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Induction of Interferon in Mice by Mycoplasmas

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Interferon was induced in mice after intraperitoneal inoculation with four different mycoplasmas. Peak levels of between 100 and 300 U of interferon per ml were attained by 6 h postinfection with each of the mycoplasmas except *Mycoplasma arthritidis*, which induced higher titers (400 to 11,800 U/ml) by this time. A fifth mycoplasma, *M. pulmonis*, induced interferon inconsistently and at a later (72 to 96 h) time. *Mycoplasmatales* virus MVL51 and sterile mycoplasmal broth did not stimulate interferon production in vivo. All of the mycoplasmas and MVL51 failed to induce interferon in murine spleen cell, peritoneal exudate cell, or peripheral blood leukocyte cultures. Preinfecting the mycoplasmas with MVL51 or treating the organisms with trypsin or dilutions of specific antisera did not enhance their ability to induce interferon in vitro.

Recent studies from these laboratories (18, 19) have demonstrated the induction of interferon in ovine leukocytes by several species of *Mycoplasma*. Numerous investigators had previously shown that mycoplasmas failed to induce interferon in chicken embryo (21, 28), mouse L (1), hamster embryo (20), and human embryonic kidney (1) cells. The possibility was considered that the ability of mycoplasmas to induce interferon may not be restricted to ovine reticuloendothelial, lymphoid, and blood cells in vitro. The present studies, therefore, were conducted to determine whether mycoplasmas could induce interferon in mice or in murine cells.

MATERIALS AND METHODS

Cells and media. The mouse L cells used originated from cloned continuous lines (L929) obtained from the American Type Culture Collection cell repository (Rockville, Md.). Fetal lamb kidney cells were prepared as has been previously described (18, 19). The cells were maintained in Eagle minimal essential medium containing 10% (vol/vol) heat-treated (56 C for 30 min) fetal calf serum (GIBCO, Grand Island, N.Y.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 μg of tylosin tartrate per ml (19).

Animal viruses. The Herts strain of Newcastle disease virus (NDV), donated by S. Baron (National Institutes of Health [NIH], Bethesda, Md.), was propagated in embryonated chicken eggs that had been injected by the allantoic route and titered 3.5×10^9 plaque-forming units (PFU) per ml when assayed on primary chicken embryo cells (19). Detailed descriptions of the vesicular stomatitis virus (VSV) (19) and the encephalomyocarditis virus (24) pools and their assay have been reported.

Mycoplasmas. Reference cultures of and antisera to *Mycoplasma pneumoniae* M710-001-084 and *M. pulmonis* M717-001-084 were obtained from the NIH by courtesy of M. F. Barile (Food and Drug Administration, Rockville, Md.). The virulent *M. pulmonis* strain JB, obtained from J. G. Tully (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), was used for the in vivo studies. *M. arthritidis* strain 158 p10 resulted from an animal passage experiment (5). The mycoplasmas were grown in mycoplasmal broth (Difco, Detroit, Mich.) supplemented to final concentrations of 20% (vol/vol) horse serum, 5% (vol/vol) fresh yeast extract, and 1,000 U of penicillin per ml (4, 9). *Acholeplasma laidlawii* strain U2, originally isolated in our laboratories (19), and *A. laidlawii* strain BN1-Nal^r, obtained from J. Maniloff (University of Rochester, School of Medicine and Dentistry, Rochester, N.Y.), were cultured as previously described (18, 19).

Mycoplasmal suspensions were titered for colony-forming units (CFU) by methods previously outlined (19). The identity of the various mycoplasmal species used in this study was confirmed by the growth inhibition test (22) using NIH reference antisera.

Mycoplasmatales virus. Pools of *Mycoplasmatales* virus MVL51, donated by J. Maniloff, were prepared and assayed on lawns of *A. laidlawii* strain BN1-Nal^r as described by Liss and Maniloff (14). The *A. laidlawii* was removed from the viral suspension by successive passage through a 0.22-μm and a 0.1-μm membrane filter supported in a Swinny holder (Millipore Corp., Bedford, Mass.).

Interferon induction in mice. Six-week-old female Swiss Webster random-bred albino mice (Simonsen

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Laboratories, Gilroy, Calif.) were used for these experiments. Animals were injected intraperitoneally with 0.2 to 0.5 ml per mouse containing 1×10^9 to 5×10^9 CFU of *A. laidlawii* strain U2, 8×10^8 to 1×10^{10} CFU of *A. laidlawii* strain BN1-Nal^r, 1.4×10^8 to 2×10^9 CFU of *M. pneumoniae*, 6×10^8 to 1.3×10^{10} CFU of *M. pulmonis*, or 5×10^9 to 2.6×10^{10} CFU of *M. arthritidis*. Mice were injected with MVL51 at a concentration of 2×10^8 PFU per mouse. Serum for interferon assays was collected from blood samples obtained by cardiac puncture at timed intervals. Controls consisted of serum from uninjected mice and animals injected with NDV (0.1 ml of the viral pool resulting in 3.5×10^8 PFU per mouse) or mycoplasmal broth (0.2 to 0.5 ml per mouse).

Interferon induction in murine cells. Peripheral blood leukocyte cultures were derived from the blood of Swiss Webster mice by the following procedures. Mice were bled by cardiac puncture using heparinized syringes. Ten-milliliter portions of the blood pooled from several mice were carefully layered onto 2.5-cm-high columns of a 16:10 ratio of a 1.5% aqueous solution of methylcellulose (Methocel MC, 15 centapoises; Dow Chemical Co., Midland, Mich.) to sodium diatrizoate (aqueous hypaque sodium, 50% [wt/vol]; Winthrop Laboratories, New York) in plastic 50-ml conical tubes (Falcon Plastics, Oxnard, Calif.) (15). After 90 min of incubation at 37 C and gravity sedimentation, the top layer of leukocyte-rich plasma was removed. The combined leukocyte preparations were centrifuged at $200 \times g$ for 10 min, and the cell pellet was treated with a 0.83% (wt/vol) NH_4Cl-tris(hydroxymethyl)aminomethane (Tris) buffer solution (3) for 15 min to lyse residual erythrocytes. After centrifugation, the leukocytes were resuspended in RPMI 1640 media (GIBCO) supplemented with 100 U of penicillin per ml, 2% (vol/vol) heat-inactivated human plasma, and $NaHCO_3$ as a buffer. Cell viability, as detected by trypan blue dye exclusion, was approximately 96%.

For spleen cell preparations, mice were sacrificed by cervical dislocation and the spleens were removed and placed in complete RPMI 1640 media. The spleens were teased with sterile forceps and the cells were filtered through a sterile, stainless-steel wire mesh. After centrifugation at $200 \times g$ for 10 min, the cell preparations were treated with Tris-buffered NH_4Cl, centrifuged, and resuspended in complete RPMI 1640 media. Cell viability was approximately 90%.

Peritoneal exudate cells were harvested from mice that had been injected intraperitoneally 5 days previously with 2 ml of sterile thioglycolate broth (Difco). After centrifugation, the peritoneal exudate cells were resuspended in medium 199 (GIBCO) supplemented with 20% (vol/vol) heat-inactivated fetal calf serum, 100 U of penicillin per ml, and $NaHCO_3$ as a buffer. Viability of the peritoneal exudate cell preparations ranged from 82 to 90%.

All of the murine cell preparations were suspended to a final concentration of 2×10^6 cells/ml in their respective media. The cells were infected with the various mycoplasmas at an inoculum of 0.5 CFU/cell and with NDV or MVL51 at a multiplicity of infection of 1.0 PFU/cell. Controls consisted of uninfected murine cells with and without sterile mycoplasmal broth. The infected cell cultures were distributed in 2-ml portions into glass culture tubes and incubated at 37 C in 5% CO_2 and humidity. Samples were harvested and assayed for mycoplasma as has been reported (19).

Interferon assay. Interferon samples were harvested as previously described (19) and were assayed by the 50% plaque reduction technique on mouse L cells, with VSV as the challenge virus (24). An internal, laboratory reference interferon preparation was included with each assay and consistently titered between 3,000 and 6,000 U/ml. The international reference mouse interferon obtained from NIH titered approximately 350 U/ml in our system as compared with the designated titer of 500 U/ml. Since occasional interfering activity was observed with serum from uninfected mice at dilutions of less than 1:50, all negative results were expressed as $<1:50$.

RESULTS

Induction of interferon in mice. Four of five different types of mycoplasmas were capable of inducing interferon in mice after intraperitoneal inoculation (Table 1). The two strains of *A. laidlawii* and the strain of *M. pneumoniae* induced peak levels of interferon of approximately 100 to 300 U/ml by 6 h post-inoculation. *M. arthritidis* appeared to be a more potent inducer of interferon than the other mycoplasmas, inducing from 400 to 11,800 U/ml by 6 h. Reducing the inoculum size of *M. arthritidis* resulted in progressively lower levels of interferon in the serum of mice obtained 6 h after injection (Table 2). *M. arthritidis* at a concentration of 6×10^6 CFU still induced 150 U of interferon per ml, whereas 6×10^5 CFU did not induce detectable levels. Although *M. pulmonis* did not induce interferon in the first 48 h in any of the four experiments, 280 U/ml was observed in the serum at 72 h in experiment 2 and 80 U/ml at 96 h in experiment 4 (Table 1).

All of the mycoplasmas except *A. laidlawii* strain BN1-Nal^r have previously been demonstrated to induce interferon in sheep leukocytes (18). In additional experiments, *A. laidlawii* strain BN1-Nal^r has been shown to be unable to induce interferon in blood leukocytes from different sheep donors, in contrast to its ability to stimulate interferon production in mice.

The peak production of interferon induced by NDV in mice is known to be at 6 h (24). In three separate experiments, serum samples from NDV-infected mice contained between 1,650 and 16,450 U of interferon per ml by this time. Interferon was not detectable in the sera of mice after injection with *Mycoplasmatales* virus MVL51 or mycoplasmal broth.

TABLE 1. *Interferon production in mice after intraperitoneal inoculation with mycoplasmas*

Inducer	Expt no.	Serum interferon level (U/ml of serum)				
		2[a]	6	12	24	48
Acholeplasma laidlawii strain U2	1	—[b]	100	—	<50	<50
	2	180	300	<50	<50	<50
	3	<50	<50	<50	<50	—
A. laidlawii strain BN1-Nal[r]	1	—	260	—	<50	<50
	2	<50	340	290	90	<50
	3	<50	240	150	<50	—
Mycoplasma pneumoniae	1	—	<50	—	<50	<50
	2	60	230	260	100	<50
	3	<50	220	110	<50	—
M. arthritidis	1	—	11,800	—	100	<50
	2	<50	3,400	3,750	90	<50
	4	600[c]	500	160	—	—
	5	<50	400	80	—	—
M. pulmonis	2	<50	<50	<50	<50	<50
	3	<50	<50	<50	<50	<50
	4	<50	<50	<50	<50	<50
	5	<50	<50	<50	<50	<50
Mycoplasmatales virus MVL51	1	—	<50	—	<50	<50
	2	<50	<50	<50	<50	<50
Newcastle disease virus	1	—	1,650	—	—	—
	2	—	6,600	—	—	—
	3	—	16,450	—	—	—

[a] Hours postinoculation.
[b] No sample taken.
[c] Sample taken at 2.5 h after mycoplasmal inoculation.

TABLE 2. *Interferon production in mice 6 h after intraperitoneal inoculation of serial dilutions of M. arthritidis in broth*

Amt of *M. arthritidis* inoculated (CFU/mouse)	Serum interferon level (U/ml of serum)
6×10^9	3,400
6×10^8	700
6×10^7	165
6×10^6	150
6×10^5	<50

The mycoplasma-induced antiviral substance in the serum of mice was characterized as interferon by the following criteria: (i) inactivation by trypsin treatment; (ii) resistance to pH 2 for 24 h at 4 C; (iii) no detectable activity in fetal lamb kidney cells; (iv) stability at 56 C for 30 min; (v) activity against both VSV and encephalomyocarditis virus in L cells; (vi) inability to inactivate VSV directly; and (vii) lack of activity in L cells treated with dactinomycin.

Absence of interferon production in mycoplasma-infected murine cell cultures. In repeated experiments, the five mycoplasmas used in these studies failed to induce detectable levels of interferon through 3 days of incubation in murine peripheral blood leukocyte, spleen cell, and peritoneal cell cultures. All of the mycoplasmas replicated in the mouse cell cultures as witnessed by a rise in mycoplasmal titers over the 3 days of infection. As a control, known inducer of interferon, NDV induced from 100 to 700 U of interferon per ml in the mouse cells by 24 h post-inoculation. No interferon was detectable in uninfected murine cells or in cell cultures inoculated with MVL51.

Attempts were made to enhance the ability of mycoplasmas to induce interferon in murine cells. The possibility was considered that a *Mycoplasmatales* virus might increase the induction of interferon by mycoplasmas. Therefore, the mycoplasmas were infected with MVL51 at a multiplicity of infection of approximately 1 PFU per mycoplasma, incubated at room temperature for 1 h to allow adsorption, and added to the mouse peritoneal exudate or spleen cells. Identical numbers of untreated mycoplasmal and viral suspensions were concurrently tested as controls. Interferon was not detected in these infected cell cultures over 3 days of incubation.

Other investigators have demonstrated that treatment of *M. pulmonis* with trypsin (12) or specific antiserum (11) greatly increased the ingestion of the mycoplasma by murine macrophages in vitro. Therefore, in these same experi-

ments the various mycoplasmas were centrifuged at 27,000 × *g* for 25 min at 4 C and the mycoplasmal pellets were treated with 100 μg of a trypsin sodium citrate-potassium chloride solution for 15 min at 37 C. After recentrifugation, the cell pellets were resuspended to the original volume in mycoplasmal broth and added to the mouse peritoneal exudate or spleen cell cultures. In addition, all of the mycoplasmas with the exception of *A. laidlawii* strain BN1-Nal^r were treated with a 1:50 and 1:500 dilution of specific antisera for 15 min at 37 C and added to the mouse cells. Neither of these mycoplasmal treatments resulted in the induction of interferon in the murine cell cultures.

Recently two types of mouse interferon, types I and II, have been distinguished by differences in their properties of characterization (29). Studies were performed to demonstrate whether the mycoplasmas were inducing the production of acid-labile, type II interferon in the murine cells that was being inactivated by the treatment at pH 2 during the standard assay procedures. Serial dilutions of non-acid-treated, 72-h samples from each mycoplasma-mouse cell combination were assayed for interferon. Interferon was not detected in any of these samples.

DISCUSSION

The data presented indicate that three different species of *Mycoplasma* and *Acholeplasma* can induce interferon in mice. These results demonstrate that animal species other than the sheep (18, 19) are capable of producing interferon in response to mycoplasmal infection. Peak levels of interferon were detected in the serum by 6 h postinfection, confirming earlier, unpublished data of W. R. Stinebring and J. S. Youngner (personal communication) in which low levels of interferon were induced in mice 6 h after intravenous inoculation of *M. pneumoniae*. However, other investigators have recently shown (B. Fauconnier and J. Wroblewski, personal communication) that an *Acholeplasma* species isolated from plants induced peak titers of circulating interferon in mice by 48 h post-inoculation that were comparable with the titers observed in our study by 6 h post-inoculation.

The present results show that the kinetics of the interferon response to mycoplasmal infection are quite similar to that observed with nonreplicating viral inducers of interferon in mice (2, 24). This suggests that similar mechanisms of induction of interferon in vivo by these agents may be occurring.

M. pulmonis induced interferon in the serum of mice inconsistently and at a delayed time period (72 h in one experiment and 96 h in another) when compared with the other mycoplasmas and NDV. Although titrations of the mycoplasmas were not performed on the serum samples, it is possible that the late induction of interferon by *M. pulmonis* is the result of replication of this mycoplasma in mouse tissues. Further studies need to be performed, however, to determine whether mycoplasmal replication or other mechanisms are responsible for the late induction of interferon.

The failure of mycoplasmas to induce interferon in murine peripheral blood leukocytes, spleen cells, and peritoneal exudate cells is of significance, especially in view of the ability of mycoplasmas to induce interferon in sheep blood leukocytes (18, 19) and spleen cells (unpublished data). In this regard, other studies have shown that tilorone hydrochloride can induce interferon in mice but cannot stimulate interferon production in mouse cell cultures (7, 25). Similarly, the interferon response induced by endotoxin in vitro is undetectable (26) or relatively low (8) compared with the levels of interferon induced in vivo (23).

Several possible factors may be involved in the inability of mycoplasmas to induce interferon in murine cells. First, it is reasonable to postulate that the cultural conditions used in these studies may not have been ideal for the induction of interferon by the mycoplasmas. However, all of the mycoplasmas replicated in the cell systems, indicating that the cultural conditions provided sufficient nutritional requirements for the organisms. The tissue culture media used in these experiments are also known to support optimal growth and functional capacity of macrophages (27) and lymphocytes (16). As evidence for this, NDV routinely induced between 100 and 700 U of interferon per ml in these cells.

Whereas the mechanism of interferon induction by mycoplasmas remains unknown, present information does not suggest a correlation between the induction of interferon and either attachment to cells or ingestion of the mycoplasmas by cells. For example, it has been shown that *M. pulmonis* attaches to murine cells (B. C. Cole, unpublished data) and can be ingested by mouse peritoneal macrophages (6, 11) more readily than is *M. arthritidis* (6). Both of these mycoplasmas failed to induce interferon in mouse cells in vitro, and only *M. arthritidis* regularly induced mouse interferon in vivo.

Attempts were made to enhance the capacity of the mycoplasmas to induce interferon in vitro. Treatments of the mycoplasmas with trypsin and specific antisera, which have been shown to increase the ingestion of *M. pulmonis*

by murine macrophages (11, 12), failed to result in interferon production in spleen or peritoneal exudate cells. Furthermore, preinfecting the mycoplasmas with *Mycoplasmatales* virus MVL51 or using the virus alone did not lead to the induction of interferon in the murine cells. These studies do not eliminate the possibility that further attempts at varying the cultural conditions and altering the mycoplasmas may lead to interferon production in mouse cells.

In comparing the present results with those published earlier (18), it is clear that some specificity is operative in that those species of mycoplasma that induce high or moderate levels of interferon in one host do not necessarily induce similar levels in another host. Whether this phenomenon is related to specific adsorption sites on the animal cell, to ability of the mycoplasmas to penetrate or replicate in the host cell, or to some other mechanism remains to be determined.

The induction of interferon by mycoplasmas in vivo may have significant implications in host resistance to viral infection. Data concerning the effects of mycoplasmas on the susceptibility of the host to viral infection are limited. Investigations have shown that dual infection with mycoplasmas and animal viruses produce a more severe disease in avian (10, 17) and porcine (13) hosts. The possible effects of mycoplasma-induced interferon production on viral pathogenesis remain to be elucidated.

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