Effect of Viral Double-Stranded RNA on Mammalian Cells in Culture: Cytotoxicity Under Conditions Preventing Viral Replication and Protein Synthesis

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Noninfectious bovine enterovirus double-stranded RNA induces cytopathic effects when added to mammalian cells in culture. This is demonstrated by ⁵¹Cr release from prelabeled murine lymphoma cells and trypan blue uptake. Also, the induction of cell death by this viral RNA occurs in the presence of inhibitors of protein synthesis (cycloheximide and puromycin). The possible role and mechanism of viral, double-stranded RNA as a cytopathic agent in virally infected cells are discussed.

We have previously reported that purified bovine enterovirus double-stranded RNA induces cytopathogenicity when added to mammalian cells in culture (10). Although no virus production could be detected, as either free virus in the culture medium or by infectious center assay of the treated cells, it was not completely determined whether the induced cytopathic effect was mediated solely by the viral double-stranded RNA molecule or by a viral RNA-induced protein. In order to test whether the observed cytopathogenicity was due to the double-stranded RNA or to the de novo synthesis of a cytopathic protein from a defective or partial replication of this viral intermediate, the cytopathic activity of this RNA was examined in the presence of inhibitors of protein synthesis (puromycin and cycloheximide). The results, to be described in this paper, further support our original observation that the viral, double-stranded RNA is not infectious and that, in the absence of protein synthesis, this molecule is capable of causing rapid and extensive cell destruction.

MATERIALS AND METHODS

Cells, virus, and viral infection. The two mouse cell lines, L1210 lymphoma and L cells, are routinely maintained in this laboratory. L1210 cells were grown in suspension in F12 medium supplemented with 10% fetal calf serum. L cells were grown as monolayer

cultures in minimum essential medium (MEM) with Hanks salts containing 5% fetal calf serum. All tissue culture media were supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml).

Ehrlich ascites tumor cells were maintained by intraperitoneal passage of approximately 10⁸ cells at 8-day intervals in adult Swiss Webster mice.

Bovine enterovirus 1 propagation and infection have been previously described (10). Titrations of the virus were performed on L cell monolayers.

⁵¹Cr labeling of L1210. L1210, growing in F12 medium plus 10% fetal calf serum, were gently centrifuged and suspended to a concentration of 5 × 10⁶ cells/ml in F12 medium without serum. The cell culture was incubated in 5 µCi of ⁵¹CrO₄⁻ (134 µCi/ml) per ml for 30 min at 37 C. After the incubation period, the cells were washed twice with fresh media and then were resuspended in fresh F12 medium containing 10% fetal calf serum.

Isolation and purification of viral double-stranded RNA. At 7 h postinfection with bovine enterovirus 1, Ehrlich ascites tumor cells were harvested by centrifugation, suspended in phosphate-buffered saline (pH 7.2) containing 0.001 M MgCl₂, and homogenized gently in a glass Dounce homogenizer. Cell debris and nuclei were removed by centrifugation at 700 × g for 20 min, the pellet was washed twice in the same buffer, and the washes and supernatant fluids were pooled. Sodium dodecyl sulfate (SDS) was added to the supernatant fluid to a final concentration of 0.1% and shaken vigorously for 5 min at room temperature. RNA was extracted at 37 C with a 1:1 mixture of buffer-saturated phenol and chloroform. The resulting aqueous layer was removed, and the interface was re-extracted. Pooled, aqueous layers were extracted twice with chloroform and then were ethanol precipitated. The viral double-stranded

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RNA was isolated by differential salt precipitation by the method of Bishop and Koch (6). Total extracted RNA was dissolved in 0.02 M sodium phosphate, pH 7.2, at a concentration of 1 mg/ml. NaCl was added to a final concentration of 1 M. The RNA solution was frozen at -20°C , then thawed at 4°C . The precipitate was collected by centrifugation at 2,000 rpm for 20 min at 0°C and was washed once with 1 M NaCl. The supernatant fluid and wash, containing viral replicative form, were pooled and ethanol precipitated. The precipitate was then suspended in 15 mM Tris-hydrochloride (pH 7.4) buffer containing 50 mM NaCl, 2 mM MgCl_2 . DNase was added (20 $\mu\text{g}/\text{ml}$), and the solution was incubated for 30 min at 23°C . The NaCl concentration was increased to 0.3 M, RNases A (20 $\mu\text{g}/\text{ml}$) and T_1 (2 $\mu\text{g}/\text{ml}$) were added, and the incubation was continued for 30 min at 37°C . At the end of this time, Pronase (preincubated for 2 h at 37°C) was added to a final concentration of 1 mg/ml, and incubation was continued for 2 h at 37°C (21). The double-stranded RNA was extracted with chloroform and purified through a 15 to 30% (wt/vol) sucrose gradient (0.15 M NaCl, 5×10^{-3} M Tris-hydrochloride, pH 7.4) by centrifugation for 20 h or longer at 25,000 rpm in a Spinco SW15.1 rotor. The purified RNA finally was dialyzed against 0.15 M NaCl and 0.001 M EDTA for 12 h at 4°C (or for melting temperature studies was dialyzed against $0.01 \times \text{SSC}$ with 0.001 M EDTA; $\text{SSC} = 0.15 \text{ M NaCl}$, 0.015 M sodium citrate, pH 7.0). Radioactive double-stranded RNA was obtained by adding 1 to 3 μCi of ^3H -uridine per ml to the viral infection medium 1 h postinfection in the presence of 5 μg of actinomycin D per ml.

Infectivity assay of viral double-stranded RNA.

To test infectivity, double-stranded RNA, appropriately diluted in 1 M NaCl containing 100 μg of DEAE-dextran per ml, was added in a volume of 0.05 ml to an L cell monolayer which previously had been washed with saline and then with 0.6 M NaCl. After 30 min of incubation at 37°C , the monolayers were overlaid with a 1:1 mixture of 1.2% Noble agar and $2 \times$ Earle MEM. The dishes (Falcon, 60 mm) were incubated for 48 h, after which the agar overlay was removed, and the cell sheet was stained with 0.8% crystal violet in 20% ethanol in order to enumerate plaques.

Gel electrophoresis. Agarose (0.5%)-acrylamide (2.0%) gels were prepared by the method of Peacock and Dingman (18) as modified by substituting E buffer (5) (0.02 M sodium acetate, 0.04 M Tris, 0.001 M EDTA, pH 7.2, with glacial acetic acid) and by incorporating 0.2% SDS into the gels. Gels were allowed to polymerize in plastic tubes (0.7 by 12.5 cm) bearing a piece of dialysis tubing secured with a rubber band at the bottom. After polymerization, the dialysis tubing was perforated, and the gels were prerun for 1 h at a constant 8 mA/gel in E buffer plus 0.2% SDS at room temperature. Samples of viral double-stranded RNA were loaded in volumes of 15 to 25 μl , and electrophoresis was carried out at a constant 8 mA/gel for 3.0 h at room temperature. At this time, gels were removed from the tubes and sliced into 2-mm slices; then they were digested in 0.5 ml of a solution composed of Biosolv-3 (Beckman) diluted 1:2 with toluene-2,5-diphenyloxazole (PPO; New

England Nuclear Corp., Boston, Mass.) scintillation fluid at 60°C for 12 to 15 h. Radioactivity was counted after the addition of 5 ml of toluene-PPO scintillation fluid.

Measurement of thermal denaturation. Viral double-stranded RNA samples were first dialyzed against $0.01 \times \text{SSC}$ with 0.001 M EDTA. The melting profiles of the RNA were obtained by using a Gilford spectrophotometer, model 2000, coupled to a Colara ultrathermostat and a thermosensor unit. Results are presented as optical densities at the ambient temperature without correction for solvent expansion.

Radioactive materials and counting. Radioactive materials were [$5\text{-}^3\text{H}$]uridine: 20 to 30 Ci/mmol (New England Nuclear Corp., Boston, Mass.) and ^{51}Cr ($^{51}\text{CrO}_4^-$, 134 $\mu\text{Ci}/\text{g}$; Amersham/Searle, Arlington Heights, Ill.). Radioactive samples of ^3H -uridine and ^{51}Cr were counted in a Beckman liquid scintillation counter in either toluene-PPO scintillation cocktail or Aquasol (New England Nuclear, Boston, Mass.) as specified in the text. By using a wide-window fixed ISO-SET, ^3H was counted at 35% efficiency, and ^{51}Cr was counted at 15% efficiency.

Inhibitors and enzymes. Inhibitors and enzymes were as follows: puromycin (Sigma Chemical Co., St. Louis, Mo.); cycloheximide, grade B (Calbiochem, San Diego, Calif.); DNase, electrophoretically pure (Sigma Chemical Co., St. Louis, Mo.); RNases A and T_1 (Worthington Biochemical Co., Freehold, N.J.); Pronase, grade B (Calbiochem, Los Angeles, Calif.); and actinomycin D (Schwarz/Mann, Orangeburg, N.Y.)

RESULTS

Characterization and purity of bovine enterovirus 1 double-stranded RNA. That the material being used in this study is viral double-stranded RNA is indicated by the following characteristics; it is resistant to the enzymatic action of DNase, Pronase, and both RNase A and RNase T_1 , at high ionic strengths, although it is sensitive to RNase treatment at low ionic strengths. That it is of viral origin, and not of host origin, is indicated by its synthesis in the presence of 5 μg of actinomycin D per ml. The molecule is denatured both by acid and alkali hydrolysis. The single, sedimenting peak of RNase-resistant RNA at approximately 20S upon zonal centrifugation in sucrose density gradients (Fig. 1) is characteristic of the sedimentation pattern obtained for picornavirus replicative form (6, 7). This species migrates as a single, homogeneous peak in 2.0% acrylamide gels to a position characteristic of picornavirus double-stranded replicative form under similar conditions (22) (Fig. 2). The thermal denaturation curve of this viral intermediate clearly reflects its double-stranded structure and purity (Fig. 3). The sharp thermal transition at approximately 73 to 74.5°C in $0.01 \times \text{SSC}$ is typical of a completely base-paired double-

FIG. 1. Sedimentation of bovine enterovirus 1 double-stranded RNA through sucrose gradients. Viral infections, labeling, and isolation of bovine enterovirus 1 double-stranded RNA were carried out as described in Materials and Methods. RNA was layered over 15 to 30% sucrose gradients and centrifuged for 20 h or more at 25,000 rpm. After centrifugation, the gradient was fractionated into 1-ml samples by removal from the top, and 0.2 ml of each sample was assayed for radioactivity. Measurement of radioactive material was obtained by precipitating each sample with 10% cold trichloroacetic acid plus 100 μ g of yeast t-RNA as carrier by collecting and washing on membrane filters. Sedimentation coefficients were estimated with peaks of L cell ribosomal RNA as points of reference. Solid line, double-stranded RNA; broken line, after treatment of gradient fractions with T_1 and A RNases (2 and 20 μ g/ml, respectively, for 30 min at 37 C). Bottom of the gradient is to the right.

stranded molecule. The low $\sigma^{2/3}$ or transition dispersion of about 8.6, as well as a hyperchromicity of 23 to 26%, indicates that this is indeed a double-stranded structure. Similar results have been obtained for poliovirus replicative form (6). We have calculated that approximately 1.4×10^4 molecules of viral double-stranded RNA are produced per cell during a typical infectious cycle.

Infectivity of viral double-stranded RNA.

From the same batch of infected cells, one-half of the total RNA initially isolated was prepared as described in Materials and Methods, and the other half was isolated in an identical fashion without RNase treatment. Both $20s_{20,w}$ sedimenting RNAs were then tested for infectivity by the plaque assay method outlined in Materials and Methods. It can be seen from Table 1 that treatment of the double-stranded viral RNA with RNase drastically reduced (5 log

FIG. 2. Analysis of bovine enterovirus 1 double-stranded RNA by agarose acrylamide gel electrophoresis. 3 H-uridine-labeled viral double-stranded RNA was prepared from Ehrlich ascites infected tumor cells as outlined in Materials and Methods, as well as from the gel procedures. RNA, (50 μ g) in a volume of 20 μ liters, was loaded on 2.0% acrylamide gels and run at 8 mA/gel for 3 h at room temperature. The arrow indicates the point of migration of the dye marker, bromophenol blue.

FIG. 3. Thermal denaturation of bovine enterovirus 1 double-stranded RNA. The melting temperature profile of purified viral double-stranded RNA was carried out in $0.01 \times$ SSC. Optical density was recorded at 260 nm. At 20 C, initial values (E_{260}) were between 0.3 to 0.5, and optical density was recorded with a 25-s dwell time for temperatures up to 96 C.

TABLE 1. Infectivity of double-stranded RNA^a

Determination	PFU/ μ g of RNA (RNase treatment)	PFU/ μ g of RNA (untreated)
Isolation 1	<1	3.2×10^5
Isolation 2	<1	5.3×10^5

^a Viral double-stranded RNA was isolated and prepared as described in Materials and Methods except, where indicated, RNase treatment was eliminated. The 20S_{20, w} sedimenting RNAs were plated on confluent L cell monolayers as described in Materials and Methods. Each value presented represents the average of 10 plates at three different dilutions of RNA.

units) the plaque-forming ability of the RNA. However, the untreated double-stranded RNA had a low efficiency of infectivity. By using a value of 4×10^6 as the molecular weight of double-stranded, bovine enterovirus 1 RNA, 1 μ g of double-stranded RNA should contain 1.5×10^{11} molecules; however, infectivity assays gave a value of 4×10^5 PFU/ μ g.

Effect of viral double-stranded RNA on ⁵¹Cr release. We have previously reported that viral double-stranded RNA is cytopathic when added to cells in culture (10). These observations were made by direct microscopy visualization of the treated cells as determined by trypan blue staining, the dye being excluded from viable cells. In an effort to assure that this method of cell death assessment is valid, a similar study was performed by using ⁵¹Cr release. ⁵¹Cr is routinely employed in assays for cell-mediated cytolysis in immunological studies (8, 9) and has recently been used for viral-induced cytopathic determination (15). Cells prelabeled with ⁵¹Cr release the label into the culture medium only when these cells are undergoing degeneration and lysis; thus release of ⁵¹Cr is a direct radioactive assay of cytopathic effect. This technique was used to assay the cytopathic effect of the viral double-stranded RNA. The data in Fig. 4 would suggest that there is a valid correlation between ⁵¹Cr release and trypan blue uptake in cells treated with viral double-stranded RNA. Similar results for both ⁵¹Cr release and trypan blue uptake were obtained with bovine enterovirus 1-infected cells (data not shown). It should also be noted that, in control cultures treated with an identical concentration of the synthetic homopolymer, poly (I) · poly (C), increased levels above an untreated control background were also obtained (1,200 counts/min and 23% stained cells at 14 h postinfection). However, the cytopathic effect is much more extensive with the natural, viral double-stranded RNA than with poly (I) · poly (C).

FIG. 4. A comparison of bovine enterovirus 1 double-stranded, RNA-induced cytopathogenicity as measured by ⁵¹Cr release and trypan blue uptake. Purified viral double-stranded RNA, in a small volume of 2 $\times$ SSC, was added to L1210 cells prelabeled with ⁵¹Cr and suspended in F12 media with 10% fetal calf serum at a concentration of 5×10^6 cells/ml. The cell culture was incubated at 37 C, and at various time intervals, 0.2-ml samples were removed from the culture, and the cells were gently removed by centrifugation at 1,000 rpm. The resulting supernatant fluid was centrifuged at 1,500 rpm, after which 0.1-ml of the supernatant fluid was pipetted into 15 ml of Aquasol for radioactive counting. The cell pellet was resuspended in 0.2-ml of saline to which 40 μ liters of 1% trypan blue stain was added for direct microscopy determination of cell death in a hemocytometer. Symbols: $\circ$, Δ , control culture to which an equal volume of 2 $\times$ SSC was added; $\bullet$, $\blacktriangle$, viral, double-stranded RNA-treated culture at a concentration of 15 μ g/ml.

Effect of inhibitors of protein synthesis on bovine enterovirus 1 double-stranded RNA-induced cell death. Although it is demonstrated in Table 1 that the double-stranded RNA employed in these studies is not infective, one cannot preclude the possibility of a defective or partial translation of this viral intermediate leading to the production of a cytopathic protein. In order to eliminate this possibility, the effect of cytopathic induction by the viral double-stranded RNA was examined in the presence of inhibitors of protein synthesis. L1210 cells pretreated with either cycloheximide (25 μ g/ml) or puromycin (100 μ g/ml), concentrations which sufficiently inhibited all host-cell protein synthesis (13, 17), were treated with bovine enterovirus 1 double-stranded RNA. Cytopathic effect occurred irrespective of the presence of the inhibitor (Fig. 5 and 6). The rate of cytopathogenicity induced by the viral RNA was as rapid as in the absence of the

FIG. 5. Effect of cycloheximide on viral double-stranded RNA-induced cytopathic effects in L1210 cells. Cycloheximide was added to a concentration of 25 $\mu\text{g/ml}$ to one of two identical cultures of L1210 cells (4×10^5 cells/ml), after which incubation was continued at 37 C. After 20 min of incubation, each culture was then divided equally and bovine enterovirus 1 double-stranded RNA was added to one-half of the divided cultures at a final concentration of 20 $\mu\text{g/ml}$ in $2 \times \text{SSC}$. An equal volume of $2 \times \text{SSC}$ was added to the other half of the culture. Incubation at 37 C was continued, during which 20- μl samples were removed, and cells were stained with 4 μl of 1% trypan blue. Viable-cell counts were made directly in a hemocytometer. Symbols: ▲, viral double-stranded RNA (20 $\mu\text{g/ml}$) + cycloheximide (25 $\mu\text{g/ml}$); ●, viral double-stranded RNA only (20 $\mu\text{g/ml}$); △, cycloheximide only (25 $\mu\text{g/ml}$); ○, untreated.

inhibitors, nearly 100% cell death 10 h after treatments. Figure 5 shows that cycloheximide alone is not deleterious to cells for up to 10 h, and even for up to 24 h no deleterious effect was noted because of the presence of this inhibitor. However, puromycin (Fig. 6) has a definite lethal effect on the cells, which can be seen to commence 10 h after addition of the inhibitor and which results in greater than 60% cell death by 20 h. This toxicity may reflect the different modes of action of the inhibitors.

Cell lysis. The effect of viral double-stranded RNA on cell growth was monitored (Fig. 7). The addition of viral double-stranded RNA to an actively growing culture of L1210 in suspension led to a decrease in the cell population in 10 h. This reduction was most likely due to cell lysis, for cell fragments were visually apparent. The untreated control culture showed a continuous growth pattern with a generation time of 16 h.

DISCUSSION

The results reported in this paper confirm in more detail the cytotoxic effect of bovine en-

FIG. 6. Effect of puromycin on viral double-stranded RNA-induced cytopathic effects in L1210 cells. The experimental procedure is the same as described in the legend of Fig. 5, except that 100 μg of puromycin per ml was added in place of 25 μg of cycloheximide per ml. Symbols: ■, viral double-stranded RNA (20 $\mu\text{g/ml}$) + puromycin (100 $\mu\text{g/ml}$); ●, viral double-stranded RNA (20 $\mu\text{g/ml}$); □, puromycin only (100 $\mu\text{g/ml}$); ○, untreated.

FIG. 7. Effect of viral double-stranded RNA on L1210 cell growth. L1210 cells in suspension culture at approximately 3×10^5 cells/ml were treated with 20 μg of bovine enterovirus 1 double-stranded RNA per ml in $2 \times \text{SSC}$. A culture of L1210 was treated identically with an equal volume of $2 \times \text{SSC}$. The cultures were incubated at 37 C. At various time intervals, 20- μl samples were removed, and direct microscopy cell counts were made with a hemocytometer. Symbols: △, viral double-stranded RNA (20 $\mu\text{g/ml}$); ▲, untreated control.

terovirus double-stranded RNA on mammalian cells in culture. The method used for isolation of this toxic RNA, specifically treatment with RNases A and T₁, has greatly reduced the intrinsic infectivity of this viral intermediate without destroying its toxicity. The infectivity was reduced by 5 log units in plaque-forming ability as compared with double-stranded RNA isolated without RNase digestion. This undigested double-stranded RNA is at the same level of infectivity as that reported for poliovirus double-stranded RNA by Bishop and Koch (6). These authors also observed that the infectivity of poliovirus double-stranded RNA was reduced after treatment with 1 μ g of RNase A per ml at high ionic concentrations. Both these results and those reported here would suggest that nicks might have occurred in one strand of the RNA or that a single-stranded region, such as poly A may have been degraded.

The characteristics of the cytotoxic material are typical of picornavirus double-stranded RNA. We have demonstrated here that the viral double-stranded RNA alone is capable of inducing cytopathic effects in treated cells. This conclusion is based on the observations that the RNA-induced effects occur under conditions preventing viral protein synthesis, i.e., in the presence of cycloheximide or puromycin. These data would preclude the possibility of the *de novo* synthesis of a viral cytopathic protein as mediator of cell death in these treated cells.

We have previously shown (11) that 20% of the viral double-stranded RNA at the concentration used here is incorporated into the intracellular regions of the cells. This is equivalent to 1.5×10^6 molecules per cell. This is 100-fold higher than the level of double-stranded RNA produced per cell during virus infection (1.4×10^4). The addition of increasing amounts of double-stranded RNA shortens the onset of cell death (10).

That viral double-stranded RNA or replicative form may be directly responsible for virus-induced cell death during picornavirus infection is consistent with other reported results. It is clearly established that a functional viral genome is required for cytopathic induction, as well as for the inhibition of cellular macromolecular synthesis (16, 19). Both of these effects require viral-specific protein synthesis (4), hence eliminating a virion structural component as a responsible cytopathic agent. Viral protein synthesis is necessary for the production of a RNA-dependent-RNA-polymerase in order to synthesize the double-stranded replicative form (20).

Studies with poliovirus by Bablanian et al. with guanidine-hydrochloride have led these

authors to the conclusion that cell death is a result of the accumulation of a cytopathic protein (1, 2, 3). However, their observations can be reinterpreted by using the data presented in this paper showing viral double-stranded RNA as the possible mediator of cell death as well as the inhibitor of cellular protein synthesis.

Koschel has recently concluded that the onset of cell death is related to the viral-induced inhibition of cellular protein synthesis (15). Ehrenfeld and Hunt have reported that poliovirus double-stranded RNA inhibits cellular protein synthesis *in vitro* (12, 14). We have subsequently shown that bovine enterovirus double-stranded RNA inhibits protein synthesis in intact cells and that the inhibition is specific for cellular protein synthesis insofar as picornavirus-specific polypeptide synthesis is unaffected by the addition of viral double-stranded RNA (11). It is possible that the inhibition of cellular protein synthesis and cell death induction are directly related through the action of double-stranded RNA. The amount of double-stranded RNA taken up by the cells may be sufficient to bind completely to a specific initiation factor or class of polysomes, thus inhibiting host protein synthesis. However, it should be pointed out that large amounts of viral double-stranded RNA are not accumulated until late in the replicative cycle of the virus, past the time of inhibition of host protein synthesis.

It seems significant that interferon is induced by toxic substances, for viral double-stranded RNA is the most active inducer. Stewart et al. (22) have shown that interferon-primed cells are much more sensitive to the toxic effect of synthetic double-stranded RNA, poly (I)·poly (C), than are unprimed cells. The concentrations of poly (I)·poly (C) used by Stewart et al. were similar to those described in this paper.

One cannot exclude the possibility that the addition of noninfective dsRNA to the cells produces a cytotoxic effect that is quite independent of events that occur during picornavirus infection. Treatment of the viral RNA with RNase may have created a toxic molecule that normally does not exist in the cell. This possibility is currently being investigated.

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