Beta-Glucuronidase Response of Cells Infected with Adenovirus Types 5 and 12

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Beta-glucuronidase activity was investigated during a 48-h period in which virus replication and changes in cell morphology occurred. Infection of an established line of chimpanzee liver cells with either nononcogenic adenovirus 5 or highly oncogenic adenovirus 12 under one-step growth conditions produced differing patterns of enzyme activity. There was an increase in total activity and also enhanced leakage of beta-glucuronidase from cells infected with adenovirus 12. In contrast, the enzymatic pattern of cells infected with adenovirus 5 was similar to that of uninfected cells. Hydrocortisone prevented the abnormal release of beta-glucuronidase from adenovirus 12-infected cells. The compound had no effect on total enzyme activity or on virus replication and the development of cytopathology.

After replication adenoviruses do not have an immediate cytopathic effect and progeny virions are not readily released from host cells (2, 8, 12). However, when assaying for the presence of extracellular lactate dehydrogenase, as an indicator of cell damage, it was found that adenovirus 12 caused a significant increase in lactate dehydrogenase activity of cell culture fluid, but the activity of fluid from adenovirus 5-infected cells was like that of uninfected cells (2). Further experiments demonstrated that the increased accumulation of extracellular lactate dehydrogenase is the result of membrane labilization.

To investigate the possible mechanism of membrane labilization caused by adenovirus 12, but not adenovirus 5, a study was made on the effect of these viruses on beta-gluconuridase, a representative hydrolytic enzyme normally found within cell lysosomes.

MATERIALS AND METHODS

Cell cultures. An established line of chimpanzee liver (CL) cells, derived from a biopsy specimen taken from a female chimpanzee (9), were grown at 37°C on L15 medium containing 5% fetal calf serum, and maintained on Eagle minimal essential medium with 0.5% fetal calf serum. Maintenance medium without phenol red pH indicator was used for investigations involving biochemical analysis of cell culture fluids. All media contained 100 U of penicillin and 100 µg of streptomycin per ml. The cells were examined at frequent intervals for mycoplasma contamination. BBL mycoplasma agar plates and broth were inoculated with culture medium and cells and incubated under aerobic and anaerobic conditions. Broth cultures were passaged three times and subcultured on agar after each passage. Positive and negative controls gave fitting results, and the cells were free from contamination. Mycoplasma orale cultures isolated from HeLa cells and obtained from the American Type Culture Collection were used as positive controls. Investigations to reveal latent viruses in CL cell cultures gave negative results. Cell culture fluid and cell extracts were inoculated into cultures of primary African green monkey kidney and primary human embryonic kidney cells as indicator systems to detect adventitious viruses and adenovirus helpers such as simian virus 40, simian adenovirus type 7, and chimpanzee viruses. Uninoculated and inoculated cultures were maintained for at least 21 days, after which supernatant fluid and cell extracts were blind passaged in new cultures.

Viruses. Preparations of adenovirus 5 and adenovirus 12 (Huie strain) obtained from the American Type Culture Collection were propagated by serial passage in CL cells. Infected cells showing advanced cytopathic effect (CPE) were treated with a MSE 100-W ultrasonic disintegrator at maximum output for 2 min at 5°C, gross particulate matter was removed by centrifugation at 650 × g for 10 min, and the virus-containing supernatant was stored at −70°C.

Viruses were titrated in tubes containing monolayer cultures of CL cells. Serial 10-fold dilutions of virus in maintenance medium were prepared, and 0.1 ml of each dilution was inoculated into the tubes. At least six tubes were inoculated for each dilution. The final reading for CPE was made on day 7 postinfection, and the mean tissue culture infective dose (TCID₅₀) was calculated.

Inoculation of monolayers. Confluent monolayers of CL cells in 30-ml Falcon flasks were inoculated with adenovirus 5 or 12 at an input multiplicity of 3.0 and
0.1 TCID<sub>50</sub> units per cell, respectively. The multiplicity of infection resulted in one-step growth of both virus types, and in a simultaneous appearance of CPE in all cells of the monolayer. After an adsorption period of 4 h at 37 C, the inoculum was removed, and the monolayers were washed three times with 5.0 ml of phosphate-buffered saline and reincubated with 4.0 ml of maintenance medium.

**Enzyme assays.** At designated intervals after infection, maintenance fluid was removed from uninfected and virus-infected monolayers and centrifuged at 650 × g for 10 min at 5 C to remove any cells that may have detached from the surface of the culture vessel. The amount of extracellular beta-glucuronidase released from 10<sup>6</sup> cells per ml of maintenance medium was measured.

For the measurement of total cellular and extracellular beta-glucuronidase activity, a suspension of cells at a concentration of 10<sup>6</sup> in 1.0 ml of their maintenance fluid was prepared, and then sonically treated and clarified with respect to particulate matter as described above. A ratio of 10<sup>6</sup> cells per ml of maintenance medium was a constant feature of all experiments reported herein. Cell counts were done in duplicate using a hemocytometer.

Beta-glucuronidase activity was determined by the method of Fishman and Bernfeld (11). Owing to the weak activity of enzyme preparations used in this investigation, especially that of cell culture fluid, the incubation period was increased from 1 to 6 h. One unit of beta-glucuronidase activity is the amount required to liberate 1 µg of phenolphthalein from phenolphthalein glucuronide under the specified conditions, and was determined by comparison against a phenolphthalein standard.

The substrate concentration and other experimental conditions were optimal and allowed linear enzyme activity for periods greater than the reaction time used in the assay.

Phenolphthalein glucuronide was obtained from Sigma Chemical Co., St. Louis, Mo.

**RESULTS**

**Virus replication.** One-step growth of adenovirus types 5 and 12 in monolayer cultures of CL cells has been described (2). Briefly, the latent period was 15 h for adenovirus 5 and 21 h for adenovirus 12. The incremental period of propagation was approximately 9 h for both virus types, after which the yield of infectious virus particles leveled off at 10<sup>7.9</sup> and 10<sup>4.4</sup> TCID<sub>50</sub> units per 0.1 ml for adenovirus 5 and 12, respectively. It has been reported that in KB cells highly oncogenic adenoviruses have a slower growth cycle than nononcogenic adenoviruses and that the Huie strain of adenovirus 12 produces low yields of infectious virus (14).

**Extracellular beta-glucuronidase activity during infection with adenovirus 5 and 12.** Beta-glucuronidase activity in culture fluids from uninfected and adenovirus 5-infected cells showed similar results both before and after the appearance of CPE induced by virus infection. In contrast, there was a progressive increase in enzyme activity of culture fluid from adenovirus 12-infected cells starting at 24 h after inoculation and prior to evidence of CPE (Fig. 1). Typical CPE consisting of a complete rounding and aggregation of cells into grape-like clusters was first observed at 24 h after inoculation with adenovirus 5 and at 30 h with adenovirus 12.

**Total beta-glucuronidase activity of cultures infected with adenovirus 5 or 12.** To learn whether the enhanced release of beta-glucuronidase from adenovirus 12-infected cells was due to a redistribution of the normal enzyme content of host cells, or if an increase in enzymatic activity might be involved in these changes, the total activity of cellular and extracellular fractions of uninfected and infected cultures was measured. Infection with adenoviruses 5 and 12 produced differing patterns of total beta-glucuronidase activity. The activity of adenovirus 5-infected cultures was like that of uninfected cultures throughout the experimental period. However, the activity of adenovirus 12-infected cultures showed an increase starting at 12 to 24 h after inoculation, and it remained elevated until termination of the experiment (Fig. 2).

**Effect of hydrocortisone on extracellular and total beta-glucuronidase activity.** Hydrocortisone has the property of stabilizing lysosomal and cytoplasmic membranes against a variety of injuries in vivo and in vitro (4, 20). Therefore, an experiment was performed to
From this generality it appears unlikely that adenovirus infection would induce a lytic enzyme response, because they are not cytolytic viruses (2, 8, 12). Cytochemical methods have demonstrated an increase in acid phosphatase activity during early stages of infection of human kidney cells with adenovirus 5 (7). Cytochemical examination of HeLa cells infected with adenovirus 5 revealed acid phosphatase staining and an increase in uptake of neutral red dye by lysosomes at approximately 6 h postinfection (1). The significance of these observations is not known, but it is possible that lysosomal enzymes might be involved in the uncoating of the virus during early infection. There is evidence suggesting that host lysosomes are involved in the uncoating of several

**DISCUSSION**

It has been observed that virus-induced cytopathology is sometimes associated with the leakage of hydrolytic enzymes from lysosomes of infected cells (10, 15). In general, cytolytic viruses stimulate leakage of lysosomal enzymes, whereas lysosomes retain their integrity after infection with nonlytic viruses.

<table>
<thead>
<tr>
<th>Hours postinfection</th>
<th>Units of beta-glucuronidase activity</th>
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<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>0</td>
<td>22.9</td>
</tr>
<tr>
<td>12</td>
<td>26.1</td>
</tr>
<tr>
<td>24</td>
<td>35.5</td>
</tr>
<tr>
<td>36</td>
<td>39.6</td>
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<td>48</td>
<td>38.7</td>
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virus 5 was...in about 4 h, after which the activity of infected cells was less than that of uninfected cells. Chardonnet (5), employing biochemical methods, observed that there was a decrease in acid phosphatase activity of human kidney cells infected with adenovirus 5 starting at 24 h postinfection, but at 72 h the activity was the same in both infected and uninfected cells.

The differential leakage of lactate dehydrogenase from CL cells infected with adenovirus 5 or 12 and inhibition of enhanced leakage from adenovirus 12-infected cultures by membrane stabilizing procedures (2) suggested that hydrolytic enzymes might be involved in this phenomenon. The increased levels of beta-glucuronidase activity in the extracellular phase of adenovirus 12-infected cultures observed in the present investigation and prevention of this by treatment with hydrocortisone at a concentration which had no effect on total beta-glucuronidase activity are evidence of cell membrane labilization. This could be the result of lytic enzyme activity and would allow leakage of soluble cytoplasmic constituents such as lactate dehydrogenase.

A study was made to elucidate the abnormal pattern of extracellular beta-glucuronidase activity of adenovirus 12-infected cultures. It was possible that adenovirus 12 might cause a redistribution of the normal enzyme content of host cells. In this event, the total cellular and extracellular beta-glucuronidase activity would be the same in uninfected and adenovirus 5- and 12-infected cultures. It was also possible that other factors might be involved in addition to a redistribution of enzymes. Experiments revealed an increase in total beta-glucuronidase activity of adenovirus 12-infected cultures as well as enhanced leakage of this enzyme from host cells. The results of adenovirus 5-infected cultures were like those for uninfected cultures. Both the increase in activity and redistribution of beta-glucuronidase induced by adenovirus 12 were moderate but consistent. A more extreme response would undoubtedly cause rapid and severe cell damage. Whether the hydrolytic enzyme response is related to the oncogenic nature of adenovirus 12, or is incidental, is not known. With respect to this observation, comparative experiments utilizing electron microscope procedures revealed differences involving lysosomes during the initial stages of infection of HeLa cells by nononcogenic and oncogenic adenoviruses. Adenoviruses replicate in the host nucleus. After cell penetration, nononcogenic adenovirus types 1 and 5 were rapidly transferred to the region of the nucleus and there was a conversion of dense particles to empty capsids. By comparison, relatively few particles of oncogenic adenovirus types 7 and 12 penetrated to the perinuclear zone of the cytoplasm. The majority of adenovirus 7 and 12 particles were sequestered in host lysosomes (6). The reason why certain adenoviruses are entrapped by lysosomes, whereas others are not, has yet to be elucidated.

The difference in beta-glucuronidase response observed between adenovirus 5 and 12 is not a function of the multiplicity of infection. Reducing the input of adenovirus 5 to that used with adenovirus 12 did not alter the enzyme pattern of adenovirus 5-infected cultures. It did delay the appearance of adenovirus 5-induced CPE until 48 h postinfection instead of at 24 h as observed with the higher multiplicity of infection (unpublished data).

Studies have indicated that the characteristic cytopathic changes observed in adenovirus-infected cells that have been fixed and stained and also in living unstained cells are caused by synthesis and accumulation of virus-specific deoxyribonucleic acid and proteins (13). However, the mechanism by which accumulated macromolecules bring about cell injury is not known. The results of the present investigation indicate that hydrolytic enzymes do not have a role in the production of distinctive adenovirus cytopathology. Both adenovirus 5- and 12-infected cells developed typical CPE, but only adenovirus 12-infected cells showed an abnormal beta-glucuronidase response.

Underwood (18) reported that treatment of L-929 cells with hydrocortisone resulted in reduced CPE and in a higher yield of herpes simplex virus than that obtained from untreated cells. The Huie strain of adenovirus 12

<table>
<thead>
<tr>
<th>Virus type</th>
<th>Virus titer (TCID₅₀/0.1 ml)</th>
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<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>Adeno 5</td>
<td>10⁷.50</td>
</tr>
<tr>
<td>Adeno 12</td>
<td>10⁷.47</td>
</tr>
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*One-step growth conditions were employed. The 4-h values are for virus present after a 4-h adsorption period at 37 C. Virus was harvested at 48 h after inoculation.

Table 2. Adenovirus yield after replication in CL cells incubated in the presence or absence of 50 μg of hydrocortisone per ml of culture fluid

activity of adenovirus 12-infected cultures as well as enhanced leakage of this enzyme from host cells. The results of adenovirus 5-infected cultures were like those for uninfected cultures. Both the increase in activity and redistribution of beta-glucuronidase induced by adenovirus 12 were moderate but consistent. A more extreme response would undoubtedly cause rapid and severe cell damage. Whether the hydrolytic enzyme response is related to the oncogenic nature of adenovirus 12, or is incidental, is not known. With respect to this observation, comparative experiments utilizing electron microscope procedures revealed differences involving lysosomes during the initial stages of infection of HeLa cells by nononcogenic and oncogenic adenoviruses. Adenoviruses replicate in the host nucleus. After cell penetration, nononcogenic adenovirus types 1 and 5 were rapidly transferred to the region of the nucleus and there was a conversion of dense particles to empty capsids. By comparison, relatively few particles of oncogenic adenovirus types 7 and 12 penetrated to the perinuclear zone of the cytoplasm. The majority of adenovirus 7 and 12 particles were sequestered in host lysosomes (6). The reason why certain adenoviruses are entrapped by lysosomes, whereas others are not, has yet to be elucidated.

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produces low yields of infectious virus in established cell lines as compared to other members of the adenovirus group (2, 14, 17, 19). Treatment with hydrocortisone under conditions which prevented enhanced leakage of betaglucuronidase from adenovirus 12-infected CL cells did not result in greater yields of virions. The compound had no effect on replication of adenovirus 5 or 12, and did not delay the time of appearance or inhibit the development of CPE induced by either virus type.

ACKNOWLEDGMENTS

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LITERATURE CITED