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Incorporation of Exogenous Uracil by *Cryptosporidium parvum* In Vitro†

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Oocysts of *Cryptosporidium parvum* were used to infect Madin-Darby bovine kidney cells. Cultures were incubated in a reduced-oxygen atmosphere in candle jars or in a 5% CO_2–95% air atmosphere. At 72 h, parasites were quantitated microscopically and found to be enhanced 5.5-fold in the reduced-oxygen atmosphere. Using candle jars, we then determined that *C. parvum* was amenable to [^{3}H]uracil incorporation assays and easily quantitated with this method.

Cryptosporidium parvum is a protozoan that infects the gastrointestinal tracts of a wide variety of mammals, including humans (6). The parasite is widespread in the environment, and 30 to 35% of the U.S. population is seropositive (20). In some third world countries, the seroprevalence may be as high as 65% (20–22).

In immunocompetent individuals, infections of *C. parvum* are generally short lived and result in moderate to severe diarrhea and weight loss over a 4- to 8-day period (6, 20). The parasite has an autoinfective cycle, however, and immunoincompetent individuals, such as those with AIDS, may experience persistent infections leading to dehydration, electrolyte imbalance, and eventual death (6).

Studies on *C. parvum* are hampered by the absence of adequate in vitro models. The parasite develops poorly with conventional cell culture techniques, and few developmental stages are produced (2). Therefore, methods of enhancing development of *C. parvum* in culture are important not only to facilitate studies on the basic biology of the parasite but also for large-scale pharmaceutical testing. We present results demonstrating that development of *C. parvum* can be enhanced in vitro by using a reduced oxygen atmosphere and that quantitation of parasite development can be easily performed by using [^{3}H]uracil incorporation.

Oocysts of *C. parvum* were purified from the feces of experimentally infected kid goats on CsCl gradients and stored at 4°C in 0.01 M (PBS) (pH 7.2) phosphate-buffered saline as described previously (2, 15, 16, 18). Animal care was in accordance with institutional guidelines. Before use, oocysts were surface sterilized with 5% (vol/vol) Clorox bleach in deionized water and washed three times in deionized water and twice in PBS. Oocysts were then suspended in RPMI 1640 medium with supplements at 22 to 24°C (see below) and adjusted to a final concentration of 1.667×10^6 oocysts per ml. All oocysts were ≤2 months old at the time of use.

Madin-Darby bovine kidney (MDBK) cells were grown to 50% confluency on 22-mm^2 glass coverslips in 6-well tissue culture plates with complete RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer, sodium bicarbonate to pH 7.4, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of Fungizone per ml (17). After removing media from each well, 3 ml of new media containing unexcysted oocysts (see above) was added to each well (final concentration, 5.0×10^6 oocysts per well). Plates were incubated at 37°C with 100% humidity either in a 5% CO_2–95% air atmosphere or a reduced-oxygen atmosphere in desiccator jars utilized as candle jars (8, 17, 19). At 72 h, plates were removed from incubation, each well was washed twice with PBS to remove cell debris and nonphagocytosed oocysts, and coverslips were examined with a ×100 oil objective lens and Nomarski interference contrast optics. Fifty oil immersion fields were examined from each coverslip, and the types and numbers of each developmental stage, based on previously reported morphologic characteristics, were determined (2, 4, 6).

After 72 h, cultures plated originally to 50% confluency had become a monolayer. Preliminary studies revealed that plating cells to a higher density resulted in sloughing of cells in candle jar cultures. Pilot studies also revealed that inoculation of cultures with unexcysted oocysts resulted in enough organisms in each developmental stage to be quantitated easily and thus eliminated the time and variability resulting from prolonged exposure of sporozoites to room atmosphere and from having both sporozoites and oocysts in the same inoculum.

Table 1 shows a comparison of numbers of parasites in each developmental stage at 72 h postinoculation resulting from incubation in both 5% CO_2–95% air and reduced-oxygen atmospheres. Figures 1 through 3 show typical developmental stages at 72 h under the reduced-oxygen atmosphere. Although organisms in early developmental stages and some macrogametes were found in the 5% CO_2–95% air atmosphere, the reduced-oxygen atmosphere enhanced parasite development by a factor of 5.5-fold. By calculating the area in one ×100 objective oil field and the area in a six-chambered well, the possible number of cells in various developmental stages in a confluent monolayer under reduced oxygen conditions was determined to be about 106,247 (versus 19,282 for the 5% CO_2–95% air atmosphere; $P < 0.05$ with the Wilcoxon Mann-Whitney U Test). Although it is plausible that the reduced-oxygen atmosphere enhanced excystation, previous studies with other coccidia have shown that development is enhanced under lower-oxygen atmospheres (14, 17).

Because coccidian parasites such as *Toxoplasma gondii* and *Eimeria tenella* readily incorporate free uracil, whereas host cells cannot (12–14), we also examined whether *C.*

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TABLE 1. Comparative development of *C. parvum* in aerobic and reduced-oxygen atmospheres[a]

Developmental stage	No. of organisms per field[b]					
	5% CO_2–95% air (n = 8)			Reduced oxygen (n = 7)		
	Mean	Range	SD	Mean	Range	SD
Undifferentiated	17.6	10–29	6.5	41.7	22–68	14.4
Type I meronts	1.6	1–2	0.5	21.1	7–48	14.2
Type II meronts	0			0.9	0–2	0.9
Microgametocytes	0			1.0	0–4	1.4
Macrogametes	0.5	0–2	0.8	44.4	13–90	24.3

[a] Monolayers of MDBK cells on 22-mm^2 coverslips were each inoculated with 5.0×10^6 CsCl-purified oocysts. Incubation time was for 72 h in RPMI 1640 supplemented with 10% fetal bovine serum.

[b] Mean number of cells in 50 oil immersion fields per test well. The mean total projected numbers of cells in the entire well, provided that the monolayer is confluent throughout, are as follows: 19,282 in the 5% CO_2–95% air atmosphere and 106,247 in the reduced-oxygen atmosphere.

parvum was also amenable to [^{3}H]uracil incorporation assays. Cell cultures were grown and inoculated with *C. parvum* oocysts as described above, except that coverslips were added only to the wells used for autoradiography. To each well, [5,6-^{3}H]uracil was added (12, 13) at a concentration of 1.0 μCi/ml of medium (specific activity, 47 Ci/mmol). At days 1, 2, and 3 postinoculation, cultures were washed five times with PBS, and organisms in various developmental stages were solubilized by using three 400-μl changes of 1.0% aqueous sodium dodecyl sulfate. Solubilized cells were placed in 20-ml scintillation vials containing 2.4-cm glass fiber filters and dried overnight at 37°C. Scintillation fluor (10 ml) was added to each vial, and the cells were counted with a Packard Tri-Carb model 1500 scintillation counter. For autoradiography, coverslips were fixed in 100% methanol, air dried, stained with toluidine blue, and coated with Kodak NTB2 nuclear track emulsion. Emulsions were exposed for 3 to 24 h in the dark, developed with Kodak D-19 developer, and examined and photographed by using bright-field microscopy.

Figures 4 through 6 show that *C. parvum*, like *T. gondii* and *Eimeria* spp. (12, 13), is capable of incorporating exogenous uracil. Uracil incorporation leveled off between 48 and 72 h postinoculation in the infected cultures, suggesting either that recycling of asexual stages within the culture

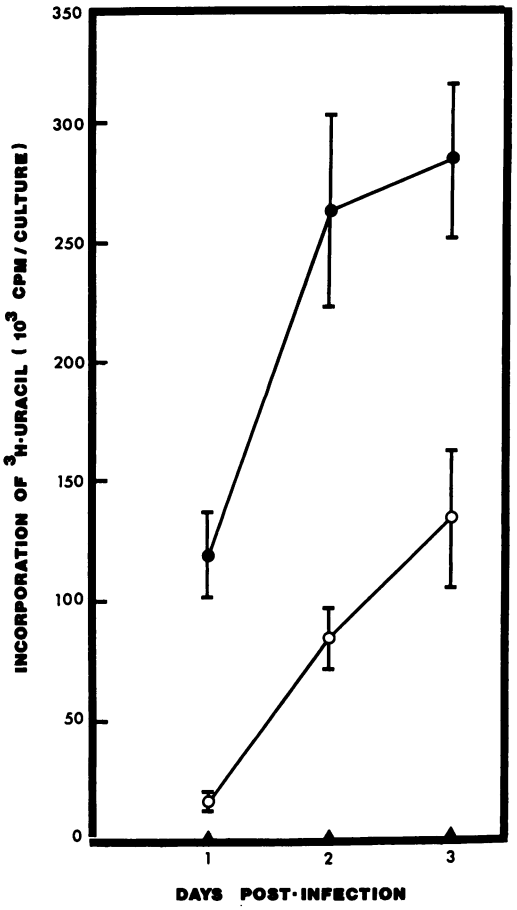

FIG. 4. Incorporation of [^{3}H]uracil by *C. parvum* in various developmental stages over 72 h in MDBK cells in candle jars. Each data point represents the mean of six repetitions and the standard deviation about the mean. Symbols: ●, monolayers infected with *C. parvum* and incubated with [^{3}H]uracil; ○, uninfected monolayers incubated with [^{3}H]uracil (unbound cytoplasmic uracil in host cells); ▲, infected monolayers without [^{3}H]uracil.

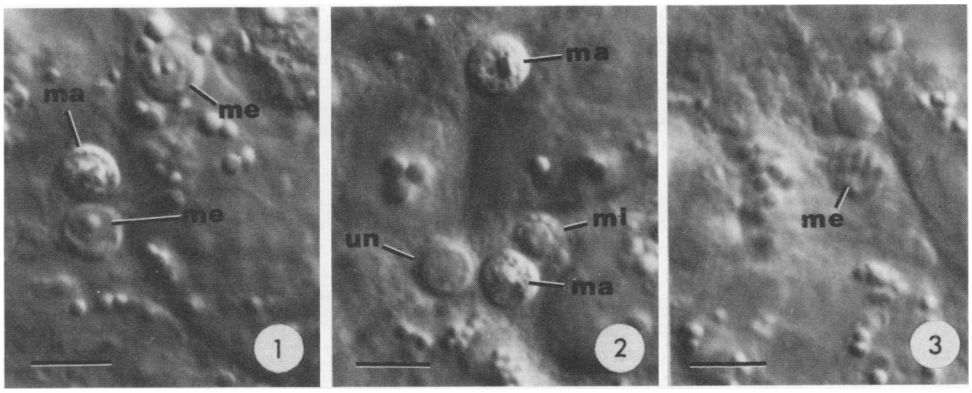

FIG. 1–3. Nomarski interference contrast photomicrographs of typical developmental stages of *C. parvum* in MDBK cells 72 h after cultivation at 37°C in candle jars. Scale bars, 5.0 μm. Abbreviations: ma, macrogamete; me, meront; mi, microgametocyte; un, undifferentiated stage.

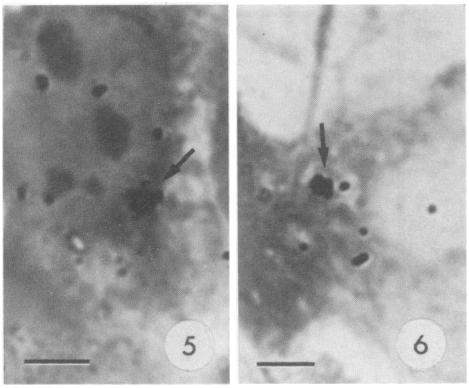

FIG. 5 and 6. Autoradiographs of MDBK cell cultures incubated for 72 h with *C. parvum* and [^{3}H]uracil (3-h exposure). Scale bars, 5.0 μm. Note uracil incorporation by cells (arrows).

system did not occur to a significant degree or that mortality of asexual stages was high.

Previous studies have shown *C. parvum* to be capable of developing to some degree in a variety of cell lines, including human fetal lung, human rectal tumor, human foreskin, human CAC02, rat LGA carcinoma, baby hamster kidney, primary chicken kidney, porcine kidney, mouse L929, human HT29 enterocyte, and Madin-Darby canine kidney cells (1, 3, 5, 7, 9–11, 23, 24). Our studies show that MDBK cells also provide a suitable host cell for *C. parvum* and that adequate numbers of developmental stages can be obtained for quantitation purposes in the reduced-oxygen atmosphere. The importance of using cells in developmental stages other than the oocyst stage for quantitation is that adequate methods of differenting phagocytosed cells versus newly formed oocysts are not available. By examining cells at 72 h, phagocytosed oocysts and the remaining free oocysts can be ignored, since sporogony occurs later than 72 h postinoculation (4, 6).

We suggest that [^{3}H]uracil incorporation be used as a means of counting *C. parvum* cells in various developmental stages whenever possible. The method is considerably faster than visually counting cells, and we were able to simplify the assay even more by eliminating automated methods of cell harvesting and nucleic acid precipitation. Although this results in counting free cytoplasmic uracil within host cells, the difference between infected and noninfected cultures is apparent, and even a moderately equipped laboratory should be able to utilize the assay to study *C. parvum*. The free cell cytoplasmic [^{3}H]uracil data in Fig. 4 also provide evidence that the cells continued to divide through 72 h in the reduced-oxygen atmosphere; we found further evidence for this by using Nomarski interference-contrast microscopy (Fig. 1 to 3).

Most pharmaceutical testing against *C. parvum* involves animal models, and nearly 100 pharmaceutical agents have been examined for efficacy against cryptosporidosis in vivo (6). However, few compounds reduce oocyst output, and at a nontoxic level none is effective at eliminating an active infection. One study utilized in vitro cultivation for pharmaceutical screening against this pathogen and found monensin and halofuginone to be effective in reducing parasite numbers by 90%, but only at high concentrations (10). These authors also incubated cultures in candle jars and counted the parasites in 50 oil immersion fields, but used mouse L929 fibroblasts as host cells and both excysted sporozoites and oocysts in the same inoculum.

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