Growth of Host Cells and *Chlamydia trachomatis* in Medium Containing Serum from 16-Week-Old Calves

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We compared fetal bovine serum with five batches of serum from calves of various ages. HeLa and McCoy cells grown in only one batch of calf serum (from 16-week-old calves) had morphology, growth kinetics, and cloning efficiency similar to those of cells grown in fetal bovine serum. Cells maintained in calf serum from this batch supported the growth of two laboratory strains of *Chlamydia trachomatis*, a genital strain (E/UW-5/Cx) in McCoy cells and a lymphogranuloma venereum strain (440L) in HeLa cells. McCoy cells maintained in calf serum also supported the growth of *C. trachomatis* from clinical specimens. The batch of serum from 16-week-old calves was an effective alternative to fetal bovine serum for the growth of cells and of *C. trachomatis*. Other laboratories may be able to use calf serum for the maintenance of cells and for the isolation of *Chlamydia* spp. Before use, however, each batch of calf serum will have to be carefully evaluated to ensure that it is equivalent to fetal bovine serum.

Chlamydia trachomatis organisms are leading causes of sexually transmitted diseases, with an estimated three million new cases occurring each year in the United States (14). Infection with these agents can lead to serious sequelae, including infertility, epididymitis, and infantile pneumonia (13). Isolation of the organism is the best diagnostic criterion. Because chlamydiae are obligate intracellular parasites, the isolation procedures require growth of the organism in host cells. Most laboratories use McCoy cells maintained in growth medium supplemented with 10% fetal bovine serum (FBS). HeLa and baby hamster kidney cells are also used. FBS is obtained from the fetuses of pregnant cattle brought to market for slaughter. In the past few years, there has been a decrease in the number of these cattle being slaughtered. This has resulted in a decrease in the availability of FBS, with a concomitant dramatic increase in its cost (1). Accordingly, we decided to examine calf sera (CSs) for the growth of cells and for the cultivation of *C. trachomatis*. We examined five sera. Cells cultured in one batch of serum (from 16-week-old calves) had growth and morphological characteristics similar to those of cells maintained in FBS. We found this serum and FBS to be equivalent for the growth of cultured cells and laboratory strains of *C. trachomatis* and for

the isolation of *C. trachomatis* from clinical specimens.

MATERIALS AND METHODS

Sera. Newborn serum and CS were obtained from three distributors: M. A. Bioproducts, Walkersville, Md.; Flow Laboratories, Inc., McLean, Va.; and Sterile Systems, Inc., Logan, Utah. These included two newborn calf sera and three sera from calves 6 to 16 weeks old. Hyclone FBS and hyclone CS were obtained from Sterile Systems. The CS used for comparative studies was obtained at 16 weeks from calves fed a special, formulated diet (Sterile Systems). The FBS and the CS cost \$162 and \$28 per 500 ml, respectively. Both sera passed the same quality assurance tests. Chlamydial antibodies were determined by complement fixation by Victor Berardi of the Virology Laboratory of the Massachusetts State Laboratory Institute, Boston. These antibodies were not detectable in the FBS and were detected at a titer of 1:4 in the CS.

Growth of host cells. Monolayers of HeLa 229 cells and McCoy cells were maintained in Eagle minimal essential medium supplemented with 0.2% sodium bicarbonate, 10 µg of gentamicin per ml, and 10% heat-inactivated FBS (6). For the growth of *C. trachomatis* from clinical specimens, McCoy cell monolayers were maintained in a growth medium of minimal essential medium with Hanks salts supplemented with 200 mM L-glutamine, 0.05% sodium bicarbonate, 50 µg of vancomycin and 10 µg of gentamicin per ml, and 10% heat-inactivated FBS. Cells were adapted to heat-inactivated CS by increasing the percentage of CS from 25 to 100% of the serum content over a 2-week

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FIG. 1. Rate of growth of HeLa and McCoy cells in medium containing FBS or CS. At day zero, 5×10^4 cells were placed in tissue culture flasks in medium with FBS or CS. At 2-day intervals, two flasks of cells grown in each serum were trypsinized, and the total number of cells was counted. The symbols represent the average number of cells in two flasks. -----, HeLa cells; —, McCoy cells. ○, FBS; □, CS.

period. This adaption was completed one month before all experiments in which the two sera were compared.

Growth and harvest of laboratory strains of chlamydiae. *C. trachomatis* strain lymphogranuloma venereum 440L (LGV) was grown in cycloheximide-treated HeLa cells as previously described (6). After 42 h of incubation, the chlamydiae were harvested from the cells.

A genital strain of *C. trachomatis* (UW-5, serotype E) which requires centrifugation onto host cells to enhance infectivity was grown in cycloheximide-treated McCoy cells, following the protocol of Lee (9). The chlamydiae were harvested from the cells after 72 h of incubation.

Chlamydiae were harvested from infected cells by procedures for *Chlamydia psittaci* (6) with modifications (9, 10). The chlamydial preparations were frozen at -70°C .

Titration of the infectivity of chlamydiae. The number of infectious units of LGV was determined by a modification of a previously published protocol (6). A total of 2×10^5 HeLa cells were placed in Leighton tubes. At 4 to 8 h later, after all the cells were attached and spread on the cover slip, the medium was removed, and 0.2 ml of phosphate-buffered saline containing various dilutions of an LGV strain 440L of *C. trachomatis* was added to the tube. After 2 h of incubation at 37°C on a shaker (100 strokes per min), the inocula were removed, and 1 ml of minimal essential medium was added to each tube. At 24 h after infection, the cells were stained with Giemsa, and 800 cells were examined for the presence of chlamydial

inclusions.

For non-LGV strains of *C. trachomatis*, an inclusion count method was followed (9). Monolayers of McCoy cells were infected with various dilutions of *C. trachomatis* in sucrose-phosphate buffer (2). After incubation at 37°C for 60 min with shaking (100 strokes per min), the inocula were aspirated, and minimal essential medium containing $0.5 \mu\text{g}$ of cycloheximide per ml was added to the monolayers. After incubation for 48 h at 35°C , the cells were examined for chlamydiae as outlined above for the LGV strain.

Growth of chlamydiae from clinical specimens. Three-day-old McCoy cell monolayers were trypsin dispersed and suspended to a concentration of 2×10^5 cells per ml of growth medium. One milliliter of this suspension was added to a 1-dram, flat-bottomed shell vial (15 by 45 mm) containing a cover slip. The vials were incubated for 24 h at 37°C in a 5% CO_2 incubator. Just before inoculation of the specimens, the medium was aspirated, the cells were rinsed once with phosphate-buffered saline, and 1 ml of inoculation medium was added to each vial. Inoculation medium was growth medium with 0.06% glucose and $0.6 \mu\text{g}$ of cycloheximide per ml (12). This concentration was chosen because higher cycloheximide concentrations are toxic to these McCoy cells. For inoculation, clinical specimens were thawed at 37°C and vibrated with glass beads on a Vortex mixer for 2 min; one-half of the specimen, approximately 0.25 ml, was added to each of two vials which were centrifuged at $4,000 \times g$ for 1 h (15). After 2 h of incubation at 37°C , the inoculation medium was aspirated, and 1 ml of fresh inoculation medium was added to each vial. The vials were then incubated at 37°C for a further 63 h and stained with iodine, and the total number of inclusions on each cover slip was counted.

Clinical specimens. Genital specimens were obtained with calcium alginate swabs (Inolex Corp., Glenwood, Ill.) from patients who were being evaluated for various sexually transmitted diseases. The specimens were placed in holding medium composed of 0.5 ml of sucrose phosphate glutamate (3), kept on wet ice for no more than 6 h, frozen in dry ice and alcohol, and stored at -70°C .

RESULTS

Growth characteristics of cells in medium containing CS or FCS. HeLa and McCoy cells were propagated in medium supplemented with five different CS for 1 month. Serum from 16-week-old calves (Sterile Systems) was chosen for further evaluation because the cells grown in this serum were the most similar to those grown in medium supplemented with FBS, as was evidenced by light microscopic examination and by the number of times the cells divided per week.

Figure 1 shows the growth kinetics of HeLa and McCoy cells maintained in medium containing either 10% CS or 10% FBS. Over a 10-day period, there was no significant difference in the rate of doubling of either cell line. Another measure of the viability of cells is the ability of single cells to form colonies (8). When 10 or 100

TABLE 1. Effect of type of serum used in growth medium on the colony-forming ability of cultured cells

Serum content of growth medium (10%)	Visible colonies formed after 10 days of incubation ^a			
	10 cells		100 cells	
	HeLa 229	McCoy	HeLa 229	McCoy
FBS	4	7	41	53
CS	7	5	60	52

^a Numbers represent the average number of colonies in four tissue culture flasks.

host cells were cloned in medium containing either CS or FBS, there was little difference in the number of cells able to form visible colonies after incubation at 37°C for 10 days (Table 1).

Growth of *C. trachomatis* in cells propagated and maintained in medium containing CS or FBS. HeLa cells propagated in medium containing CS or FBS were infected with dilutions of an LGV strain of *C. trachomatis*. Table 2 shows the number of cells that contained chlamydial inclusions and the percentage of cells that were infected at each dilution. The infection rates were similar in cells maintained with either serum, although there were slightly fewer inclusions in CS-maintained cells.

McCoy cells were inoculated with a *C. trachomatis* strain that is usually centrifuged onto the cells to increase infectivity. Although no centrifugation was used in these experiments, inclusions were noted with equal frequency in McCoy cells maintained in the two media (Table 2).

Isolation of *C. trachomatis* from clinical specimens with cells propagated and maintained in medium containing CS or FBS. We compared the ability of cells grown and maintained in CS and FBS to support the growth of chlamydiae from clinical specimens. Twelve batches of specimens were evaluated over 9 months. The inclusions in CS-maintained cells were larger, possibly a reflection of the greater cytoplasmic area of these cells as compared with cells maintained

in FBS. *C. trachomatis* was found in 57 (18.4%) and 59 (19.0%) of 310 clinical specimens inoculated onto McCoy cells grown and maintained in medium containing FBS and CS, respectively. The bacterium was found in 63 (20.3%) of these specimens inoculated onto and grown in cells maintained in either medium. There were a few more isolations in the CS-maintained cells. This was not a statistically significant difference. Of the 10 discordant specimens (positive in only one type of serum), 6 had three or fewer inclusions, and the other 4 specimens were accounted for by damage to cells due to cytotoxic effects or fungi. The number of chlamydial inclusions seen in cells maintained in CS or FBS is shown in Table 3. Similar numbers of inclusions were detected in cells maintained in the two types of sera. *C. trachomatis* serotype D/E run as a control with each batch of specimens produced an average of 62 and 48 inclusions per cover slip in the media containing FBS and CS, respectively.

DISCUSSION

One batch of serum from 16-week-old calves (CS) was shown to be an effective alternative to FBS for the support of host-cell and chlamydial growth. Since *C. trachomatis* was first isolated in cell culture by Gordon and Quan (5), workers have supplemented growth media with 5 to 10% FBS. The requirement for FBS has gone unchallenged, and other available serum sources have not been examined for the isolation of chlamydiae from clinical specimens.

McCoy cells supported in CS were equal to cells supported in FBS for isolation of chlamydiae from clinical specimens. The ability of CS-maintained cells to support serial passages of these isolates was not examined. For these studies, the cultured cells were gradually transferred from 100% FBS to 100% CS. This technique was used to avoid the possibility of shock to the cells and to avoid selecting a subset of the population of cultured cells which grew well on CS. This technique is recommended for other

TABLE 2. Effect of type of serum used for growth and maintenance on inclusion formation by laboratory strains of *C. trachomatis* in cultured cells

Dilution of chlamydial inoculum	No. (%) of cells containing <i>C. trachomatis</i> inclusions ^a			
	Strain LGV 440L		Strain E/UW-5/Cx	
	FBS	CS	FBS	CS
10 ⁻¹	408 (51)	392 (49)	760 (95)	760 (95)
10 ⁻²	152 (19)	88 (11)	392 (49)	376 (47)
10 ⁻³	ND ^b	ND	144 (18)	112 (14)

^a A total of 800 cells were examined. Inclusions were counted 24 and 48 h after incubation of LGV with HeLa cells and of E/UW-5/Cx with McCoy cells, respectively.

^b ND, No inclusions detected.

TABLE 3. Number of inclusions of *C. trachomatis* from clinical specimens inoculated onto McCoy cells grown and maintained in medium containing FBS or CS

Serum in medium (10%)	No. of specimens with following no. of inclusions:				
	1-10	11-100	101-1,000	1,001-10,000	>10,000
FBS	20	18	11	7	1
CS	23	17	15	4	—

workers for analyzing other batches of CS.

Chlamydiae are obligate intracellular bacteria requiring the cytoplasm of the host cell for their development. The role of serum in host-cell interactions has not been clearly defined. Horse serum has been used to support the growth of *C. trachomatis* in suspensions of L-929 cells (4) and in monolayers of irradiated McCoy cells (5), and serum-free medium containing sodium oleate and bovine serum albumin has been used to propagate chlamydiae in L929 cells (11). Karayiannis and Hobson (7) noted that, when FBS is omitted from maintenance medium, the number, size, and quality of chlamydial inclusions are altered. They found that a macromolecular fraction of FBS could reverse this effect. Serum may contain growth-promoting factors necessary for cell maturation and chlamydial growth or factors that have a nonspecific effect on host cells. Our findings and those of others (7) suggest that FBS does not contain a unique factor necessary for host-cell or chlamydial growth.

Chlamydiae are one of the most prevalent causes of sexually transmitted diseases. Thus, there are increasing requests for isolation of the organism and increasing amounts of chlamydial research. With fluctuations in the price and availability of FBS, it is desirable to have good serum alternatives which support host-cell and chlamydial growth. Since we found one batch of serum from 16-week-old calves to be useful, it is possible that other laboratories may also be able to use calf serum. Before use, however, it is

necessary that each batch of CS be carefully evaluated to ensure that it is equivalent to FBS.

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