IN VITRO
Vol. 8, No. 2, 1972

GROWTH OF *CHLAMYDIA PSITTACI* STRAIN MENINGOPNEUMONITIS IN MOUSE L CELLS CULTIVATED IN A DEFINED MEDIUM IN SPINNER CULTURES

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SUMMARY

L cells were grown in spinner cultures in a defined medium consisting of Waymouth medium MB752/1 (19) supplemented with 2 mg of fatty acid-free bovine serum albumin (BSA) per ml and 5 μg of oleate per ml (WO_5 medium). Growth in WO_5 medium was comparable to spinner L cell growth in two serum-containing media. The optimal concentration of oleate in the WO medium was 5 to 10 μg per ml. The use of 20 to 80 μg of oleate per ml of medium resulted in lower peak populations and earlier declines in viable cell counts. Cell death occurred rapidly in WO_{100} medium. Cell growth in WO medium containing 5 to 80 μg of oleate per ml was well above the level of growth observed when no oleate was present in the medium. Since the total lipid and fatty acid compositions of the BSA used in this study have been characterized by the authors, the WO medium may be considered a defined medium. L cells have been continuously maintained in spinner cultures in WO_5 medium for over 50 passages with no major variation in the growth pattern. A 1000-fold increase in *Chlamydia psittaci* strain meningopneumonitis, with a peak titer of 9.3×10^7 plaque-forming units per ml, was observed when the chlamydial agents were grown in spinner L cells in WO_5 medium.

Key words: defined medium; L cells; spinner culture; *Chlamydia psittaci* strain meningopneumonitis; Waymouth oleate medium.

INTRODUCTION

Historically, it has been difficult to grow mammalian cells and obligate intracellular microorganisms in well defined media, particularly in suspension cultures. There is a limited number of reports on the cultivation of mammalian cells in semi-defined or defined media using spinner cultures (1, 2) or shaker cultures (3–11).

Chlamydial agents have been grown in mouse L cells cultivated in spinner cultures by using serum-containing media (12, 13). Growth of chlamydiae occurred in monkey kidney cells (LLC-MK2; 14) cultivated in monolayers in a defined medium composed of Waymouth medium MB 752/1 (19) supplemented with fatty acid-poor bovine serum albumin (2 mg per ml) and sodium oleate (10 to 20 μg per ml), with initiation of cell growth by a 1% serum medium (15, 16). However, this system was not convenient for use in suspension culture, because the cells often formed large clumps. The cultivation of chlamydial agents in mammalian cells grown in serum-free defined medium in suspension cultures has not been reported.

A defined medium system with the advantages of suspension cultures was desired for studying lipid metabolism of normal and chlamydiae-infected cells. Therefore, we investigated the cultivation of L cells in spinner cultures in a defined, supplemented Waymouth medium and the growth of a chlamydial agent in this cell system.

MATERIALS AND METHODS

Cells. Mouse L cells (17, 18) clone 5b (12) were obtained from J. W. Moulder (Department of Microbiology, University of Chicago).

Growth media. Waymouth medium (19; imMEDIAte Dry Tissue Culture Medium MB 752/1, Schwarz/Mann, Orangeburg, N. Y.), pH 7.2, was supplemented with 2 mg of fatty acid-

free bovine serum albumin (BSA; Pentex, Miles Laboratories, Inc., Kankakee, Ill.) per ml and various concentrations of oleic acid. The oleic acid (Lipids Preparation Laboratory, The Hormel Institute, Austin, Minn.) was added as the sodium salt prepared by the method previously described (20). Henceforth, this Waymouth-BSA-oleate medium will be referred to as WO medium with a subscript number designating the number of micrograms of oleic acid per milliliter of medium. Cells were also grown in Waymouth medium containing 10% heat-inactivated fetal calf serum (FCS_{10}), pH 7.2, and in Medium 199 (21; Instant Tissue Culture Powder Medium, Grand Island Biological Company, Grand Island, N. Y.), plus heat-inactivated FCS_{10}, pH 7.2. Vancomycin hydrochloride (Eli Lilly & Co., Indianapolis, Ind.) and streptomycin sulfate were each used at concentrations of 100 μg per ml of medium. All media were sterilized by membrane filtration (0.45-μ porosity; Millipore Corporation, Bedford, Mass.)

Cell cultivation. The L cells were propagated in WO_5 medium in spinner cultures in a 5% CO_2, 95% air incubator at 37°C. Cells were grown in 250-ml centrifuge bottles equipped with sampling ports and suspended magnetic stirring bars, rotated by a magnetic stirrer. Every 3 to 5 days the L cell populations were determined and the cultures were centrifuged (200 × *g*, 10 min). The supernatant fluid was decanted, and the cells were resuspended in fresh medium with a starting population of approximately 5×10^5 cells per ml.

Sterility of the cultures and media was ascertained by using blood agar slants, trypticase soy broth, and thioglycollate broth. The cell culture and meningopneumonitis (MN) agent were determined to be free of mycoplasmas by T. F. Smith, Microbiology Section, Mayo Clinic, Rochester, Minn.

L cell growth curves. The growth of L cells in Waymouth-FCS_{10} medium, Medium 199-FCS_{10}, unsupplemented Waymouth medium, and in Waymouth-BSA medium containing concentrations of oleic acid ranging from 0 to 160 μg per ml of medium was compared, by using the cultivation procedures described above. Two to six spinner cultures were used to determine each growth curve. Duplicate 1.5-ml samples were withdrawn from each bottle at 12- or 24-hr intervals, and duplicate counts of each cell sample were made microscopically with the aid of a hemacytometer (15). Viable cells were determined by the trypan blue exclusion technique (22).

Meningopneumonitis agent. Chlamydia psittaci strain MN (23), used to infect spinner L cells, was serially propagated in L cell monolayers grown in WO_5 medium. The stock inoculum of MN was prepared in L cells as described by Jenkin (24). MN in its 3rd to 7th L cell passage was pooled and used as inoculum for the experiment.

Growth of meningopneumonitis agent in defined medium L cell spinner cultures. L cells were grown in spinner cultures in WO_5 medium for 80 hr at 37°C. The cell suspension was centrifuged (200 × *g*, 10 min), the supernatant medium was discarded, and the cells were resuspended in 30 ml of WO_5 medium. A cell count was made and 1.8×10^5 cells were distributed to each spinner bottle. L cells were infected using the procedure of Tribby (12). The MN agent [6.8×10^5 plaque-forming units (PFU)] was added to each bottle, and the total volume was brought to 20 ml with WO_5 medium. The cells and agent were kept in suspension during the 2-hr absorption period which was carried out at 37°C in a 5% CO_2, 95% air atmosphere. At the end of the absorption period, the cell suspension was centrifuged (200 × *g*, 10 min, 4°C), and the supernatant medium containing unabsorbed MN was decanted. The infected L cell culture was resuspended in 120 ml of WO_5 medium. A starting cell population of approximately 1.5×10^6 cells per ml was used in an attempt to establish a nonmultiplying cell population (25). Duplicate infected and uninfected L cell spinner cultures were used.

Triplicate Leighton tubes containing cover slips and 0.5 ml of WO_5 medium were inoculated with 0.5 ml of cell suspension for each spinner culture and incubated at 37°C for 24 hr. The L cells were stained with May-Grünwald-Giemsa stain and examined to determine the percentage of infected L cells (13). Greater than 90% of the L cells contained one or more inclusions.

At 6-hr intervals, from 0 to 96 hr, duplicate 1.5-ml samples were withdrawn from each spinner bottle and centrifuged (200 × *g*, 10 min, 4°C) to sediment the cells. One 0.5-ml aliquot of supernatant medium from each sample was

stored at 4°C for no longer than 15 hr before a plaque assay was performed. L cell counts from each spinner culture were made every 24 hr as described above.

Plaque assay of meningopneumonitis agent. Chlamydiae were assayed by a modification of the plaque assay procedure of Banks et al. (26). Cell monolayers for plaquing were prepared by using LLC-MK2 cells grown in monolayers with Eagle's minimal essential medium (27) prepared with Hanks' balanced salt solution (28) and 10% newborn calf serum (MEM-NCS_{10}). Streptomycin (150 μg per ml) and vancomycin (150 μg per ml) were added to the medium. Cells were distributed to give 3×10^5 to 4×10^5 cells per tissue culture well (35 × 10 mm; 6 wells per plate; Linbro Chemical Co., Inc., New Haven, Conn.) in 2.5 ml of MEM-NCS_{10}. The monolayers were used after 1 to 2 days of incubation at 37°C.

Cell monolayers were washed once with phosphate-buffered saline. Ten-fold serial dilutions of MN were prepared in Brain Heart Infusion (Difco Laboratories, Detroit, Mich.), and triplicate 0.1-ml amounts of appropriate dilutions were added to the MK2 cell monolayers. Absorption was carried out at room temperature for 2 hr on a rocker platform. Each monolayer was then overlaid with 2.5 ml of overlay medium (26) containing 200 μg each of streptomycin and vancomycin per ml. Plates were incubated at 37°C in a 5% CO_2, 95% air atmosphere with 95% humidity. After 9 days of incubation, 2.5 ml of overlay medium (26) containing 0.01% neutral red were distributed to each well. The plates were incubated for 2 more days at 37°C before the plaques were counted.

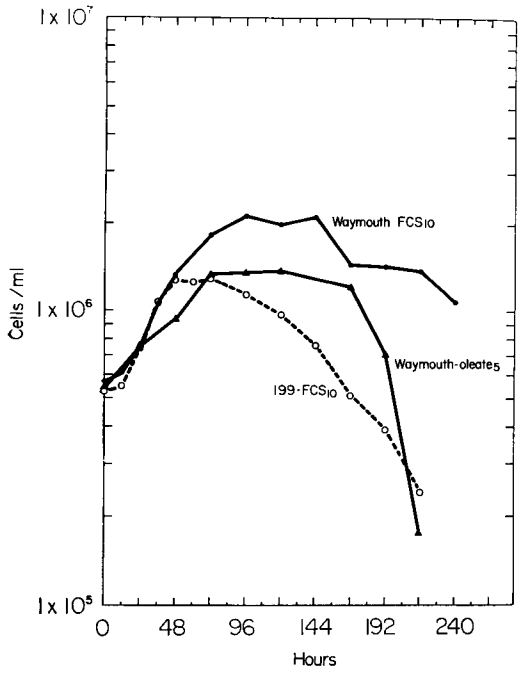

FIG. 1. Growth of L cells in spinner culture in three different media. ●——●, Waymouth medium MB752/1 + 10% fetal calf serum; ▲——▲, Waymouth medium MB752/1 + 2 mg of fatty acid-free bovine albumin per ml and 5 μg of oleate per ml; ○- - -○, Medium 199 + 10% fetal calf serum.

Analysis of the lipid content of bovine albumin. One gram of BSA was extracted twice at 25°C for 10 min with 40 ml of $CHCl_3$-MeOH (2:1, v/v) and two more times with 40 ml of $CHCl_3$-MeOH (1:1) at 40°C for 20 min. The extract was filtered after each extraction. The extracts were pooled, washed with water to remove nonlipid material, and concentrated by evaporation of the solvent under N_2. The BSA residue remaining after four extractions was subjected to alkaline hydrolysis and tested for residual lipid (16). Thin-layer chromatographic (TLC) analyses of the two lipid samples were done by the procedures of Makino et al. (16). Total lipids were methylated, and the fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) as previously described (29). Fatty acid methyl esters were measured by comparison to an internal standard in GLC (29).

RESULTS AND DISCUSSION

Comparison of growth of L cells in various media. The growth of L cells cultivated in spinner cultures in serum-containing media (199-FCS_{10} and Waymouth-FCS_{10}) and in defined medium (WO_5) are compared in Fig. 1. Mouse L cells grown in Medium 199-FCS_{10} and WO_5 medium increased 2.5- to 3-fold in number, whereas the population of cells cultivated in Waymouth-FCS_{10} medium increased 4-fold. The stationary phase of L cell growth was reached at 48 hr when the cells were cultivated in Medium 199-FCS_{10}, at 72 hr when cells were grown in WO_5 medium, and at 72 to 96 hr when cells were grown in Waymouth-FCS_{10} medium. In Medium 199-FCS_{10}, decrease of the viable cell population occurred after a 24-hr stationary phase. Cells

remained in stationary phase for 72 to 96 hr in Waymouth-FCS_{10} and for 96 hr in WO_5 medium before the viable cell population decreased. The pH of the medium did not drop below pH 7 during the course of the experiment.

The growth of L cells was supported as well by the defined medium as by the undefined 199-FCS_{10} medium, which is routinely used to grow these cells in spinner cultures (12, 13). Although cells cultivated in Waymouth-FCS_{10} medium reached a slightly higher population than did cells cultivated in Waymouth medium supplemented with BSA and sodium oleate, the latter system provides the defined medium desired.

Effect of various concentrations of sodium oleate in the defined medium. Mouse L cells were grown in Waymouth-BSA medium containing various concentrations of oleic acid to determine the concentration that would produce optimal cell growth (Fig. 2). The peak yield of cells and the persistence of viability were highest in WO_5 and WO_{10} media. The maximum cell densities reached in WO_{20}, WO_{40}, and WO_{80} media were somewhat lower than in WO_5 or WO_{10} media. Increasing the concentration of sodium oleate in the medium also resulted in an earlier decrease in viable cell population. The majority of cells died within 15 min when placed in WO_{160} medium. Cells suspended in unsupplemented Waymouth medium gradually decreased in number. The addition of only fatty acid-free bovine albumin to the Waymouth medium permitted approximately a 50% increase in the cell population within 72 hr before the number of viable cells gradually decreased. L cells grown in WO_1 and $WO_{2.5}$ media (not shown in Fig. 2) showed a slight stimulation in cell growth above that obtained by cells grown in WO_0 medium.

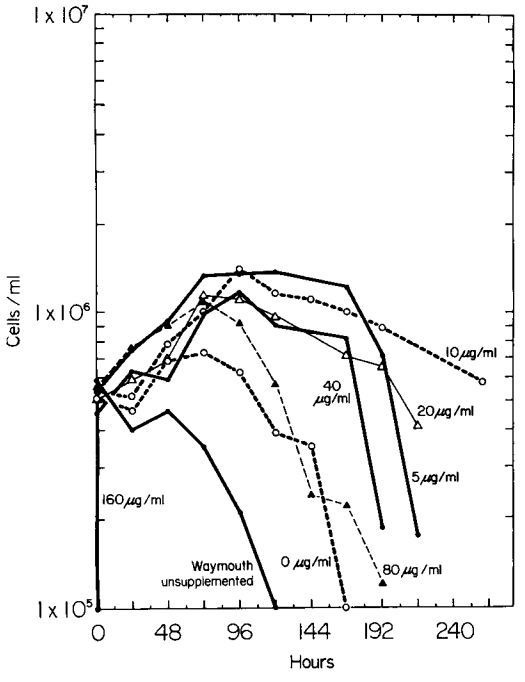

FIG. 2. Growth of L cells in spinner culture in Waymouth MB752/1 medium supplemented with 2 mg of fatty acid-free bovine albumin per ml and various designated concentrations of oleate.

TABLE 1
ANALYSIS OF FATTY ACIDS IN "FATTY ACID-FREE" BOVINE SERUM ALBUMIN

Fatty Acid	Relative Percentage Composition of Fatty Acids	
	Total extractable lipid*	Residue lipid†
14:0‡	1.9	0.2
15:0	0.5	
16:0	32.7	23.7
16:1	6.7	5.1
18:0	15.9	42.3
18:1	11.2	20.0
18:2	23.6	7.0
18:3		1.9
22:0	2.5	
20:4	4.1	

* 249 µg of fatty acid per g of BSA. See "Materials and Methods" for extraction procedure.

† 50 µg of fatty acid per g of BSA. Refer to "Materials and Methods" for alkaline hydrolysis and extraction procedures.

‡ Number of carbon atoms in acid:number of double bonds.

Fatty acid content of fatty acid-free bovine albumin. Because bovine albumin has been reported to contain lipid contaminants (30), a lipid analysis of the fatty acid-free bovine albumin was performed. One gram of BSA contained 249 µg of fatty acid in the extractable lipid. An additional 50 µg of fatty acid per g of BSA remained in the residue after extraction and was removed by alkaline hydrolysis. The relative percentage compositions of the fatty acid methyl esters in the BSA from a typical analysis are presented in Table 1. The addition of 2 mg of BSA per ml to the medium results in the addition of 0.6 µg of fatty acid per ml of medium. The contribution of 18:1 fatty acid by the BSA to the medium is 0.058 µg per ml, which is

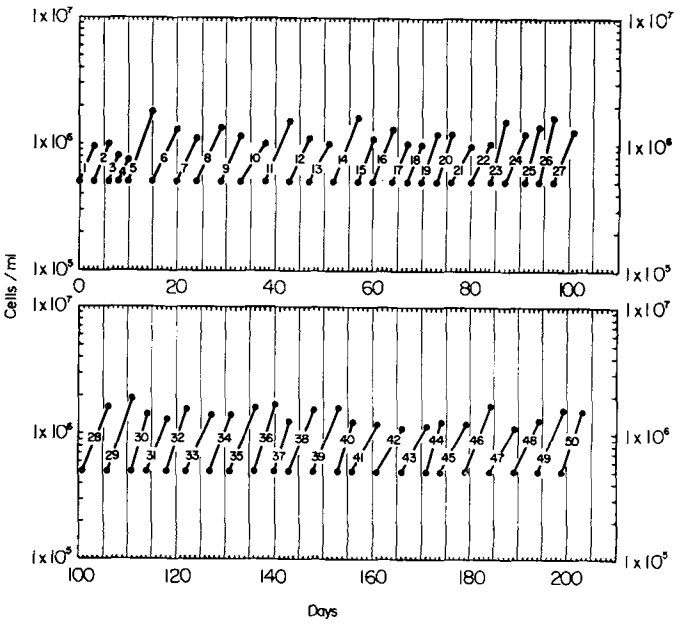

FIG. 3. Continuous passage of L cells in spinner cultures in Waymouth-bovine albumin (2 mg per ml)-oleate (5 μg per ml) medium. Numbers represent passage number.

approximately 1.2% of the oleate added in WO_5 medium. The lipid contaminants in the BSA may contribute to the slight stimulation of growth of spinner L cells in Waymouth medium supplemented only with BSA. However, the levels of cell growth achieved with the addition of various concentrations of oleate to the Waymouth-BSA medium are significantly above the level of growth of L cells in WO_0 medium. The effects of the other fatty acids present in BSA on the growth of spinner L cells have not been investigated. The total lipid was estimated to comprise 0.1 to 0.15% of the bovine albumin. The lipid classes in the total lipid were tentatively identified by TLC as cholesterol, cholesteryl ester, phospholipids, free fatty acids, and triglyceride (listed in order of decreasing quantity based on intensity of the staining reaction). Although small amounts of lipid contaminants were present in the fatty acid-free BSA which was added to the WO medium, the lipid composition has been defined and the fatty acids have been measured quantitatively.

Continuous passage of L cells in spinner culture with WO_5 medium. Fig. 3 illustrates the growth of L cells during the first 50 passages in spinner cultures with WO_5 medium. Cell densities at the time of transfer were usually in the range 1×10^6 to 1.5×10^6 cells per ml. Variations in the rate of growth and final cell population may be due to the differences in ages and stage of growth of the cultures at the time of transfer. Also, variation in the starting population undoubtedly accounted for some differences in final cell populations. No major variations in the growth patterns have been observed during the period of continuous passage of L cells in spinner culture in the WO_5 defined medium.

Growth of meningopneumonitis agent in spinner L cells grown in WO_5 medium. The growth curve of MN agent in L cell spinner cultures in WO_5 medium is shown in Fig. 4. The total input of MN was 5.6×10^6 PFU per ml. The residual titer of 5.8×10^5 PFU per ml observed at 0 hr may be attributed in part to the fact that cells were not washed before suspension in the full volume of medium after absorption. A decrease in MN titer in the medium was observed until 18 hr. Release of chlamydiae into the medium began at 18 hr and continued until 60 hr. The peak MN agent titer was 9.3×10^7 PFU per ml, which was approximately a 1000-fold increase in chlamydial titer between 18 and 60 hr.

Schechter (13) reported that the release of MN from L cells in spinner cultures with Medium 199-CS_{16} began at 10 hr and reached a

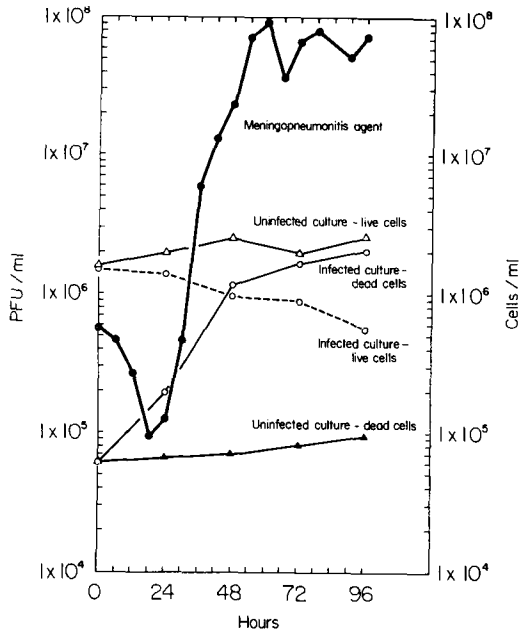

FIG. 4. Growth of *Chlamydia psittaci* strain meningopneumonitis in L cell spinner cultures with Waymouth-bovine albumin (2 mg per ml)-oleate (5 μg per ml) medium: ●——●, meningopneumonitis agent (PFU per ml). Cell counts per ml: △——△, live cells in uninfected culture; ▲——▲, dead cells in uninfected culture; ○- - -○, live cells in infected culture; ○——○, dead cells in infected culture.

peak titer at 40 hr, indicating a more rapid cycle of infection than observed in WO_5 medium. The peak titer of extracellular MN reported by Schechter (13) was approximately $10^{8.5}$ calculated as the egg-LD_{50}, which was slightly higher than that seen for MN cultivated in spinner L cells in WO_5 medium. Based on this comparison and unpublished observations in our laboratory, the defined medium supports growth of MN agent in L cells as well as a serum-containing medium.

The persistence of the initial residual MN and of the released chlamydiae may be due to a stabilizing effect of the WO_5 medium. A similar observation for Japanese encephalitis virus in WO medium was previously made and was attributed to the BSA content of the medium (S. Makino and H. M. Jenkin, submitted for publication).

Counts of the viable and dead cells in both the infected and uninfected L cell spinner cultures are also shown in Fig. 4. There was little death of cells in the uninfected culture during the experiment. There was approximately a 50% increase in cell population in the uninfected culture, indicating that attempts to establish a nonmultiplying cell population were only partially successful. The failure of cells to exclude trypan blue corresponded with cell infection, as was expected.

The techniques described in this report now permit investigation of the lipid metabolism of normal and chlamydiae-infected L cells grown in a defined medium and utilizing spinner cultures.

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The authors thank Tze-ken Yang for the lipid analysis and Lloyd E. Anderson for his excellent technical assistance.

This investigation was supported by Public Health Service Research Grant HE 08214 from the Program Projects Branch, Extramural Programs, National Heart and Lung Institute; The World Health Organization; and The Hormel Foundation.