Short communication

Use of Percoll for the infection of cells in vitro with *Cryptosporidium parvum* oocysts

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Abstract

A method for the infection of non-adherent THP-1 cells and adherent MDBK cells with *Cryptosporidium parvum* oocysts using isotonic Percoll solutions was developed. Excystation was maximal after 2 h, but toxicity increased with the oocyst/cell ratio and the incubation time. The infection rates did not increase with the oocyst/cell ratio and both cell types were equally parasitized.

Keywords: *Cryptosporidium parvum*; Culture; In vitro; MDBK; Percoll; THP-1

At present, there is still no ideal method available for the in vitro cultivation of the enteric protozoan *Cryptosporidium parvum*. We previously developed a technique using non-adherent monocytic cells (Lawton et al., 1996) that displays many advantages over the use of monolayers. However, the infection of cells involves either direct contact of the cells with oocysts or addition of purified free sporozoites for various periods of time. When *C. parvum* is cultured in adherent monolayers, high numbers of oocysts are used in order to achieve a correct invasion (Upton et al., 1994a,b; Woods et al., 1995; Griffiths et al., 1998; Theodos et al., 1998; You et al., 1998). Our original protocol, which involved mixing oocysts and THP-1 cells in the culture wells

for at least 4 h (Lawton et al., 1996), resulted in many oocysts and sporozoites remaining in suspension while the host cells quickly sedimented, unless periodically resuspended. Thus, to optimize infection, we designed a protocol ensuring that both excysting oocysts and host cells were in close contact, thereby reducing the amount of oocysts needed and the invasion time.

The myelomonocytic cell line THP-1 (ECACC 88081201; Sophia Antipolis, France) was continuously maintained as previously described (Lawton et al., 1996). Madin Darby Bovine Kidney cells (MDBK; ATCC 6071, Rockville, MD) were grown as monolayers in 75-cm² Falcon culture flasks (Becton Dickinson, Meylan, France) in the same conditions, except that they were detached with trypsin-EDTA solution (Sigma, L'Isle d'Abeau, France) for routine passaging and prior to the experiments. *C. parvum* oocysts were passaged in neonatal (age 24–48 h) male calves (INRA, Nouzilly, France) and

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purified as previously described (Lawton et al., 1996) except that the pooled fecal pellet was allowed to sediment twice in 25-ml cold tap water in order to remove larger debris. Sterile isotonic Percoll was prepared by mixing one part of $10 \times$ Hanks balanced salts solution (HBSS; Sigma H-1641) with nine parts Percoll (Sigma P-4937) and stored at 4°C . Solutions of 30% and 60% were made by diluting the isotonic Percoll with RPMI-1640 and growth medium, respectively. The solutions were stored at 4°C and prewarmed at 37°C before use.

1–4 h prior to infection, the cells were collected and pelleted for 4 min at $160 \times g$. They were suspended in 5-ml growth medium and kept in two wells of a 24-well Falcon microplate (Becton-Dickinson; Meylan, France) (mean concentration: $6.9 \pm 2.2 \times 10^6 \text{ ml}^{-1}$) in the incubator until required. The MDBK cells were periodically resuspended to minimize their adherence to the bottom of the wells.

The oocysts (approximately $15\text{--}50 \times 10^6$) were processed, as previously described (Lawton et al., 1996), and were mixed with the cell suspension from one well (oocyst to cell ratio was 1:1–3:1). The suspension was carefully layered on top of 2.5 ml of the 60% Percoll solution and centrifuged for 10 min at $1500 \times g$ in a 15-ml Falcon centrifuge tube. Excystation was allowed to proceed for 1.5–3 h at 37°C in the parasite–cell layer at the interface between the Percoll solution and the medium. This layer was then retrieved with a Pasteur pipette and washed once in RPMI-1640 for 9 min at $600 \times g$. After dilution with the cells from the remaining well, the parasitized cells were layered on top of a 30% Percoll solution and centrifuged at $1500 \times g$ for 10 min. The pellet was subsequently washed once at $600 \times g$ and thrice at $160 \times g$ as above and distributed at an approximate density of $6 \times 10^6 \text{ cells ml}^{-1}$ under reduced oxygen in a candle jar for 24 h. When MDBK cells were used, the 30% Percoll step was omitted. After invasion, the parasitized cells were mixed with the remaining cells, washed thrice at $160 \times g$, distributed in six-well microplates fitted with $22 \times 22\text{-mm}$ glass coverslips and allowed to settle and adhere.

After 24 h, the percentage of THP-1 cells infected with *C. parvum* was assessed on thin smears, fixed in 100% methanol and stained with 20% Giemsa and 1% Alcian blue, as previously described (Lawton et

al., 1996). The parasite growth in the adherent MDBK cells was also evaluated 24 h post-infection, after the removal of the coverslips from the bottom of the wells. When needed, the cell suspension was diluted 1:10 in saline and counted in a Malassez cell. Toxicity was assessed by Trypan blue dye exclusion. The excystation yield was evaluated by counting the remaining unexcysted oocysts after infection of the cells. The Student's *t*-test was used to assess the statistical significance of the results.

The excystation yield depends on the viability of the oocysts and on the duration of incubation at 37°C . According to Robertson et al. (1993), maximum excystation occurs after 4 h, but the maximum sporozoite yield is achieved after 30 min. Upton et al. (1994b) observed a maximum between 90 and 120 min, and Joe et al. (1998) reported that the attachment of sporozoites to Caco-2 cells reached a maximum after 2 h of incubation. Whatever the oocyst/cell ratio, the percentage of excystation increased when the invasion duration was increased from 1.5 to 2 h, then significantly decreased ($p < 0.05$) at 3 h (Fig. 1). This might be due to the acidification of the medium (pH 6.5) and/or close contact with toxic molecules. However, the damage to the THP-1 cells was significantly more important ($p < 0.05$) as the oocyst/cell ratio increased ($6.1 \pm 2.5\%$, $19.2 \pm 5.1\%$ and $39.2 \pm 4.3\%$ dead cells at oocyst/cell ratios of 1:1, 2:1 and 3:1, respectively).

The infection rates at 24 h p.i. were independent from the oocyst/cell ratio, with about 4.5% of the host cells harboring developing parasites. The infection rates of MDBK cells were comparable to those of THP-1 cells (Fig. 2). The initial percentages of infected cells depend on the availability of host cells and on the invasiveness of the sporozoites. Whatever the cells used in our experiments, monocytic or epithelial, the invasion rates were lower than expected. Several authors have reported comparable infection rates (Slifko et al., 1997; Chen and LaRusso, 2000). This may also be compared to experiments with human volunteers (Chappell et al., 1996) where a minimum of 30 oocysts was necessary to initiate an infection. It is thus possible that only a small fraction of sporozoites are able to successfully multiply into the host cells.

This technique provides maximum contact between host cells and parasites on short periods of

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180 Fig. 1. Efficiency of excystation as a function of incubation time. The maximum excystation yield was achieved after 2 h. THP-1 cells and
an 1:1 oocyst/cell ratio were used. Results from three different experiments.

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182 time at an acceptable oocyst/cell ratio. Accordingly,
our current standard procedure uses an incubation

time of 1.5 h at a 1:1 oocyst/cells ratio. The subse-
quent use of 30% Percoll also allows the elimination

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187 Fig. 2. Percentage of parasitized cells 24 h p.i., with an incubation time of 1.5 h. The mean number of cells harboring developing parasites
188 slightly increased with the amount of oocysts added to the host cells. The infection rates of THP-1 cells and MDBK cells were similar with
the same oocyst/cell ratio. Results from three different experiments.

- 189 of most of the cell debris and of the oocysts shells,
 190 as previously reported (Nesterenko and Upton, 1996)
 191 and may also be used in the course of cultivation in
 192 order to eliminate dead cells, which can accumulate
 193 during these rather stringent culture conditions.
 194 However, this step is omitted with MDBK cells
 195 because these cells have a wider size heterogeneity,
 196 resulting in significant amounts remaining at the
 197 surface of the 30% Percoll solution. The present
 198 technique designed for non-adherent cells is applica-
 199 ble to epithelial cells and allows the use of the same
 200 parasitized adherent cell suspension and its even
 201 distribution in culture wells for drug testing.
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