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Effect of Double-Stranded Viral RNA on Mammalian Cells in Culture

(bovine enterovirus/picornavirus/interferon/tumor regression)

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ABSTRACT **During bovine enterovirus infection of Ehrlich ascites tumor cells, large amounts of double-stranded RNA accumulate. Addition of this double-stranded RNA to uninfected cells leads to rapid cell death. This is not a result of infectious virus production. Neither single-stranded RNA nor heat-denatured double-stranded RNA has this effect. Similar experiments with synthetic double-stranded polymers, poly(I)·poly(C) and poly(A)·poly(U), show that they are only slightly toxic at the concentrations used. The effect of the double-stranded RNA is nonspecific for cells of different origins. The implications of this finding in relation to the cytopathic effects of picornavirus and to cancer chemotherapy are discussed.**

During the replication cycle of picornaviruses, double-stranded RNA is formed as an intermediate (1). In polio- and mengovirus infected cells, this double-stranded RNA is detectable 3–5 hr after infection (2, 3). In bovine enterovirus (BEV) replication, double-stranded RNA is detectable 2 hr after infection (manuscript in preparation). Unlike mengovirus- and poliovirus-infected cells, in which only 1–5% of the total amount of RNA is double-stranded, in BEV-infected cells, double-stranded RNA accumulates throughout the infection cycle; 7 hr after infection, just before cell death, it is the major species of viral RNA in the cells. In order to test whether there was any relationship between the accumulation of double-stranded RNA and the cytopathic effect, we have examined the effect of this RNA on various cells in culture. Our data suggest that viral double-stranded RNA is very toxic to such cells.

MATERIALS AND METHODS

Cells and virus

Monolayer cultures of WI38 were purchased from GIBCO (Grand Island, N.Y.) and were maintained in F10 medium supplemented with 5% fetal calf serum. Mouse L-cells and Maden bovine kidney cell line are routinely carried in this laboratory and were grown in Eagle's Minimum Essential Medium (MEM) containing 5% calf serum. The leukemic cultures, CRM-CEM (human) and L1210 (mouse) were the gift of Dr. A. Granoff, St. Judes Children's Hospital. These cells were grown in suspension in MEM-suspension medium supplemented with 10% fetal calf serum. All tissue culture media were supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (5 units/ml).

Abbreviations: MEM, minimal essential medium; SDS, sodium dodecyl sulfate; BEV, bovine enterovirus; SSC, standard saline citrate (0.15 M NaCl–0.015 Na citrate); MDBK, Maden bovine kidney cells.

Ehrlich ascites tumor and Sarcoma-1 were maintained as ascites tumors by intraperitoneal passage of about 10^8 cells at 8-day intervals in adult Swiss-Webster mice.

Bovine enterovirus-1 (BEV) was grown as described (4). Titrations of the virus were performed on either L-cells or MDBK cells.

Virus infection

Suspensions of 10^7 cells per ml were infected with BEV at a multiplicity of infection of 20. All viral-RNA labeling was done in the presence of actinomycin D. Virus adsorption was allowed to proceed for 1 hr at 37°C with low-speed rotary shaking. After the adsorption period, cells were washed and resuspended to 2×10^6 cells/ml in MEM with 2% chicken serum and 5 μg/ml of actinomycin D. To label viral RNA, we added [^{3}H]uridine (1 μCi/ml; 20–30 Ci/mmol, New England Nuclear Corp., Boston, Mass.) to the culture.

Preparation of doubled-stranded viral RNA

After 7–7.5 hr of infection, cells were harvested by centrifugation and viral RNA was extracted by differential salt precipitation (5). The double-stranded viral RNA was treated with 10 μg/ml of pancreatic RNase at 37°C for 30 min and further purified on an Agarose 2B column eluted with sodium dodecyl sulfate (SDS)–Tris buffer (0.1 M NaCl–0.01 M Tris·HCl–0.001 M EDTA–0.5% SDS, pH 7.4) or Sephadex G-200 eluted with 0.2 M NaCl–0.02 M Tris·HCl–0.002 M EDTA, pH 7.5. Double-stranded RNA was precipitated with ethanol, dried thoroughly, and resuspended in either saline or saline containing 30 μg/ml of protamine sulfate or 50 μg/ml of DEAE-dextran.

Denaturation of double-stranded RNA

Samples of double-stranded viral RNA, suspended in either 0.1× SSC or 2× SSC (1× SSC = 0.15 M NaCl–0.015 M sodium citrate, pH 7) were heat-denatured at 100°C for 60 min, after which the samples were rapidly cooled at 0°C. Absorbance was measured to observe any hyperchromic effect. Samples of double-stranded RNA were also denatured by incubation of the RNA in a solution of low ionic strength, 0.1× SSC, in the presence of 10 μg/ml of pancreatic RNase for 30 min at 37°C.

Cell treatment with double-stranded RNA

Double-stranded RNA was added to small volumes of cells (1×10^6 cells) in phosphate-buffered saline (0.02 M Na-PO_4–0.15 M NaCl) at various concentrations. At various times after addition of the RNA, viable and dead cells were counted and discriminated by their ability to exclude trypan blue, dead

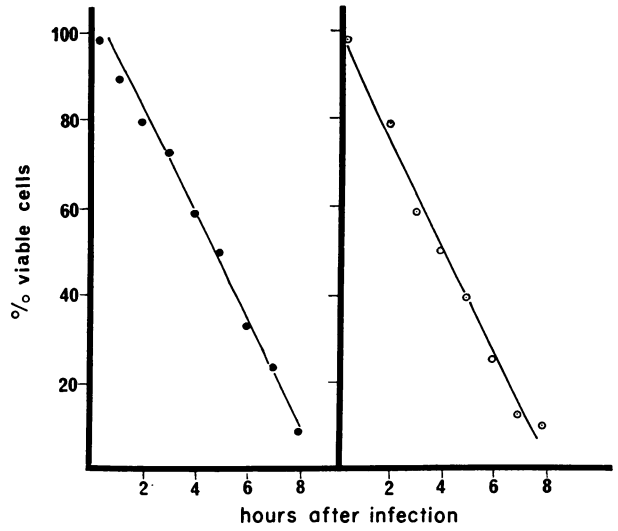

FIG. 1. Rate of cell killing after BEV-1 infection. Cells were infected with 20 plaque-forming units/cell. Samples of 10^6 cells were extracted from the infected culture at different intervals and stained with a 1% trypan blue solution. The percentage of viable cells was determined by direct microscopic count. Uninfected cells are no more than 20% dead after 8 hr in suspension culture. *Left*, Ehrlich ascites tumor; *right*, L-cells.

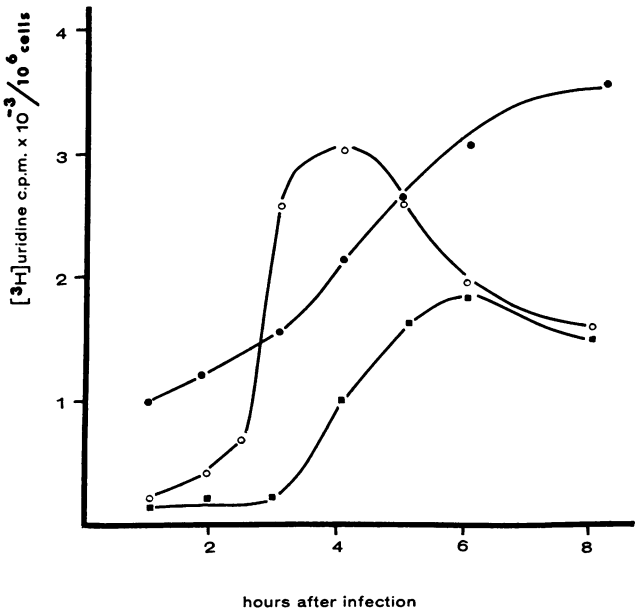

FIG. 2. Uridine incorporation into single-stranded RNA, double-stranded RNA, and virus in Ehrlich ascites tumor cells infected with BEV-1. At the time of viral infection, 10 μg/ml of actinomycin D was added. 1 hr after infection, 1 μCi/ml of [5-^{3}H]uridine (30 Ci/mmol) was added. Samples were withdrawn and analyzed for [^{3}H]uridine incorporation by the method of Plagemann (19). Symbols: —O—, single-stranded RNA; —●—, double-stranded RNA; —■—, RNA in virions.

cells being permeable to the dye. Similar experiments were done with heat denatured RNA, poly(I)·poly(C), and poly(A)·poly(U) (Miles Lab., Elkhart, Ind.).

Infectious center assay

Cells infected with double-stranded RNA were placed on monolayers for 1 hr, then covered with a 0.6% washed agar–MEM layer. After 48 hr, the monolayers were stained with crystal violet for detection of plaques.

Labeled samples were collected by precipitation with trichloroacetic acid on Millipore filters, or else the precipitated material was solubilized in Beckman Biosolv-3 for 12 hr at 37°C.

RESULTS

The replication cycle of BEV is very rapid, as indicated by the kinetics of cell killing (Fig. 1). Both hosts, Ehrlich ascites tumor and L-cells, show the same rapid rate of cell destruction after infection with this virus; all cells are dead by 8 hr. This rate of killing is directly proportional to the multiplicity of infection (manuscript in preparation).

We have reported (4) that the amount of total-viral RNA accumulated in BEV-infected cells shows increased RNA synthesis after 1–4 hr of infection, with maximum synthesis between 6–8 hr. This RNA accumulation was further examined by following the synthesis of specific classes of viral RNA. The results in Fig. 2 show [^{3}H]uridine incorporation into single- and double-stranded viral RNA and the RNA of free virions. Single-stranded RNA is seen to accumulate up to 4 hr after infection, after which its synthesis decreases, most probably because of packaging of single-stranded RNA into mature virions (which increase simultaneously). Double-stranded RNA, however, is synthesized early and continues to be synthesized up to the time of cell death, at which stage it accounts for the majority of viral RNA in the cell. Purified double-stranded RNA from BEV was used to infect cells in order to examine whether there was a correlation between onset of cell death and accumulation of RNA. The results in Fig. 3 and 4 indicate that the addition of double-stranded viral RNA to growing cells leads to cell death. Cell destruction is rapid; 100% of the cells are dead 2 hr after the addition of the double-stranded RNA. This effect does not seem to be due to the replication of the input double-stranded RNA, since infectious virus could not be recovered during these infections nor did infectious-center assay reveal the presence of any viral particles. It would appear, therefore, that this double-stranded RNA is not reproductively active and that such activity is not necessary to produce cell death. In the experiment shown in Fig. 3, DEAE-dextran was used to increase the infectivity of the double-stranded RNA (6), however, DEAE-dextran alone is toxic to cells. Other polybasic compounds were also tested, but since these also killed cells, they were not used for assays of RNA infectivity.

If the double-stranded viral RNA is denatured by either heat or treatment with 0.1× SSC and RNase, its ability to kill cells is eliminated (Fig. 4).

The effect of the concentration of input double-stranded RNA on cell killing was examined (Fig. 5). The rate of cell destruction is directly related to the amount of double-stranded RNA used to infect the cells. At 1 mg of RNA per 10^7 cells, all cells are dead 0.75–1 hr after the addition of the RNA. The time required to kill all the cells becomes increasingly longer as the input concentration of double-stranded viral RNA is decreased. These curves suggest that several sites in the cells are affected by the double-stranded RNA. 1 mg of RNA, by our calculations, may be equivalent to 10^{10}–10^{11} total particles of virus. At this cell:RNA ratio,

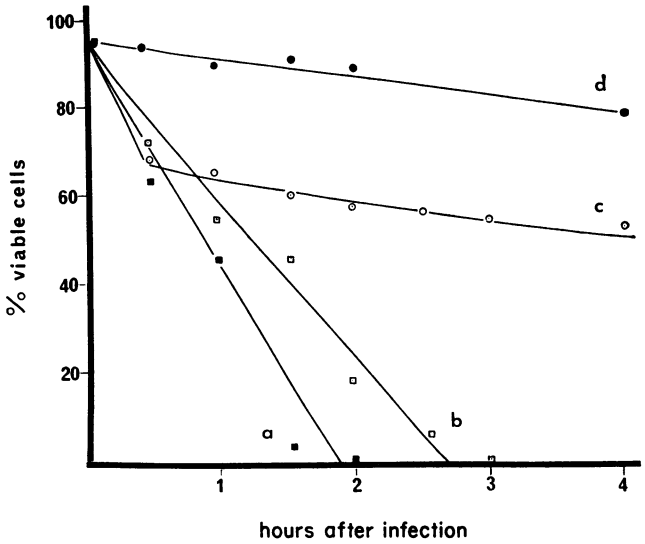

FIG. 3. Rate of cell killing with viral double-stranded RNA. Four aliquots, of 6×10^5 Ehrlich ascites cells each were washed and suspended in: (*a*) 0.06 ml of RNA solution (0.05 mg/ml in DEAE-dextran–saline solution); (*b*) 0.06 ml of RNA solution (0.05 mg/ml in saline); (*c*) 0.06 ml of glycogen (0.05 mg/ml; ethanol-precipitated and dried in the same manner as the RNA in DEAE-dextran–saline solution); and (*d*) 0.06 ml of glycogen (0.05 mg/ml in saline). RNA was measured by its A_{260}, 1 mg/ml = 25 A_{260} units/ml. The infected cultures were incubated at 37°C with slight rotary shaking. Samples of 0.01 ml were treated with 0.002 ml of 1% trypan blue, and viable-cell counts were made directly in a hemocytometer.

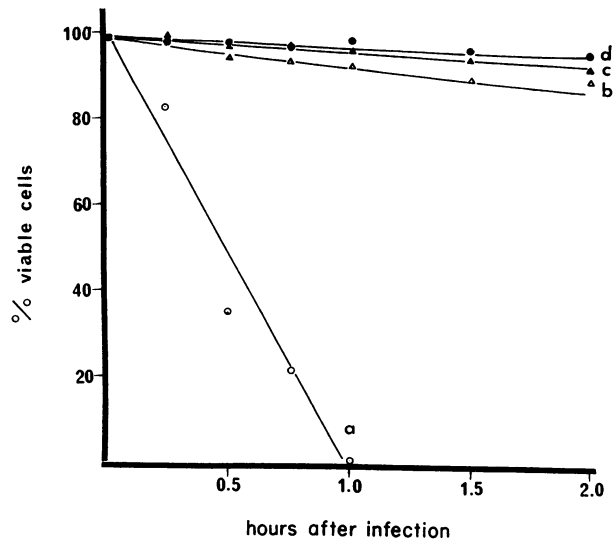

FIG. 4. Rate of cell killing with denatured, viral double-stranded RNA. Four aliquots of L1210 cells (6×10^5 each) were washed and suspended in: (*a*) 0.06 ml of RNA (1.0 mg/ml in saline); (*b*) 0.06 ml of heat-denatured RNA (originally 1.0 mg/ml in saline); (*c*) 0.06 ml of RNA (originally at 1.0 mg/ml, treated with RNase in 0.1 × SSC); and (*d*) 0.06 ml of glycogen (1.0 mg/ml in saline) and incubated at 37°C with low-speed rotary shaking. 0.01-ml samples were treated with 0.002 ml of 1% trypan blue, and viable-cell counts were made directly in a hemocytometer.

the maximum amount of RNA used is about 10 times that which is produced by each cell in a normal infection. If we assume that some of the RNA is degraded, this amount is well within physiological concentrations. Holland (7) has shown that infection with as many as 10^6 poliovirus particles per cell does not lead to an immediate cytopathic effect.

It is known that foreign nucleic acids are readily incorporated into cells (8). In order to test whether double-stranded RNA acts from without, or by association with the cell, we used labeled double-stranded RNA to induce cell death. After 100% of the cells were killed, the infected cells were collected and assayed for radioactive acid-insoluble material. The data of Table 1 indicate that the double-stranded RNA is associated with the cell. Whether the double-stranded RNA is attached to the cell membrane or is in association with some internal structure is not now known.

Synthetic double-stranded polymers, poly(I)·poly(C) and poly(A)·poly(U), were also tested for their ability to induce cell death at concentrations equivalent to the viral double-stranded RNA (Fig. 6). Up to 30% of the cells are killed by concentrations equivalent to those that usually produce 100% cell death 1.5 hr after infection with double-stranded RNA from BEV.

The effect of double-stranded RNA from BEV on different cells was tested, and is presented in Table 2. In every cell type tested, this RNA killed cells, but the rate of killing

TABLE 1. *Incorporation of [^3H]RNA*

	Acid-insoluble material (cpm) Sample	
	A	B
Cell-associated	1290	1300
Supernatant and wash	278	214

Double-stranded [^{3}H]RNA was prepared as described in *Methods* (1.0 mg/ml with 23,400 cpm/mg). L1210 cells (6×10^5) were washed and suspended with 0.06 ml of this RNA in saline. The infected culture was incubated with low-speed rotary shaking for 1.0 hr, after which the cells were centrifuged and washed three times in saline. The supernatant and washes were pooled. Both the cell pellet and supernatant were analyzed for acid-insoluble material by precipitation onto Millipore filters.

TABLE 2. *Cell response to double-stranded RNA*

Cell type	Origin	Hours to reach 100% death
Ehrlich ascites tumor	mouse	1.5
SA1	mouse	1.75
L1210	mouse	1.5
L-cells	mouse	2.0
CRF-CEM	human	1.0
WI38	human	1.25
Kidney	bovine	1.5

6×10^5 cells of each cell type were washed, resuspended with RNA as in Table 1, and incubated at 37°C. Samples of 0.01 ml were mixed with 0.002 ml of a 1% trypan blue solution at 0.25-hr intervals. Viable-cell counts were made directly in a hemocytometer. All results are ±0.5 hr.

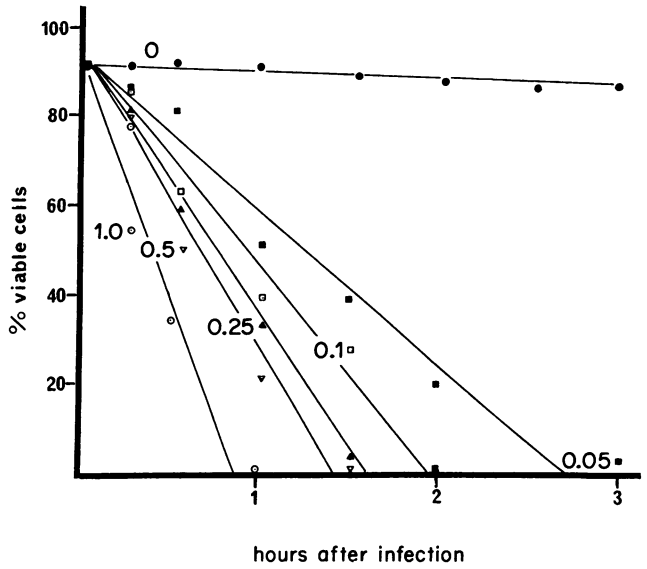

FIG. 5. Rate of cell killing with different concentrations of double-stranded RNA. Six aliquots of L1210 cells (6×10^5 each) were washed and suspended in 0.06 ml of saline containing different quantities of RNA. Cells were incubated at 37°C with low-speed rotary shaking. Samples of 0.01 ml were removed and treated with 0.002 ml of a 1% trypan blue solution; viable-cell counts were made directly in a hemocytometer. The curves are labeled with the concentration of double-stranded RNA added, in mg/ml (from left to right, 1.0, 0.5, 0.25, 0.1, and 0.05).

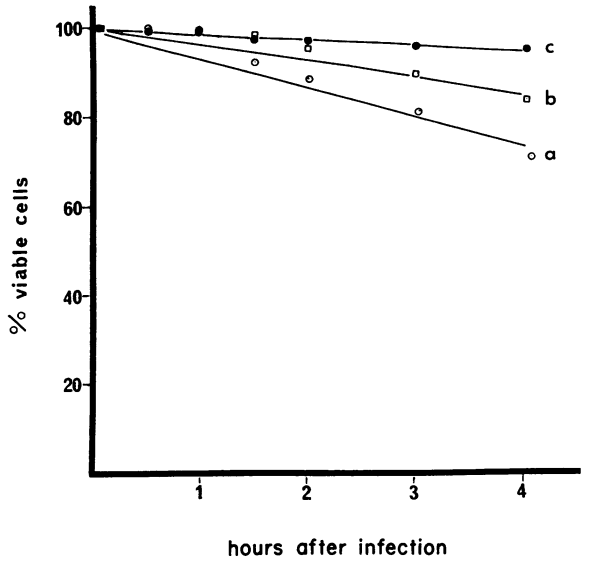

FIG. 6. Rate of cell killing by synthetic double-stranded RNA. Three aliquots of L1210 cells (6×10^5 each) were washed and suspended in: (*a*) 0.06 ml of poly(I)·poly(C) (0.5 mg/ml in saline), (*b*) 0.06 ml of poly(A)·poly(U) (0.5 mg/ml in saline), and (*c*) 0.06 ml of glycogen (0.5 mg/ml in saline), and incubated at 37°C with low-speed rotary shaking. Samples of 0.01 ml were treated with 0.002 ml of a 1% trypan blue solution and viable-cell counts were made directly in a hemocytometer.

varied between cell types. These results imply that this killing action is nonspecific.

Since the addition of RNA can change the ionic strength and pH of the media, all cultures were tested for changes in pH. The addition of RNA, at the concentration used, did not affect pH.

DISCUSSION

Cell death occurs very rapidly after infection with picornaviruses. This is accompanied by extensive vacuolation of the cell, degradation of the nuclear membrane, and the appearance of crystalline viral forms within 6–8 hr after infection (9, 10). Amako and Dales (11) have suggested that mengovirus-induced cytopathic effect is the result of virus-directed protein synthesis. They observed that streptovitacin A (an inhibitor of protein synthesis), applied to infected cells up to 4 hr after infection, reduced the viral yield and prevented cell death. Haase *et al.* (12) confirmed these observations and reported that cells treated with cyclohexamide 4 hr after infection died, as did untreated infected cells. These workers suggested that protein synthesis is essential for cell death. Although the evidence suggests that a late viral antigen is involved in cell death, it is by no means clear that a direct interaction of viral protein with a specific cell component is involved.

In Maden bovine kidney cells, a restrictive host for mengovirus replication, cell death occurs at the same time as in mengovirus-infected L-cells, a nonrestrictive host; although viral RNA synthesis is equivalent, but viral protein synthesis is minimal and very few complete virions are formed. The restrictive event involves the rapid degradation of newly synthesized viral RNA, although double-stranded RNA remains intact (3, 13). It is difficult to explain the rapid cytopathic effect in the restrictive host on the basis of a protein that is synthesized late in the infection.

Interferon, at a concentration that reduces yields of mengovirus by 90% in L-cells, fails to prevent cell death in these infected cells (12). There is evidence which suggests that interferon acts by modifying host ribosomes (or ribosome-bound factors) so that they can distinguish host mRNA from viral mRNA (14–16). One would expect, therefore, that any late-synthesized viral protein involved in cell death would not be synthesized. Haase *et al.* (12) have suggested that some forms of viral mRNA may be less sensitive to interferon than others, or that the particular putative protein that leads to cell death is only required in trace amounts.

The data presented in this paper demonstrate that the addition of double-stranded RNA isolated from cells infected with bovine enterovirus kills uninfected cells. That the "killing" material is double-stranded RNA and of viral origin was demonstrated by (*a*) its synthesis in the presence of actinomycin D, (*b*) its resistance to pancreatic RNase under standard conditions, (*c*) its susceptibility to RNase at low ionic strength, paralleled with a loss of its killing ability, and (*d*) subsequent loss of its "killing" effect upon denaturation with heat. There was also a pronounced effect of dilution that suggested that the number of copies of the RNA was important to the rate of killing.

There are reports of infective double-stranded RNA being isolated from poliovirus- and mengovirus-infected cells (1). These RNAs were isolated from cells at early stages of the viral replication cycle, whereas the double-stranded RNA from BEV-infected cells was isolated at the end of the replication period; this BEV RNA did not appear to produce viral particles.

Unlike poliovirus and mengovirus, large amounts of double-stranded RNA are accumulated during infection with

BEV. This situation has also been observed for foot-and-mouth disease virus (17), which also has a rapid and extensive cytopathic effect on its host. Whether this is a general characteristic of bovine viruses is not known.

It is well established that foreign nucleic acids are rapidly taken up by mammalian cells. The data in Table 1 show that labeled double-stranded RNA either enters the cell or is bound by the cell membrane. The *in vitro* work of Ehrenfeld and Hunt (18) suggests that double-stranded viral RNA prevents the initiation of protein synthesis. It is possible that the double-stranded RNA blocks translation. However, it is also possible that it leads to the breakdown of lysosomes, membranes, or other cellular organelles. As might be expected, this double-stranded RNA is nonspecific and kills cells that are normally not susceptible to BEV infection, for example, WI38 and CRF-CEM, two human-cell lines.

We have previously reported that BEV is a relatively efficient oncolytic agent (19). We feel this efficiency is due to the propensity of this virus to produce large amounts of double-stranded RNA. Viral double-stranded RNA has been demonstrated to cause tumor regression in experimental animals with established syngeneic tumors (P. Alexander, personal communication). A number of different types of viral double-stranded RNA were compared as to their ability to induce interferon and their anti-tumor activity, but no correlation could be shown. The use of synthetic polynucleotides, usually poly(I)·poly(C), to cause tumor regression has been investigated (20–22); it has been suggested that their mechanism of tumor destruction is by interferon production. Our results supports the hypothesis that regression of tumor cells might be due to the direct action of the copolymer on the cell. The need for repeated injections of the synthetic double-stranded RNA to cause tumor regression may be due to the efficiency of cell killing.

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