Poliovirus-induced Cellular Injury

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Protein leakage was used as a quantitative measure of poliovirus-induced cellular injury under suspended cell culture conditions. The requirements for protein leakage were studied in detail and it was established that events early in the infectious cycle which depend upon viral protein synthesis were responsible for cell damage. Extralysosomal β -glucuronidase appeared in infected cells before the onset of protein leakage and release of newly synthesized virus. Hydrocortisone treatment of infected cells resulted in only a slight delay in the release of β -glucuronidase from lysosomes and protein and virus from cells. These results suggest that events associated with poliovirus synthesis trigger the release of lysosomal hydrolases which in turn injure the plasma membrane, allowing cytoplasmic proteins and virus to leak out of the cell.

Virus-induced cytopathic changes are characteristically studied from the morphologic standpoint. Beyond detecting gross alterations in cellular architecture, the morphologic approach provides little quantitative information as to the degree of cell injury conditioned by virus infection. Methods currently used to investigate cytopathic processes include subjective classification of cytopathic effects (CPE) into various stages (4, 18), determination of the permeability of cells to dyes, or the determination of the proportion of cells remaining attached to culture vessels as a function of time after virus infection (9, 19). Often attempts are made to relate such quantal responses to the amount of virus present in cells or culture fluids (a population response) at the time morphological observations are made. In examining the mechanisms which underlie virus-induced cellular injury, it would be highly desirable to compare all parameters selected for study on the basis of a population effect.

Some years ago, Gilbert (14) showed that cytoplasmic enzymes were released into culture fluids of virus-infected cells. This suggested a preliminary study (7) which established the usefulness of protein leakage from mengovirus-infected cells as a quantitative measure of cellular damage. It was also apparent from this study that protein leakage could be viewed as a population response to virus infection.

In the experiments to be described in this paper, we examined the conditions which lead to the release of intracellular proteins from poliovirus-infected cells. Many virological investigations now use suspended cell cultures, and under these circumstances morphologic details of cellular

alterations are obscured by the tendency of cells to retain a spherical shape. Therefore, an additional goal of this investigation was to establish the feasibility of using protein leakage as a parameter for measuring cellular injury in a suspended culture system.

MATERIALS AND METHODS

Cell culture. All experiments were performed with HEP-2 cells obtained from Albert Sabin and adapted to growth in suspension culture. Cells were propagated in Eagle's minimum essential medium (MEM-F12, Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% calf serum, 200 units of penicillin, 200 μ g/ml of streptomycin, and 15 centipoise of Methocel (methylcellulose; Dow Chemical Co., Midland, Mich.) at a final concentration of 0.2%. Cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of culture fluid in a gyratory, shaker-incubator (New Brunswick Scientific Co., New Brunswick, N.J.) rotating at 125 rev/min at 37 C. Cultures were seeded initially with 8×10^5 cells/ml, and were harvested when cell concentrations reached 2×10^6 cells/ml. Cells were transferred to fresh medium 18 hr prior to initiation of an experiment.

Virus. Poliovirus (type 1, Mahoney) was passaged three times in HEP-2 suspension cultures prior to preparation of a single pool which was used throughout this study. Stock virus was prepared from 24-hr infected cell suspensions by three cycles of rapid freezing and thawing. These fluids were clarified by low-speed centrifugation and stored at -20 C.

Measurement of extracellular protein was accomplished as follows: Samples (25 ml) of uninfected or infected cell cultures (containing approximately 8×10^5 cells/ml) were centrifuged at $17,000 \times g$ for 20 min. Such high centrifugal forces were necessary to insure complete sedimentation of particulate materials. The supernatant fluids were carefully removed and

chilled in an ice bath. To 3.0 ml of this material was added cold 60% perchloric acid (PCA) to give a final concentration of 0.5 M. The resulting precipitates were washed twice with cold 0.5 M PCA and assayed for protein content by the method of Lowry et al. (17), using crystalline, bovine-serum albumin (Pentex, Inc., Kanakee, Ill.) as the protein standard.

Cell fractionation. Cells in the desired concentration (1 to 2×10^8) were suspended in 2.5 ml of 0.125 M sucrose solution and homogenized with 100 strokes of a Dounce homogenizer at 1 C. After centrifugation of $17,000 \times g$ for 20 min, the supernatant fluids which contained free lysosomal enzymes were removed. To the pellets (containing intact lysosomes and other sub-cellular particulate materials) was added 0.2% Triton X-100, and the suspension was rapidly frozen and thawed to release lysosomal-bound enzymes. The percent of extralysosomal enzyme was calculated from the ratio of free enzyme ($17,500 \times g$ supernatant fluid) to the total amount of enzyme available (free enzyme plus that released upon disruption of lysosomes; 22).

β -Glucuronidase assays were performed according to the method of Allison and Sandelin (1).

Streptovitacin A (StA, lot no. 4931RRH-7), the 4-OH derivative of cycloheximide, was obtained through the courtesy of M. G. Savage (The Upjohn Co., Kalamazoo, Mich.). Stock solutions of 250 $\mu\text{g}/\text{ml}$ were made in serum-free culture medium and stored at -20°C .

Hydrocortisone. The water-soluble sodium succinate ester of hydrocortisone (Solu-Cortef, The Upjohn Co.) was dissolved in serum-free medium just prior to use in each experiment.

Infectivity titrations were carried out by plating 0.3 ml of an appropriate virus dilution on HEp-2 cell monolayers grown in 6-cm plastic culture dishes. After a suitable adsorption period, 5.0 ml of an MEM-F12 agar overlay was added. After 48 hr of incubation at 37°C , 1.5 ml of 1:5000 neutral red in 0.16 M NaCl containing 0.01% acetic acid was added to each dish, and plaques were counted at 60 hr after infection.

RESULTS

Maintenance of suspended cell cultures in serum-free medium. A prerequisite for measuring extracellular protein is the ability to maintain cell cultures in serum-free medium for periods of time dictated by the experimental plan. HEp-2 cells were harvested by centrifugation, washed twice with serum-free medium with or without 0.2% Methocel. The cells were distributed in 25-ml amounts into 125-ml flasks and incubated at 37°C in a gyratory shaker (see Materials and Methods). These cultures were then incubated in the presence or in the absence of 0.2% Methocel. At selected intervals thereafter, the contents of a flask were removed, and the supernatant fluids were assayed for protein content as described in Materials and Methods. Results of such an experiment are shown in Fig. 1. The difference between the two curves demonstrates the protective effect of Methocel when this material was present during

FIG. 1. Release of intracellular proteins from uninfected HEp-2 cells in suspension culture. Eighteen-hr cultures of HEp-2 cells were divided into equal portions. One half of the cells were washed twice with serum-free medium, transferred to fresh medium lacking serum, and incubated as described in Materials and Methods. The remaining cells were washed twice with serum-free medium containing 0.2% Methocel (15 centipoise) and incubated in fresh medium of the same composition. At the indicated times, appropriate samples were centrifuged at $17,000 \times g$, and the supernatant fluids were examined for acid-insoluble protein content.

the washing and centrifugation steps. By the 2nd hr of incubation, the kinetics of protein release either in the presence or in the absence of Methocel were similar. After incubation for 24 hr in serum-free medium with Methocel, the cells appeared morphologically normal and exhibited viability in excess of 98% as measured by dye exclusion tests. As a result of these findings, Methocel was routinely present in all media and washing fluids.

Release of protein from poliovirus-infected cells. Experiments were carried out to measure the extent and reproducibility of protein leakage from poliovirus-infected cells maintained in suspension culture. Cells were infected in suspension with an input multiplicity of 10 plaque-forming units (PFU)/cell, washed, and incubated under the conditions described above. At 2 and 18 hr after infection, supernatant fluids were examined for protein content. The data in Table 1 show that significant amounts of acid-insoluble protein were released only from poliovirus-infected cells.

The kinetics of protein leakage and the release of virus were examined next, and it is clear from Fig. 2 that protein leakage and virus release were concomitant events. The simultaneous release of intracellular materials of such great size variation is evidence of a drastic alteration in the functional capacity of the plasma membrane and may be considered a real manifestation of cellular injury.

TABLE 1. Release of protein from poliovirus-infected HEP-2 suspension cultures

Experiment	Protein in culture fluids ($\mu\text{g/ml}$)			
	Infected cells		Uninfected cells	
	2 hr ^a	18 hr	2 hr	18 hr
1	1.0	116	1.0	1.0
2	3.7	146	3.4	1.0
3	3.6	128	1.1	3.6

^a Hours after infection.

FIG. 2. Kinetics of protein and virus release from HEP-2 cells. Cells were infected with poliovirus as described in the text, and were incubated in serum-free Methocel medium. At selected intervals after infection, cells were removed by centrifugation and the supernatant fluids were assayed for extracellular virus and protein content.

Some information was sought concerning the nature of the proteins released from infected cells. Lactic dehydrogenase (LDH) and glutamic oxalacetic-transaminase (GOT) were readily detected in culture fluids from infected cells, but were absent in fluids from uninfected cells. Since these enzymes appear in soluble fractions of tissue homogenates (6, 11) it was considered likely that at least a portion of the extracellular protein in virus-infected cultures was derived from soluble cytoplasmic constituents. As a result of these findings, the presence of extracellular proteins in culture fluids was used as an operational definition of cellular injury.

Requirements for protein release. The relationship between the amount of virus synthesized and the extent of protein leakage was investigated by the use of StA, a potent inhibitor of protein and virus synthesis (8, 10, 12, 20). HEP-2 cells were infected with poliovirus, incubated for 18 hr in the presence of selected StA concentrations, and assayed for total virus and extracellular protein

content. Appropriate uninfected control cultures were included in each experiment. Poliovirus synthesis was completely suppressed by StA concentrations in the range of 1.0 $\mu\text{g/ml}$ (Table 2). Also, the amount of protein which was released from infected, inhibitor-treated cells was directly related to the amount of virus synthesized, since StA by itself did not cause protein leakage in addition to that routinely observed for uninfected cells.

The relationship between protein leakage and virus synthesis was investigated in detail by the addition of StA to cultures at 30-min intervals after infection with virus. After addition, the inhibitor was present during the remainder of the experiment, which was terminated 18 hr after infection. At that time, extracellular fluids were assayed for protein content. StA added during the early phase of virus synthesis effectively suppressed virus synthesis and protein leakage (Fig. 3 and Table 3). Beyond the 5th hr of infection, the addition of StA had little effect on the release of intracellular protein or the synthesis of virus. These data suggest that virus-induced reactions associated with cellular injury were initiated before the 3rd hr of infection and were completed by the 5th hr.

Mechanism of poliovirus-induced protein leakage. The experiments presented thus far had established a relationship between virus synthesis and protein release, and it was our interest to consider the mechanism underlying the protein leakage reaction. Previous investigators have attempted to relate the activation of lysosomal hydrolases to cellular injury and the development of virus-induced CPE (1, 13, 22). As a working hypothesis, it was considered possible that lysosomal enzymes effect an alteration in cell-plasma

TABLE 2. Effect of streptovitacin A (StA) on poliovirus synthesis and protein leakage

StA ($\mu\text{g/ml}$)	Poliovirus (PFU/ml) ^a	Protein in culture fluids ($\mu\text{g/ml}$) ^b	
		Infected cells	Uninfected cells
0	3.3×10^8	145	3
0.05	1.9×10^8	130	1
0.10	4.0×10^7	102	5
0.15	8.3×10^6	74	5
0.20	3.3×10^5	10	4
0.50	1.0×10^3	12	1
1.0	1.0×10^3	5	5
5.0	1.0×10^3	5	3

^a Total virus per milliliter of cell suspension 18 hr after infection.

^b 18 hr after infection.

FIG. 3. Effect of StA on the release of protein from poliovirus-infected HEP-2 cells. Infected cells were incubated in serum-free medium containing Methocel. At the indicated times ($\downarrow$) 1 μ g of StA per ml was added to a suspension culture, and the inhibitor was allowed to remain in contact with cells until 18 hr after infection. Extracellular fluids were then assayed for protein content. The points on the curve represent protein values 18 hr after infection. The percentage of maximal protein leakage was calculated from data comparing infected, StA-treated cells with infected cells maintained in the absence of the inhibitor.

TABLE 3. Effect of streptovitacin A (StA) on poliovirus synthesis

StA added (1.0 μ g/ml)	Poliovirus (PFU/ml) ^a
hr	
2.0	1.0×10^3
2.5	1.0×10^3
3.0	1.0×10^3
3.5	1.1×10^3
4.0	3.3×10^3
4.5	2.0×10^6
5.0	2.0×10^7
5.5	5.9×10^7
6.0	2.5×10^8
None	4.0×10^8

^a Total virus per milliliter of cell suspension, 18 hr after infection.

membrane function which is manifested by the release of intracellular proteins and virus. Suggestive evidence to support this hypothesis was derived from experiments which established the temporal relationship between the release of lysosomal enzymes and the leakage of protein and virus from cells. For this purpose, β -glucuronidase was selected as a marker for lysosomal enzymes (11). The data shown in Fig. 4 reveal that increased extralysosomal β -glucuronidase activity was detectable from the 4th hr, and reached

maximal proportions by the 11th hr after infection. The appearance of β -glucuronidase preceded the release of protein and virus by approximately 2 hr, allowing the speculation that the latter events were the direct result of injury to the plasma membrane by lysosomal hydrolases.

Effect of hydrocortisone on cellular injury. A number of compounds stabilize isolated lysosomes (21). Of these, hydrocortisone and cortisone have been tested for their effects on cell lysis and lysosomal enzyme distribution during virus infection (3, 13, 15). Since the results of these studies were somewhat varied, we examined the effect of hydrocortisone on extra-lysosomal β -glucuronidase levels during virus infection, intra- and extracellular virus levels, and protein leakage. HEP-2 cells were infected with virus and incubated in the presence or absence of 100 μ g/ml of hydrocortisone. At selected intervals, replicate normal and infected cell samples were collected; the cells were removed by centrifugation and examined for extralysosomal β -glucuronidase content as previously described. Supernatant fluids were assayed for the presence of extracellular virus and protein. Infected cells exposed to hydrocortisone displayed consistently lower levels of extralysosomal β -glucuronidase than did untreated cells (Table 4). The simplest explanation of this data is that some stabilization of lysosomal membranes had occurred as the result of exposure to hydrocortisone. However, microscopic examination of cell homogenates revealed that drug-treated cells were less easily disrupted than untreated cells under comparable conditions of homogenization.

FIG. 4. Temporal relationship between the appearance of extralysosomal β -glucuronidase ($\bullet$), extracellular virus ($\times$), and extracellular protein ($\blacktriangle$). Infected HEP-2 cells were harvested, Dounce-homogenized, and fractionated, as described in Materials and Methods. Appropriate subcellular fractions were examined for β -glucuronidase content. Supernatant fluids from these cells were assayed for the presence of extracellular virus and protein.

TABLE 4. Extralysosomal β -glucuronidase levels in poliovirus-infected HEP-2 cells incubated in the presence or absence of hydrocortisone

Hours after infection	Per cent extralysosomal β -glucuronidase ^a	
	Hydrocortisone-treated	Untreated
2	7.4	13.7
4	11.0	14.6
6	17.5	20.0
8	17.3	26.4
10	18.8	27.2
12	21.8	31.3

^a Calculated as described in Materials and Methods.

TABLE 5. Effect of hydrocortisone treatment on extracellular poliovirus levels

Hours after infection	Extracellular virus (PFU/ml)	
	Hydrocortisone-treated	Untreated
2.5	2.0×10^3	2.0×10^3
5.5	1.8×10^4	2.1×10^4
7.0	1.4×10^6	1.6×10^6
8.5	4.0×10^6	6.9×10^6
9.5	5.9×10^6	7.6×10^6
10.0	6.2×10^6	9.6×10^6
11.0	8.5×10^6	2.1×10^7
12.5	1.7×10^7	2.6×10^7

Consequently, the view that hydrocortisone stabilizes lysosomes must be tempered by the observation that the plasma membrane also may have been stabilized by this drug. Virus release (Table 5) and protein leakage (Fig. 5) were slightly delayed by hydrocortisone treatment. However, the magnitude of the observed differences cannot argue strongly for a stabilizing effect of this drug on virus-induced injury to lysosomes. Taken as a whole, our results are best interpreted as a direct effect of hydrocortisone on the plasma membrane of the cell.

DISCUSSION

Poliovirus infection of HEP-2 cells in suspension culture resulted in the release of proteins into the culture medium. In this respect, poliovirus behaved in a manner analogous to mengovirus (7). In relation to the results reported here, it was of importance to consider the nature and origin of these proteins. LDH and GOT, enzymes which are freely soluble in the cytoplasmic sap, were detectable only in extracellular fluids from infected cells, and represented an unknown por-

tion of the total amount of acid-insoluble proteins which were measured. Thus, the leakage of intracellular proteins was taken as direct evidence of an extensive deterioration in the functional permeability of the plasma membrane, and was employed as an operational definition of cellular injury.

The reactions which result in protein leakage are most likely due to the direct action of virus-specific proteins and not the result of suppression of host cell function by the viral genome. This is inferred by the experiments which showed that StA alone did not cause protein leakage, but did protect virus-infected cells from injury when added early in the infectious process. Furthermore, a detailed study of the temporal relationship between virus synthesis and protein leakage showed that the degree of cellular injury was quantitatively related to the amount of virus synthesized. In this regard, the effect of StA on protein leakage is analogous to the action of puromycin and guanidine hydrochloride in preventing morphological changes in poliovirus-infected cells (5). It is also important to note that poliovirus-induced cellular injury is a self-limiting process. Thus, interruption of virus synthesis by StA at any time before the 5th hr after infection prevented further protein leakage for the duration of the experiment.

Of particular interest is the mechanism which underlies the protein leakage reaction. An attempt was made to associate an alteration in cell permeability with the release of β -glucuronidase from lysosomes. In the absence of direct evidence for the participation of lysosomal enzymes in damaging plasma membranes, it was important

FIG. 5. Effect of hydrocortisone on protein leakage from virus-infected cells. Infected cells were incubated in serum-free Methocel medium or such medium containing 100 μ g/ml of hydrocortisone. At selected intervals after infection, extracellular fluids were examined for protein content.

to demonstrate that extralysosomal β -glucuronidase appeared about 2 hr prior to the onset of protein and virus release. This order of events permits the speculation that lysosomal hydrolases attack the plasma membrane and effect changes in membrane permeability. The experiments of Allison et al. (2) have some bearing on this point. They showed that photosensitized lysosomes of cells cultured in vitro release lysosomal enzymes upon subsequent exposure to light. This event was followed by the leakage of potassium ion from cells and the eventual deterioration of plasma membrane integrity. Thus, taken as a whole, the cited studies and those presented in this paper lend some support to the concept that lysosomal enzymes play an integral role in cytolytic virus infections in which injury to the plasma membrane is a predominant feature. A study is now in progress on the effects of lysosomal enzymes on isolated plasma membrane fractions.

Treatment of poliovirus-infected cells with hydrocortisone had little effect on the development of cellular injury as measured by β -glucuronidase release from lysosomes and virus and protein from cells. Since hydrocortisone-treated cells were more resistant to mechanical homogenization than were infected, untreated cells, our results are probably the result of a direct stabilization of the plasma membrane by this drug. That hydrocortisone can stabilize cell membranes has been reported (16).

Finally, it must be emphasized that the measurement of extracellular proteins is a convenient and reliable procedure for quantitating cellular injury, especially under conditions where the usual morphologic criteria could not be applied. The appearance of extracellular proteins resulting from poliovirus infection constituted a population response which could be related effectively to other parameters having a similar base.

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