JOURNAL OF CLINICAL MICROBIOLOGY, June 1990, p. 1098–1100
0095-1137/90/061098-03$02.00/0

Vol. 28, No. 6

Amino Acid Requirements for Growth of *Chlamydia pneumoniae* in Cell Cultures: Growth Enhancement by Lysine or Methionine Depletion

CHO-CHOU KUO[1]* AND J. THOMAS GRAYSTON[1,2]

Departments of Pathobiology[1] and Epidemiology,[2] University of Washington, Seattle, Washington 98195

Received 11 December 1989/Accepted 15 February 1990

Amino acid requirements for growth of two isolates of *Chlamydia pneumoniae* were studied and compared with those for one strain of *Chlamydia trachomatis* in a HeLa 229 cell culture. It was shown that among 13 amino acids in Eagle minimum essential medium, *C. pneumoniae* required all amino acids except lysine. A true requirement for arginine, isoleucine, leucine, threonine, and valine could not be determined because depletion of 100% of these amino acids caused cell detachment. Consequently, a requirement for these amino acids was based on 90% depletion. *C. trachomatis* biovar *trachoma* required all amino acids except threonine, which was indeterminable. Depletion of 100 and 90% of the lysine and 90 to 70% of the methionine was shown to enhance the growth of *C. pneumoniae*. This phenomenon was shown to be a property of *C. pneumoniae* because the effect of lysine and methionine reduction was also demonstrated in another human line, HL cells, and a mouse line, McCoy cells.

Chlamydia pneumoniae is a newly identified *Chlamydia* species of human origin that causes acute respiratory infections (5, 6). It has been estimated that about 10% of community-acquired pneumonias are due to *C. pneumoniae* (7). *C. pneumoniae* grows poorly in cell cultures compared with the growth of the other two species of *Chlamydia* (10). We have used HeLa 229 cells for isolation and propagation of *C. pneumoniae* (10). However, continuous cell culture passage of *C. pneumoniae* isolates is often not possible. To improve the cell culture growth of *C. pneumoniae*, we have been studying the nutritional requirements of *C. pneumoniae* and have been making an effort to find a more sensitive cell line. This study concerns the amino acid requirements of *C. pneumoniae* and findings on the growth enhancement of *C. pneumoniae* by lysine or methionine depletion.

MATERIALS AND METHODS

***Chlamydia* organisms.** *C. pneumoniae* TWAR strains TW-183, AR-39, and AR-388 (10) and *C. trachomatis* E/UW-5/Cx (14) were used in this study. TW-183 was isolated from human conjunctiva; all other TWAR isolates were obtained from the human respiratory tract. These strains were grown in HeLa 229 cell culture to contain 10^8 inclusion-forming units per ml (11). Dilutions of 10^{-3} were used for the experiments.

Cell lines. Two human cell lines, HeLa 229 (9) and HL (2) cells, and one mouse line, McCoy cells (4), were tested.

Reagents. Cell culture-tested amino acids in crystal form were obtained from Sigma Chemical Co., St. Louis, Mo. These were L- arginine, L-cysteine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, and L-valine.

Preparation of amino acid mixture and culture medium. A 50× amino acid mixture, a complete formula, or a complete formula minus a single amino acid depleted 100 or 90% was prepared as described by Eagle (3). The mixture or formula was freshly prepared for each experiment by dissolving amino acid powders in 0.1 N HCl. The preparation was filter sterilized. Glutamine (100×) was prepared separately in distilled water. Culture medium was then freshly prepared each time by the addition of amino acid mixture, glutamine, vitamins (GIBCO Laboratories, Santa Clara, Calif.), 10% heat-inactivated fetal bovine serum, and antibiotics (streptomycin and vancomycin, 100 μg/ml each) to Hanks balanced salt solution; and the pH was adjusted to neutrality with sodium bicarbonate. Fetal bovine serum was dialyzed against normal saline and was filter sterilized before use.

Assay of amino acid requirements. Assays of amino acid requirements were done in vial cultures. The vial used was a disposable, flat-bottomed, 1-dram (4-ml) glass vial shell containing a glass cover slip (diameter, 12 mm) (11). The vial was capped with a no. 0 rubber stopper. A 1-ml suspension of 10^5 cells was inoculated into each tube to obtain a confluent cell layer. Cell monolayers were used 1 day after preparation. Inoculation of organisms was done by first removing the culture medium. Cell monolayers were washed once with Hanks balanced salt solution containing 30 μg of DEAE-dextran per ml. A 0.1-ml amount of organisms was then added. The inoculated vial was centrifuged at 2,200 rpm ($900 \times g$) for 60 min at room temperature. The inoculum was removed. One milliliter of complete or test medium was added. The vials were incubated at 35°C. After incubation for 3 days, the cover slips from two vials were removed, fixed with methanol, and stained with May-Greenwald Giemsa for assessment of cell condition, inclusion morphology, and inclusion counts. Cells from the remaining two vials were harvested by scraping the infected cells off the cover slip. The titers in the harvest were assayed by passage into new cultures, with three vials used for each dilution. Eagle minimum essential medium (GIBCO) containing 10% undialyzed, heat-inactivated fetal bovine serum and 0.9 μg of cyclohexamide per ml was used for culture. After 3 days of incubation, cover slips were fixed with methanol and stained with fluorescein-conjugated, genus-specific monoclonal antibody CF-2 for inclusion counts (10). Titers in terms of inclusion-forming units per milliliter were determined and were used for calculation of the growth rate in the test medium relative to that in the complete medium. Values of

* Corresponding author.

TABLE 1. Requirement of essential amino acids by *C. pneumoniae* and *C. trachomatis* in HeLa 229 cell cultures

Amino acid omitted or reduced	Ratio of infectivity titers[a]		
	C. pneumoniae		*C. trachomatis* biovar *trachoma* E/UW-5/Cx
	AR-39	TW-183	
None	1.0	1.0	1.0
Arginine	(0.4)	(0.2)	(0.002)
Cysteine	0.003	0.00009	0.3
Glutamine	0.8	0.5	0.004
Histidine	0.00006	0.00007	0.0000001
Isoleucine	(0.4)	(1.0)	(0.03)
Leucine	(0.0001)	(0.000007)	(0.00001)
Lysine	15	31	0.0003
Methionine[b]	0.004	0.001	0.0000001
	(3)	(39)	(0.0000001)
Phenylalanine	0.00002	0.000007	0.0000006
Threonine	(0.6)	(0.5)	(0.8)
Tryptophan	0.003	0.00002	0.0000006
Tyrosine	0.0001	0.00001	0.0000007
Valine	(0.0001)	(0.000009)	(0.0000007)

[a] Ratios relative to the complete medium. Values in parentheses are those from 90% amino acid depletion when 100% depletion of amino acids resulted in cell detachment.

[b] Note the inhibition at 100% depletion and the enhancement at 90% depletion with *C. pneumoniae*.

greater than 1 indicated an increased growth rate, and values of less than 1 indicated a decreased growth rate.

RESULTS

Requirements for 13 essential amino acids in Eagle minimum essential medium were assayed by using two TWAR strains, TW-183 and AR-39, and were compared with those of a strain of *C. trachomatis* biovar *trachoma* E/UW-5/Cx. Because complete removal of some amino acids caused cell detachment, assays were done by depleting 100 and 90% of a single amino acid. The following amino acids, when depleted 100%, caused detachment of cell monolayers: arginine, isoleucine, leucine, threonine, and valine. Therefore, a 90% reduction in amino acid concentration, which did not impair the cells, was used to determine whether these amino acids are essential for growth. TWAR strains required all amino acids except lysine for growth (Table 1). Omission of cysteine, histidine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine resulted in greater than 2-log-unit decreases in infectivity titers. Arginine, isoleucine, and threonine seemed to be required because titers decreased 50 to 80%, even when they were depleted 90%. Glutamine was marginally required; only 50 to 80% titer decreases were observed on omission. The *C. trachomatis* biovar *trachoma* strain was shown to require all amino acids except threonine, which was indeterminable. Omission or 90% depletion resulted in greater than 2-log-unit decreases in titers of all amino acids except cysteine. If cysteine was omitted, the titer of UW-5 was reduced by 70%.

It was found that TWAR strains grew better when lysine was totally (Table 1) or partially (see below) depleted. In addition, although methionine was required, growth was enhanced when the concentration was reduced (Table 1). Both phenomena were true for strains TW-183 and AR-39, as well as for an additional strain, AR-388, which is not shown in Table 1. Growth enhancement was observed only when lysine was depleted 90 or 100%. No differences in growth rates were observed when lysine concentrations were reduced 80% or less. The effect of methionine was seen over a range of concentrations. Growth rates were two to three times greater when methionine concentrations were reduced 80 to 60%. An additive effect was not observed when both lysine and methionine concentrations were reduced from 90 to 70% at various combinations. The enhancing effects of lysine and methionine depletion were abolished when HeLa cell metabolism was inhibited with cycloheximide at 0.9 μg/ml, which is the maximum nontoxic concentration for HeLa 229 cells.

To determine whether growth regulation by lysine and methionine was a property of strain TWAR and not a host factor, the effect of reducing lysine and methionine on the growth of TWAR was examined in another human line, HL cells, and one mouse line, McCoy cells. All treatments had the same effect in all three cell lines tested, although the effects were greater in HeLa and HL cells and were less in McCoy cells. The average ratios of infectivity titers relative to those in complete minimum essential medium in the three cell lines (HeLa, HL, McCoy) for the two strains tested were 23, 17.5, and 3, for 100% depletion of lysine; 26, 13, and 2.7 for 90% depletion of lysine; and 21, 8, and 2.1 for 90% depletion of methionine, respectively.

DISCUSSION

Results of this study indicate that growth of *C. pneumoniae* and *C. trachomatis* in cell cultures requires most or all of the 13 essential amino acids in Eagle minimum essential medium. The study also showed that it is not possible to assay for the requirements of all amino acids because complete depletion of some amino acids caused cell death. These amino acids were arginine, isoleucine, leucine, threonine, and valine. We assumed that these amino acids were required if 90% depletion resulted in a significant reduction in growth.

Allan and Pearce (1) studied the amino acid requirements of 10 serovars of *C. trachomatis* and four strains of *C. psittaci* in McCoy cells. The study was based on inclusion counts with Giemsa stain in the first passage at 48 h with *C. trachomatis* and at 26 or 48 h with *C. psittaci* and was based on the percentage of cells bearing inclusions. The toxicity caused by omission of amino acids was not mentioned. Their results were somewhat different from ours in that fewer amino acids were required. For example, they found *C. trachomatis* serovars D through I required only histidine, isoleucine, phenylalanine, valine, and possibly, glutamine. They also found differences in amino acid requirements among strains and suggested that the patterns of amino acid requirements may be used for strain classification. The discrepancy may have arisen from differences in the methods of the infectivity assays that were used in the two studies.

Our finding of growth enhancement by depletion of lysine and methionine seems to be unique for *C. pneumoniae*. The mechanisms of growth regulation of *C. pneumoniae* by lysine and methionine are unknown. It has been shown that the transport of lysine by some extracellular (12) and intracellular (13) bacteria is mediated by membrane potential. Hatch et al. (8) showed that lysine transport in chlamydial reticulate bodies is dependent on exogenous ATP and is inhibited by an inhibitor of ATPase, oligomycin, and a proton ionophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which collapses the membrane potential. It would be of interest to see whether use of these inhibitors produces the same effect as that of depletion of lysine.

Since lysine and methionine depletion enhanced the growth of *C. pneumoniae*, we explored this treatment for cell culture growth of *C. pneumoniae*. However, no additional enhancement was observed when host cell metabolism was inhibited with cycloheximide, a commonly used method for enhancing chlamydial growth in cell culture. Thus, the application of lysine and methionine depletion for routine isolation of *C. pneumoniae* does not appear to have any advantage over use of Eagle minimum essential medium when cycloheximide is used.

ACKNOWLEDGMENTS

This study was supported by Public Health Service research grant AI-21885 from the National Institutes of Health.

We thank Amy Lee for technical assistance.

LITERATURE CITED

1. **Allan, I., and J. H. Pearce.** 1983. Amino acid requirements of strains of *Chlamydia trachomatis* and *C. psittaci* growing in McCoy cells: relationship with clinical syndrome and host origin. J. Gen. Microbiol. **129**:2001–2007.
2. **Cavallaro, J. J., and A. S. Monto.** 1972. HL cells, a sensitive line for the isolation and propagation of respiratory syncytial virus. Proc. Soc. Exp. Biol. Med. **140**:507–510.
3. **Eagle, H.** 1959. Amino acid metabolism in mammalian cell culture. Science **130**:432–437.
4. **Gordon, F. B., H. R. Dressler, A. L. Quan, W. T. McQuilkin, and J. I. Thomas.** 1972. Effect of ionizing irradiation on susceptibility of McCoy cell cultures to *Chlamydia trachomatis*. Appl. Microbiol. **23**:123–129.
5. **Grayston, J. T., C.-C. Kuo, L. A. Campbell, and S.-P. Wang.** 1989. *Chlamydia pneumoniae* sp. nov. for *Chlamydia* sp. strain TWAR. Int. J. Syst. Bacteriol. **39**:88–90.
6. **Grayston, J. T., C.-C. Kuo, S.-P. Wang, and J. Altman.** 1986. A new *Chlamydia psittaci* strain called TWAR from acute respiratory tract infections. N. Engl. J. Med. **315**:161–168.
7. **Grayston, J. T., S. P. Wang, C. C. Kuo, and L. A. Campbell.** 1989. Current knowledge on *Chlamydia pneumoniae*, strain TWAR, an important cause of pneumonia and other acute respiratory diseases. Eur. J. Clin. Microbiol. Infect. Dis. **8**: 191–202.
8. **Hatch, T. P., E. Al-Hossainy, and J. A. Silverman.** 1982. Adenine nucleotide and lysine transport in *Chlamydia psittaci*. J. Bacteriol. **150**:662–670.
9. **Jenkin, H. M.** 1966. The continuous passage of agents of trachoma in cell culture. I. Characteristics of TW-3 and Bour strains of trachoma cultivated in serial passage in HeLa 229 cells. J. Infect. Dis. **116**:390–399.
10. **Kuo, C.-C., H.-H. Chen, S.-P. Wang, and J. T. Grayston.** 1986. Identification of a new group of *Chlamydia psittaci* strains called TWAR. J. Clin. Microbiol. **24**:1034–1037.
11. **Kuo, C.-C., S.-P. Wang, and J. T. Grayston.** 1977. Growth of trachoma organisms in HeLa 229 cell culture, p. 328–336. *In* D. Hobson and K. K. Holmes (ed.), Nonogonococcal urethritis and related infections. American Society for Microbiology, Washington, D.C.
12. **Niven, D. F., and W. A. Hamilton.** 1973. Valinomycin-induced amino acid uptake by *Staphylococcus aureus*. FEBS Lett. **37**:244–248.
13. **Smith, D., and H. H. Winker.** 1977. Characterization of a lysine-specific active transport system in *Rickettsia prowazekii*. J. Bacteriol. **129**:1349–1355.
14. **Wang, S.-P., and J. T. Grayston.** 1970. Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. Am. J. Ophthalmol. **70**:367–374.