The Influence of Mycoplasma Infection on the Induction of the Antiviral State by Polyinosinic:Polycytidilic Acid (36374)

S. H. SINGER AND M. FORD
(Introduced by S. Baron)
Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

We have previously reported that chronic mycoplasma infection of hamster cell cultures impairs the ability of these cells not only to synthesize and respond to interferon, but also to respond to a potent interferon inducer, polyinosinic:polycytidylic acid (poly I·poly C) (1). Since the effects of poly I·poly C on a variety of cell cultures are being studied in many laboratories and mycoplasma contamination is widespread (2), we have examined other cell systems. The possible mechanisms by which mycoplasma infection decreases the ability of poly I·poly C to stimulate the antiviral state were also explored.

Materials and Methods. Cell Cultures. Primary mouse embryo fibroblast (MEF) cultures were prepared in two ounce bottles by the Cell Biology Section, Laboratory of Virology and Rickettsiology, DBS, from 15–17-day-old embryos. WI-38 cells were obtained commercially from HEM Research, Rockville, MD. Cell cultures were maintained in Eagle's minimal essential medium (MEME) containing 4.8 mmoles/ml of glutamine to which was added 1–5% fetal calf serum. The serum was obtained commercially from Microbiological Associates, Bethesda, MD and filtered before use in a Nalgene filter unit (10^{-2} micron plain membrane) since filtration was found to reduce the risk of contamination by bovine mycoplasma in cell cultures (3). Antibiotics were omitted from the medium.

Mycoplasma Species. Preparation of pools of *Mycoplasma arginini*, strain G230, and *Mycoplasma hyorhinis*, strain 545 have been described elsewhere (1). Mycoplasma infection was initiated by inoculating approximately 10^5 colony forming units (cfu's) of the appropriate mycoplasma onto complete monolayers. At time of use no cytopathology due to the mycoplasmas was noted in either the MEF or WI-38 cells.

Chemicals. Poly I·poly C was obtained from P-L Biochemicals, Inc., Milwaukee, WI as the double stranded polymer in a physiological salt solution in the lyophilized state and was reconstituted with pyrogen-free water. In experiments with diethylaminoethyl dextran (DEAE-dx), a concentration of 200 μg/ml was employed, and in those with neomycin sulfate, 300 μg/ml was used.

Interferon Assays. These were performed as previously described using a yield reduction method with VSV as the challenge virus (1). The inhibitor was characterized as interferon by its resistance to degradation at pH 2, its species specificity, and its broad antiviral spectrum.

Results. Two or three days after mycoplasma infection the supernatant fluids were decanted and the cultures were exposed for 24 hr to 2.0 ml of maintenance medium containing 50 μg of poly I·poly C/ml. Following this they were challenged with a high multiplicity of vesicular stomatitis virus (VSV), Indiana strain, large plaque variant (MOI 1-10). This high MOI was used since it has been previously reported that mycoplasmas can affect virus replication in mouse cells, but that high MOI minimizes or abolishes this effect (4, 5). Supernatant fluids were collected 16–18 hr after viral challenge and assayed for virus content.

Poly I·poly C readily induced the antiviral state in MEF cultures. However, in cells which had been previously infected with either *M. arginini* or *M. hyorhinis* the degree of viral inhibition was significantly less, being in a series of experiments .025–11% of the

TABLE I. The Effects of Mycoplasmas on the Antiviral State Induced by Poly I·Poly C.

	No poly I·poly C	With poly I·poly C[b]	Log_{10} protection
		Mouse embryo fibroblasts	
No mycoplasma	5.7[a]	1.4	4.3
M. hyorhinis	5.6	4.9	0.7
M. arginini	5.3	4.6	0.7
		WI-38 cells	
No mycoplasma	7.0	5.9	1.1
M. hyorhinis	6.8	6.1	0.7
M. arginini	7.1	6.9	0.2

[a] Titer of VSV yield expressed as $\log_{10}$ PFU/0.2 ml.
[b] Poly I·poly C concentration = 50 μg/ml.

inhibition achieved in mycoplasma free cells. A typical experiment in MEF is demonstrated in Table I. Virus inhibition induced by poly I·poly C was 4.3 $\log_{10}$, but in the presence of either mycoplasma, the degree of inhibition induced by poly I·poly C was 0.7 $\log_{10}$.

In WI-38 cells (Table I) *M. hyorhinis* did not significantly influence the action of poly I·poly C consistently, but in a series of experiments, inhibition in the presence of *M. arginini* was 12–40% of that induced in mycoplasma-free cells.

It has been reported that addition of DEAE-dextran to the culture medium enhances poly I·poly C to stimulate antiviral activity where poly I·poly C by itself is inactive (6, 7). Therefore, MEF were exposed to poly I·poly C as above except that DEAE-dx was also added. As seen in Table II, the degree of viral inhibition induced by poly I·poly C plus DEAE-dx in mycoplasma-infected cells (2.1 and 2.4 $\log_{10}$) was within the range induced in mycoplasma-free cells (2.3 $\log_{10}$). Thus, a complete reversal of the effects of mycoplasma on the action of poly I·poly C was achieved if DEAE-dx is also present.

Supernatant fluids were collected from MEF exposed to poly I·poly C as described above and were assayed for interferon content after acidification at pH 2 for 72 hr (which in a control experiment effectively eliminated any activity of residual poly I·poly C in MEF). The results (Table III) parallel those described above. The amount of interferon found in the supernatant fluids of poly I·poly C treated mycoplasma-free cells was 300 units. In the presence of *M. hyorhinis* and *M. arginini*, 10 units and less than 10 units were found, respectively. The addition of DEAE-dx with poly I·poly C boosted the interferon production to 10,000 units not only in mycoplasma-free cells, but also in the *M. hyorhinis* and *M. arginini*-infected cells with the result that equal amounts of interferon were produced in all cases.

Experiments were performed as above

TABLE II. Reversal by DEAE-dextran of the Effect of Mycoplasmas on Poly I·Poly C in MEF.[a]

	DEAE-dx[b] only	DEAE-dx + poly I·poly C	Log_{10} protection
No mycoplasma	4.3[c]	2.3	2.0
M. hyorhinis	4.4	2.3	2.1
M. arginini	4.5	2.1	2.4

[a] Poly I·poly C concentration = 50 μg/ml.
[b] DEAE-dx concentration = 200 μg/ml.
[c] Titer of VSV yield expressed as $\log_{10}$ PFU/0.2 ml.

TABLE III. Interferon Induced by Poly I • Poly C With and Without DEAE-dx in Mycoplasma Free and Infected MEF Cells.

	Interferon titers[a]
Mycoplasma free + poly I • poly C	300
Mycoplasma free + poly I • poly C + DEAE-dx[b]	10,000
M. hyorhinis + poly I • poly C	10
M. hyorhinis + poly I • poly C + DEAE-dx	10,000
M. arginini + poly I • poly C	<10
M. arginini + poly I • poly C + DEAE-dx	10,000

[a] Units/1 ml.
[b] Poly I • poly C concentration = 50 μg/ml; DEAE-dx concentration = 200 μg/ml.

using neomycin (at a concentration of 300 μg/ml) instead of DEAE-dx since neomycin plus poly I·poly C has been reported to produce effects similar to those seen with the poly I·poly C DEAE-dx complex (8). However, in contrast to the results seen with DEAE-dx and poly I·poly C, the addition of neomycin (Table IV) did not reverse the effect of mycoplasma infection on the action of poly I·poly C. Identical results were obtained when neomycin was used at a concentration of 600 to 900 μg/ml.

Discussion. The results indicate that, as with hamster cells, infection with mycoplasma can also impair the response of mouse and human cells to poly I·poly C. This impaired response, however, has been shown to be reversible in MEF if DEAE-dx is also added. This implies that mycoplasma infection does not impair the cell's ability to respond to poly I·poly C. Since we have never found that infecting cells with mycoplasma alone can produce interferon, the data presented are not likely to be due to the hyporesponsiveness of cells previously stimulated to produce interferon.

TABLE IV. Failure of Neomycin to Reverse the Effect of Mycoplasmas on Poly I • Poly C in MEF.

	Neomycin[a] only	Neomycin + poly I:poly C[b]	Log_{10} protection
No mycoplasma	5.3[c]	3.3	2.0
M. hyorhinis	5.7	5.4	0.3
M. arginini	5.4	5.4	0.1

[a] Neomycin sulfate concentration = 300 μg/ml.
[b] Poly I • poly C concentration = 50 μg/ml.
[c] Titer of VSV yield expressed as log_{10} PFU/0.2 ml.

It has been postulated that DEAE-dx can potentiate the effect of poly I·poly C by either protecting the nucleic acids from being degraded (9) or by an effect of DEAE-dx on cells (7). Ribonuclease production by mycoplasmas has been described (10) and it is possible that degradation of the RNA prior to uptake could be occurring. However, experiments (data not reported) wherein poly I·poly C was exposed to broth cultures of mycoplasma and then tested for activity indicated little or no impairment of the ability to stimulate the antiviral state. This would imply no direct degradation of the complex by cell-free mycoplasma and that DEAE-dx plays no significant role in preventing extracellular degradation of poly I·poly C in this system.

Another possibility is that DEAE-dx itself was toxic to the mycoplasmas thereby producing mycoplasma-free cultures. However, no such effect was found, and recovery of mycoplasma from cultures with DEAE-dx was the same as from cultures without DEAE-dx.

Neomycin has also been reported to enhance the action of poly I·poly C and protect against nucleases (9), and yet, in contrast to DEAE-dx, it did not reverse the effect of mycoplasma contamination on the action of poly I·poly C. This suggests an additional mechanism of action of DEAE-dx which is not provided by neomycin.

This observation of the effect of mycoplasmas on the induction of the antiviral state by poly I·poly C is of significance in any assay

where unsuspected mycoplasma contamination may be present, and it may be part of the explanation as to why different laboratories often get divergent results when testing the same lot of poly I·poly C *in vitro*.

Summary. Mouse embryo fibroblast cells infected with *M. arginini* or *M. hyorhinis* showed a decreased ability to produce interferon and achieve the antiviral state following exposure to a synthetic polynucleotide, poly I·poly C. This effect by the mycoplasmas was reversed by the addition of DEAE-dx but not by neomycin, suggesting a mechanism of action of DEAE-dx not shared by neomycin. WI-38 cells infected with *M. arginini* also showed a decreased ability to respond to poly I·poly C.

The authors wish to thank Drs. Ruth Kirschstein and Michael Barile for their constructive suggestions and Mr. Philip Noguchi for his technical assistance.

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

2. Stanbridge, E., Bacteriol. Rev. **35**, 206 (1971).

3. Barile, M. F., personal communication.

4. Singer, S. H., Ford, M., Barile, M., and Kirschstein, R. L., Proc. Soc. Exp. Biol. Med. **139**, 56 (1972).

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

6. Dianzani, F., Cantagelli, P., Gagnoni, S., and Rita, G., Proc. Soc. Exp. Biol. Med. **128**, 708 (1968).

7. Dianzani, F., Baron, S., Buckler, C. E., and Levy, H. B., Proc. Soc. Exp. Biol. Med. **136**, 1111 (1971).

8. Billiau, A., Buckler, C. E., Dianzani, F., Uhlendorf, C., and Baron, S., Proc. Soc. Exp. Biol. Med. **132**, 790 (1969).

9. Dianzani, F., Gagnoni, S., and Cantagelli, P., presented 2nd Conf. Antiviral Substances, N.Y. May 1969; Ann. N.Y. Acad. Sci. **173**, 727 (1970).

10. Russell, W. C., Nature (London) **212**, 1537 (1966).

Received Nov. 24, 1971. P.S.E.B.M., 1972, Vol. 139.