The Relationship Between Cytotoxicity and Interferon Induction in L-M Strain Mouse Fibroblasts (37297)

S. S. ASCULAI[1] AND R. J. KUCHLER
(Introduced by A. B. Kupferberg)
Department of Bacteriology, Rutgers University, New Brunswick, New Jersey 08903

Interferon is induced *in vitro* and *in vivo* by the double-stranded polymer of inosinic and cytidylic acids (poly I:C). However, in L-M cell cultures little interferon is produced without adding DEAE-dextran which enhances the binding of poly I:C to cells (2).

Since DEAE-dextran as well as poly I:C are moderately cytotoxic for cultured cells (3), the present study was designed to examine the relationship between cell damage and interferon production.

Materials and Methods. Inducing reagents. Appropriate dilutions of the sodium salt of poly I:C (P-L Biochemicals, Milwaukee, WI) were prepared from a stock solution containing 1 mg/ml distilled water. DEAE-dextran (approx mol wt 2×10^6, Pharmacia Corp., Piscataway, NJ) was diluted from a stock solution containing 40 mg/ml distilled water.

Cell growth. Three hundred milliliter suspensions of L-M cells were grown in KLM3 medium in 1 liter screw-cap, saline bottles (McGaw Laboratories, Milledgeville, GA) on a gyrotory shaker incubator (New Brunswick Scientific Co., New Brunswick, NJ) at 110 rpm and 35°. KLM3 medium is composed of auto-pow MEM for suspension cultures (Flow Laboratories, Rockville, MD) supplemented with yeast extract (0.05%), peptone (0.5%) and lactalbumin hydrolysate (0.25%). Other additions include $NaHCO_3$ (0.17%) and methylcellulose (0.12%).

Virus growth. Five million L-M cells growing in logarithmic phase as cell sheets on 30 ml plastic flasks were infected with mengovirus at a multiplicity of 10 PFU. After a 30-min adsorption period at room temperature, the excess fluid was removed and 5 ml of medium 199 supplemented with 0.5% peptone (199P) was added. After an 18-hour incubation period at 35°, the supernates from a number of flasks were harvested and pooled. This virus pool was clarified by centrifugation at 800*g* for 10 min. The virus pool retained the same infectivity titer for extended periods when stored at 2°.

Plaque assay. Five milliliter volumes of L-M cells from suspension cultures containing 1.5×10^6 cells/ml were pipetted onto 60 mm diameter plastic petri dishes. A confluent sheet of cells forms in 0.5 to 1.5 hr if 10% calf serum is included. At this time, the medium bathing the cells was discarded and the cell sheets were washed with 5 ml of 199P. Five-tenths milliliter of diluted virus was adsorbed to the sheet for 30 min at 35°. The excess fluid was removed from the plates and an overlay medium consisting of either 1.5% noble agar, DEAE-dextran, and minimal essential medium (MEM), or 1.5% Seakem agarose (Marine Colloids, Inc., Springfield, NJ) and MEM, was added. The plaques developed in 48 hr and were counted after staining with neutral red.

Viable cell counts. Cell viability was determined by dye exclusion. The dye used was 0.4% erythrosin B in Hanks' salt solution.

Experimental results. DEAE-dextran titration for cytotoxicity. DEAE-dextran alone is cytotoxic for animal cells grown in stationary cultures. In this study cells in suspension cultures were exposed to DEAE-dextran and combinations of DEAE-dextran and poly I:C to evaluate accurately cytotoxicity as it influences interferon production. Initally, increasing amounts of DEAE-dextran were added to 100 ml suspension cultures. After 3

[1] Present address: Ortho Research Foundation, Raritan, NJ 08869.

hr each culture was split into two 50 ml portions. The medium was changed on one portion by packing the cells and resuspending them in fresh media. The extent of cell damage was examined after 15 hr. Cytotoxicity was encountered when the concentration of DEAE-dextran was 50 μg/ml (Fig. 1). At this concentration, 35% of the cells were damaged in the culture where the medium was unchanged. Only 9% of the cells were damaged in the duplicate culture in which the medium was changed. Increasing the concentration of DEAE-dextran increased the incidence of cell damage. In all cases less damage was evident when the culture medium was changed. None of the supernates contained inhibitory substances for the test mengovirus.

Poly I:C and DEAE-dextran cytotoxicity. Dianzani *et al.* (4) have reported that for optimal induction of interferon in L-M cell cultures, a combination of poly I:C at 10

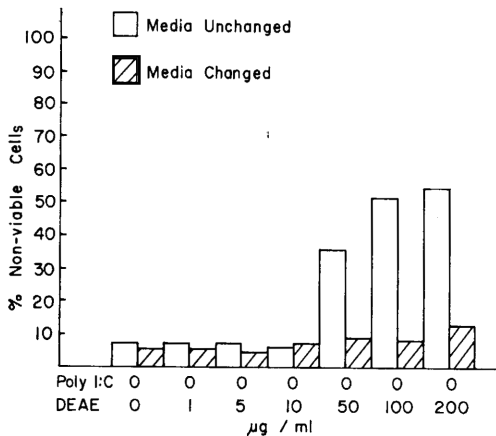

FIG. 1. Seven, 100 ml, suspension cultures in 250 ml Erlenmeyer flasks containing cells in late log or early stationary phase, were exposed to 0, 10, 25, 50, 100, and 200 μg DEAE-dextran/ml, respectively. After 3 hr at 35°, 50 ml of each culture were aseptically removed and centrifuged at 500*g* for 5 min. The supernate was discarded and the cells were resuspended in 50 ml fresh KLM3 medium. The cell suspension was transferred to sterile 250 Erlenmeyer flasks. Incubation was contained for an additional 12 hr. Viable cell counts were taken of each culture. The remainder was clarified by centrifugation at 800*g* for 5 min. The cell-free medium diluted 1:10 in 199P was assayed for interferon activity by the plaque reduction method.

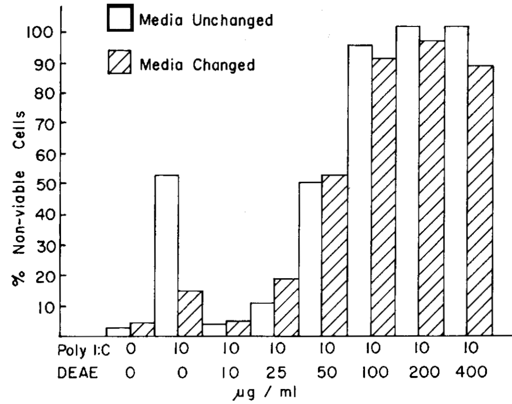

FIG. 2. Suspension cultures containing 7 × 10^5 cells/ml were incubated overnight at 35°. These cells, growing in a logarithmic fashion, were divided into eight replicates, each containing 100 ml cell suspension in 250 ml Erlenmeyer flasks. To seven of these cultures was added poly I:C at 10 μg/ml, respectively. The eighth flask remained as a control containing neither DEAE-dextran nor poly I:C. After 3 hr at 35°, 50 ml of each culture were aseptically removed and centrifuged at 500*g* for 5 min. The supernate was discarded and the cells resuspended in 50 ml fresh KLM3 medium. The cell suspension was transferred to sterile 250 ml Erlenmeyer flasks. Incubation was continued for an additional 12 hr. Viable cell counts were taken of each culture.

μg/ml, and DEAE-dextran at 400 μg/ml is required. In view of the cytotoxicity of DEAE-dextran at levels above 50 μg/ml, an experiment was designed to evaluate the combined toxic effect on L-M cells in the log phase of the growth cycle. The cells were exposed to poly I:C at 10 μg/ml and increasing concentrations of DEAE-dextran. Figure 2 shows that poly I:C alone caused cell damage which could be neutralized by the addition of 10 μg/ml of DEAE-dextran. However, as the concentration of DEAE-dextran was increased a cytotoxic effect was noted which was potentiated by the combination of poly I:C and DEAE-dextran. For example, at 200 μg/ml of DEAE-dextran 55% of the cells were damaged in cultures where the medium was not changed, and 15% of the cells were damaged in cultures where the medium was changed. When 10 μg/ml of poly I:C was included, cell damage was increased to 98% in the culture without a medium change and 90% in the culture

with a medium change. Essentially, the same result was obtained when L-M cells were exposed during the log phase or the plateau phase under the same conditions.

Interferon production. The supernates from all cultures exposed to poly I:C and DEAE-dextran were titered for viral inhibitory substances by a plaque reduction assay. Figure 3 illustrates that the plaque reducing activity of supernates from log phase cell cultures was related to the extent of cell damage (shown in Fig. 2). The only place that this was not observed was when poly I:C alone was used. At 10 μg/ml, poly I:C was extremely toxic but little antiviral material was produced. When DEAE-dextran at 10 μg/ml was added, the poly I:C cytotoxic effect was neutralized and an amount of interferon was produced comparable to the cultures in which poly I:C alone was added. Cytotoxicity comparable to that seen in cultures exposed to 10 μg/ml of poly I:C was only achieved when the DEAE-dextran concentration was 50 μg/ml. However, in this instance more antiviral material was evident. At 400 μg/ml of DEAE-dextran all the cells were damaged and a 1:10 dilution of the supernate completely inhibited plaque formation.

The product induced in L-M suspension cultures by 10 μg/ml poly I:C, 400 μg/ml DEAE-dextran was characterized as an interferon by testing for pH stability and trypsin sensitivity. The product was sensitive to trypsin action and was stable at pH 2 and pH 10.

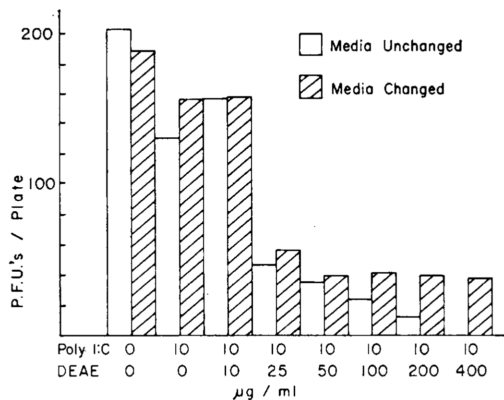

FIG. 3. The cell suspensions from cultures exposed to poly I:C and DEAE-dextran were clarified by centrifugation at 800*g* for 5 min. The cell-free medium, diluted 1:10 in 199P, was assayed for interferon activity by the plaque reduction method.

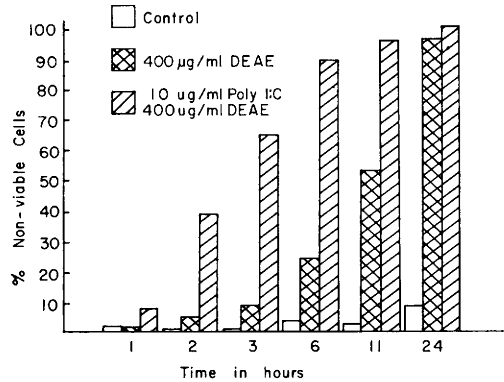

FIG. 4. Three, 100 ml, suspension cultures in 250 ml Erlenmeyer flasks, containing cells in logarithmic phase of growth were exposed to 10 μg/ml poly I:C and 400 μg/ml DEAE-dextran, 0 μg/ml poly I:C and 400 μg/ml DEAE-dextran, and 0 μg/ml poly I:C and 0 μg/ml DEAE-dextran. At 1, 2, 3, 6, 11, and 24 hr, 10 ml samples were withdrawn aseptically from each culture. Viable cell counts were done on an aliquot of each.

Cytotoxicity and interferon production with time. Since the cytotoxic reaction was related to the production of viral inhibitor, an experiment was designed to follow the extent of cell damage with time in cell cultures exposed to DEAE-dextran and the combination of poly I:C and DEAE-dextran. Figure 4 shows that after 1 hr some cells were damaged in those cultures exposed to the combination of 10 μg/ml of poly I:C and 400 μg/ml of DEAE-dextran. The rate of damage occurred more rapidly in the cell cultures exposed to the combined treatment. Analysis of the supernates indicated that after one hour an antiviral substance was produced that completely abolished plaque formation at a 1:10 dilution. When the supernates were tested at a 1:50 dilution an increasing amount of inhibitor was produced with time (Table I).

Discussion. The results presented here confirm observations on the cytotoxic action of both DEAE-dextran and poly I:C (3, 5, 6). When L-M cells are growing in suspension cultures, DEAE-dextran is toxic at concen-

TABLE I. At the Time a Culture Was Tested for Cell Viability, Another Portion of the Suspension Was Clarified by Centrifugation at 800g for 5 Min.[a]

Time (hr)	% Plaque reduction of supernate diluted 1:50
1	20
2	23
3	30
6	32
11	36
24	42

[a] The cell-free medium, diluted 1:50 in 199P, was assayed for interferon activity by the plaque reduction method.

trations greater than 50 μg/ml. This toxic reaction can be reversed if L-M cells are transferred at the third hour to media without DEAE-dextran. At 10 μg/ml poly I:C is toxic for L-M cells and this toxic reaction can also be reversed by transferring cells to media without this nucleic acid polymer before the third hour. In addition, poly I:C toxicity can be neutralized by adding 10 μg/ml of DEAE-dextran. However, when DEAE-dextran at 50 μg/ml or greater is combined with 10 μg/ml of poly I:C, the toxicity cannot be reversed by transferring cells into fresh media at the third hour. The reason for this is that cell toxicity is greatly amplified at an early time by the combined polymer treatment. When 400 μg/ml of DEAE-dextran is combined with 10 μg/ml of poly I:C, approximately 70% of the cells are irreversibly damaged by the third hour. Since DEAE-dextran facilitates the uptake of poly I:C, the amplification of the toxicity at an early time could occur because poly I:C damages the cell membrane more rapidly when in direct contact with it. This is unlikely because cell damage is not apparent when only 10 μg/ml of DEAE-dextran is used. Alternatively, the binding of the poly I:C could in turn facilitate the binding of additional molecules of DEAE-dextran. Thus, the results could reflect an amplification of the DEAE-dextran toxicity.

Since interferon is induced at an early time in these L-M suspension cultures, and increases with time, the data suggest an integral relationship between the magnitude of cell membrane damage and the degree of antiviral activity. Thus, it is likely that poly I:C after being taken up by the cell attaches to an intracellular receptor to modify a substance which is released as interferon through the damaged membrane (7).

Summary. The incidence of cell damage in L-M cell cultures exposed to DEAE-dextran increased with an increase in the concentration of the polycation. Poly I:C alone exerted a toxic effect which could be neutralized by 10 μg DEAE-dextran/ml. Cytotoxicity, however, was potentiated by poly I:C at higher DEAE-dextran concentrations. The plaque reducing activity of the supernates from cultures exposed to poly I:C and DEAE-dextran increased with an increase in the incidence of cell damage. The antiviral substance, when induced by 10 μg/ml poly I:C and 400 μg/ml DEAE-dextran, was evident after an exposure of one hour. The potency of the material increased with the duration of exposure. The material was characterized as an interferon by its pH stability and trypsin sensitivity.

The authors acknowledge the excellent technical assistance of Mrs. Patrica Thomson. This work was supported by U.S. Public Health Service Grant GM-T-505 and a Biomedical Science Support Grant from Rutgers University.

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Received Sept. 19, 1972. P.S.E.B.M., 1973, Vol. 143.