Enhanced Virus Yields and Decreased Interferon Production in Mycoplasma-Infected Hamster Cells (34053)

STANLEY H. SINGER, MICHAEL F. BARILE, AND RUTH L. KIRSCHSTEIN
(Introduced by S. Baron)

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

Mycoplasma contamination of cell cultures and of virus suspensions is a widespread phenomenon (1–3). When one of our virus pools was found to be contaminated with mycoplasma, studies were initiated to investigate what effects such contamination might have on virus–cell interaction. It was found and subsequently reported (4) that hamster embryo fibroblasts (HEF), infected with *Mycoplasma arginini*, were capable of yielding significantly greater amounts of vesicular stomatitis virus (VSV) than cells free of mycoplasma. It was then decided to investigate the effect of mycoplasma infection on the production of interferon and its assay in primary hamster cells.

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 cell cultures were prepared by the Tissue Culture Section, Laboratory of Virology and Rickettsiology, DBS, by seeding cells into 2 oz bottles. The cell cultures were used when confluent monolayers were obtained, usually in 3–5 days. The average cell count in hamster cultures at the time of their use in the following experiments was 3.5×10^6 cells. Chick embryo fibroblast (CEF) monolayers were prepared from 10–12-day-old embryos and seeded in a similar manner.

Media. Eagle's basal medium containing 2% fetal calf serum and 4.8 mmoles glutamine was used for maintenance of the chick and hamster cultures, and to prepare all dilutions. When the cultures developed as confluent monolayers, the antibiotics were omitted from the media unless otherwise stated.

Viruses and virus titration. Pools of Semliki Forest Virus (SFV) and a large plaque variant of VSV, Indiana strain, were prepared in CEF. Titrations of viruses were performed in CEF using a modified Dulbecco's plaque technique. The final overlay consisted of equal portions of Eagle's plaque base and 1.8% Noble agar with a final concentration of 1:30,000 parts neutral red. VSV and SFV plaques were counted at 48 and 72 hr respectively.

Mycoplasma species. Mycoplasma arginini, strain G230, an arginine utilizer originally isolated from the brain of a scrapie-infected mouse (5), 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 by continuous serial passage in hamster cells.

Mycoplasma titrations. The titers of mycoplasma were determined by the dilution plate count method using the Hayflick type medium (6) for *M. hyorhinis* and the BYE medium (7) for *M. arginini*. Tenfold dilutions were made and duplicate plates containing colony counts between 30–300 were used to establish the mycoplasma titer.

Establishment of mycoplasma infection. To initiate mycoplasma infection in HEF, 1 ml of the particular mycoplasma pool was added to 7 ml of the maintenance media in each bottle. Fluids were changed every 2 days. Noninfected cell cultures used as controls were treated in an identical manner except that maintenance medium was inoculated in place of the mycoplasma.

Interferon assays. Samples for interferon assay were first acidified to pH 2.0 for 48 hr

and then neutralized to pH 7.0. This procedure effectively destroyed the infectivity of all residual virus and mycoplasma. Beginning with a 1:4 dilution, subsequent twofold dilutions were prepared and 3 ml of a sample were incubated in triplicate on homologous cells overnight. After overnight incubation the cell sheets were washed and then challenged with $10^{6.3}$ plaque forming units (PFU) of VSV (as determined on HEF). Eighteen–twenty hr thereafter, cells were observed for cytopathic effect (CPE) and determinations of VSV and mycoplasma yields were performed on aliquots of the supernatant fluids. A decrease in virus yield of 0.5 $\log_{10}$ in treated bottles when compared to that in untreated bottles was considered significant ($p \geqq 0.05$) (8). Interferon titers were expressed as the reciprocal of the highest dilution showing this 0.5 $\log_{10}$ decrease. This material was characterized as interferon by its acid stability (pH 2.0 for 48 hr), heat stability (56° for 1 hr), species specificity, and effect on more than one virus.

Results. Mycoplasma infection of HEF. Infection by either *M. arginini* or *M. hyorhinis* caused a diffuse granularity of the cells which became apparent 2–3 days after infection. Increased acidity was noted in the supernatant fluids of cultures infected with the fermenter, *M. hyorhinis*, but not with *M. arginini*, the nonfermenter. No other overt change in the appearance of the cultures was seen. Viable cell counts performed on mycoplasma-infected and uninfected cultures indicated that the number of cells constituting the monolayer was usually slightly higher in the control bottles after the third day of infection. Therefore, tests were performed generally within the first 2 days after mycoplasma infection when cell counts did not differ significantly. Both mycoplasmas grew well in HEF. Cultures which had been inoculated with 10^5–10^7 cfu of *M. arginini* or 10^4–10^7 cfu of *M. hyorhinis* showed a decrease in titer during the first 24 hr, followed by a rise to a level equal to or greater than that of the inoculum. This level was maintained for periods of more than 1 week despite frequent changes of the media.

Induction of interferon; Mycoplasma. Mycoplasma infection alone did not induce any detectable levels of interferon in the supernatant fluids of infected cells.

SFV. To determine the effect of mycoplasma infection on subsequent interferon induction by a virus, mycoplasma-infected and uninfected cultures in triplicate containing an average of $10^{6.5}$ cells were inoculated with $10^{5.7}$ PFU (as determined on CEF) of SFV. Approximately 18 hr later, when 3–4+CPE (75–100%) was seen in all cultures, aliquots were removed for SFV titration and the remaining material was prepared and assayed for interferon. The results in two typical experiments, 24 and 48 hr after mycoplasma infection, indicate that cells infected with either *M. arginini* or *M. hyorhinis* did not produce as much interferon as mycoplasma free cultures (Table I). The amount of interferon produced in the virus-infected cells varied from test to test with a range of 1:64–1:256. In each case, the interferon levels produced in the mycoplasma-infected cultures were found to be lower, with a range of 1:4–1:8 in *M. arginini*-infected cultures, and a consistent titer of $<$1:4 in *M. hyorhinis*-infected cultures. These results were also seen when excess arginine (0.8 mmole instead of 0.2 mmole) was added to *M. arginini*-infected cultures indicating that arginine depletion in these cultures was not responsible for the decreased interferon production.

SFV replication in mycoplasma-free and infected cells. Table I also shows the titer of the interferon-inducing virus, SFV, at the time that samples were collected for the interferon studies reported above. SFV was able to replicate equally well in both the mycoplasma-free and infected cells and there was no significant difference in yields of virus. However, since the amount of interferon induced by SFV in mycoplasma-treated cells was lower than that induced in control cells, it seemed reasonable to conclude that mycoplasma infection should have an effect on SFV yield. Cultures producing less interferon should yield more virus than cultures producing higher levels of interferon if multiple growth cycles are allowed to occur. Experiments were designed to explore this further by using a much lower input of SFV to

TABLE I. Induction of Interferon by Semliki Forest Virus in Mycoplasma-Free and Mycoplasma-Infected Hamster Cells.

Days after Mycoplasma infection	Cells	Titer SFV (log_{10}) after interferon induction[a]		Titer of interferon induced	
		a[b]	b[b]	a	b
1	Control	7.6	7.3	1:128	1:64
	M. arginini-infected	7.4	7.3	1:8	1:8
	M. hyorhinis-infected	7.6	7.3	<1:4	<1:4
2	Control	7.3	7.5	1:256	1:64
	M. arginini-infected	7.5	7.7	1:8	1:4
	M. hyorhinis-infected	7.5	7.3	<1:4	<1:4

[a] PFU/0.1 ml.

[b] a and b represent two separate experiments on two lots of cells.

initiate the infection. Thus, there would be enough time for the interferon produced during early cycles of virus growth to affect later cycles of virus replication. In these experiments, approximately 10^2 PFU of SFV were inoculated onto *M. hyorhinis*-infected and control cells instead of $10^{5.7}$ PFU. After incubation at 37° for 1 hr, the cell cultures were washed three times to remove residual virus, refed, and then incubated for an additional 24 hr. A significant increase in SFV yield resulted in cultures previously infected with mycoplasma. The best results were seen with *M. hyorhinis*-infected cultures (Table II). Significant increases in virus yield were also seen in *M. arginini*-infected cultures, but the results were less consistent (3 out of 6 tests), possibly reflecting the fact that the decreases in the interferon yields were less pronounced in the *M. arginini*-infected cells than in the *M. hyorhinis*-infected cells.

TABLE II. Increased Semliki Forest Virus Yields from *M. hyorhinis*-infected Hamster Cells.

Titer SFV log_{10}[a,b]			
Control	*M. hyorhinis*-infected	Log_{10} difference from control	% Control
(a) 6.4	(a) 7.3	(a) +0.9	(a) 790
(b) 6.4	(b) 7.3	(b) +0.9	(b) 790
(c) 6.4	(c) 7.1	(c) +0.7	(c) 500

[a] PFU/0.1 ml.

[b] a, b, and c represent three separate experiments on three lots of cells.

Induction of resistance by Poly I • Poly C. Since the synthetic double-stranded RNA, Poly I • Poly C, has been shown to be a potent inducer of interferon in a variety of cells (9, 10), it was decided to attempt to induce intracellular resistance in hamster cells with this synthetic double-stranded polynucleotide and then to determine what effect mycoplasma infection had on the induction of this resistance. HEF were found to be relatively insensitive to induction of viral resistance by Poly I • Poly C. However, significant resistance could be induced at a concentration of 100 μ/ml if the potentiating substance, neomycin (300 μ/ml) (11), was also present, but this concentration of Poly I • Poly C was ineffective if neomycin was omitted from the media. Cultures were overlaid with 3 ml of media containing the Poly I • Poly C and neomycin for 24 hr after which time the media was decanted, the cell sheets were washed three times, and the cultures were challenged with $10^{6.3}$ PFU of VSV. Table III shows that there were significant decreases in VSV yields (1.2-1.9 log_{10}) from mycoplasma-free cells treated with Poly I • Poly C, but insignificant or borderline decreases in yields from the Poly I • Poly C-treated cells which had been previously infected with mycoplasma.

Sensitivity to exogenous IF. A standard hamster interferon preparation was prepared and was assayed on mycoplasma-infected and mycoplasma-free cells. Table IV shows that the mycoplasma-infected cells do possess the

TABLE III. Induction of Viral Resistance by Poly I • Poly C in Mycoplasma-Free and Mycoplasma-Infected Hamster Cells.

Cells	Treatment with Poly I • Poly C[a]	VSV titers (log_{10})[b]	Log_{10} difference from matched control
Control	No	(a) 7.1	
		(b) 6.8	
		(c) 6.5	
Control	Yes	(a) 5.2	(a) —1.9
		(b) 5.1	(b) —1.7
		(c) 5.3	(c) —1.2
M. arginini-infected	No	(a) 7.2	
		(b) 7.1	
		(c) 6.9	
M. arginini-infected	Yes	(a) 6.7	(a) —0.5
		(b) 6.8	(b) —0.3
		(c) 6.6	(c) —0.3
M. hyorhinis-infected	No	(a) 7.2	
		(b) 6.8	
		(c) 6.9	
M. hyorhinis-infected	Yes	(a) 7.1	(a) —0.1
		(b) 6.9	(b) +0.1
		(c) 6.7	(c) —0.2

[a] 100 μ/ml Poly I • Poly C with 300 μ neomycin/ml.

[b] a, b, and c represent three separate experiments on three lots of cells.

ability to respond to exogenous interferon. However, they were found to be four to eight times less able to respond than control cells.

Discussion. The relationship of mycoplasma infection to interferon production has been reported by others. Armstrong and Paucker (12), using *M. hominis*, the Negroni agent, *M. pneumoniae*, and strain F12 were not able to show any effects of these mycoplasmas on interferon production, its induction, or its assay in L or HEK cells. Yershov and Zhdnov (13), found that *M. laidlawi*, *M. agalactiae*, *Mycoplasma* KB, and *Mycoplasma* HeLa did not induce interferon by themselves in chick embryo fibroblasts. However, 24 hr after mycoplasma infection of CEF about four times more interferon could be induced by eastern or Venezuelean equine encephalitis viruses in mycoplasma-infected CEF than in mycoplasma-free CEF. More prolonged mycoplasma infection prior to viral inoculation led to the production of less interferon. Since CPE was apparently seen with these mycoplasmas in chick cells, the authors attributed the decreased interferon levels to the general deterioration of the cultures. No data on the titers of the inducing virus taken at the time of harvest of the supernatant fluids for interferon studies were presented.

TABLE IV. Sensitivity of Mycoplasma-Infected Cells to Exogeneously Applied Interferon.

Cells	Interferon titer[a]		
	a	b	c
Control	1:32	1:32	1:32
M. arginini-infected	1:8	1:4	1:4
M. hyorhinis-infected	1:4	1:4	1:4

[a] a, b, and c represent three different experiments on three separate cell lots.

The results presented in this report indicate that at least two species of *Mycoplasma* can cause a pronounced decrease in the ability of HEF to produce or respond to interferon. Viral transcription, however, was able to proceed unimpaired and, in fact, when a low input of SFV was used to initiate interferon, enhanced virus yields could be obtained from the mycoplasma-infected cells.

If the impaired interferon production was responsible for the viral enhancement, this would explain why this enhancement was seen when a low input of virus was used, but not when a high input was used. With a low input, only a small number of cells would be initially infected, leaving most of the cells available to react to any interferon generated during the first and subsequent growth cycles. In mycoplasma-free cultures, the interferon generated, thus, would have an influence on the net yield of virus obtained, but, in cultures having an impaired ability to produce interferon or react to it (mycoplasma-infected cultures) the effect of interferon would be minimal and higher levels of virus could result. With a high input of virus, a large number of cells would be initially infected, leaving only a relatively small number able to react subsequently with the interferon generated. Thus, there would be little opportunity for the presence or absence of inter-

feron to have an effect on the net virus yield in either the mycoplasma-free or the mycoplasma-infected cells.

The concept that interferon produced during early growth cycles can inhibit later growth cycles and thus have an effect on net virus yields has been discussed by others. Glasgow and Habel (14) using vaccinia virus in mouse embryo cells, and Wong, Baron, and Ward (15) using rubella virus in African green monkey kidney cells have described this. Additional support for this concept comes from experiments in which interferon production was intentionally decreased or blocked and effects on virus yields were evaluated. Hermodsson (16) showed that parainfluenza virus, Type 3, (PIV_3) inhibited interferon production in calf kidney cultures and that dual infection with PIV_3 and Newcastle disease virus (NDV) led to enhanced NDV yields. Heller (17) blocked interferon production in CEF with actinomycin D and showed marked enhancement of Chikungunya virus yields.

The effect of mycoplasma on interferon induction and subsequently on virus yields conceivably could be used to advantage. It may provide a tool to explore situations where the presence of interferon is suspected but not certain. Also, by decreasing interferon production and enhancing virus yields these agents may have a role in the detection of latent viruses.

Results have been presented with two species of *Mycoplasma* in one cell system, HEF. It is pertinent to recall that the genus *Mycoplasma* consists of at least 37 distinct and separate species with widely divergent properties. These species are not only antigenically different, but also have marked differences in their ability to produce disease *in vivo* and CPE *in vitro*, replicate in a given cell line, and utilize substrates (urea, dextrose, or arginine). Different combinations of mycoplasmas and cell lines will have to be studied before it is known to what degree the results presented here can be extended and generalized. However, in at least this one cell system; namely, hamster embryo fibroblast cultures, mycoplasma contamination can have significant effects on virus–host cell interrelationships. Thus, it is clear that unless continuous monitoring is employed, the use of cells inadvertently and unknowingly infected with certain mycoplasmas (*M. hyorhinis*, *M. arginini*, and perhaps other species) could have profound effects on studies dealing with virus growth, interferon production, or interferon assay.

Summary. The effects of mycoplasma infection on interferon production were studied in hamster embryo fibroblasts. Both mycoplasmas chosen for investigation, *M. arginini* and *M. hyorhinis*, were able to replicate and establish a chronic infection in these cells. Neither mycoplasma was able to induce interferon by itself. However, both caused a pronounced decrease in the amount of interferon induced by a virus (Semliki Forest virus) and in the amount of viral resistance induced by a synthetic complexed RNA (polyinosinic and polycytidilic acid). In addition to depressing interferon production, mycoplasma infection rendered the cells less sensitive to exogenously supplied interferon. Viral transcription, however, was not impaired. Rather, under the appropriate conditions, enhanced SFV yields could be obtained from cultures previously infected with mycoplasma.

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