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Induction of Interferon in Ovine Leukocytes by Species of Mycoplasma and Acholeplasma[1] (38158)

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In a recent study from this laboratory (1) it was shown that infection of ovine leukocyte cultures with *Acholeplasma laidlawii* was associated with the induction of interferon. Previously it had been demonstrated that mycoplasmas did not induce interferon in animal cell cultures (2–5). An important question posed by our initial study was whether species of mycoplasma other than *A. laidlawii* are capable of inducing interferon. The present report confirms and extends these earlier data and documents the ability of a number of species of *Mycoplasma* and *Acholeplasma* to induce interferon in sheep leukocytes.

Material and Methods. Cells and media. Procedures for the culture of fetal lamb kidney (FLK) cells and other cell types employed in these studies have been previously described (1). The cells were maintained in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum, 100 units/ml of penicillin, 25 μg/ml of tylosin tartrate, and 100 μg/ml of streptomycin. The FLK cells were cultured in 250 ml tissue culture flasks (Falcon Plastics, Oxnard, Calif.) and used between the tenth and sixtieth passage.

Animal viruses. A detailed description of the bluetongue virus (BTV), vesicular stomatitis virus (VSV), and Sindbis virus pools and their assay has been reported (1).

Mycoplasmas. The following reference cultures and antisera were obtained from the NIH, Bethesda, Maryland, by courtesy of Dr. M. F. Barile (Division of Bacterial Products, FDA, Rockville, Maryland): *M. pneumoniae* M710-001-084, *M. hominis* M711-002-084, *M. salivarium* M712-002-084, *M. fermentans* M713-002-084, *M. orale* type 1 M714-001-084, *M. orale* type 2 M714-012-084, *M. pulmonis* M717-001-084, *M. hyorhinis* M718-001-084, *A. granularum* M719-001-084, *M. gallisepticum* M722-001-084, *M. neurolyticum* M723-001-084, *M. arginini* M732-001-084. *Mycoplasma mycoides* var. *capri* (used by permission of the USDA) was originally obtained from Dr. D. G. ff Edward (Public Health Lab, Dulwich Hospital, London), *M. hyosynoviae* 25591 from Dr. R. F. Ross (Veterinary Medical Research Inst., Ames, Iowa), and *A. axanthum* S-743 from Dr. J. G. Tully (NIAID, Bethesda, Maryland). *Acholeplasma laidlawii* U2 was isolated in our own laboratory as previously described (1) and *M. arthritidis* strain 158 P10 resulted from an animal passage experiment (6). Unless stated otherwise, mycoplasmas were grown in mycoplasmal broth (Difco) supplemented to final concentrations of 20% horse serum, 5% fresh yeast extract, and 1000 units/ml of penicillin (7, 8). *Acholeplasma laidlawii* was cultured as described previously (1). In order to obtain sufficiently high counts of *M. pneumoniae,* the organisms were harvested from cultures on mycoplasmal agar (7, 8) incubated for 6 days at 37°.

Mycoplasmal suspensions and leukocyte cultures were titered for viable mycoplasmas by methods previously outlined (1). The identity of the various mycoplasmal species used in this study was confirmed by use of the growth inhibition test (9) employing NIH reference antisera.

Mycoplasmatales virus. The Mycoplasmatales virus, MVL51, and its host *A. laidlawii*

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strain BN1-Nal^r (10, 11) were obtained from Dr. J. Maniloff (Univ. of Rochester, Sch. of Medicine and Dentistry, Rochester, N.Y.). Viral pools were prepared and assayed on laws of *A. laidlawii* strain BN1-Nal^r as described by Liss and Maniloff (10). The *A. laidlawii* was removed from the viral suspension by successive passage through a 0.22 μm and a 0.1 μm MF membrane filter supported in a Swinny holder (Millipore Corp., Bedford, Mass.).

Interferon induction and assay. Ovine leukocytes were derived from the blood of adult sheep (1) and were suspended to a final concentration of 2×10^6 cells/ml in MEM containing 10% fetal calf serum and 100 units/ml of penicillin, but no streptomycin or tylosin tartrate. In most cases, the leukocyte cultures were infected with the various mycoplasmal species at an inoculum of 0.5 colony forming units (CFU)/cell. In certain experiments, however, the range of inoculum was from 0.05 to 5.0 CFU/cell, dependent upon the titers of the mycoplasmal pools utilized. Leukocytes were infected with BTV at a multiplicity of infection (m.o.i.) of 0.15 plaque forming units (PFU)/cell and with Mycoplasmatales virus MVL51 at a m.o.i. ranging between 0.05–5.0 PFU/cell. The infected cell cultures were incubated at 37° in 5% CO_2 and humidity, and samples were harvested each day and assayed for mycoplasma and interferon. The interferon samples were assayed by the 50% plaque reduction technique on FLK cells using VSV as the challenge virus (1).

Results. Induction of interferon by mycoplasmas. Table I summarizes the results from a series of experiments which demonstrate that 12 different species of *Mycoplasma* and *Acholeplasma* are capable of inducing interferon in ovine leukocyte cultures. Based on our earlier studies using *A. laidlawii* (1) the most suitable inoculum was found to be approximately 10^6 CFU/ml of the leukocyte cultures. In general, certain mycoplasmal species (e.g., *M. pneumoniae*) appear to be more potent inducers of interferon than others (e.g., *M. hominis*), but none were as effective inducers as BTV. Mean levels of interferon induced by the mycoplasmas reached peak amounts by 24–48 hr postinoculation, whereas those induced by the animal virus, BTV, were achieved by 24 hr. For these experiments, leukocytes were taken from two different animals, and no difference was found in the relative capacity of different mycoplasmal species to induce interferon in the cells from either

TABLE I. Induction of Interferon by *Mycoplasma* and *Acholeplasma* Species in Ovine Leukocytes.

Inducer	No. of experiments	Mean interferon levels after 24 hr	48 hr	72 hr
Bluetongue virus	4	1210[a]	1240	1150
M. pneumoniae	4	235	960	480
A. laidlawii	4	135	395	245
M. orale type 1	3	115	285	455
M. pulmonis	4	65	280	190
M. fermentans	4	100	205	180
M. arthritidis	4	55	115	135
M. hyorhinis	4	<20	90	165
M. orale type 2	3	20	60	70
M. gallisepticum	2	60	60	55
A. granularum	3	30	55	55
M. salivarium	2	40	40	25
M. hominis	3	30	25	35
M. neurolyticum	2	<20	<20	20
M. arginini	4	<20	<20	<20
A. axanthum	3	<20	<20	<20
M. hyosynoviae	2	<20	<20	<20
M. mycoides var. *capri*	3	<20	<20	<20
MVL51 virus	3	<20	<20	<20

[a] Mean interferon titers are expressed as units/ml.

TABLE II. Experimental Variation in Interferon Production and Mycoplasmal Replication in Ovine Leukocyte Cultures Infected with *M. pneumoniae.*

Expt	Assay	Time postinoculation (hr) 0	24	48	72
A	Mycoplasma	4.02[a]	4.41	4.52	5.08
	Interferon	—[b]	20[c]	35	25
B	Mycoplasma	4.33	5.63	6.36	6.90
	Interferon	—	20	400	200
C	Mycoplasma	6.10	6.50	6.96	7.30
	Interferon	—	110	220	130
D	Mycoplasma	6.14	7.23	7.99	8.11
	Interferon	—	835	3180	1570

[a] Mycoplasmal titrations expressed as $\log_{10}$ CFU/ml.

[b] No sample taken.

[c] Interferon titers expressed as units/ml.

of these sheep.

Two mycoplasmal species, *M. neurolyticum* and *M. arginini,* induced only low levels of interferon (20–40 units/ml) in one experiment and failed to induce interferon in the other experiments performed. *A. axanthum, M. hyosynoviae* and *M. mycoides* var. *capri* did not induce detectable levels of interferon in any of these experiments.

The interferon titers obtained with each mycoplasmal species varied considerably from experiment to experiment although similar inocula were used. Data in Table II show the experiment-to-experiment variation in the interferon response with a representative mycoplasmal inducer, *M. pneumoniae.* A similar variation was also noted with the other 11 mycoplasmas which consistently induced interferon.

Attempt to induce interferon with a Mycoplasmatales virus. We have previously postulated that Mycoplasmatales viruses may play a role in the induction of interferon by mycoplasmas (1). The present studies indicate that a Mycoplasmatales virus, MVL51, failed to induce interferon in three separate experiments using leukocytes from one ewe (Table I). There was neither evidence of a significant decrease in PFU/ml nor detectable replication of the Mycoplasmatales virus in the leukocyte cultures throughout the course of the experiments.

Mycoplasmal replication and leukocyte viability. All 17 species of mycoplasma were capable of replicating in the sheep leukocyte cultures, although some species demonstrated this inconsistently. Using *M. pneumoniae,* a correlation was found between the degree of replication and the amount of interferon produced (Table II). Higher levels of interferon were induced in those experiments where the greatest amount of mycoplasmal replication occurred. This correlation was also demonstrated for *A. granularum.* With the remaining 15 mycoplasmas, the range of inoculum did not vary enough between experiments to allow such relationships between replication and interferon production to be drawn. Evidence was obtained from several experiments that, providing the initial inoculum was sufficiently high, further replication of the mycoplasmas was not necessary for induction of interferon. For example, in one experiment using *M. pulmonis,* the mycoplasmal titers decreased from $10^{3.6}$ CFU/ml initially to undetectable numbers by 24 hr of incubation, yet interferon titers had reached 105 units/ml by this time period.

On the other hand, *M. mycoides* var. *capri,* a natural pathogen of sheep (12), multiplied quite well in the cell cultures yet failed to induce detectable interferon (Table I). The titer of *M. mycoides* var. *capri* rose rapidly from the initial inoculum of $10^{4.8}$–$10^{6.8}$ CFU/ml to greater than $10^{8.7}$ CFU/ml by 48 hr in several experiments. As the replication of this mycoplasma routinely changed the media to pH 6 by 48 hr, it is possible that the organism

TABLE III. Leukocyte Viability in *M. mycoides* var. *capri* and *M. pneumoniae* Infected and Control Cultures.

Expt	Inducer added	m.o.i.[a]	% Leukocyte viability after 0 hr	24 hr	48 hr	72 hr
B	None	—	95[b]	84	83	69
	Broth	—	95	78	73	74
	M mycoides var. *capri*	1.30	95	86	65	62
	M pneumoniae	0.70	95	84	75	59
D	None	—	92	88	65	62
	Broth	—	92	90	75	66
	M. mycoides var. *capri*	0.03	92	79	84	47
	M. mycoides var. *capri*	3.00	92	76	79	53
	M. mycoides[c] var. *capri*	3.00	92	74	69	47
	M. pneumoniae	0.01	92	94	75	45

[a] Multiplicity of infection.

[b] % Viable leukocytes detected by the trypan blue dye exclusion technique.

[c] The pH in this sample was maintained at 7.4 with $NaHCO_3$.

damaged or killed the leukocytes before they could produce detectable interferon. To examine this possibility, leukocyte viability was monitored in portions of the daily samples using trypan blue due exclusion. In addition, in one experiment (Table III, Expt D), cultures infected with *M. mycoides* var. *capri* were maintained at pH 7.4 with 1 *N* $NaHCO_3$. Data from two experiments (Table III, Expts B and D) illustrate that although *M. mycoides* var. *capri* infection did not result in a substantial loss in leukocyte viability through 48 hr as compared with uninfected controls, interferon was not induced by this mycoplasma. Furthermore, maintaining the pH at 7.4 (Table III, Expt D) did not enhance leukocyte viability and did not result in the induction of interferon by *M. mycoides* var. *capri*. The data in Table III show that infection with *M. pneumoniae*, the most potent mycoplasmal inducer of interferon in this system, also did not result in major differences in leukocyte viability compared with *M. mycoides* var. *capri* or controls. Similar results showing no difference in leukocyte viability through 48 hr were obtained with the other 15 mycoplasmas used in these experiments. These investigations suggest that the interferon produced in the leukocyte cultures during mycoplasmal infection is not likely to be related to a cytotoxicity phenomenon as has been reported for induction of interferon *in vitro* by poly I:C (13).

Characterization of interferon. The antiviral substance induced by each of the various mycoplasmas was characterized as interferon by the following criteria: (i) inactivation by trypsin treatment, (ii) resistance to pH 2 for 24 hr at 4°, (iii) no detectable activity in mouse L cells, (iv) stability at 56° for 30 min, (v) activity against both VSV and Sindbis viruses in FLK cells, (vi) inability to inactivate VSV directly, and (vii) no activity in FLK cells treated with actinomycin D. To further eliminate the possibility of antiviral interference of noninterferon origin, viable untreated and nonviable acid-treated suspensions (10^6 CFU/ml) of each mycoplasmal species were added to FLK cells and used as in the interferon assay (1). Although viable and nonviable samples of *M. neurolyticum* resulted in a very low level of interference at a one-tenth dilution, none of the other 16 mycoplasmas displayed interfering activity against VSV.

Discussion. These studies demonstrate that 12 different species of *Mycoplasma* and *Acholeplasma* are capable of inducing interferon in ovine peripheral blood leukocyte cultures. Certain of these species induced relatively low levels of interferon (e.g., *M. hominis*) whereas others induced higher levels of interferon (e.g., *M. pneumoniae*). Two other mycoplasmal species, *M. neurolyticum* and *M. arginini*, were able to induce low levels of interferon in sheep leukocytes on only one occasion, while

three mycoplasmas (*A. axanthum*, *M. hyosynoviae*, and *M. mycoides* var. *capri*) did not induce interferon in any of the experiments performed. Other investigators have recently demonstrated (B. Fauconnier and J. Wroblewski, personal communication) that an *Acholeplasma* species isolated from infected plants induced circulating interferon in mice, while two other *Acholeplasma* species failed to induce interferon *in vivo*. These results confirm and extend those presented in an earlier report (1) from this laboratory in which it was initially shown that a mycoplasma, *A. laidlawii*, was associated with the induction of interferon *in vitro*.

There are several possible mechanisms by which mycoplasmas lead to interferon production in ovine leukocytes. An interaction of the mycoplasma with the host cell membrane would appear to be a necessary prerequisite for interferon induction. There does not seem to be a direct correlation between the reported ability of mycoplasmas to attach to animal cells and their capability of inducing interferon in sheep leukocytes under the conditions in our laboratory. Several investigators have studied mycoplasma–animal cell interactions using electron microscopic techniques (14–16) and the ability of animal cells to adhere to mycoplasmal colonies (17–19). These studies have demonstrated, for example, that *M. pneumoniae* (14, 15, 17–19) and *M. gallisepticum* (14, 15, 17–19) attach to the membranes of cells from several animal species including sheep erythrocytes (19), while *M. fermentans* (18, 19), *M. orale* type 2 (18, 19), and *M. salivarium* (17–19) apparently do not. However, all of these mycoplasmas were capable of inducing interferon to varying degrees in ovine leukocytes. Further studies are necessary to determine the possible relationship between the capacity of mycoplasma to attach to ovine leukocytes and other host cells and their ability to induce interferon.

Assuming that attachment to the leukocytes does occur, the mycoplasmas may act at the host cell surface to initiate interferon production, as has been postulated for interferon induction by poly I:C (20–22). This concept is supported by the evidence that mycoplasmas may alter the morphology of the animal cell membrane after adsorption (14, 23). On the other hand, if the process of interferon induction is similar to that of animal viruses (24), ingestion of the organisms by the cells may be required. Although there is no data concerning the interaction of mycoplasmas and sheep leukocytes, electron microscopic studies have demonstrated the uptake of certain mycoplasmas by human leukocytes (14, 15) and murine macrophages (16).

The data presented in this report further suggest that immunological mechanisms (25) are not involved in the process of interferon induction by mycoplasmas, since it is unlikely that the ovine donors used in these experiments were sensitized to 12 different mycoplasmal species.

Previously we postulated that Mycoplasmatales viruses may be of significance in the induction of interferon by *A. laidlawii* (1). Viruses have been isolated from *A. laidlawii* (10, 26), *M. pneumoniae* (27), *A. granularum* (28), and several other mycoplasmas (A. Liss and J. Maniloff, personal communication), and have been observed in *M. hominis* (29). The present studies indicate that the Mycoplasmatales virus, MVL51, survived in the ovine leukocyte cultures yet failed to induce interferon. Although this virus appears not to induce interferon by itself, it remains to be determined whether the presence of Mycoplasmatales viruses has any effect on the induction of interferon by mycoplasmas.

In recent years, mycoplasmal contamination of cell cultures has become of increasing concern to investigators working with tissue culture (30). For example, *M. orale* type 1, *M. arginini*, *M. hyorhinis*, *A. laidlawii*, and *M. hominis* have been isolated with high frequency from tissue cultures (30). These organisms have been shown to induce interferon to varying degrees in ovine leukocytes. In view of the capacity of these mycoplasmal species to induce interferon *in vitro*, investigators working with cell cultures, leukocytes, and macrophages should consider the possible influence of unsuspected mycoplasmal contamination on their experimental results.

Summary. Twelve different species of *Mycoplasma* and *Acholeplasma* were shown to induce interferon in ovine peripheral blood leukocyte cultures. Interferon production reached mean peak levels ranging from 35

units/ml for *M. hominis* to 960 units/ml for *M. pneumoniae* by 24–48 hr after infection. Two other mycoplasmas, *M. arginini* and *M. neurolyticum,* were able to induce low levels of interferon on only one occasion. *Acholeplasma axanthum, M. hyosynoviae, M. mycoides* var. *capri,* and Mycoplasmatales virus MVL51 failed to induce interferon in the leukocyte cultures. All 17 mycoplasmal species tested were capable of replicating in the sheep leukocyte cultures, although certain species (e.g., *M. pulmonis*) failed to multiply in every experiment. There was no consistent correlation between the degree of replication and the amount of interferon induced, and interferon production did not appear to relate to leukocyte cytotoxicity secondary to the mycoplasmal infection. The antiviral substance induced by the mycoplasmas was characterized as interferon by the usual criteria.

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