PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 859–863 (1975)

The Effect of Mycoplasmas on Replication and Plaquing Ability of Herpes Simplex Virus (38648)

J. E. MANISCHEWITZ, B. G. YOUNG, AND M. F. BARILE

University of Maryland, College Park, Maryland and Food and Drug Administration, Bethesda, Maryland 20014

Mycoplasmas have been shown to alter the yield of certain viruses grown in cell culture (1). They can increase virus yields by inhibiting interferon production and interferon activity, and thereby decrease cell resistance to infection (2), and they can decrease virus yields by either depleting the medium of an essential amino acid (arginine), or by inhibiting cell metabolism or cell growth or by causing cytopathic destruction of the culture. Most mycoplasmas derive energy either from dextrose or from arginine but rarely from both substrates (1). Some of the arginine utilizing mycoplasmas (3) have been shown to decrease the growth of certain arginine requiring DNA viruses (4, 5). This report presents data demonstrating the effect of *Mycoplasma arginini*, on the growth of Herpes simplex virus, type 1 (HSV). The data will show that *M. arginini* can decrease the growth of HSV and that supplementing the medium with additional arginine will reverse this effect.

Materials and Methods. Mycoplasmas. An arginine-utilizing species [(*M. arginini*, strain G-230(7)] and a nonarginine utilizing, but fermenting species (*Acholeplasma laidlawii*, strain PG-8) were used. Pools of mycoplasmas grown in standard broth medium (1) and containing from 10^7 to 10^8 colony-forming units (cfu)/ml were stored at −70° until used.

Cell cultures. The African green monkey kidney (Vero) cells used were prepared by the Tissue Culture Branch, Bureau of Biologics, Bethesda, MD. Cells were monitored routinely for the presence of mycoplasma contamination.

Media and arginine content. Vero cells were grown in 32 oz bottles containing Eagle's Minimal Essential Medium with Earle's balanced salt solution (EMEM) and 10% heat inactivated fetal bovine sera (FBS). After 48 hr of incubation, cells were maintained in Eagle's Basal Medium with Earle's balanced salt solution (EBME) plus 10% FBS.

Media containing three different concentrations of arginine were used. The standard EBME medium containing 0.1 m*M* L-arginine hydrochloride (referred to as "low level") was also supplemented to contain either 0.3 m*M* ("medium level") or 0.6 m*M* ("high level") of arginine. The EMEM medium contains approximately 0.6 m*M* arginine and is comparable to the "high level" EBME medium.

Mycoplasma infection of Vero cells. Five 32 oz bottles were infected with 1 ml of *M. arginini* and five bottles were infected with 1 ml of *A. laidlawii*. A chronic mycoplasma infection developed within 10 days. Five bottles of mycoplasma-free cells were also maintained, treated in the same manner and served as controls. The cells were subcultured periodically to 2 oz flasks for study.

Herpes simplex virus (HSV). The large plaque strain of HSV used was obtained from H. Hopps, Bethesda, MD. A virus pool was prepared in EBME plus 2% FBS and stored at −70° until used. Virus titers were obtained by using a modification of the plaque assay described by Roizman and Roane (8). Briefly, 10^2 plaque forming units (pfu) of HSV/0.5 ml was inoculated into 2 oz flasks of mycoplasma-free Vero cells. After 1 hr, the cells were fed with standard EMBE plus 10% FBS and 1.5% human immune serum globulin (Merck, Sharp and Dohme, West Point, PA) and incubated for 3 days. The cells were then washed three times with phosphate buffered saline (PBS) and stained with a solution (1:4 parts) of saturated alcoholic crystal violet in aqueous ammonium oxalate. The excess stain was removed by washing and the number of plaques were counted. A decrease in HSV titer of 0.5 $\log_{10}$ or greater was considered significant (9).

Copyright © 1975 by the Society for Experimental Biology and Medicine
All rights reserved.

HSV yields in mycoplasma-infected Vero cells. One hundred and fifty 2 oz flasks containing Vero cells were examined: 50 flasks chronically infected with *M. arginini*, 50 flasks chronically infected with *A. laidlawii* and 50 flasks maintained free of mycoplasmas were inoculated with 10^2 pfu of HSV. Cell cultures free of HSV and of mycoplasmas were also examined. After 1 hr of incubation the fluids were decanted and each group of cells was fed with medium containing either a low, medium or high level of arginine. The cells were then incubated and three flasks from each group were titered for HSV periodically during a 3-day period. For virus titration studies, cells were freeze–thawed twice, and then sedimented by centrifugation at 1500 *g* for 10 min. The supernates were collected and stored at −70° until assayed for HSV. All assays were performed in duplicate with three flasks used for each dilution.

Results. The effect of mycoplasmas on Vero cell growth. Viable cell counts were determined using the trypan blue dye exclusion procedure. The results shown in Table I indicated that there was no significant difference in the number of viable Vero cells in the mycoplasma-free vis/a/vis the mycoplasma-infected cell cultures during the period of study. However, the cell density of the *M. arginini*-infected cells was slightly less than that of the mycoplasma-free and *A. laidlawii*-infected cells. Microscopically, the *M. arginini*-infected and the *A. laidlawii*-infected cell cultures were slightly more granular than the mycoplasma-free cells. In addition, the *M. arginini*-infected cells produced a slightly more alkaline and the *A. laidlawii*-infected cells a slightly more acid medium than the mycoplasma-free cells.

The effect of arginine on HSV yields. The HSV titers obtained in mycoplasma-free and mycoplasma-infected Vero cells fed with media containing low, medium and high concentrations of arginine are shown in Fig. 1. When the "low level" medium containing 0.1 m*M* arginine was used, the HSV titer was at least 100-fold lower in *M. arginini*-infected cells than in mycoplasma-free cells. However, when medium containing "high levels" (0.6 m*M*) of arginine was used, there was no significant difference in HSV titer yields in *M. arginini*-infected and mycoplasma-free cells. The data indicate that *M. arginini* decreases the yield of HSV by depleting arginine from the medium and that supplementing the medium with additional arginine can reverse this effect. *A. laidlawii*, the nonarginine user, had no effect on yields of HSV.

Effect of mycoplasmas on plaquing ability of HSV. Plaque assays were performed in both the mycoplasma-free and mycoplasma-infected cells. Results of a typical experiment shown in Table II indicate that *M. arginini* can cause a statistically significant

TABLE I. EFFECT OF MYCOPLASMA INFECTION ON CELL VIABILITY OF VERO CELL CULTURES.

		Number of viable cells ($\log_{10}$)		
Time[a] (days)	Arginine concentration (m*M*)	Mycoplasma-free cells	*A. laidlawii*-infected cells	*M. arginini*- infected cells
1	0.1	6.22	6.28	5.96
2	0.1	6.32	6.39 (−0.06)[b]	6.10 (−0.24)
3	0.1	6.42	6.42	6.19
1	0.3	6.22	6.33	6.03
2	0.3	6.32	6.42 (−0.09)	6.20 (−0.18)
3	0.3	6.42	6.47	6.18
1	0.6	6.24	6.29	6.01
2	0.6	6.34	6.45 (−0.04)	6.19 (−0.21)
3	0.6	6.44	6.42	6.21

[a] = Cells were chronically infected with mycoplasmas and viable cell counts were made for 3 consecutive days.
[b] = Average $\log_{10}$ difference from mycoplasma-free cells.

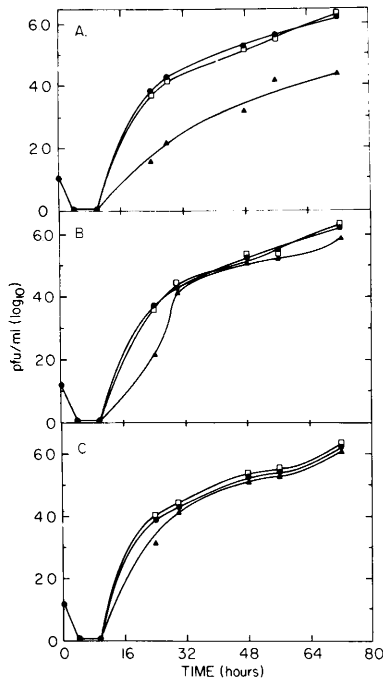

FIG. 1. The effect of arginine concentrations [0.1 mM (A), 0.3 mM (B), 0.6 mM (C)] on yields of HSV grown in *M. arginini* (▲——▲)-infected, *A. laidlawii* (□——□)-infected and mycoplasma-free cells (●——●).

TABLE II. EFFECT OF ARGININE CONCENTRATION ON PLAQUING ABILITY OF HERPES SIMPLEX VIRUS.

	Number of plaques ($\log_{10}$)		
Arginine concentration (mM)	Mycoplasma-free cells	*A. laidlawii*-infected cells	*M. arginini*-infected cells
0.1	2.24	2.23 (−0.01)[a]	1.54 (−0.7)
0.3	2.23	2.22 (−0.01)	1.45 (−0.78)
0.6	2.24	2.24 (0)	1.97 (−0.27)

[a] = Log_{10} difference from mycoplasma-free cells.

reduction in the number of HSV plaques and that supplementing medium with additional arginine can reverse this effect. A significant reduction was seen in Vero cells grown in media with low (0.1 m*M*) and medium (0.3 m*M*) levels of arginine but not in cells grown in medium with high levels of arginine. *M. arginini* also produced a reduction in the size of HSV plaques but it could not be established whether additional arginine alone could reverse this effect (Fig. 2). *A. laidlawii*, the nonarginine utilizer, had no effect on the number or size of HSV plaques produced in Vero cells.

Discussion. Schimke and Barile (10) have shown that certain mycoplasmas (3) can utilize arginine for energy. Accordingly, mycoplasma infection or contamination of cell cultures can result in the rapid depletion of arginine from the medium, depriving the cell culture of an essential amino acid (11) and producing profound effects on cell morphology (1, 12–14) and cell function (15, 16). Miller *et al.* (17) have shown that *M. arginini* can cause cellular lysis of human lymphoblastoid cell cultures, and that supplementing medium with arginine can reverse this effect. Arginine-utilizing mycoplasmas can also inhibit phytohemagglutinin (PHA) stimulation of lymphocyte cultures (18, 19), and Barile and Leventhal (20) showed that arginine supplementation can reverse this effect. In addition, it has been shown that arginine-utilizing mycoplasmas can decrease the yields of several arginine-requiring DNA viruses, e.g., adenovirus type 2 (4) and vaccinia virus (5). Arginine is not required for the early steps of virus replication, including viral DNA synthesis (21, 22), but it is essential for the synthesis of viral coat protein and the production of complete infectious virions.

In this report, we have shown that *M. arginini* (an arginine user) but not *A. laidlawii* (a nonarginine user) can reduce the yield of HSV, and that arginine supplementation can reverse the effect. It would seem reasonable to suggest that arginine-utilizing mycoplasmas may also effect the replication of many other arginine-requiring DNA viruses and that the requirement for arginine by these DNA viruses could be easily and readily established by the *M. arginini* effect. These findings also indicate that the investigators working with HSV are obliged to examine cells for the presence of mycoplasma contamination in order that they might properly interpret the results of study.

Summary. M. arginini, an arginine utilizer, can decrease the yield of Herpes simplex virus, type 1 grown in Vero cells. *M.*

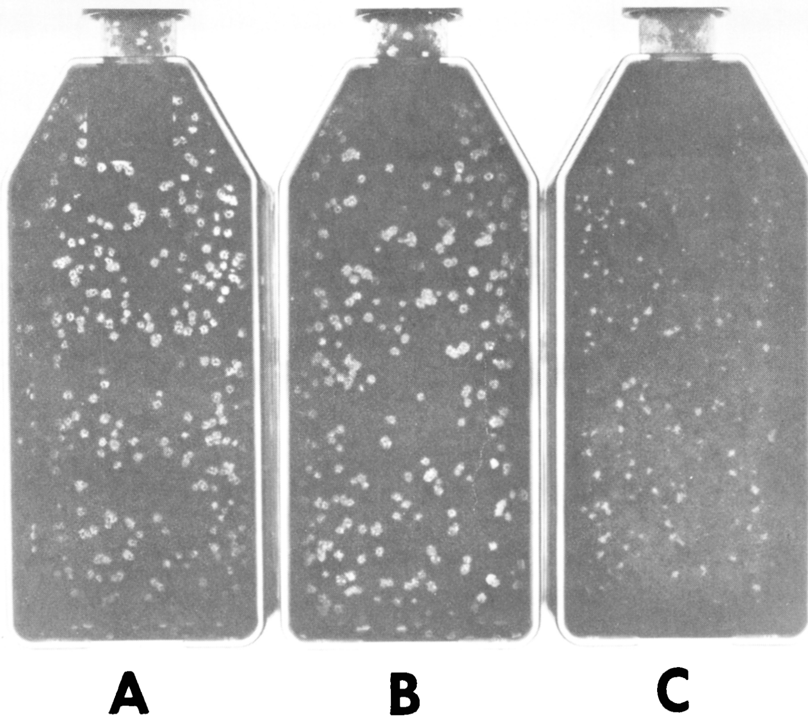

FIG. 2. The effect of *M. arginini* (C) on the reduction in size and number of plaques produced by HSV in Vero cells. *A. laidlawii* (B) had no effect on size of plaques which were similar to those produced in mycoplasma-free Vero cells (A).

arginini can also cause a reduction in number and size of plaques produced by HSV. The reduction in titer and plaque size produced in *M. arginini*-infected cells can be reversed by supplementing medium with additional arginine. *A. laidlawii*, a non-arginine utilizing mycoplasma, had no effect on the growth of HSV.

1. Barile, M. F., *in* "Contamination in Tissue Culture" (J. Fogh, ed.), p. 131. Academic Press, New York (1973).
2. Singer, S. H., Barile, M. F., and Kirschstein, R. L., Proc. Soc. Exp. Biol. Med. **131,** 1129 (1969).
3. Barile, M. F., Schimke, R. T., and Riggs, D. B., J. Bacteriol. **91,** 189 (1966).
4. Rouse, H. C., Bonifas, V. H., and Schlesinger, R. W., Virology **20,** 357 (1963).
5. Singer, S. H., Fitzgerald, E. A., Barile, M. F., and Kirschstein, R. L., Proc. Soc. Exp. Biol. Med. **133,** 1439 (1970).
6. Tankersley, R. W., Jr., J. Bacteriol. **87,** 609 (1964).
7. Barile, M. F., DelGiudice, R. A., Carski, T. R., Gibbs, C. J., and Morris, J. A., Proc. Soc. Exp. Biol. Med. **129,** 489 (1968).
8. Roizman, B., and Roane, P. R., J. Immunol. **87,** 714 (1961).
9. Baron, S., Buckler, C. E., Levy, H. B., and Friedman, R. M., Proc. Soc. Exp. Biol. Med. **125,** 1320 (1967).
10. Schimke, R. T., and Barile, M. F., J. Bacteriol. **86,** 195 (1963).
11. Morgan, J. F., Morton, H. E., and Pasieka, A. E., J. Biol. Chem. **233,** 664 (1958).
12. Kenny, G. E., and Pollock, M. E., J. Infec. Dis. **112,** 7 (1963).
13. Pollock, M. E., Treadwell, P. E., and Kenny, G. E., Exp. Cell Res. **31,** 321 (1963).
14. Fogh, J., Holmgren, N. B., and Ludovici, P. P., *In Vitro* **7,** 26 (1971).
15. Levanthal, B. G., Smith, C. B., Carbone, P. P., and Hersch, E. M., in "Proceedings of the Third Annual Leucocyte Culture Conference" (W. O. Rieke, ed.), p. 519. Appleton, New York (1969).
16. Cherry, J. D., and Taylor-Robinson, D., Bacteriol. Proc., P. 54 (1972).

17. Miller, G., Emmons, J., and Stitt, D., J. Infec. Dis. **124**, 322 (1971).
18. Copperman, R., and Morton, H. E., Proc. Soc. Exp. Biol. Med. **123**, 790 (1966).
19. Cochrum, K. C., Dykman, L., Najarian, J. S., and Fudenberg, H. H., in "Proceedings of the Third Annual Leucocyte Conference" (W. O. Rieke, ed.) p. 169. Appleton, New York (1969).
20. Barile, M. F., and Levanthal, B. G., Nature (London) **219**, 751 (1968).
21. Becker, Y., Olshevsky, V., and Levitt, J., J. Gen. Virol. **1**, 471 (1967).
22. Russell, W. C., and Becker, Y., Virology **35**, 18 (1968).

Received November 6, 1974. P.S.E.B.M. 1975, Vol. 148.