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Inhibition of Interferon Induction in Mice by Mycoplasmas and Mycoplasmal Fractions (39996)

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A single injection or repeated injections of an interferon inducer may result in a state of hyporeactivity in which the host produces progressively less interferon with each successive challenge (15, 16, 22, 28). This phenomenon has limited the usefulness of interferon inducers in the treatment of viral disease.

Recent studies in our laboratories have shown that a variety of mycoplasmas induce a pH-stable interferon following inoculation of sheep and human leukocyte cultures *in vitro* and that the lymphocyte appears to be the active interferon-producing cell. Other investigations have shown that mycoplasmas can also induce interferon *in vivo* in mice. Further studies demonstrated that mice became hyporeactive to interferon induction by NDV or poly(I:C) after inoculation with viable *Mycoplasma arthritidis* (5). This mycoplasma species induces an acute arthritis in rats (9) and a chronic arthritis in mice (7). It has also been reported to be immunosuppressive *in vivo* (2, 17) as well as inhibiting lymphocyte mitosis *in vitro* (1, 20, 26).

The present investigation was undertaken to determine whether mycoplasma-induced hyporeactivity was specific for *M. arthritidis* and to further characterize the mycoplasma component responsible.

Materials and methods. Cultivation of mycoplasmas. Mycoplasma arthritidis strain 14124 p10 (12) and *M. pulmonis* JB, designated "TR" (obtained from D-Taylor-Robinson), were grown on mycoplasma agar or in broth (Difco Laboratories, Detroit, Mich.) supplemented to final concentrations of 15% (v/v) horse serum, 5% (v/v) fresh yeast extract, and 1000 U of penicillin/ml (3, 13). *Mycoplasma pulmonis* received an additional supplement of 1% (v/v) of a 2 mg/ml solution of diphosphopyridine nucleotide (DPN-102, Sigma Chemical Co., St. Louis, Mo.). *Acholeplasma laidlawii* strain BNl-Nal^r was obtained from Dr. A. Liss (Storrs, Conn.) and strain 1305 was obtained from Dr. R. N. Gourlay (Compton, Surrey, England). Both acholeplasmas were cultured in tryptose broth supplemented with 1% (v/v) serum fraction, 1% (w/v) glucose, and 1000 U of penicillin/ml (19).

Mycoplasma pools were harvested by centrifugation, assayed for colony-forming units (CFU), and stored at −70° as previously described (12). Sonified mycoplasma suspensions were prepared by washing the organisms ×3 in phosphate-buffered saline (PBS), resuspending in deionized water, and exposing to 1-min periods of ultrasonic vibration using a Model S75 Branson sonifier until free of viable organisms. Dilutions of sonified suspensions were prepared in PBS.

Crude membranes were removed from sonified suspensions by centrifugation at 20,000 rpm using an SS34 rotor (Sorvall RC5 centrifuge). The membrane sediment was resuspended in PBS, washed ×3 by additional centrifugation, and finally resuspended to the volume of the original mycoplasma suspension. In experiments to compare the hyporeactive-inducing ability of whole organisms, sonified organisms, or membranes, all preparations were made from aliquots of the same mycoplasma suspension.

Virus cultivation. The Hertz strain of NDV (Courtesy of Dr. S. Baron, University of Texas, Galveston, Tex.) was grown in embryonated chicken eggs and titered 10^9 plaque-forming units (PFU) in primary chicken embryo cells (28).

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Mice. Seven- to eight-week-old female Swiss-Webster mice (Simonsen Laboratories, Gilroy, Calif.) were used throughout these studies.

Cell culture and interferon assays. Mouse interferon was assayed on mouse L cells (L929, obtained from the American Type Culture Collection, Rockville, Md.) grown in Eagles minimal essential medium (MEM) supplemented to final concentrations of 10% (v/v) fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The 50% plaque reduction technique employing vesicular stomatitis virus (VSV) as the challenge virus was used for interferon assays (21). An internal mouse interferon standard consisting of a known poly(I:C)-induced serum interferon was included with each set of assays. This internal standard was compared with an international reference mouse interferon obtained from the National Institutes of Health. The international interferon standard (designated titer of 6500 units) titered 6847 ± 2664 units in our assay system (10 determinations).

Demonstration of hyporeactivity in vivo. Appropriate dilutions of the mycoplasma suspensions were injected intraperitoneally (ip) in 0.2-ml amounts into groups of three mice each. Uninjected mice or mice injected with PBS or mycoplasma broth medium served as negative controls. For the demonstration of hyporeactivity the injected mice were challenged with 0.2 ml of NDV 1, 3, and 6 days later. Mice were exsanguinated 6 hr after inoculation of the virus, about the time of maximum interferon induction by NDV. The resulting sera from each group of three mice were pooled. In a previous study (5) we showed that inhibition of interferon induction by *M. arthritidis* was not caused by an alteration in the kinetics of induction by NDV but was in fact due to an actual inhibition of the response. As a positive control for hyporeactivity, mice were given a preliminary injection of NDV and were rechallenged with NDV 1, 3, and 6 days later. Prior to interferon assay, all serum samples were treated at pH 2.0 for 5 days at 4°.

Results. Inhibition of the interferon response by M. arthritidis. Lack of requirement for viable organisms and kinetics of the hyporeactive response. The ability of various concentrations of viable and nonviable, sonified *M. arthritidis* to induce hyporeactivity to interferon induction by NDV in mice was compared. The results are summarized in Table I. Viable mycoplasmas induced significant hyporeactivity at a concentration of 5×10^9 CFU per mouse 1 and 3 days postinoculation (200 and 630 units of interferon, in comparison with control values of 4800 and 3200 units, respectively). In contrast, a dose of 5×10^8 CFU per mouse induced only minimal hyporeactivity at 1 day, and 5×10^7 CFU per mouse did not result in any detectable inhibition of the interferon response to NDV. In this experiment, nonviable sonified preparations of the same *M. arthritidis* pool induced a more striking hyporeactive effect than did the viable preparations. Thus, a complete inhibition of the interferon response (<50 units of interferon) was apparent 1 day after injection of 5×10^7 sonified organisms, and a suppressed interferon response was still apparent by 3 days. A dose of 2×10^{10} disrupted organisms per mouse virtually eliminated all interferon production through 6 days (data not shown).

Additional experiments were conducted to determine how soon after the injection of *M. arthritidis* the hyporeactive state developed. Mice were injected ip with 10^9 nonviable sonified or 5×10^9 viable suspen-

TABLE I. INHIBITION OF THE INTERFERON RESPONSE OF MICE TO NDV FOLLOWING INOCULATION WITH VIABLE AND NONVIABLE *M. arthritidis*.

First injection	Interferon response[a] of mice to NDV challenge, Days after first injection		
	1	3	6
M. arthritidis			
5×10^7 CFU, viable	4200	5500	4300
5×10^8 CFU, viable	1400	3200	3200
5×10^9 CFU, viable	200	630	3200
5×10^7 CFU, sonified[b]	<50	800	3200
5×10^8 CFU, sonified	200	50	3200
5×10^9 CFU sonified	<50	<50	800
NDV, 10^8 PFU	100	<50	1300
Mycoplasma broth	4800	3200	3500

[a] Interferon titers measured 6 hr post-NDV injection.

[b] Mycoplasma counts performed prior to sonification.

sions of *M. arthritidis* in PBS. Mice injected with PBS served as negative controls for hyporeactivity. At 2, 6, 14, or 24 hr, mice were challenged with NDV and serum was collected 6 hr later for interferon assays. To control for interferon induction by the mycoplasmas themselves, additional mice were bled without NDV challenge 8 and 12 hr after injection of the mycoplasmas. These times represent the peak of the interferon response of mice to mycoplasmas (24). The results are summarized in Table II. Sonified mycoplasmas did not induce a detectable interferon response in mice 8 or 12 hr postinjection. The 200 units of interferon induced in mice by viable mycoplasmas 8 hr postinjection was insufficient to significantly alter the interferon titers resulting from NDV challenge at the 2- and 6-hr time periods. Thus there was no evidence of hyporeactivity in mice 2 or 6 hr postinoculation with sonified or viable mycoplasmas. In contrast, mice injected 14 or 24 hr previously with *M. arthritidis* exhibited a marked suppression of the interferon response to NDV challenge.

Hyporeactivity induced by M. pulmonis and A. laidlawii. Since mycoplasmas exert a variety of diverse effects on cell functions *in vitro* and *in vivo*, experiments were next conducted to determine whether the observed suppression of interferon induction by *M. arthritidis* could also be obtained by other species of mycoplasma. In the first experiment, mice received primary injections of (i) mycoplasma broth, (ii) NDV, (iii) *M. pulmonis* JB (1.8×10^9 CFU), or (iv) *M. pulmonis* JB (1.8×10^8 CFU). Uninjected mice served as controls. After 1, 3, or 6 days, all animals were challenged with NDV and were exsanguinated 6 hr later. The interferon levels obtained are summarized in Fig. 1. Mice receiving a primary injection of mycoplasma broth produced interferon levels in response to challenge with NDV similar to those seen in uninjected mice challenged with NDV. Mice injected with NDV produced <50 units of interferon when challenged with NDV 1 and 3 days later, and produced only 150 units of interferon when challenged 6 days later with NDV. Mice receiving 1.8×10^9 CFU per mouse of *M. pulmonis* exhibited a marked hyporeactivity to NDV-induced interferon through 3 days, but appeared normal by 6 days. Decreasing the dose of *M. pulmonis* 10-fold resulted in no significant hyporeactivity. In a repeat experiment, a primary injection of *M. pulmonis* JB (2×10^9 CFU per mouse) decreased the interferon response to NDV from 1800 units of interferon in control

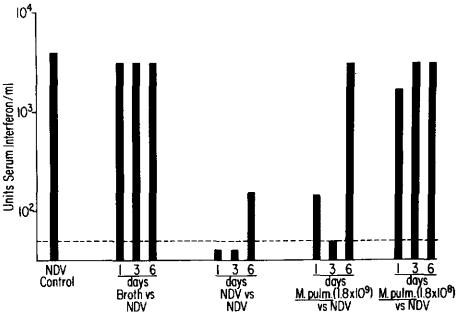

FIG. 1. Effect of *M. pulmonis* on the interferon response of mice to NDV. NDV control: Serum was collected 6 hr after the ip injection of NDV into normal mice. In the other groups, mice receiving a primary ip injection of mycoplasma broth, NDV, or 1.8×10^9 and 1.8×10^8 CFU of *M. pulmonis* strain JB were challenged ip 1, 3, and 6 days later with NDV. Serum for interferon assay was collected 6 hr post-NDV injection. The dotted line represents the level of sensitivity of the assay system, i.e., 50 U of interferon.

TABLE II. KINETICS OF THE HYPOREACTIVE RESPONSE.

First injection	Interferon response to first injection after		Interferon response[a] to NDV challenge, Hours after first injection			
	8 hr	12 hr	2	6	14	24
M. arthritidis, 10^9 CFU, sonified[b]	50	50	1900	5200	575	226
M. arthritidis 5×10^9 CFU, viable	200	NT[c]	8000	9500	90	200
PBS			7500	7400	NT	2300

[a] Interferon titers measured 6 hr post-NDV injection, i.e., at 8, 12, 20, and 30 hr post-mycoplasma injection.
[b] Mycoplasma counts performed prior to sonification.
[c] Not tested.

mice to 130 units after 1 day and 710 units after 3 days. By 6 days after injection of *M. pulmonis*, interferon levels increased in response to NDV, i.e., 980 units. Similar results were also obtained when *M. pulmonis* strain TR was tested for its ability to induce hyporeactivity.

Experiments were also conducted using *A. laidlawii* strain 1305 (Fig. 2). A primary injection of mice with tryptose broth (used for the culture of *A. laidlawii*) did not affect the ability of mice to produce interferon in response to a challenge injection of NDV. In contrast, primary injections of NDV and 5×10^9 CFU per mouse of *A. laidlawii* resulted in complete hyporeactivity through 3 days to interferon induction by a challenge injection of NDV. When approximately 10-fold fewer acholeplasmas were administered, hyporeactivity was complete at 24 hr, but only partial at 72 hr postinoculation. The interferon-producing capacity of the animals was normal 6 days postinoculation. The experiment was repeated with *A. laidlawii* strain BNl-Nal^r, using primary injections of 10^7 or 10^9 CFU/ml. The development of hyporeactivity followed a similar time sequence and was again shown to be dose dependent.

Further characterization of the mycoplasma component inducing hyporeactivity. In the next series of experiments viable, sonified, and membrane preparations of *A. laidlawii* Nal^r and *M. arthritidis* 14124 p10 were compared for their ability to suppress the interferon response of mice to NDV. The results are summarized in Table III. Both viable and sonified preparations of *A. laidlawii* and *M. arthritidis* resulted in a decreased interferon response to NDV. The crude membrane fractions prepared from *A. laidlawii* induced a striking hyporeactive effect through 6 days; those prepared from *M. arthritidis* induced hyporeactivity through 3 days.

To further characterize the component of mycoplasmas responsible for hyporeactivity, mice were injected ip with a sonified preparation of *M. arthritidis* 14124 p10 which had been heated to 56° for 1 hr or to 120° for 15 min. Mice injected with a sonified unheated preparation served as controls. The results are summarized in Table IV. In the first experiment, mice treated with unheated, sonified mycoplasmas were markedly hyporeactive to interferon induction in response to NDV through 3 days, whereas mice pretreated with heated material exhibited a normal interferon response to NDV at all time periods of challenge. This observation was confirmed in the second experiment since heat treatment of sonified *M. arthritidis* prior to injection ($5 \times$

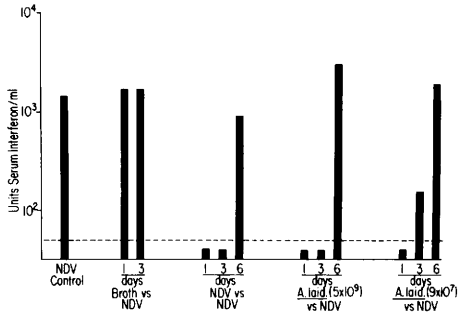

FIG. 2. Effect of *A. laidlawii* on the interferon response of mice to NDV. NDV control: Serum was collected 6 hr after the ip injection of normal mice with NDV. In the other groups, mice receiving a primary ip injection of tryptose broth, NDV, or 5×10^9 and 9×10^7 *A. laidlawii* strain 1305 were challenged ip 1, 3, and 6 days later with NDV. Serum for interferon assay was collected 6 hr post-NDV injection.

TABLE III. DEVELOPMENT OF HYPOREACTIVITY TO INTERFERON INDUCTION IN MICE FOLLOWING THE INJECTION OF VIABLE, SONIFIED, AND CRUDE MEMBRANE PREPARATIONS OF VARIOUS MYCOPLASMAS.

First injection	Interferon response[a] to NDV challenge, Days after first injection		
	1	3	6
A. laidlawii Nal^r			
Viable, 1.6×10^9 CFU	<50	400	600
Sonified, 1.6×10^9 CFU[b]	<50	<50	200
Membranes from 1.6×10^9 CFU	80	50	70
M. arthritidis 14124 p10			
Viable, 1.6×10^9 CFU	<50	<50	550
Sonified, 1.6×10^9 CFU	200	90	3600
Membranes from 1.6×10^9 CFU	50	290	3200
Tryptose broth	3000	3200	NT[c]
Mycoplasma broth	1600	3200	NT

[a] Serum interferon levels were determined 6 hr post-NDV challenge.
[b] CFU prior to sonification.
[c] Not tested.

TABLE IV. EFFECT OF HEAT TREATMENT ON THE STABILITY OF THE HYPOREACTIVITY-INDUCING COMPONENT OF *M. arthritidis*.

First injection	Interferon[a] response of mice to NDV challenge. Days after the first injection		
	1	3	6
Expt. I			
M. arthritidis, 8×10^8 CFU,[b] unheated	50	<50	1900
M. arthritidis, 8×10^8 CFU, 56° 1 hr	4500	6600	9000
M. arthritidis, 8×10^8 CFU, 120° 15 min	4100	7100	6400
NDV	<50	<50	4500
PBS	6300	NT[c]	NT
Expt. II			
M. arthritidis, 5×10^9 CFU, unheated	<50	<50	800
M. arthritidis, 5×10^9 CFU, 56° 1 hr	1200	800	3200
M. arthritidis, 2×10^{10} CFU, unheated	<50	<50	70
M. arthritidis, 2×10^{10} CFU, 56° 1 hr	<50	<50	<50
NDV, 10^8 PFU	100	<50	1300
Mycoplasma broth	4800	3200	3500

[a] Serum interferon levels were determined 6 hr post-NDV challenge.
[b] CFU before sonification.
[c] Not tested.

10^9 CFU per mouse) markedly decreased the ability of the preparation to induce hyporeactivity. However, hyporeactivity was not abolished by heating (56°, 1 hr) when the initial injection of *M. arthritidis* was increased to 2×10^{10} CFU per mouse.

Discussion. The present studies have shown that three biochemically distinct mycoplasma species are capable of inhibiting the interferon response of mice to NDV. Disrupted mycoplasmas were also active in inducing the hyporeactive state, and the mycoplasma component responsible was heat labile and appeared to be associated with membranes.

The mechanism of the mycoplasma-mediated inhibition of the interferon response *in vivo* could be related to a general toxic effect on host functions since some mycoplasmas, including membrane preparations, have been reported to exhibit toxic properties (6, 10, 11, 30). However, the ability of mycoplasmas to induce interferon (8, 24) prior to the development of the hyporeactivity state is not consistent with this hypothesis.

Since *M. arthritidis* is known to inhibit lymphocyte mitogenesis and antibody synthesis *in vitro* by arginine depletion of culture media (1, 20, 26), it might be argued that a similar mechanism is responsible for the immunosuppressive and interferon-inhibiting properties of *M. arthritidis in vivo* (2, 5, 17). In the latter regard it is relevant that only lymphocytes produce interferon in response to *in vitro* infection with mycoplasmas (4). However, significant arginine depletion *in vivo* is not a likely event. Our present findings that two glucose-utilizing mycoplasmas inhibit the interferon response *in vivo* indicates that another mechanism is operative.

Recent work by Stringfellow and Glasgow (29) has shown that serum taken from mice made hyporeactive to interferon production by injection with encephalomyocarditis virus (EMC) contains a factor which confers the hyporeactive state to mouse embryo fibroblasts *in vitro*. Furthermore, this factor was distinct from interferon and was produced at a time when the interferon response had abated (27). Preliminary experiments in our laboratories (unpublished data) indicate that serum taken from mice 24 to 72 hr postinoculation with *M. arthritidis* also contains a factor which inhibits the *in vitro* interferon response of mouse embryo fibroblasts to NDV. The physicochemical relationship of this factor to interferon and to the hyporeactive factor described by Stringfellow (27) is currently under study.

The finding that a variety of mycoplasmas can induce interferon and hyporeactivity to interferon induction *in vivo* clearly provides the potential for altered host susceptibility to infection with viruses. Thus an increased resistance to viral infection could occur during the early phase of mycoplasma infection in which serum interferon was present. In this regard, Fauconnier and Wroblewski (8) presented evidence that a primary injection of mice with an interferon-inducing acholeplasma species protected the animals against infection with Semliki Forest virus. In contrast, a decreased resistance to viral infection might occur during the hyporeactive state induced by mycoplasmas. It is of inter-

est that in certain circumstances mixed mycoplasma/virus infections of avian (14, 23) and porcine (18) hosts appear to result in more severe disease.

Summary. The injection of both viable and sonified nonviable preparations of *M. arthritidis, M. pulmonis,* and *A. laidlawii* into mice suppressed the interferon response of the animals to challenge with Newcastle disease virus (NDV). The inhibitory effect was dependent upon the numbers of organisms injected. The suppression of the interferon response was first apparent 14 hr postinjection and reached a maximum 1 and 3 days postinjection. High doses of organisms produced an inhibitory effect through 6 days. The mycoplasma component responsible for induction of hyporeactivity appeared to reside in the membrane fraction of the organisms. Heat treatment of mycoplasma preparations abolished the effect except at very high inocula.

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