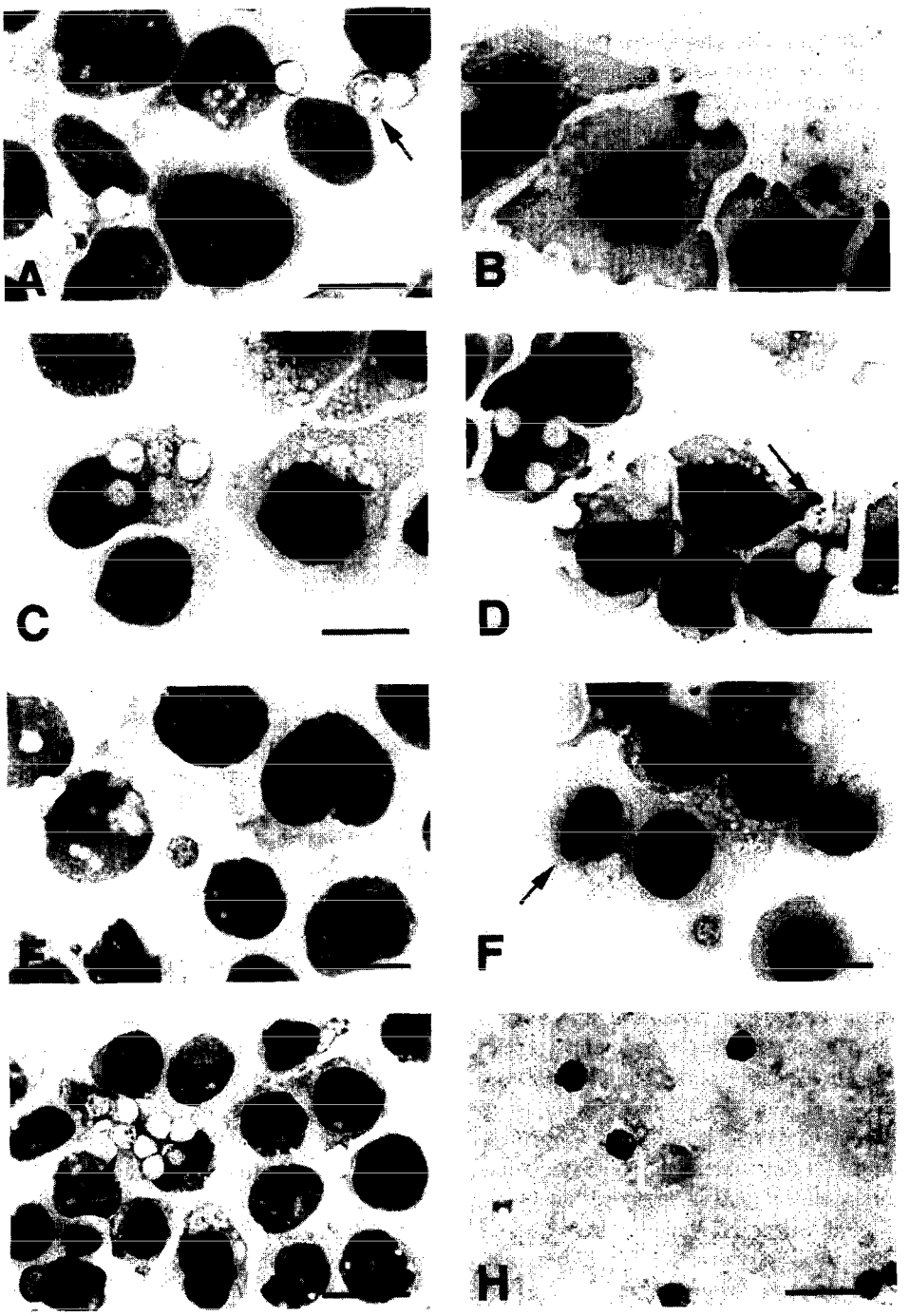


Fig. 1. Bright field microscopy of THP-1 cells infected with *C. parvum*. (A) Undifferentiated stages and late trophozoites (arrows) at 24 h p.i. (B) Trophozoite developing in a dividing cell. (C) Multiple infections of a THP-1 cell with undifferentiated stages and a developing eight-nuclei type I meront. (D) Four-nuclei type II mature meront (arrow) at 24 h p.i. (E) Macrogamont at 55 h p.i. (F) Microgamont and mature oocyst (arrow) at day 14 p.i. (G) Multiple infections and mature type II meront at day 8 p.i. (arrow). (H) Culture supernatant after centrifugation at 160×*g* for 4 min at day 10 p.i. showing numerous oocysts and cellular debris. Alcian blue-giemsa staining. Bar: 10 μm.

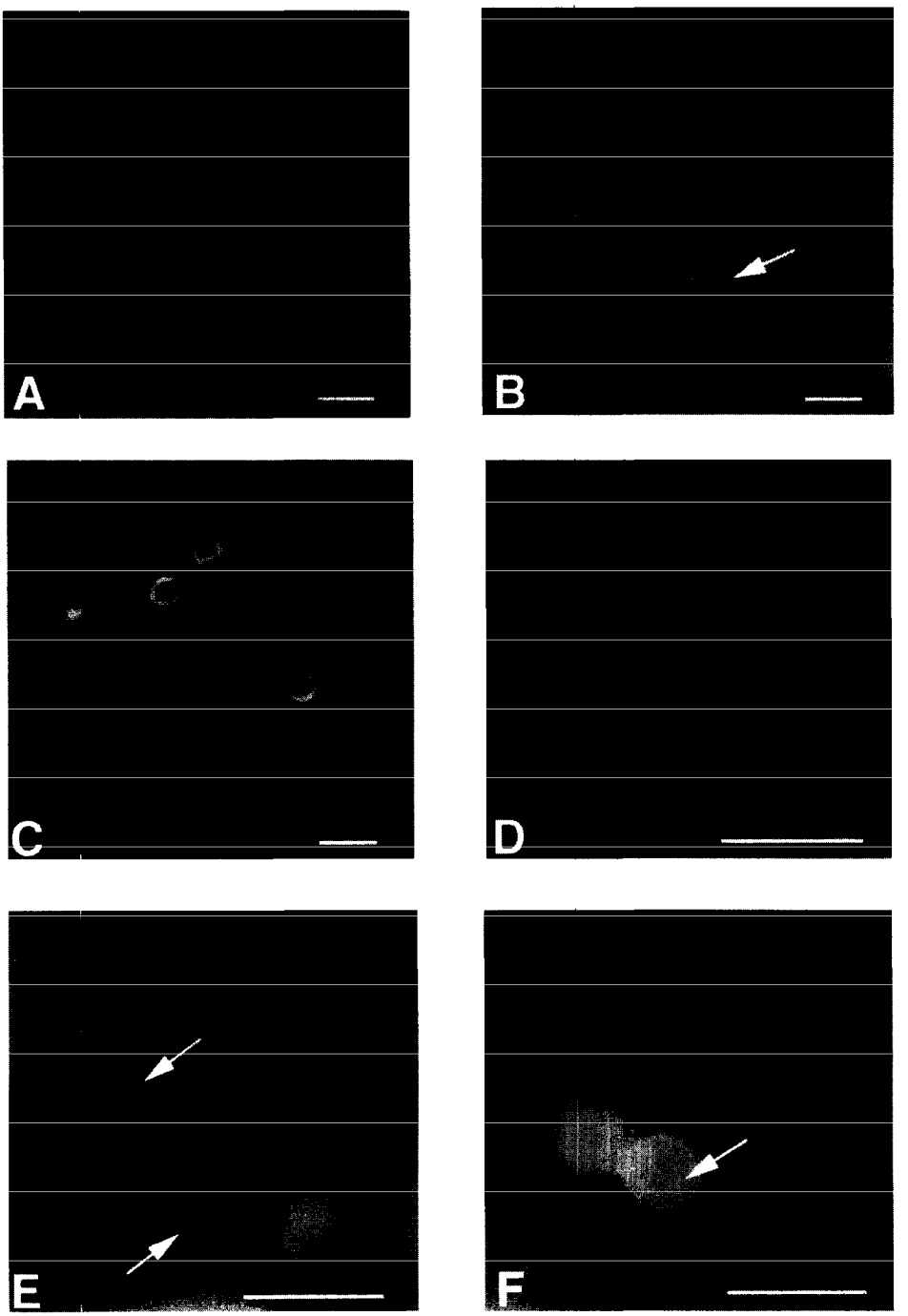


Fig. 2. Immunofluorescence and propidium iodide staining of THP-1 cells infected with *C. parvum*. (A) Culture after removal of the residual oocysts, 4 h after inoculation. Scarce oocysts are visible. (B) Culture at 24 h p.i. No positive staining is seen. A developing trophozoite is visible (arrow). (C) Supernatant after centrifugation at 160×*g* for 4 min at day 6 p.i. Positive staining of oocyst walls. (D) Sporulated oocyst at day 6 p.i. (E) Multiple infections of a THP-1 cell at day 17 p.i., showing FITC-negative asexual parasites and two meronts (arrows). (F) Oocyst with 4 sporozoites (arrow) at day 23 p.i. Bar: 10 μm.

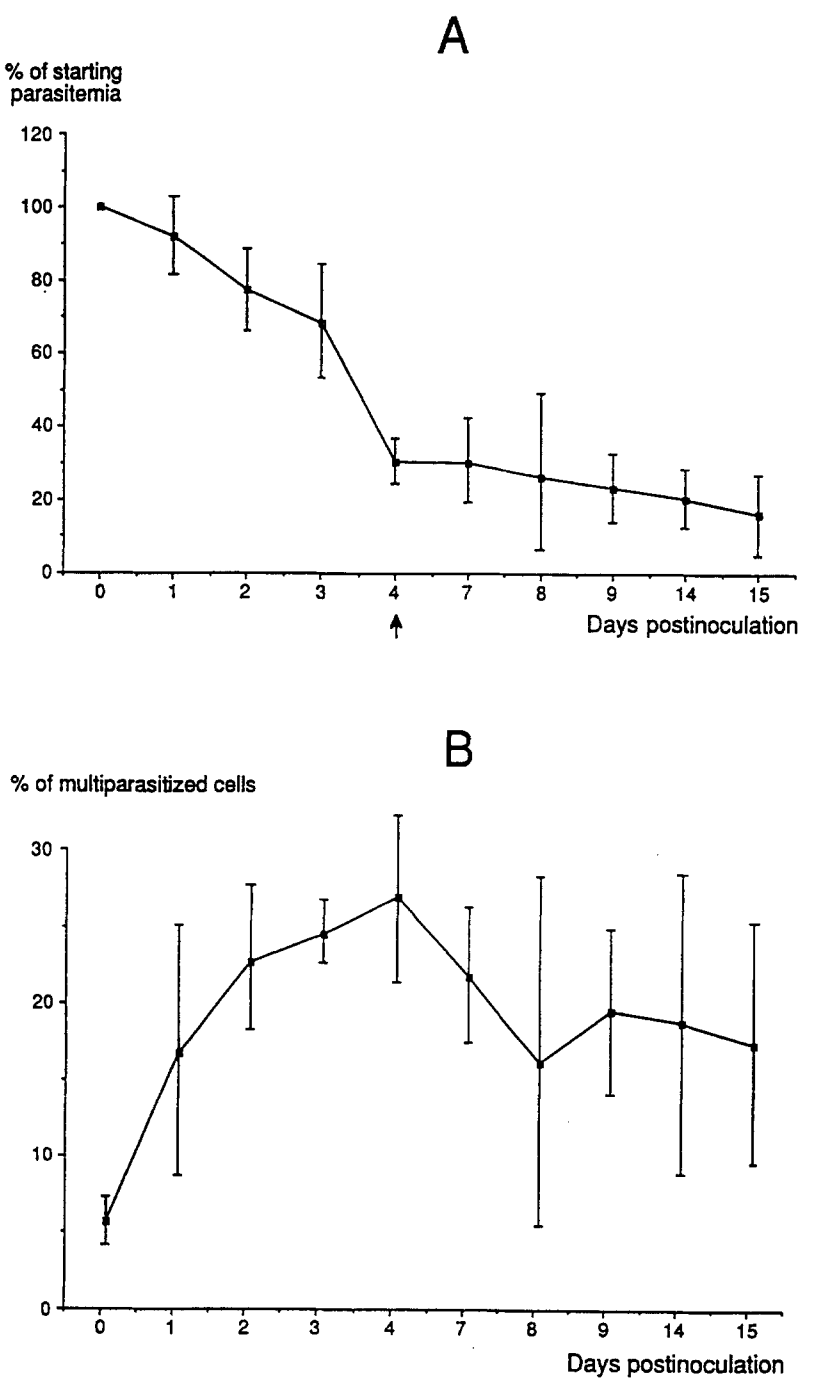


Fig. 3. Growth and development of *C. parvum* in THP-1 cells during 15 days of cultivation. (A) Parasite development expressed as a percentage of the relevant starting parasitemias (range 12–26%) from three separate experiments. Multiple infections (3 to 24 parasites) account for one infected cell. The arrow indicates a 1:1 dilution of the cultures with fresh cells. (B) Percentage of multiparasitized THP-1 cells (harboring 3 to 24 parasites, average: 8) related to the total number of infected cells. Results from the same three separate experiments.

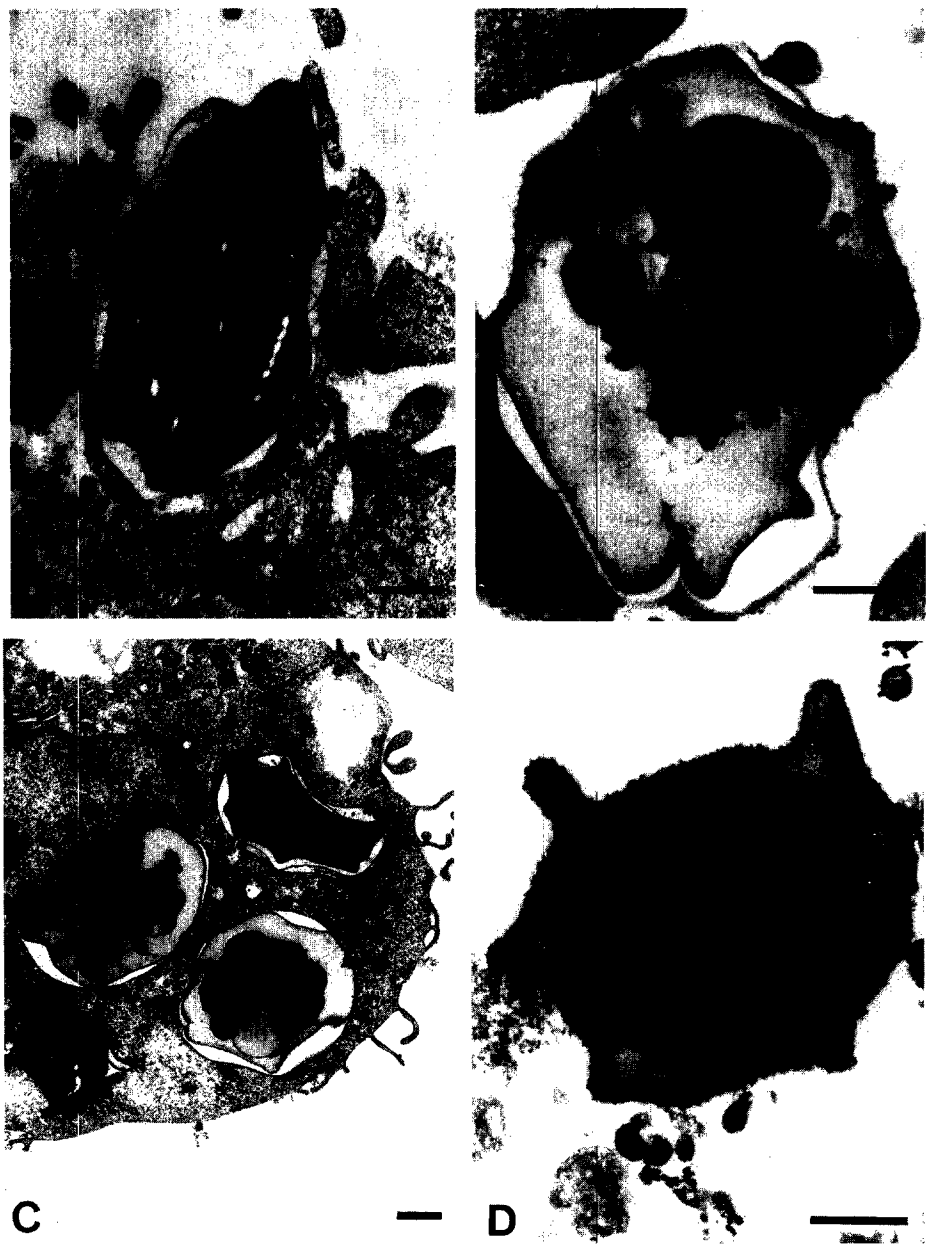


Fig. 4. Electron microscopy of THP-1 cells infected with *C. parvum*. (A,B) Mature type II meronts classically located at the cell surface at 24 h p.i. Bars: 0.5 μm. (C) Multiple infections of a THP-1 cell with developing meronts located in the cytoplasm at day 3 p.i. Bar: 1 μm. (D) Mature type II meront released from its host cell during processing at day 7 p.i. Bar: 0.5 μm.

procedure but requiring experience to differentiate the various parasitic stages. In addition, histological staining on monolayers requires stopping of the cultures and the use of as many wells as the samples.

We used the candle jar procedure because it provides a good environment, closer to the in vivo oxygen concentration [24,25]. Furthermore, previous studies had emphasized its use for *C. parvum*

cultivation [3,5] and we also noted that host cell proliferation decreased, thus partly preventing a mechanic decrease in parasitemia.

Our results show that THP-1 cells were fully susceptible to infection with *C. parvum* oocysts. The subsequent decrease in parasitemia must be corrected by the high number of multiparasitized cells, the developing parasites being more numerous than the inoculum during the first 3 days of culture. The direct infection with oocysts [16] provides efficient, rapid and time-saving inoculations. The major drawback of this technique would be their adherence or entrapment by the host cells. As a matter of fact, most of the residual free oocysts were removed by mild washings. Rare phagocytized oocysts can be seen and controls with anti-oocyst monoclonal antibodies showed their scarcity after washing of the cultures and their absence at 24 h p.i. This simple control procedure is more secure than other techniques such as the Ziehl-Nielsen stain, which was reported to react only with the dead oocysts and grossly underestimate their number [9]. Cultivation experiments never lasted less than 15 days, some of them up to 30 days; to our knowledge, only Current and Haynes [1] found oocysts after 31 days of cultivation.

All asexual stages were seen. Type I eight nuclei meronts were the first stages to appear but lasted less than 2 days, whereas type II four nuclei meronts appeared at 15–16 h p.i. and were seen throughout the experiments (up to 25 days at present), indicating that an asexual cycle occurred. Electron microscopy confirmed that the developing parasites were localized on the cell membrane but sometimes also deep into the cell cytoplasm, often near the nucleus as previously described in vivo [22] and in vitro with macrophages [21] and MDCK cells [19].

The sexual stages appeared at day 2 p.i. and were seen throughout long term (15 to 30 days) cultivation. Oocysts were also present, their appearance in light microscopy was often that of punctated forms and may be compared to the pitted surface described by Rasmussen et al. [20], a sign that a complete cycle takes places in our system. Despite the presence of rare sporulated oocysts as late as 23 days p.i., no successful subculturing has been achieved at present. The reason is probably the low amount of these oocysts, often still intracellular. This apparent difficulty of oocyst shedding and perhaps microgamete release [4] could explain the gradual decrease in parasitemia, the asexual cycle being less prominent.

The overall in vitro infection was consistent with the in vivo infection, with a prepatent period of 3 days and maximal oocyst production beginning at day 3 through day 11 p.i. [26,27].

The present culture system is easy to standardize, it was designed to remain as simple and time-saving as possible. It utilizes the easy and efficient invasion procedure of Upton et al. [16] with elimination of almost all of the residual oocysts by gentle washing. The starting parasitemia is easily adjustable simply by diluting the cultures with new cells to obtain the desired parasite load. For drug susceptibility tests, a reproducible distribution in microplates is possible, with the possibility of taking aliquots at given time intervals, allowing time-dependent data. For routine monitoring, thin smears and the simple Giemsa-alcian blue staining provided results in 30 min. When needed, electron microscopic studies are easy to perform, with high parasitemias and no need for handling adherent cells and their substratum.

Our system can also be improved. The supplementation with various substances [4] may further enhance its efficiency, as well as gentle shaking of the cultures, especially during the first few days, when the asexual stages are predominant. Improving the microgametes and oocysts release from their host cells should also facilitate long-term cultivation of *C. parvum*. The main feature of the present culture system is the concentration of host cells into a small volume, thus reaching cell concentrations of $3–6\times 10^6$ per ml. This cultivation technique is somewhat unorthodox, but as underlined by Strout and Schmatz [25]: "the models used for propagating parasites have been biased by using methods developed for culturing the host cells with little regard for the [atmospheric] conditions required for the parasites".

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