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Effects of Select Medium Supplements on In Vitro Development of *Cryptosporidium parvum* in HCT-8 Cells†

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Surface-sterilized oocysts of *Cryptosporidium parvum* were applied to subconfluent monolayers of human adenocarcinoma (HCT-8) cells grown on coverslips in six-well cluster plates. Parasite-infected cultures were then incubated in RPMI 1640 with 10% fetal bovine serum, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, and antibiotics at 37°C in a 5% CO_2–95% air incubator for 2 h to allow sporozoites to excyst and enter cells. After cultures were washed free of debris, fresh cell culture media containing select supplements were added and cultures were reincubated. Parasite growth was assessed 66 h later by counting the number of parasite developmental stages in 25 random ×100 oil fields by Nomarski interference-contrast microscopy. Four vitamin supplements, calcium pantothenate, L-ascorbic acid, folic acid, and 4-(*para*)-aminobenzoic acid, each resulted in a significant increase in parasite numbers in vitro. The addition of insulin and the sugars glucose, galactose, and maltose also had a positive effect on parasite growth, although the effect was less pronounced than with any of the vitamins. Using the above information, we developed a supplemental medium formulation consisting of RPMI 1640 with 10% fetal bovine serum, 15 mM HEPES, 50 mM glucose, and 35 μg of ascorbic acid, 1.0 μg of folic acid, 4.0 μg of 4-aminobenzoic acid, 2.0 μg of calcium pantothenate, 0.1 U of insulin, 100 U of penicillin G, 100 μg of streptomycin, and 0.25 μg of amphotericin B (Fungizone) per ml (pH 7.4). The growth of *C. parvum* in this medium was found to be enhanced approximately 10-fold compared with that in control medium without additional glucose, insulin, or vitamins.

Cryptosporidium parvum is an enteric apicomplexan capable of causing moderate to severe diarrheal illness in immunocompetent individuals and life-threatening disease in immunocompromised individuals (4, 40). Although growth of this parasite in vitro was achieved a decade ago (5), a culture system capable of supporting growth of high numbers of this parasite has yet to be obtained. Recently, we developed a simple and reproducible method of obtaining viable, surface-sterilized oocysts for in vitro use (46). Additional studies revealed that the human adenocarcinoma (HCT-8) cell line supported superior development of this parasite in a conventional 5% CO_2–95% air incubator compared with 10 other cell lines and six other atmospheres (43, 44). This study was designed to develop a cell culture medium capable of enhancing parasite development in HCT-8 cells in vitro.

MATERIALS AND METHODS

Parasite preparation. Oocysts of *C. parvum* were passaged in 5-day-old neonate calves (*Bos taurus*). Oocysts in feces were strained through a graded series of wire mesh sieves to a final exclusion of 53 μm, concentrated by centrifugation, and stored in 2.5% (wt/vol) aqueous potassium dichromate ($K_2Cr_2O_7$) solution at 4°C until use (3). Prior to use, oocysts were purified on CsCl gradients as described previously (3, 35, 38). Once pipetted from the gradients, the oocyst-CsCl suspension was mixed with 2 volumes of ice-cold double-distilled water and pelleted by centrifugation at 1,500 × *g* for 20 min. Oocysts were transferred to 1.5-ml conical Eppendorf microcentrifuge tubes and washed again in ice-cold double-distilled H_2O by centrifugation at 5,000 × *g* for 2 min in a Costar model 10 microcentrifuge. Once the supernatant was removed, a 10% (vol/vol) ice-cold aqueous Clorox bleach solution was used to surface sterilize oocysts (46). Once added, the mixture was vortexed and the suspension was allowed to sit on ice for 10 min. Oocysts were then pelleted by centrifugation for 2 min at 5,000 × *g*, the supernatant was removed, and oocysts were washed once following resuspension in sterile, ice-cold phosphate-buffered saline (PBS) by centrifugation for 2 min at 5,000 × *g*. The supernatant was again removed, and oocysts were resuspended in 1 ml of cell culture medium. A small aliquot of the oocyst suspension was diluted 1:100 in water, and oocysts were quantitated with a hemacytometer. Oocysts were then diluted in cell culture medium to a final concentration of 5×10^5 oocysts per ml.

Cell culture. Human adenocarcinoma (HCT-8) cells (ATCC CCL 244) were maintained in 75-cm^2 tissue culture flasks (43, 44). Cells were tested bimonthly for contamination with *Mycoplasma* spp., using 4′,6-diamidino-2-phenylindole (DAPI) stain (21, 29, 41). Cells were lifted from the surface of flasks by using a solution of 0.25% (wt/vol) trypsin and 0.53 mM EDTA in PBS. The cell culture medium consisted of RPMI 1640 with L-glutamine (Sigma R-5382, lot 33H46291), 15 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin B (Fungizone) per ml. For routine cell passage, 5% fetal bovine serum (FBS) was used (maintenance medium), whereas 10% FBS was used whenever parasites were employed (growth medium), except when different concentrations of FBS were examined.

Fourteen hours prior to parasite inoculation, HCT-8 cells were plated onto 22-mm^2 glass coverslips in six-well cluster plates at a concentration of 5×10^5 viable cells in a total volume of 3 ml. Cell viability was assessed by using trypan blue exclusion (0.02% [wt/vol] in PBS), and cell numbers were quantitated with a hemacytometer (49). Plates were incubated at 37°C in a 5% CO_2–95% air humidified incubator.

For infection of monolayers, all maintenance medium was removed and 10^6 oocysts in 2 ml of growth medium were added (5×10^5 oocysts per ml). Cluster plates were then placed at 37°C in a 5% CO_2–95% air humidified incubator. After 2 h, unexcysted oocysts, oocyst walls, and other toxic materials that may have been liberated from oocysts were washed from the monolayers with PBS (8), and 3 ml of new growth medium with or without additional supplements (see below) was added. Cluster plates were then placed back into the 37°C incubator.

Parasite growth in vitro was assessed 68 h postinfection by removing coverslips from cluster plates and viewing parasite-infected monolayers under Nomarski interference-contrast optics. A ×10 ocular lens and ×100 objective oil immersion lens were employed. Twenty-five oil fields per coverslip were randomly examined, and total numbers of parasite developmental stages were assessed (43–46). Each experiment involving parasite development in cell culture was performed in replicates of four to six, and data are expressed as the means followed by the

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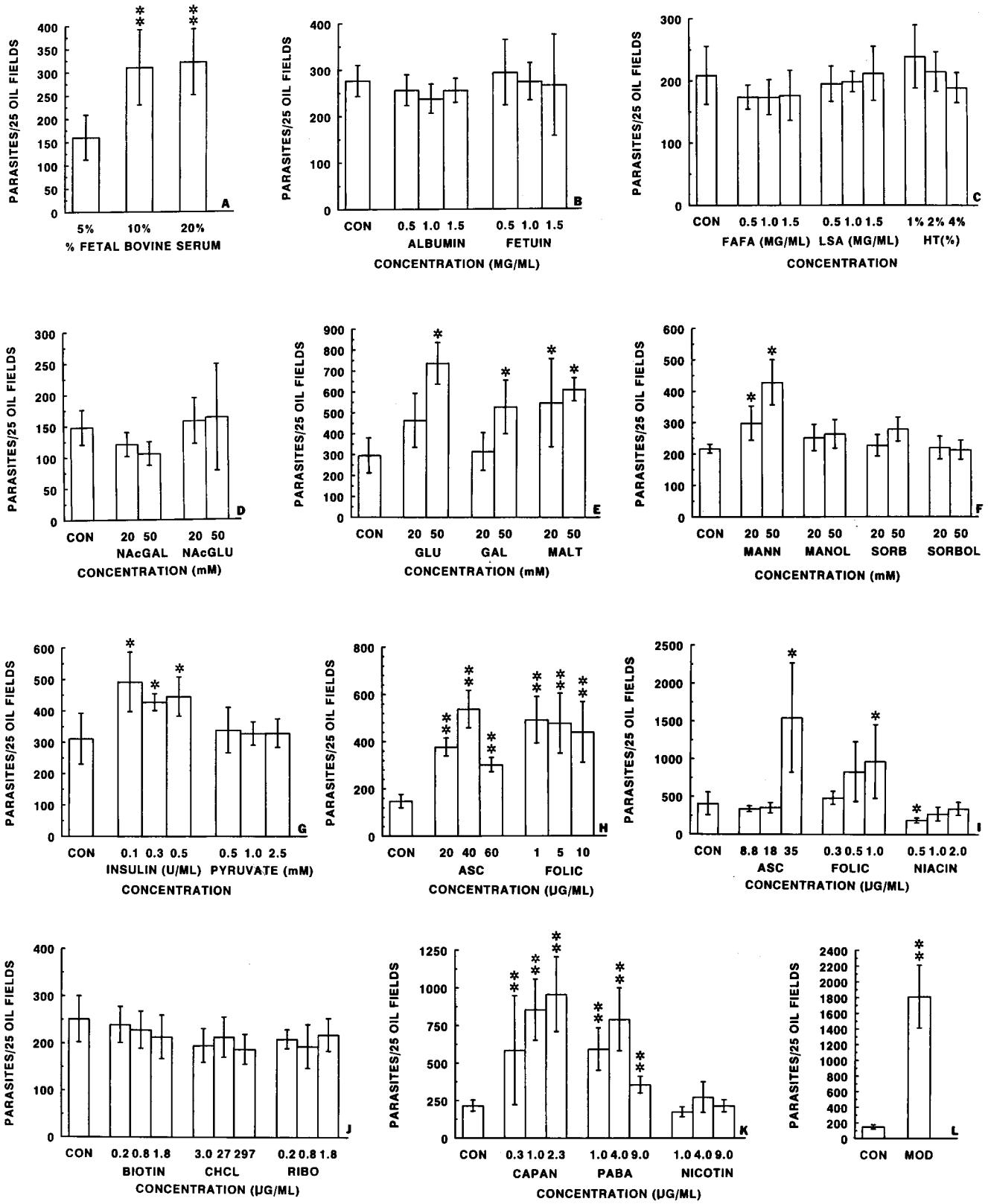

FIG. 1. Effects of select medium supplements on development of *C. parvum* in human adenocarcinoma (HCT-8) cells in vitro. Surface-sterilized oocysts were applied to subconfluent monolayers of HCT-8 cells grown on coverslips in six-well cluster plates. Parasite-infected cultures were then incubated in RPMI 1640 with 10% FBS, 15 mM HEPES buffer, and antibiotics at 37°C in a 5% CO_2–95% air incubator for 2 h to allow sporozoites to excyst and enter cells. After cultures were washed free of debris, fresh cell culture media containing select supplements were added and cultures were reincubated. Parasite growth was assessed 66 h later by counting the number of parasite developmental stages by Nomarski interference-contrast microscopy in 25 oil fields. Abbreviations: ASC, ascorbic acid; CAPAN, calcium pantothenate; CHCL, choline chloride; CON, control medium consisting of RPMI 1640 with 10% FBS, 15 mM HEPES buffer, 100 U of penicillin G per ml, 100 μg

standard deviations. Most experiments were repeated a second time to confirm results, including all supplements found to cause a statistically significant enhancement of parasite growth.

For each experiment, the percentage of *C. parvum* oocysts that excysted was determined. Aliquots of oocysts were incubated in cell culture medium at 37°C for 2 h; then at least 100 oocysts were scored as either empty or intact (unexcysted). Experiments were terminated when batches of oocysts had <70% excystation.

Medium supplements. Twenty-five different medium supplements were evaluated for their effects on the in vitro development of *C. parvum* in HCT-8 cells. Some of these were normally absent from the basal RPMI 1640 formulation, whereas others were added in addition to the concentrations already present. Concentrations of each supplement were based on concentrations utilized by other researchers or as multiples of concentrations already present in the basal formulation of RPMI 1640. All modifications of the basal RPMI 1640 formulation were made within 24 h of use, the pH was adjusted to 7.4, and the media were stored in the dark at 4°C until used. Supplements examined were 5, 10, and 20% (vol/vol) heat-inactivated FBS (Sigma F-4010 lot 32H0554); 20 and 50 mM (each) D-(+)-glucose (Sigma G-7528, lot 112H01422), D-galacose (Aldrich 11,259-3, lot 04229MP), maltose (USB 18724, lot 76401), D-mannitol (Sigma M-4125, lot 113F-0332), alpha-D-mannose (Aldrich 11,258-5, lot BT 00712BT), *N*-acetyl-D-galactosamine (Aldrich 33,591-6, lot 07908LV), *N*-acetyl-D-glucosamine (Sigma A-8625, lot 62H0064), D-sorbitol (Sigma S-1876, lot 43H0281), and L-(−)-sorbose (Sigma S-2001, lot 13H0341); 0.5, 1.0, and 1.5 mg of fraction V bovine serum albumin (BSA) (U.S. Biochemicals 10857, lot 65165) per ml; 0.5, 1.0, and 1.5 mg of fatty acid-free BSA (Sigma A-0281, lot 10H9304) per ml; 0.5, 1.0, and 2.5 mg of BSA per ml conjugated to additional linoleic acid at a molar ratio of 1:2 (Sigma L-8384, lot 21H9414); 0.5, 1.0, and 1.5 mg of fetuin (Sigma F-2379, lot 69F-9620) per ml; 0.125, 0.25, and 0.5 U of insulin (Sigma I-5500, lot 119F0586) per ml; 0.5, 1.0, and 2.5 mM sodium pyruvate (Sigma P-5280, lot 56F-07585); 1, 2, and 4% (vol/vol) hypoxanthine-thymidine medium supplement (Sigma H-0137, lot 71H-9406); 3.0, 27.0, and 297 μg of choline chloride (Sigma C-1879, lot 119C-0062) per ml; 50 μM 2-mercaptoethanol (Eastman 4196, lot D5C); 8.8, 18, 20, 35, 40, and 60 μg of L-ascorbic acid (Sigma A-4034, lot 12H07846) per ml; 0.2, 0.8, and 1.8 μg of D-biotin (Sigma B-4639, lot 92H06415) per ml; 0.3, 1.0, and 2.3 μg of calcium pantothenate (Aldrich 25,972-1, lot 07609KF) per ml; 1.0, 5.0, and 10.0 μg of folic acid (Sigma F-7876, lot 25C-0216) per ml; 0.5, 1.0, and 2.0 μg of niacin (Sigma N-0761 lot 62H01625) per ml; 1.0, 4.0, and 9.0 μg of nicotinamide (Aldrich 24,020-6, lot BF01322CJ) per ml; 0.2, 0.8, and 1.8 μg of riboflavin (Sigma R-0508, lot 49F0639) per ml; and 1.0, 4.0, and 9.0 μg of 4-(*para*)-aminobenzoic acid (Aldrich 85,291-0, lot HF02104CZ) per ml. Once all supplements were tested, a special medium consisting of the basal medium with what we believe to be near-optimal concentrations of each test supplement was developed. This final medium formulation consisted of RPMI 1640 supplemented with 10% heat-inactivated FBS, 15 mM HEPES, 50 mM glucose, and 35 μg of ascorbic acid, 1.0 μg of folic acid, 4.0 μg of 4-aminobenzoic acid, 2.0 μg of calcium pantothenate, 0.1 U of insulin, 100 U of penicillin G, 100 μg of streptomycin, and 0.25 μg of amphotericin B (pH 7.4) per ml.

Statistical analysis. Because data between individual experiments were considered independent, and because they would not necessarily be expected to follow a Gaussian distribution, experimentals and controls were analyzed by using an unpaired, two-tailed Mann-Whitney U test. The effects of each supplement on parasite development were considered to be significant only when $P = \leq 0.05$.

RESULTS

Of the 25 medium supplements examined, 10 were found to enhance development of *C. parvum* in vitro (Fig. 1). FBS, 10% (vol/vol), resulted in about double the number of parasites versus 5% serum (Fig. 1A). Increasing the concentration to 20% did not result in enhanced parasite numbers. Supplementing with additional BSA, fetuin, or linoleic acid conjugated to BSA did not enhance parasite numbers (Fig. 1B and C). The four sugars glucose, galactose, maltose, and mannose resulted in increased parasite numbers, whereas *N*-acetylglucosamine, *N*-acetylgalactosamine, mannitol, sorbose, and sorbitol had no obvious effect on parasite growth (Fig. 1D and F). Insulin supplementation resulted in a slight increase in parasite numbers (Fig. 1G), and the four vitamins ascorbic acid, calcium pantothenate, folic acid, and *para*-aminobenzoic acid greatly enhanced parasite growth (Fig. 1H and K). Supplementation with the vitamins niacin, nicotinamide, and riboflavin did not appear to affect parasite growth, nor did 2-mercaptoethanol (data not shown), choline chloride, hypoxanthine-thymidine, or sodium pyruvate (Fig. 1C, G, J, and K). When a combined medium formulation was developed and tested in vitro, a 30- to 100-fold increase in sexual stages occurred and total numbers of parasite development stages were enhanced about 10-fold (Fig. 1L and 2; Table 1).

DISCUSSION

Early studies in our laboratory with MDBK cells, an inoculating dose of 5.0×10^6 oocysts per well in six-well cluster plates, and a reduced oxygen atmosphere resulted in the development of an in vitro system capable of producing about 10^5 developmental stages per well in six-well cluster plates after 72 h (45). By optimizing host cell type, inoculating dose, and atmosphere (8, 43, 44, 46), we were able to enhance development even further. However, the use of medium supplements herein has resulted in much higher numbers of parasites, and the system now collectively produces approximately 3.8×10^6 parasites per well after 68 h when an inoculating dose of 10^6 oocysts (4.0×10^6 sporozoites per well) is employed. Considering that excystation averages 80 to 85% in these studies (46), this represents a parasite/host cell ratio of about 1:1. This is considerably higher than the 3 to 20% host cell infection rates reported by most authors previously (2, 9, 18, 20, 48), although recent reports suggest that Caco-2 cells may produce results comparable to those seen with HCT-8 cells (10, 24).

The use of HCT-8 cells and medium supplements results in numerous type I meronts, macrogametes, and microgametocytes. However, allowing cultures to develop further results in the production of few oocysts. Solely on the basis of our visual observations, we believe that most microgametes fail to leave microgametocytes. Thus, the cues necessary for proper fertilization are still lacking in our system and are actively being studied.

Supplementing media with FBS or serum proteins is known to be important for motility and development of some coccidia in vitro (1, 42). In our study, we found the optimal concentration of FBS to lie somewhere between 5 and 10%. Supplementing medium containing 10% FBS with additional albumin or fetuin did not result in increased parasite numbers. Although Upton and Tilley (42) showed that albumin and fetuin enhanced numbers of the rat coccidian *Eimeria nieschulzi* in vitro, they utilized 5% rather than 10% FBS. More recent, unpublished observations in our laboratory have shown that although numbers of *E. nieschulzi* are enhanced in vitro when albumin is used to supplement cells grown in RPMI 1640 containing 5% FBS, increased numbers of parasites do not occur over controls when a medium containing 10% FBS is supplemented.

Previous lipid analysis has shown *C. parvum* to have very high levels of linoleic acid (18:2) (22). This led the authors to postulate that 18:2 lipid supplementation may enhance para-

of streptomycin per ml, and 0.25 μg of amphotericin B per ml at pH 7.2; FAFA, fatty acid-free albumin; FOL, folic acid; GAL, galactose; GLU, glucose; HT, hypoxanthine-thymidine; LSA, 18:2 lipid-supplemented albumin; MALT, maltose; MANN, mannose; MANOL, mannitol; MOD, modified medium consisting of RPMI 1640 with 10% FBS, 15 mM HEPES, 50 mM glucose, and 35 μg of ascorbic acid, 1.0 μg of folic acid, 4.0 μg of 4-aminobenzoic acid, 2.0 μg of calcium pantothenate, 0.1 U of insulin, 100 U of penicillin G, 100 μg of streptomycin, and 0.25 μg of amphotericin B per ml at pH 7.2; NICOTIN, nicotinamide; PABA, *para*-aminobenzoic acid; RIBO, riboflavin; SORB, sorbose; SORBOL, sorbitol. $*P \leq 0.05$ compared with control, except in panel A in which the comparison was made with 5% FBS; $**P \leq 0.005$ compared with control.

site numbers in vitro. However, our results show that supplementing media with BSA loaded with 18:2 does not result in higher numbers of parasites over media with 10% FBS alone or media with 10% FBS also supplemented with delipidized BSA. These results imply that host cells provide *C. parvum* with ample 18:2 or precursors thereof.

Insulin is known to have a positive effect on development of some coccidia in vitro (11, 15, 34), and our results support these data. However, the sugars glucose, galactose, maltose, and mannose also enhanced development, similar to what has been reported for other coccidia (33, 36). Although it is likely that insulin merely enhanced the uptake of the sugars by host cells, we chose simply to include it in our final formulation rather than to determine its necessity. We also chose to utilize glucose rather than one of the other sugars in our final formulation because of its convenience and commercial availability. It is noteworthy that although the surfaces of *C. parvum* oocysts and sporozoites are known to possess *N*-acetylgalactosamine and *N*-acetylglucosamine residues (14, 17, 37–39), neither carbohydrate had any effect on parasite numbers at the concentrations employed.

Pyruvate and hypoxanthine-thymidine are sometimes used during cultivation of *C. parvum* in vitro (9, 11, 48). Pyruvate is known to enhance development of some eimerians in cell culture, perhaps by channeling pyruvate into the tricarboxylic acid cycle (31). However, our results suggest that pyruvate is an unnecessary medium supplement for *C. parvum*. These data suggest that pyruvate may not be an energy source for this parasite, supporting previous ultrastructural (6) and lipid (22) analysis showing that this parasite fails to possess mitochondria. Likewise, hypoxanthine-thymidine appeared unnecessary to *C. parvum* at the concentrations used. *Toxoplasma gondii* is known to readily incorporate hypoxanthine (32), but the intracellular pool is probably adequate so that this is not a limiting factor. Coccidia have long been known to poorly incorporate exogenous thymidine (19, 23, 25–28).

TABLE 1. Comparative development of *C. parvum* in HCT-8 cells in supplemented versus control medium[a]

Developmental stage	No. of organisms per 25 oil fields[b]					
	Control medium ($n = 6$)			Supplemented medium ($n = 6$)		
	Mean	Range	SD	Mean	Range	SD
Undifferentiated	93	45–110	31	511	303–762	172
Mature meronts	21	15–33	6	201	67–310	84
Microgametocytes	1	0–1	1	165	88–297	77
Macrogametes	34	23–50	12	935	613–1,223	218

[a] Subconfluent monolayers of HCT-8 cells on 22-mm^2 coverslips were each inoculated with 10^6 CsCl-purified oocysts of *C. parvum*. The incubation time was 68 h in RPMI 1640 with 10% fetal bovine serum. Supplemented medium also included 50 mM glucose and 35 μg of ascorbic acid, 1.0 μg of folic acid, 4.0 μg of 4-aminobenzoic acid, 2.0 μg of calcium pantothenate, and 0.1 U of insulin per ml.

[b] Numbers of parasites in 25 random oil fields per test well. The mean total projected numbers of parasites infecting host cells in the entire well, provided monolayers are confluent throughout, are 2.9×10^5 for nonsupplemented controls and 3.53×10^6 for supplemented.

Vitamin supplementation appears to have the most pronounced effect on parasite development. The importance of vitamins on development of coccidia in vitro has been known for several decades (7, 13, 16, 30, 31, 47), and the addition of at least four vitamins enhanced development of *C. parvum*. Of those tested, ascorbic acid yields the most pronounced results and is the only one absent from RPMI 1640. This vitamin may be an important antioxidant and allows for enhanced parasite survival.

It is not possible to examine all variables in this type of assay, and numerous other factors influencing parasite development in vitro remain to be tested. For instance, we chose to utilize a single lot of FBS in our assays, but different lots of FBS may have pronounced effects on parasite development. pH may also be important, and Hamer et al. (12) have shown that a pH range of 7.0 to 8.0 results in enhanced binding of *C. parvum* sporozoites to host cells. Our medium was adjusted to an initial pH of 7.4, and although HEPES buffer was used in our study, the color of the medium suggested that the pH drops below 7.0 during the 3 days of cultivation. This drop in pH may well result in reduced binding of merozoites to host cells, and studies more carefully examining the effects of pH may result in a formulation resulting in even higher numbers of parasites in culture. Nevertheless, the final medium formulation outlined in this study is now routinely used in our laboratory and provides reproducibly high levels of *C. parvum* developmental stages in vitro.

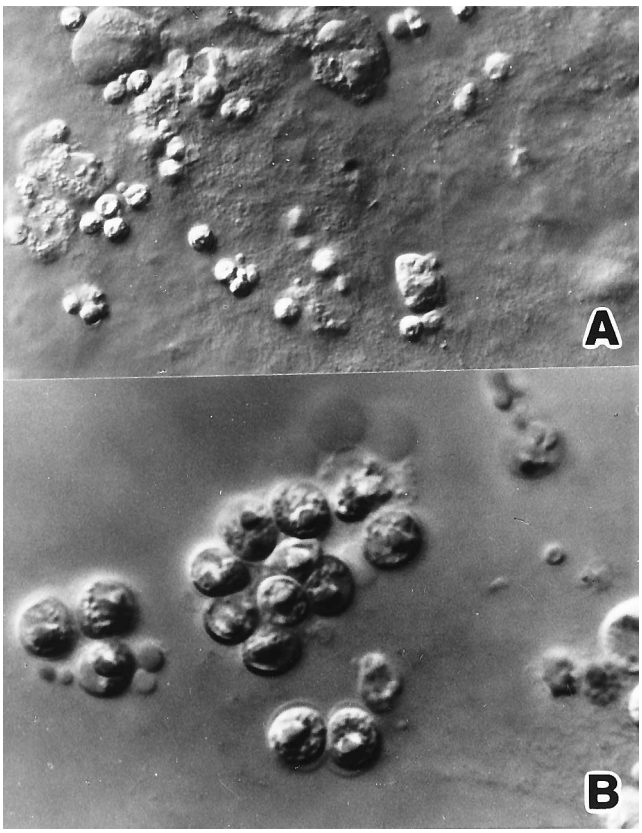

FIG. 2. Nomarski interference-contrast photomicrographs of developmental stages of *C. parvum* in HCT-8 cells in vitro 68 h after infection. The medium consisted of RPMI 1640 with 10% FBS, 15 mM HEPES, 50 mM glucose, and 35 μg of ascorbic acid, 1.0 μg of folic acid, 4.0 μg of 4-aminobenzoic acid, 2.0 μg of calcium pantothenate, 0.1 U of insulin, 100 U of penicillin G, 100 μg of streptomycin, and 0.25 μg of amphotericin B per ml at pH 7.4. Magnification: (A) ×351; (B) ×1,206.

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