Activation of Lysosomal Enzymes in Rabies-Infected Tissue Culture Cells Without Accompanying Cytopathic Effect

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Lactic dehydrogenase and β-glucuronidase were assayed in tissue culture fluids and cellular lysates, respectively, from BHK-21/13S and Iota cell cultures infected with rabies virus. Activation of lysosomal enzymes was shown from the day 4 of infection on, without any indication of cytopathic effect.

The interaction between viruses and susceptible cells in tissue cultures often results in cellular alterations referred to as cytopathic effect (CPE) with or without virus replication, polykaryocytes, and alterations in host cell deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis (9). Another response of host cells is the redistribution and release of their hydrolytic enzymes after virus infection (2). Gilbert (11) studied enzymatic levels of glutamic oxalacetic transaminase and lactic dehydrogenase (LDH) in monkey kidney cells infected with echovirus type 12, adenovirus type 7, and Sendai virus. These viruses exhibited distinctive patterns of enzyme activities when correlated with cytopathic effects. Allison and Sandelin (4) found increased levels of β-glucuronidase in rhesus monkey kidney cells infected with mouse hepatitis virus and vaccinia virus.

The present study describes the release of LDH into tissue culture fluids in rabies-infected cells and β-glucuronidase levels in cell lysates from the same cultures to determine the role of lysosomes in rabies-infected tissue culture without accompanying CPE.

MATERIALS AND METHODS

Cells. Baby hamster kidney (BHK-21 clone 13S) and hamster embryo cells (Iota or I cells; reference 8) were used. The cells were grown as monolayers in 8-oz (ca. 250 ml) Falcon plastic flasks.

The medium for the BHK cells was Eagle's minimum essential medium with double the indicated amount of amino acids and vitamins, as modified by MacPherson and Stoker (16), supplemented with 10% tryptose phosphate broth and 10% fetal calf serum (FCS). I cells were grown in Eagle's basal medium supplemented with 10% FCS. Eagle's medium with 0.1% bovalbumin bovine fraction V replacing the FCS and 0.52% sodium bicarbonate was used as the maintenance medium after virus infection.

Viruses. ERA (1) and CVS-11 (14) strains of rabies virus adapted to growth in BHK-21/13S and I cells were used. These viruses had infectivity titers of $10^{-5}$ to $10^{-6}$ and $10^{-1.3}$ to $10^{-3.5}$ LD$_{50}$ per 0.03 ml, respectively, when injected intracerebrally into weanling mice (0.03 ml/mouse).

General procedures. Twelve 8-oz flasks were seeded with 4 × 10$^6$ BHK-21/13S cells or 3 × 10$^6$ I cells and incubated for 72 hr at 37°C. The medium was then decanted, and half of the flasks were infected by adding 0.5 ml of a 10-fold dilution of the virus described above for 60 min at 37°C. The rest of the flasks received 0.5 ml of maintenance medium and were used as uninfected controls. After the 60-min period, the inoculum was removed and the cells were reincubated with maintenance medium at 35°C.

At 1-day intervals after infection, fluids from one inoculated and one control flask were decanted and centrifuged at 170 × g for 10 min. These cell-free fluids were examined for LDH activity, as described below, and samples were stored at −70°C for virus titration.

Cell monolayers were washed twice with phosphate-buffered saline (PBS), pH 7.2, at 25°C and removed with a sterile rubber policeman (18). The policeman and flask were carefully washed with PBS. The cells were centrifuged at 700 × g for 5 min. The pellets were resuspended in 1 ml of distilled water and then frozen and thawed 10 times (with an alcohol-dry ice bath). The cell lysates were examined for β-glucuronidase activity, and samples were stored at −70°C for virus titration. Total cell counts were made of each flask, and enzyme units were calculated for a constant number of cells.

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Fluorescent-antibody staining. The infection was observed by fluorescent-antibody (FA) staining of cells grown on cover slips in Leighton tubes and infected simultaneously with the 8-oz plastic flasks. We used the staining procedures described previously (7).

Fluorescence was observed with a Zeiss microscope with UG1 exciter filter and no. 65 barrier filter illuminated with an OSRAM HBO-200 high-pressure mercury vapor lamp.

Acridine orange staining. Fresh staining solution prepared each day consisted of $1.6 \times 10^{-3}$ m (1:2,000) acridine orange (AO) stock diluted to $1.6 \times 10^{-4}$ m (1:20,000) with Eagle's basal medium. Infected and normal cells grown on cover slips were stained with 10 ml of staining solution for 10 min at 25 C. They were then removed and placed in a small volume of Eagle's medium without AO for 2 or 3 hr at 37 C (20).

The same microscope was used with Corning exciter filter 5113 and barrier filter no. 50.

Assays of enzymes. Tissue culture fluids were assayed for LDH by the method of Wroblewsky and La Due (22) by using Sigma reagents. For each assay, 0.2 ml of fluid was used. Determinations were carried out with a Beckman DU spectrophotometer. One unit of LDH activity was that amount causing a decrease in optical density at 340 nm of 0.001 per min at 25 C in 3 ml of reaction mixture in a cuvette having a 1-cm light path. For β-glucuronidase determination, cell lysates were incubated for 20 min with the β-glucuronide of 0.01 M phenolphthalein at 37 C. The reaction was stopped by adding 4 ml of 0.04 glycine buffer (pH 10.7). After centrifugation for 10 min at 170 × g, the amount of phenolphthalein released was measured in a Beckman DU spectrophotometer at 545 nm (4).

One unit was reported as "micrograms of phenolphthalein released in 20 min and in $10^8$ cells" (6).

RESULTS

LDH in tissue culture fluids. Inoculation of ERA or CVS-11 virus increased the levels of LDH in tissue culture fluids and raised the virus titer (Fig. 1). The rise in titer was not accompanied by changes in enzyme levels during the first 3 days after infection. LDH activity increased moderately on the 4th day and rose to highest levels on the 6th day, at which time FA staining showed 100% of the cells to be infected, and the virus titer was highest. The increase of fluorescence followed approximately the same pattern with both viruses in both cell lines. The cell lysates showed more or less the same enzymatic β-glucuronidase curve in both cell lines infected with both viruses (Fig. 2). The difference in the levels of β-glucuronidase in lysates of normal and infected cells began to be evident during the 4th day after infection and rose to the highest levels on the 5th or 6th day. There was no significant difference between BHK and I cells in virus titer, fluorescence, or enzyme levels; moreover, both viruses gave the same reaction in both types of cells.

The vital AO staining showed numerous orange fluorescent lysosomes in the cytoplasm in normal and infected cells up to day 3 after infection; during day 4, the number of normal staining lysosomes decreased to approximately 30%, and
there was generalized green fluorescence of nuclei and cytoplasm during days 5 and 6, suggesting progressive lysosomal disruption.

**DISCUSSION**

Two stages of lysosomal enzyme activation have been reported in cells infected with viruses that produce CPE (3). In the first stage, there is increased permeability of lysosomal membranes as demonstrated by an increase in the capacity of the lysosomes to take up vital dyes and fluorochromes. The second stage of activation involves the release of lysosomal enzymes into the surrounding cytoplasm as well as accumulation of extracellular lysosomal enzymes in the tissue culture medium (2). Mallucci and Allison (17) found that chick embryo cells infected with fowl plague virus (an avian strain of influenza A that produces CPE) released lysosomal enzymes from the particulate to the supernatant fraction as early as 6 hr after infection. These changes occurred in the presence of high concentrations of p-fluorophenylalanine, suggesting that they resulted from redistribution of preformed enzymes rather than from de novo protein synthesis. No such changes were observed when the Melbourne strain of influenza virus multiplied in the same system without cytopathic effects.

According to some authors, rabies virus produces CPE, but it becomes evident only 10 or more days after infection, depending on the cell lines and type of virus strain used (5, 10, 12, 15, 21).

The experiments described in this paper showed that the growth of two strains of rabies virus in two different cell lines was accompanied by activation of lysosomal enzymes without any indication of CPE. One of the indications of viral uptake into cells is the disappearance of viral antigen (ellipse period) and the subsequent appearance of new virus, as determined by FA staining and infectivity. In rabies virus-infected BHK-21 cells to which diethylaminoethyl dextran has been added, newly formed rabies antigen appears in the cytoplasm as early as 9 hr after infection (13). In our experiment, approximately 30% of BHK and I cells were seen infected 24 hr after infection. Nevertheless, the permeability of lysosomal membranes began to increase only 3 days after infection, as demonstrated by AO staining; the lysosomes increased in size and took up the dye very well; before this time, the presence of virus did not appear to alter the lysosomal membranes. At 3 days postinfection, no enzyme changes were observed between normal and infected cells, whereas on day 4 a difference in levels was seen; at that time the rabies-infected cells appeared to begin their second lysosomal activation stage, LDH was detected in tissue culture fluids, and the $\beta$-glucuronidase concentration decreased in cell lysates. It was not possible to detect $\beta$-glucuronidase in tissue culture fluids; moreover, its concentration in the cells decreased gradually, as the enzyme apparently became inactivated in the cytoplasm or synthesis diminished. These results differ from those described by Allison and Sandelin (4) with cytopathic MHV-3 and vaccine virus. They showed an increase in enzyme activity in cell homogenates, reflecting escape of enzymes from lysosomes into the cytoplasm. LDH activity in tissue culture cells infected with ERA and CVS-11 rabies virus followed a similar curve as echovirus type 12 and Sendai virus (11). The latter viruses, however, produced increased LDH activity at 48 to 72 and 72 to 96 hr, respectively, after infection, when most of the cells presented CPE. LDH activity in rabies virus-infected cell lysates was shown to be less than in normal cell lysates; these curves, however, could not be routinely reproduced. It is interesting to note that in our experiments the Iota (1) cell line was virtually as susceptible to rabies virus as BHK cells. The virus titers in both lines were similar when they were infected with ERA or CVS-11 viruses.

**LITERATURE CITED**