INFECTION AND IMMUNITY, Apr. 1978, p. 209-214
0019-9567/78/0020-0209$02.00/0

Vol. 20, No. 1
Printed in U.S.A.

Induction of a pH-Stable Interferon in Sheep Lymphocytes by *Mycoplasmatales* Virus MVL2

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Received for publication 31 October 1977

The data presented in this report show that the enveloped *Mycoplasmatales* virus MVL2 is capable of interferon induction in sheep peripheral blood lymphocytes. The phenomenon was dose dependent, requiring approximately 10^8 plaque-forming units of virus per 2×10^6 lymphocytes. The interferon was stable to pH 2.0 treatment, was produced in moderately high levels (>1,000 units), and met many of the criteria for classification as a type I interferon. Heat-inactivated MVL2 lost its ability to induce interferon, whereas ultraviolet-inactivated virus retained its capacity to induce levels comparable to untreated virus. Whereas the MVL2 used in these studies was contaminated with several host cell proteins as determined by sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis, the contaminants probably did not play a role in the induction because isolated cell membranes or soluble cell contents from *Acholeplasma laidlawii* are inactive as inducers. Also presented in this report is a preliminary description of the structural polypeptides of MVL2.

Numerous viruses, intracellular single-celled organisms, synthetic and naturally occurring nucleic acids, endotoxins, polysaccharides, and synthetic polymers have been shown to elicit an interferon response in vivo (9). Studies in our laboratory have shown that a wide variety of *Mycoplasma* species induce interferon in mice and in cultured sheep and human peripheral blood lymphocytes (2, 18, 19). Certain strains of *Acholeplasma laidlawii*, a non-sterol-requiring member of the *Mycoplasmatales*, were also shown to induce low levels of interferon in these systems. The recent discovery that *A. laidlawii* harbors three distinct virus groups (4-7, 12), MVL1, MVL2, and MVL3, prompted us to investigate the role of these viruses in interferon induction by this organism. In preliminary experiments, MVL1 and MVL3 as well as their host strain of *A. laidlawii* consistently failed to induce detectable levels of interferon in human and sheep peripheral blood lymphocytes. In the present report we describe the ability of the enveloped deoxyribonucleic acid (DNA) virus MVL2 to induce a pH stable interferon in cultured sheep lymphocytes. Preliminary results have been presented (P. S. Lombardi and B. C. Cole, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, 623, p. 134).

MATERIALS AND METHODS

Mycoplasmas and mycoplasma viruses. *Acholeplasma laidlawii* strain 1305 was obtained from R. N. Gourlay and maintained in continuous culture in tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% (vol/vol) PPLO serum fraction (Difco) and containing 1,000 U of penicillin per ml. The acholeplasmas were assayed as colony-forming units on tryptose agar plates consisting of 2% (vol/vol) tryptose (Difco), 0.1 M NaCl, 0.85% (wt/vol) Difco purified agar, 1% (wt/vol) glucose, and 2% (vol/vol) PPLO serum fraction, and buffered to pH 8.0 with 0.04 M tris(hydroxymethyl)aminomethane. Crude membranes of 1305 were prepared by repeated sonic disruption of cells suspended in distilled water. The membranes were separated from soluble cytoplasmic components by centrifugation (48,000 × *g*). The supernatant from this centrifugation was saved and designated "cell contents." The protein concentration of the cell contents was measured by the Lowry procedure (14) and adjusted to a final concentration of 1.5 mg/ml. The membranes were washed with distilled water an additional five times, and their protein contents were adjusted to 3.0 mg/ml. Alternatively, membranes were prepared by the procedure of Razin et al. (17). Briefly, this method consisted of suspending the acholeplasmas from a log-phase culture in distilled water. The suspension was then centrifuged (20,000 × *g*) to sediment large debris and any remaining intact cells. The supernatant containing the membranes was centrifuged (30,000 × *g*) for 40 min. The sediment was then washed ×4 in distilled water and adjusted to a final protein concentration of 1.0 mg/ml.

The virus MVL2 was obtained from R. N. Gourlay and assayed as plaque-forming units (PFU) on lawns of *A. laidlawii*. The technique involved spreading 0.4 ml of a 1:10 dilution of an overnight culture of *A. laidlawii* on a 100-mm-diameter tryptose agar plate and allowing the cell suspension to absorb into the

surface of the agar. Virus was diluted in tryptose phosphate broth and added to the lawn in 0.5-ml portions. After overnight incubation at 37°C, plaques were counted.

Stock preparations of MVL2 were prepared in tryptose phosphate broth cultures by inoculating approximately 10^7 colony-forming units of *A. laidlawii* with 10^6 PFU of virus. After 24 to 48 h of incubation at 37°C, the virus, which titrated approximately 10^9 PFU/ml, was freed of any viable acholeplasma by centrifuging the suspension at 10,000 × *g* for 40 min, then passing the supernatant through a 220-nm membrane filter (Nalgene Labware Div., Nalgene/Sybron Corp., Rochester, N.Y.). Partially purified MVL2 was prepared by concentrating the crude stock virus by precipitation in 50% saturated ammonium sulfate followed by equilibrium centrifugation (20,000 rpm, 18 h, SW 27 rotor) on a 20 to 60% (wt/wt) sucrose gradient. The single prominent opaque band of virus was collected, dialyzed against 0.001 M phosphate buffer (pH 7.2), and then lyophilized. The virus was further purified by velocity sedimentation through a 5 to 20% (wt/wt) sucrose gradient for 70 min at 27,000 rpm by using an SW 27 rotor. The final virus preparations titered about 4×10^{10} PFU/ml.

Animal viruses. Bluetongue virus, vesicular stomatitis virus (VSV), and Sindbis virus were propagated as previously described (18, 19).

Preparation of peripheral blood lymphocytes. Sheep peripheral blood lymphocytes were separated from polymorphonuclear leukocytes and erythrocytes by centrifugation of a heparinized (10 U/ml) sample of peripheral blood, diluted 1:1 with Eagle minimal essential medium supplemented with 0.3% glutamine and containing 100 U of penicillin per ml, through a Ficoll-Hypaque solution (2). After centrifugation, the lymphocyte layer was removed, resuspended in 2 to 3 ml of minimal essential medium, and then centrifuged (200 × *g*) for 10 min. The cell pellet was then resuspended in 10 ml of minimal essential medium. A 60-ml amount of peripheral blood fractionated as above yielded about 7×10^7 leukocytes, of which 95 to 99% were mononuclear. Viability ranged from 98 to 99% as determined by trypan blue exclusion. The cells were diluted to 2×10^6 cells per ml in minimal essential medium supplemented with glutamine, penicillin, and 10% (vol/vol) fetal calf serum.

Interferon induction and assay. A 0.1-ml sample of each inducer was added to 1 ml of lymphocytes. Duplicate cultures were used for each determination. After the indicated incubation time at 37°C, the cells were centrifuged (200 × *g*) for 5 min, and the supernatants from duplicate samples were removed and pooled. During all experiments, lymphocyte viability did not decrease below 60%.

Interferon was assayed as previously described (19) and consisted of a 50% plaque reduction assay of VSV on a semicontinuous line of fetal lamb kidney cells. All samples of interferon were adjusted to pH 2.0 for 24 h, then returned to pH 7.0 before assay. The units of interferon were expressed as the reciprocal of the dilution of interferon that produced a 50% reduction of plaques. The lower level of detection in this assay was 25 units. A standard sheep interferon standard was included in each assay.

Characterization of interferon. A sample of MVL2-induced interferon was divided into several portions. All were adjusted to pH 2.0 for 24 h, then returned to pH 7.0 except for one that received no pH treatment. Trypsin treatment consisted of incubating the sample with trypsin (0.25% final concentration) at 37°C for 1 h. The action of the trypsin was stopped by adding fetal calf serum (40% [vol/vol] final concentration). To assure that carryover of the activity of the trypsin did not occur, thus causing cell killing in the interferon assay, a control tube containing minimal essential medium was treated in an identical manner and then added to cells. No toxicity or alteration of the ability of VSV to form plaques was observed. Actinomycin D (0.5 μg/ml) was added to several dilutions of interferon samples, and these were then added to fetal lamb kidney cells. After 5 h of incubation at 37°C, the VSV challenge was added, and the assay for interferon activity was completed as usual. As a control, interferon not containing actinomycin D was added to cells in a similar manner. In a further control, cell toxicity by actinomycin D was minimal and did not alter the ability of VSV to plaque on fetal lamb kidney cells. Activity of the MVL2-induced interferon against a heterologous virus was demonstrated by using Sindbis virus instead of VSV as challenge in the interferon assay. Species specificity was demonstrated by assaying the activity of interferon on mouse (L929) cells by using VSV as a challenge.

Inactivation of MVL2. Heat inactivation consisted of heating concentrated MVL2 to 56°C for 1 h. Virus was ultraviolet-inactivated by exposure at 5 cm from a short-wavelength ultraviolet lamp for 240 s. The energy output of the lamp was 62 ergs/mm^2 per s. Antibody treatment consisted of adding 20 μl of undiluted antiserum (prepared in rabbits against concentrated MVL2) to 0.4 ml of virus and incubating at 37°C for 1 h.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide slab gels were prepared by the procedure of O'Farrell et al. (16). The resolving gel formed between glass plates consisted of polyacrylamide ranging from 7.5 to 12.5%. A 7.5% stacking gel was cast above the resolving gel. Proteins were heated to 100°C for 1 to 2 min in the presence of 5% β-mercaptoethanol, 3% sodium dodecyl sulfate, and 0.0625 M tris(hydroxymethyl)aminomethane, pH 6.8, and then applied to wells formed in the stacking gel. A constant current of 45 mA was applied until the bromophenol marker reached the bottom of the resolving gel. Proteins were stained with Coomassie brilliant blue and destained, and the gel was then dried under vacuum. Approximate molecular weight determinations were calculated by comparison with known protein markers. Virus-specific bands were identified only after comparing the electrophoretic patterns of *Acholeplasma* components with those of the virus on gels by using the complete range of polyacrylamide concentrations.

RESULTS

Induction of interferon by MVL2 in sheep lymphocytes. Lymphocyte suspensions prepared from the whole blood of three different ewes by fractionation on Ficoll-Hypaque gra-

dients were challenged with MVL2. After 24 and 48 h of incubation, the supernatant culture fluids were assayed for interferon activity. The data of one such experiment are shown in Table 1. Both our standard stock preparation of MVL2 as well as a partially purified preparation of the virus induced an interferon, which was stable to treatment at pH 2.0. The response was clearly dose dependent with approximately 10^8 to 10^9 PFU of virus being necessary for the induction. Interferon was not detected in lymphocyte cultures infected with the host strain (1305) of *A. laidlawii* or in cultures exposed to the *A. laidlawii* growth medium. The interferon levels induced by 10^9 PFU of MVL2 virus equalled those induced by 10^5 PFU of bluetongue virus, a known potent inducer of interferon in sheep lymphocytes (19).

In another experiment shown in Fig. 1, the kinetics of interferon induction by MVL2 were examined. Interferon first appeared at 12 h, peaked at 24 h, and remained relatively stable through 48 h.

Characterization of MVL2-induced interferon. Experiments were next conducted to characterize the antiviral substance induced by MVL2 as interferon. The procedures used and data obtained are shown in Table 2. The antiviral activity was stable to pH 2.0 treatment and to heating at 56°C for 1 h. The protein nature of the substance was indicated by its inactivation by trypsin. The activity in cells could be blocked by actinomycin D, which inhibits the transcription necessary for the expression of the antiviral nature of interferon (20). The interferon also inhibited replication of Sindbis virus in fetal lamb kidney cells but was not active in heterologous (murine) cells.

Effect of loss of virus infectivity on the induction phenomenon. In preliminary experiments to determine the mechanism of interferon induction by MVL2, several treatments were used to alter the infectivity of the virus and determine whether loss of infectivity correlated with any changes in the inducing activity of the virus. The data are shown in Table 3. Heat treatment at 56°C markedly decreased the infectivity of the virus and resulted in a concomitant reduction in the capacity to induce interferon. However, infectivity could be differen-

TABLE 1. *Induction of interferon by MVL2 in sheep peripheral blood lymphocytes*

Inoculum (0.1 ml)	Units of interferon at:	
	24 h	48 h
Crude MVL2 stock: undiluted (10^8 PFU/0.1 ml)	680	340
Partially purified MVL2: undiluted (10^9 PFU/0.1 ml)	1,110	970
Partially purified MVL2: diluted 1:10	440	400
Partially purified MVL2: diluted 1:100	<25	<25
A. laidlawii (1305): 10^7 to 10^8 CFU[a]/0.1 ml	<25	<25
Tryptose phosphate broth	<25	<25
Bluetongue virus: undiluted (10^5 PFU/0.1 ml)	1,400	1,100

[a] CFU, Colony-forming units.

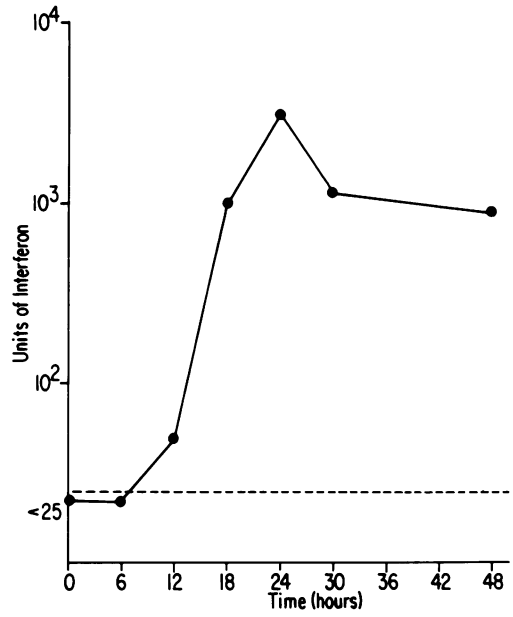

FIG. 1. *Kinetics of interferon induction by MVL2 in sheep lymphocytes. Crude MVL2 stock virus (10^8 PFU) was added to 1 ml of a sheep lymphocyte suspension (2×10^6 cells) at zero time. Supernatants were harvested at the indicated times and assayed for interferon. Each point was determined by using a pooled sample from duplicate cell cultures. The dotted line represents the lower level of detection in the interferon assay. Interferon was undetectable in control (uninfected) leukocyte cultures at any time period.*

TABLE 2. *Characterization of MVL2-induced interferon*

Characterization procedure[a]	Units of interferon
Control interferon (pH treated)	880
Interferon with no pH treatment	930
Heat-treated interferon	580
Trypsin-treated interferon	<25
Inhibition by actinomycin D	<25
Actinomycin D control (no drug)	680
Activity against Sindbis virus	850
Activity on murine cells	<25

[a] See text for procedural details.

TABLE 3. *Effect of various treatments of MVL2 on infectivity and ability to induce interferon*

Treatment	Infectivity (PFU/ml)	Units of interferon at 24 h
Untreated virus	1.0×10^{10}	875
Heat: 56°C, 1 h	2.0×10^{2}	215
Ultraviolet inactivation	1.4×10^{5}	750
Neutralization by antibody	8.0×10^{7}	1,050

tiated from interferon-inducing capacity as evidenced by the effect of ultraviolet treatment, which reduced infectivity about 100,000-fold yet had no effect on the ability to induce interferon. Partial neutralization of approximately 100-fold of infectivity by specific antiserum had no effect on the induction phenomenon.

Components of virus responsible for interferon induction. Although we showed in a previous section that intact, viable *A. laidlawii* cells did not induce interferon, the possibility exists that interferon induction by MVL2 was due to the presence of a contaminating subcellular component of the acholeplasma host cell. Contamination of the virus preparation with membranes was considered because the density of MVL2 virions (7) and that of mycoplasma membranes (21) are similar, rendering their separation during purification on sucrose gradients difficult. To examine this problem, the polypeptide composition of the virus preparation and that of *A. laidlawii* membranes and cell contents were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To adequately compare the full range of molecular weight species present in the virus and the acholeplasmas, gels ranging in concentrations from 7.5 to 12.5% were used. Figure 2 shows a representative 12.5% gel on which are indicated the polypeptides thought to be virus-specific structural proteins.

The partially purified virus used to induce interferon contains six to seven unique polypeptides ranging in molecular weights from 17,000 to 79,000. However, also present in the virus preparation are several major and minor polypeptides that appear to co-migrate with polypeptides present in both cell membranes as well as cell contents of *A. laidlawii*. Thus, the possibility exists that a contaminating acholeplasma cell component present in the virus preparation is responsible for the induction phenomenon. To test this, we examined the ability of two different preparations of *A. laidlawii* membranes as well as cell contents to induce interferon in sheep lymphocytes. As shown in Table 4, none of these preparations induced interferon, thus further suggesting that the virus is the critical component responsible for interferon induction.

Fate of virus after induction. An important question with respect to the mechanism of interferon induction by MVL2 is first, whether the virus remains intact, and second, whether the virus is able to replicate in the sheep lymphocyte cultures. In three separate experiments we measured the virus titers in the supernatants of the cell cultures at zero time and again at 48 h. In one such case the virus titer in the supernatant at the beginning of the experiment was 1.0×10^{8} PFU/ml. After 48 h, the virus titer was 8.6×10^{6} PFU/ml. In no case did we detect an increase in the virus titer from 0 to 48 h. In all three cases the virus titer dropped approximately 10-fold after 48 h. Thus, it seems clear that the virus remains relatively intact and does not undergo

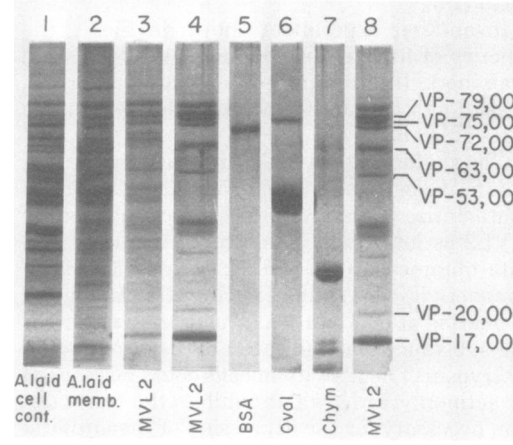

FIG. 2. *Structural polypeptides of MVL2 determined by electrophoresis on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Track 1, Soluble cell contents of A. laidlawii after removal of membranes; track 2, membranes isolated from A. laidlawii by sonic lysis of cells; track 3, partially purified MVL2; tracks 4 and 8, more highly purified MVL2 with virus-specific polypeptides (VP) indicated along with their approximate molecular weights; and tracks 5 through 7, molecular weight markers: bovine serum albumin (BSA), 68,000; ovalbumin (Oval), 43,000; and α-chymotrypsinogen (Chymo), 25,700.*

TABLE 4. *Failure of A. laidlawii components to induce interferon in sheep leukocytes*

Inoculum (0.1 ml)	Units of interferon at 48 h
A. laidlawii 1305 crude membranes prepared by sonic disruption (3.0 mg/ml)	<25
A. laidlawii 1305 membranes prepared by osmotic lysis (1.0 mg/ml)	<25
A. laidlawii 1305 cell contents (1.5 mg/ml)	<25
Bluetongue virus	1,100

a complete replication cycle in the lymphocyte cultures.

DISCUSSION

The data of this report show that MVL2, an enveloped DNA-containing virus isolated from *A. laidlawii*, is able to interact with sheep lymphocytes in vitro, resulting in the production of interferon. The interferon induced by MVL2 meets many of the standard criteria for classification as a type I (9) interferon. It is produced in moderate to high levels (>1,000 units), and its kinetics of appearance are similar to induction of type I interferon in lymphocytes by other agents (22). Sera from several sheep contained no detectable neutralizing antibody activity to MVL2 (unpublished data) making it unlikely that the interferon induction was mediated by an immune phenomenon.

Although the mechanism of induction of interferon by MVL2 is not resolved in this study, it appears reasonably clear that the virus and not a contaminant from the host acholeplasma is responsible for the phenomenon. Even though the partially purified virus preparations contained polypeptides that were probably of host origin, these are not likely to be the active agents because membranes or cell contents prepared from uninfected *A. laidlawii* did not induce interferon.

In this study, heat inactivation of MVL2 resulted in a decrease of the ability of the virus to induce interferon. Although the effect of heat on MVL2 is unknown, possibly the structural architecture of the virus was destroyed, thus decreasing the interaction of virus and lymphocyte. We were able to separate virus infectivity from interferon-inducing capacity by ultraviolet inactivation of the virus. The results of this experiment suggest that a fully functional viral genome is unnecessary for the induction process. Although we do not know the exact mechanism of the ultraviolet inactivation of MVL2, it probably resulted in an inactivation of the virus genome rather than an alteration of the structural integrity of the virus because the amount of ultraviolet irradiation used was considerably less than those levels that have been reported to alter or destroy structural proteins of other viruses (13, 15).

The literature contains several accounts of interferon induction by viruses from prokaryotic organisms and fungi. Both ribonucleic acid (RNA) and DNA-containing bacteriophages as well as RNA mycophages have been reported to induce interferon in vivo (8, 9). Most RNA phages active as interferon inducers have been viruses that contain double-stranded genomes (1, 8, 11). A notable exception was the work reported with the single-stranded RNA phage MS-2. Field and co-workers (3) showed that whereas the double-stranded replicative form of MS-2 phage RNA isolated from infected *Escherichia coli* was an active inducer in rabbits, the intact phage or single-stranded RNA extracted directly from the virus did not induce interferon. Hilleman (8) described a similar result for the MU-2 coliphage. He also reported that a DNA-RNA hybrid of F1 bacteriophage was an active inducer in rabbits. These studies appear to indicate that single-stranded viral RNA alone is inactive as an interferon inducer, but that RNA-RNA or DNA-RNA hybrid nucleic acids are active inducers. Interferon induction by DNA phages has been more limited, and the most notable example is the induction in mice by T4 phage (10). In these studies only intact virus was capable of causing induction, and phage particles devoid of DNA or the isolated DNA itself were incapable of causing the response. Although the ability of MVL2 to induce interferon may appear similar to induction by T4 phage, it should be emphasized that MVL2 induction has only been detected in vitro in lymphocytes. Mice injected intraperitoneally with 10^{10} PFU of MVL2 failed to produce detectable interferon (Lombardi and Cole, unpublished data).

The mechanism by which MVL2 induces interferon could be related to the virus genome in one of several ways. First, the viral DNA itself could cause the induction. Second, the virus could be contaminated with DNA-RNA hybrids from the infected host. Third, the virus could initiate an abortive replicative cycle in the lymphocyte, perhaps generating a nucleic acid hybrid capable of stimulating the induction process. These possibilities are currently under investigation.

The induction of interferon by MVL2 may also be dependent upon a specific interaction of the viral envelope with the membrane of the lymphocyte. Our preliminary studies on the polypeptide composition of MVL2 provide a basis for future studies to determine the nature of the envelope-membrane interaction as well as to further define the molecular structure of MVL2. A preliminary study of the structural polypeptides of MVL2 was also presented by A. L. Watkins (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, 624, p. 134). The data were similar although not identical to ours and suggested that the virus is composed of six separate polypeptides ranging in molecular weight from 14,500 to 89,000.

Thus, our observations that an intact enveloped DNA virus, MVL2, of a prokaryotic orga-

nism is able to interact with a eukaryotic cell in vitro appears unique, and elucidation of the mechanisms involved should provide valuable insights into membrane interactions between enveloped viruses and cells. These studies also raise questions as to whether interferon induction by other *Mycoplasma* species is mediated by harbored viruses and whether these viruses may alter mammalian cell functions in other ways.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to R. Kilholm and P. Caskey for their excellent technical assistance.

This investigation was supported by Public Health Service grant AI-12103 from the National Institute of Allergy and Infectious Diseases, and by a grant from the Kroc Foundation.

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