Mycoplasm-Mediated Hyporeactivity to Various Interferon Inducers

B. C. COLE,* J. C. OVERALL, JR., P. S. LOMBARDI, AND L. A. GLASGOW

Division of Arthritis,* Department of Medicine, and Departments of Microbiology and Pediatrics, University of Utah College of Medicine, Salt Lake City, Utah 84132

Received for publication 16 June 1975

Three strains of Mycoplasma arthritidis were shown to induce marked hyporeactivity in mice to interferon induction by both Newcastle disease virus and poly(I:C). In contrast, the interferon response of mice to tilorone was only partially suppressed by pretreatment of the animals with mycoplasmas. Hyporeactivity to Newcastle disease virus was maximal 1 and 3 days after mycoplasma treatment, but the interferon response returned to normal by 13 days. Hyporeactivity to poly(I:C)-induced interferon was maximal 1 day after injection of the mycoplasmas and was no longer apparent by 5 days. No relationship was found between the ability of the mycoplasmas themselves to induce interferon and the degree of hyporeactivity produced. These results suggest that mycoplasmas may alter virus-host relationships in vivo.

Many viral and synthetic interferon inducers result in a state of hyporeactivity to further interferon production after either single or multiple injections (4, 9, 14, 15, 19, 28, 29, 31). This refractory state has greatly limited the therapeutic usefulness of interferon inducers in protection against viral disease.

Recent studies in our laboratories have shown that a wide variety of Mycoplasma species are capable of inducing interferon in ovine leukocyte cultures (22, 23). Although attempts to demonstrate synthesis of interferon in mouse cells after exposure to mycoplasmas in vitro were unsuccessful, Mycoplasma pneumontae, M. arthritidis, and Acholeplasma laidlawii were found to be capable of inducing serum interferon in mice 6 h postinfection (21). These findings indicate that unsuspected mycoplasmal contamination of cell cultures or infection of animals may alter viral replication and lead to erroneous interpretations of experimental results.

The present studies were undertaken to determine whether a chronic mycoplasmal infection of mice was capable of inducing hyporeactivity to other interferon inducers, thus providing the potential for increased host susceptibility to subsequent viral infection due to a decreased interferon response against the challenge virus. The interferon inducers chosen were Newcastle disease virus (NDV), which has been extensively studied in our laboratories, polyinosinic-polycytidylic acid (poly(I:C)), which has been widely considered as a candidate for interferon induction in man, and tilorone hydrochloride, which induces a striking degree of hyporeactivity to subsequent administration of the compound (27). M. arthritidis infection of mice, which has been extensively studied in one of our laboratories, was used since the organism persists in the peripheral circulation for 1 to 2 weeks (6) and in other sites for many months (7) after inoculation.

MATERIALS AND METHODS

Mice. Seven-week-old female Swiss-Webster mice were obtained from Simonsen Laboratories (Gilroy, Calif.) and were used throughout these experiments.

Mycoplasma cultivation. The sources of M. arthritidis strains L4 Sandoz, 14124 P10, and PG6 were as previously described (8, 11). Mycoplasmas were grown in mycoplasma broth (Difco Laboratories, Detroit, Mich.) supplemented to final concentrations of 15% (vol/vol) horse serum, 1% (vol/vol) fresh yeast extract, and 1,000 U of penicillin per ml (5, 12). Mycoplasma cultures were centrifuged at 27,000 × g and resuspended in mycoplasma broth supplemented to 5% (vol/vol) horse serum. The suspensions were frozen at −70°C, assayed for colony-forming units per milliliter (13), and pretested for their ability to induce interferon in mice (21) before use in the experiments. In the hyporeactivity experiments, mice were injected intraperitoneally (i.p.) with 0.2 ml of the mycoplasma suspensions containing approximately 5 × 10⁶ colony-forming units.

Interferon inducers. The Herts strain of NDV originally obtained from S. Baron (National Institutes of Health, Bethesda, Md.) was propagated in embryonated hens eggs and titered 10⁶ plaque-forming units per ml on primary chicken embryo cells (28). For interferon induction, mice were injected...
i.p. with 0.1 ml of the virus pool (10⁶ plaque-forming units/mouse).

Poly(I:C) and tilorone hydrochloride were provided by the Antiviral Substances Program of the National Institutes of Health. Poly(I:C) was supplied as a 1-mg/ml solution and was frozen at −20°C until use. Mice were injected i.p. with 0.2 ml of the poly(I:C) solution (0.2 mg/mouse) (28). Tilorone hydrochloride was stored as a powder at 4°C until use. A solution of 50 mg/ml was prepared in phosphate-buffered saline administered orally (10 mg/mouse) by means of an infant catheter attached to a 1-ml syringe (27).

Cells and interferon assay. Interferon assays were performed by using mouse L-cells (L929) obtained from the American Type Culture Collection cell repository (Rockville, Md.). The cells were propagated in Eagle minimal essential medium supplemented to final concentrations of 10% (vol/vol) fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 μg of tylosin tartrate per ml. Interferon samples were exposed to pH 2.0 for 24 h, a procedure that eliminates mycoplasmas persisting in the serum (22). Samples taken from mice injected with NDV were treated at pH 2 for 5 days.

Interferon assays were performed by the plaque reduction technique, using vesicular stomatitis virus as the indicator (18). Interferon units represent the reciprocal of that dilution which inhibits 50% of the challenge inoculum. An internal laboratory mouse interferon standard was included with each set of assays, and the titer varied less than fourfold between different assays. The sensitivity of our assay system was periodically checked by assaying the international reference mouse interferon obtained from the National Institutes of Health (a titer of 350 U/ml in our system as compared with the designated titer of 500 U/ml).

RESULTS

Preliminary studies showed that some strains of M. arthritidis induced higher levels of interferon than did others. Thus in each set of experiments hyporeactivity was tested with both a high (A) and a low (B) interferon-inducing strain in order to determine whether there was any relationship between degree of interferon induced and degree of hyporeactivity to other interferon inducers.

Groups of three mice each were injected i.p. with either the A or B strain of M. arthritidis and were challenged 1, 3, 5, and 13 days later with either NDV, poly(I:C), or tilorone hydrochloride. Animals were exsanguinated 6 h after challenge with NDV and poly(I:C) and 24 h after tilorone, i.e., close to the time of maximum interferon induction by these inducers (28). Controls consisted of mice injected with NDV, poly(I:C), or tilorone alone as well as mice inoculated with mycoplasma broth and challenged 1 and 5 days later with these same inducers. Positive controls for the development of hyporeactivity consisted of mice treated with (i) NDV and challenged with NDV and (ii) tilorone and challenged with tilorone.

In the first series of experiments, M. arthritidis A (strain L4 Sandoz) induced 500 U of serum interferon per ml and M. arthritidis B (strain PG6) induced 100 U of serum interferon per ml 6 h postinjection.

The development of hyporeactivity to NDV is summarized in Fig. 1. Mice that received NDV alone (NDV control) produced 3,200 U of serum interferon per ml 6 h postinoculation. Mice that received an initial inoculation with mycoplasma broth followed by an injection with NDV 1 and 5 days later produced 8,400 and 8,800 U of serum interferon per ml, respectively. Mice given a primary injection of NDV followed by a second injection of NDV exhibited partial hyporeactivity on days 1 and 3 (serum interferon levels of 530 and 470 U, respectively, or 17 and 15% of control values). By days 5 and 13 the levels of interferon induced by the second injection of NDV were similar to the levels in control mice receiving a single injection of NDV. In the groups of animals given the two strains of M. arthritidis, marked hyporeactivity to the induction of interferon by NDV occurred. After an initial injection of M. arthritidis A, the high interferon-inducing strain, mice produced no detectable serum interferon on days 1 and 3 in response to NDV challenge. By days 5 and 13 the response to NDV was similar to that in the control. M. arthritidis B, the low interferon-inducing strain, resulted in complete hyporeactivity at 1 day (no detectable serum interferon) and partial hyporeactivity at 3 days (460 U of serum interferon per ml or 14% of the NDV control) and 5 days (1,000 U/ml or 31% of control). Recovery of essentially normal interferon production was apparent by 13 days.

The experiment was repeated by using a different M. arthritidis A strain (14124 P10), which induced 140 U of serum interferon per ml, and the same M. arthritidis B (strain PG6), which induced <50 U/ml. Similar hyporeactivity results were obtained. Mice that received NDV followed by NDV were completely hyporeactive on days 1 and 3, with interferon levels returning to normal on days 5 and 13. Mice that were injected with M. arthritidis A were partially hyporeactive on day 1 (interferon levels 23% of control) and completely hyporeactive on day 3, but normal on days 5 and 13. Mice receiving M. arthritidis B were completely hyporeactive on days 1 and 3 but normal in their interferon response to NDV by days 5 and 13.

The development of hyporeactivity to poly(I:C) is summarized in Fig. 2. Mice injected with poly(I:C) alone produced 4,400 U of serum interferon per ml. Mice receiving mycoplasma
**Fig. 1.** Mycoplasma-mediated hyporeactivity to interferon induction by NDV in mice. NDV control: Mice were sacrificed 6 h after i.p. injection of NDV. Broth versus NDV: Mice were challenged i.p. with NDV 1 and 5 days after a primary i.p. injection of mycoplasma broth. Mice receiving a primary i.p. injection of NDV, M. arthritidis A, or M. arthritidis B were challenged i.p. 1, 3, 5, or 13 days later with NDV. Serum interferon levels were determined 6 h after the challenge injection of NDV. The dotted line represents the level of sensitivity of the assay system, i.e., 50 U of interferon per ml of serum.

**Fig. 2.** Mycoplasma-mediated hyporeactivity to interferon induction by poly(I:C) in mice. Poly(I:C) control: Mice were sacrificed 6 h after the i.p. injection of poly(I:C). Broth versus poly(I:C): Mice were challenged i.p. with poly(I:C) 1 and 5 days after a primary i.p. injection of mycoplasma broth. Mice receiving primary i.p. injection of M. arthritidis A or M. arthritidis B were challenged i.p. 1, 3, 5, or 13 days later with poly(I:C). Serum interferon levels were determined 6 h after the challenge injection of poly(I:C).
broth followed by poly(I:C) on days 1 and 5 produced similar levels of interferon. Both strains of *M. arthritidis* resulted in complete hyporeactivity to poly(I:C)-induced interferon 1 day after injection of the organisms. At 3 days only mice receiving the low interferon-inducing strain (B) exhibited hyporeactivity. The interferon response to poly(I:C) appeared to be unimpaired 5 and 13 days after injection of the organisms. The short-lived hyporeactivity to poly(I:C) induced by *M. arthritidis* was confirmed in a repeat experiment. Thus, 1 day after injection of either strain of *M. arthritidis*, challenge with poly(I:C) resulted in undetectable interferon levels. Poly(I:C) given 3, 5, and 13 days after injection of *M. arthritidis* A and *M. arthritidis* B induced interferon levels similar to those of the control value.

The effect of *M. arthritidis* on interferon induction by tilorone was next investigated (Fig. 3). Control mice receiving tilorone alone produced 2,700 U of serum interferon per ml 1 day after oral administration. Pretreatment of mice with mycoplasma broth did not alter the interferon response to tilorone. In contrast, the primary administration of tilorone resulted in complete hyporeactivity to subsequent tilorone treatments through day 5. A refractory response was still apparent in these mice at 13 days, when only 400 U of serum interferon per ml (approximately 15% of control levels) was induced. In contrast, pretreatment of mice with either strain of *M. arthritidis* resulted in only partial hyporeactivity in response to challenge with tilorone. Thus 1 day after the injection of *M. arthritidis* A and B, 600 (22% of the control value) and 200 (7% of control) U of serum interferon per ml, respectively, were produced in response to tilorone. At most other time periods the interferon levels in mycoplasma-injected mice were similar to those in mice receiving tilorone alone. In a repeat experiment some variability was encountered in that *M. arthritidis* A induced complete hyporeactivity at 3 days, partial hyporeactivity at 5 days, but no hyporeactivity at 1 or 13 days after injection. *M. arthritidis* B did not induce noticeable hyporeactivity to tilorone-induced interferon.

All tests for interferon production by NDV and poly(I:C) were undertaken on serum taken 6 h after injection of the agent. The following experiment was designed to determine whether the decreased interferon response observed in *M. arthritidis*-treated mice represented a delay in, rather than a complete inhibition of, the ability of the host to produce interferon in response to the challenge inducers. Groups of three mice each received a primary injection of mycoplasma broth or *M. arthritidis* strain 14124 P10. After 24 h mice were challenged with NDV or poly(I:C) and were then exsanguinated after 6, 12, 24, or 48 h. The results are...
summarized in Table 1. In control mice that received mycoplasma broth, peak levels of interferon occurred 6 to 12 h after the injection of NDV or poly(I:C). These results are consistent with the interferon response of untreated mice injected with these inducers (28). Mice that received a primary injection of *M. arthritidis* and a secondary injection 24 h later of NDV or poly(I:C) failed to produce detectable interferon through 48 h. The experiment was repeated and similar results were obtained.

**DISCUSSION**

Although interferon is considered to be an important component of host defenses against the initial stages of viral disease (2), the development of a refractory or hyporeactive state during the course of a viral infection (28) may greatly limit the time during which this mechanism is effective. A wide variety of agents apart from viruses are known to induce hyporeactivity, including poly(I:C), tilorone, and endotoxin. It is notable that each of these agents also induces interferon in untreated animals.

Our results indicate that hyporeactivity may occur in the absence of detectable production of interferon. The data additionally suggest that mycoplasma infection might either protect animals against or enhance susceptibility to viral infection, depending on whether the viral infection occurred during the time of interferon production or during the time of hyporeactivity. Therefore differences in the kinetics of interferon production and in the time of development of hyporeactivity in infections with different *Mycoplasma* species could be important in determining the effect on a concomitant or subsequent viral infection. In this regard, it is interesting that *M. arthritidis*, *M. pneumoniae*, and *A. laidlawii* appear to induce the maximum levels of serum interferon in mice at 6 h postinfection, whereas a much later interferon response is apparent with *M. pulmonis* (21) and an unidentified plant *Acholeplasma* species (11). The relationship between the kinetics of the interferon response and the time of development of hyporeactivity in mice to mycoplasmas remains to be determined.

Although there is only limited published information concerning the outcome from mixed mycoplasma-viral infections in animals, the data that are available are conflicting. A plant *Acholeplasma* species has been reported to protect mice against infection with Semliki Forest virus, presumably mediated by interferon induction (10). In contrast, more severe infections have been reported in avian and porcine hosts infected with both mycoplasmas and animal viruses (13, 16, 20). In other studies *M. arthritidis* apparently enhanced Freund virus-induced leukemia (R. J. Eckner, J. Han, and V. Kumar, Fed. Proc., p. 769, 1974), whereas *A. laidlawii* protected mice against leukemia produced by Rauscher virus (17). Similarly divergent results have been obtained by using mixed mycoplasma-viral infections of cell cultures. Thus mycoplasmas have been shown to depress, enhance, or have no effect upon interferon induction by various viruses (1, 24, 30). Inhibition or enhancement of viral infections by mycoplasmas may not necessarily be dependent upon interferon or the development of interferon hyporeactivity, however, since mycoplasmas exhibit a wide range of other effects upon mammalian cells (25).

In the present studies, *M. arthritidis*-induced hyporeactivity was relatively short-lived and the interferon-producing capability of mice to NDV and poly(I:C) was at or near normal levels 5 days after injection of the mycoplasmas. This is somewhat surprising since virulent *M. arthritidis* strains (of which 14124 P10 is one) persist, although at low levels, in the peripheral circulation of mice for at least 6 days after injection (6). These results suggest that the initial interferon and hyporeactivity response may require a large number of organisms. Thus low levels of persisting mycoplasmas in either the peripheral circulation or the joints of infected mice may be insufficient to maintain the refractory state.

Whether in fact the hyporeactivity observed in these studies is due to the production of a hyporeactive protein, as occurs during viral infections (3, 26), or is perhaps due to a myco-

<table>
<thead>
<tr>
<th>First injection</th>
<th>Second injection</th>
<th>Serum interferon (U/ml) detected on given hour after second injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td><em>M. arthritidis</em></td>
<td>NDV</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Broth</td>
<td>NDV</td>
<td>2,100</td>
</tr>
<tr>
<td><em>M. arthritidis</em></td>
<td>Poly(I:C)</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Broth</td>
<td>Poly(I:C)</td>
<td>2,000</td>
</tr>
</tbody>
</table>

* Strain 14124 P10.
plasma-mediated toxicity for interferon-producing cells remains to be determined. The lack of correlation in our studies between the amount of interferon induced by the A and B strains of *M. arthritidis* and the degree of hyporeactivity would be consistent with the current opinion that interferon and hyporeactivity factor are two different entities (3, 26). Furthermore, the development of hyporeactivity in the absence of a detectable interferon response suggests that the refractory state is not due to (i) feedback inhibition by interferon or (ii) exhaustion of a preexisting interferon or interferon-precur sor pool.

ACKNOWLEDGMENTS

We thank P. Wagner and J. DeCarria for technical assistance.

This investigation was supported by Public Health Service grants AI12106 and AI10217 from the National Institute of Allergy and Infectious Diseases and grant AM02255 from the National Institute of Arthritis, Metabolism and Digestive Diseases. J.C.O. is an Investigator, Howard Hughes Medical Institute.

LITERATURE CITED


