Effect of Mycoplasmas on Vaccinia Virus Growth: Requirement for Arginine (34708)

STANLEY H. SINGER, EDWARD A. FITZGERALD, MICHAEL F. BARILE, AND RUTH L. KIRSCHSTEIN

Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

The widespread distribution of mycoplasmas in cell culture systems and virus pools has been well documented (1, 2). Although, in many cases, dual infection with both mycoplasma and virus has no observable effect on virus replication, in some instances, mycoplasmas either enhance (3–5) or depress (6) virus yield. As part of our continuing studies of these effects, the replication of vaccinia virus was studied in the presence or absence of either of two *Mycoplasma* species.

Materials and Methods. Cells. Pregnant golden Syrian hamsters (*Mesocricetus auratus*) were obtained from the Animal Production Section, Laboratory Aids Branch, NIH. Embryos were harvested on days 14–16 of gestation and cultures of hamster embryo fibroblasts (HEF) were prepared by the Cell Biology Section, Laboratory of Virology and Rickettsiology, DBS, by seeding cells into 2-oz bottles. Chick embryo fibroblast (CEF) monolayers were prepared from 10 to twelve 4-day-old embryos and seeded in a similar manner. Cell sheets were used for testing only when they had become confluent monolayers. A continuous line of human amnion (HA) cells, the FL strain, (7) was prepared in 2-oz bottles by the Cell Biology Section.

Media. Eagle's basal medium containing 2% fetal calf serum and 4.8 mmoles of glutamine was used for maintenance of the cultures, and to prepare all dilutions. This medium contains 0.02 g/liter (0.1 m*M*) of arginine. When the cultures developed as confluent monolayers, the antibiotics were omitted from the media unless otherwise stated.

Mycoplasma species. Mycoplasma arginini, strain G230, an arginine utilizer originally isolated from the brain of a scrapie-infected mouse, and *Mycoplasma hyorhinis,* strain 545, a dextrose fermenter, isolated from an adenovirus 18 pool grown in human embryonic kidney (HEK) cells, were used in these studies. Pools of each mycoplasma containing 10^4–10^7 colony-forming units (cfu)/ml, were prepared as previously described (4).

Establishment of mycoplasma infection. Details of the establishment of chronic mycoplasma infection in the hamster cells have been published elsewhere (3, 4). Identical procedures were used to establish chronic mycoplasma infection of the HA cells. Such infection with each mycoplasma was readily established.

Virus and virus titration. Vaccinia virus, strain WR, was kindly supplied by Dr. Norman Salzman, NIH, Bethesda, Maryland. Prior to seeding cultures, the stock virus was incubated at 37° for 0.5 hr with an equal volume of 0.25% trypsin to minimize aggregation, and then diluted in phosphate-buffered saline (pH 7.3) so that each bottle received approximately 10^2 plaque-forming units (pfu) of virus.

In order to determine vaccinia virus titers, samples were harvested 72 hr after infection, frozen and thawed if necessary, mixed with 0.25% trypsin as above, and then assayed on CEF using a standard plaque titration technique (8) and the modified overlay of Ho and Breinig (9). Plaques were counted 5 days later. Using 4 replicate titrations at 2 dilutions each (5 bottles/dilution) titrations were sufficiently reproducible that a decrease in virus yield of 0.5 $\log_{10}$ or greater was considered significant for these systems at the level of $p \leq 0.05$.

Results. Growth of vaccinia virus. Since it has been reported that, in certain cell culture systems, intracellular titers of vaccinia virus

TABLE I. Vaccinia Virus Yields[a] from Cell Pellets and Supernatant Fluids.

	Hamster embryo fibroblasts			Human amnion (FL) cells		
Exp.	Cell pellet	Supernatant fluid	Ratio of intracellular to extracellular virus	Cell pellet	Supernatant fluid	Ratio of intracellular to extracellular virus
1	7.4	6.6	6.3:1	6.8	6.0	6.3:1
2	7.3	6.4	8:1	6.7	5.9	6.3:1

[a] Expressed as $\log_{10}$ pfu/ml.

exceed extracellular titers by approximately 10-fold (10), we assayed both HEF and HA cells, as well as supernatant fluids of each, 72 hr after each bottle was infected with 10^2 pfu of vaccinia. As shown in Table I, 6–8 times the amount of progeny virus was found to be intracellular as extracellular in both these systems. Therefore, for all further experiments only the cell pack samples were assayed.

Dual infection with mycoplasma and vaccinia. Two to 3 days after initiation of mycoplasma infection when the titers ranged from 10^5 to 10^7 cfu/ml and after viable cell counts had indicated no significant differences between the mycoplasma free and mycoplasma infected cells, all the cultures were further infected with vaccinia. To establish the dual infection, the mycoplasma infected HEF or HA cells were inoculated with approximately 10^2 pfu of vaccinia/bottle. Following incubation at 37° for 1 hr, the monolayers were washed 3 times to remove unadsorbed virus and then refed with Eagle's basal medium. Approximately 72 hr later, when the characteristic vaccinia virus cytopathic effect appeared in approximately 75% of control cultures, the cells were detached from the glass surface by scraping, centrifuged at 1500 rpm for 15 min to form a pellet, suspended in 1 ml of medium and frozen at −70° until assayed.

Effect of chronic mycoplasma infection on vaccinia virus yield. There was a decreased yield of vaccinia virus in cultures previously infected with *M. arginini* (Table II). This

TABLE II. Vaccinia Virus Yields[a] from Mycoplasma Free and Mycoplasma Infected Cells With and Without Additional Arginine.

Cells	Additional[b] arginine	HA	$\log_{10}$ difference from control	HEF	$\log_{10}$ difference from control
Control	No	(a) 5.0[c]		(a) 5.3	
		(b) 6.0		(b) 5.8	
	Yes	(a) 5.1	+0.1	(a) 5.7	(a) +0.4
		(b) 6.6	+0.6	(b) 5.9	(b) +0.1
M. arginini infected	No	(a) 3.5	−1.5	(a) 4.4	(a) −0.9
		(b) 3.1	−2.9	(b) 3.8	(b) −2.0
	Yes	(a) 4.8	−0.2	(a) 5.3	(a) ±0
		(b) 5.6	−0.4	(b) 5.2	(b) −0.6
M. hyorhinis infected	No	(a) 5.0	±0	(a) 5.5	(a) +0.2
		(b) 6.0	±0	(b) ND[d]	(b) ND

[a] Expressed as $\log_{10}$ pfu/ml.
[b] Eagle's basal medium with 0.4 m*M* arginine instead of 0.1 m*M* arginine.
[c] (a) and (b) represent two separate experiments on two different lots of cells.
[d] ND = not done.

decrease was consistent and in a series of experiments, 2 of which are shown in Table II, averaged 1.7 $\log_{10}$ in HA cells and 1.1 $\log_{10}$ in HEF. No such decrease in yield was seen from cultures which had been previously infected with *M. hyorhinis,* the fermenter.

Effect of arginine on vaccinia virus yield. The results described above suggested that arginine utilization by *M. arginini* during the 72-hr period from the time of virus inoculation to the time of harvest might be related to the decreased titer. Experiments then were performed in a similar manner except that, in one group of bottles, medium containing 4 times the usual arginine concentration (0.08 g/liter), was added to *M. arginini*-infected cultures at the time of initiation of vaccinia infection. As shown in Table II, addition of this increased amount of arginine reversed the decrease and yields of vaccinia virus equaled or approximated the levels obtained in cultures free of *M. arginini.* Further studies by Fitzgerald (unpublished data) have shown that cells, not infected by *M. arginini,* but depleted of arginine, yielded significantly less vaccinia virus than do cells not so depleted. The virus thus produced still was 80–90% cell associated.

Discussion. The concept that depletion of available arginine by mycoplasma contamination can result in decreased viral yields has been reported by Rouse *et al.* (6) using human adenovirus type 2 in KB cells. Further studies of adenovirus type 2 (11) and type 5 (12) showed that arginine was not required during the early stages of viral replication and synthesis of viral deoxyribonucleic acid (DNA) but was essential in the final steps of adenovirus assembly. Other reports have indicated that adenovirus type 1 (13), herpes virus (14, 15), SV40 (16), polyoma virus (17) and cytomegalovirus (18) are also dependent on arginine for production of complete infectious viral progeny. Goldblum *et. al.* (16) feel that the requirement for arginine may constitute a common feature of all intranuclear DNA viruses.

This report indicates that growth of vaccinia virus, another DNA-containing virus but one which replicates in the cytoplasm of cells is also dependent on arginine and thus is inhibited by an arginine-utilizing mycoplasma, *M. arginini.* The exact point in the replicative cycle of this virus at which arginine is required, as well as the relative requirement of other amino acids, are currently under investigation.

Our findings would support a concept that arginine-utilizing mycoplasmas are capable of depressing yields of any of the arginine-requiring viruses by competing for available arginine. Many of the well-established species of *Mycoplasma* can not only utilize (19) but also rapidly and completely degradate arginine (20) and such arginine-utilizing *Mycoplasma* species are common contaminants of cell cultures (21). Thus, such contamination can be expected to affect markedly the results of studies not only with adenoviruses as reported previously (6) but now also with vaccinia virus and perhaps all other viruses requiring arginine for replication. It is suggested that investigators should examine cells for the presence of mycoplasmas so that the results of their studies can be properly interpreted.

Summary. Vaccinia virus yields were decreased from cells previously chronically infected with *M. arginini* but not *M. hyorhinis.* This decrease was found to be a result of utilization of arginine from the medium by this mycoplasma. When added arginine was incorporated, vaccinia yields were the same as in cells not infected by mycoplasma. Thus, vaccinia virus was found to be arginine dependent.

We would like to thank Dr. C. F. Maloney for performing the statistical analysis, and Mrs. M. Shaffer for her excellent technical assistance.

1. Carski, T. R., and Shepard, C. C., J. Bacteriol. 81, 626 (1961).

2. Barile, M. F., Malizia, W. F., and Riggs, D. B., J. Bacteriol. 84, 130 (1962).

3. Singer, S. H., Kirschstein, R. L., and Barile, M. F., Nature (London) 222, 1087 (1969).

4. Singer, S. H., Barile, M. F., and Kirschstein, R. L., Proc. Soc. Exp. Biol. Med. 131, 1129 (1969).

5. Gafford, L. G., Sinclair, F., and Randall, C. C., Virology 37, 464 (1969).

6. Rouse, H. C., Bonifas, V. H., and Schlesinger, R. W., Virology 20, 357 (1963).

7. Fogh, J., and Lund, R., Proc. Soc. Exp. Biol. Med. **94,** 532 (1957).

8. Salzman, N. P., Shatkin, A. J., and Sebring, E. D., Virology **19,** 542 (1963).

9. Ho, M., and Breinig, M. K., J. Immunol. **89,** 177 (1962).

10. Joklik, W. K., Bacteriol. Rev. **30,** 33 (1966).

11. Rouse, H. C., and Schlesinger, R. W., Virology **33,** 513 (1967).

12. Russell, W. C., and Becker, Y., Virology **35,** 18 (1968).

13. Dubes, G. R., Moyer, K. B., Halliburton, B. L., and Van de Bogart, R. K., Acta Virol. **13,** 8 (1969).

14. Tankersley, R. W. Jr., J. Bacteriol. **87,** 609 (1964).

15. Becker, Y., Olshevsky, U., and Levitt, J., J. Gen. Virol. **1,** 471 (1967).

16. Goldblum, N., Ravid, Z., and Becker, .Y, J. Gen. Virol. **3,** 143 (1968).

17. Winters, A. L., and Consigli, R. A., Bacteriol. Proc., 182 (1969).

18. Minashima, Y., and Benyesh-Melnick, M., Baceteriol. Proc. 170 (1969).

19. Barile, M. F., Schimke, R. T., and Riggs, D. B., J. Bacteriol. **91,** 189 (1966).

20. Schimke, R. T., and Barile, M. F., J. Bacteriol. **86,** 195 (1963).

21. Barile, M. F., Nat. Cancer Inst. Mongr. **29,** 201 (1968).

Received Dec. 18, 1969. P.S.E.B.M., 1970, Vol. 133.