Enterovirus Type 70 Virion and Intracellular Proteins

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The proteins of purified enterovirus type 70 grown in rhabdomyosarcoma cells and intracellular proteins at 4 to 5 h after infection have been examined by polyacrylamide gel electrophoresis. Virions contained four proteins: P-1 (35,000, daltons) P-2 (28,000, daltons) P-3 (27,000, daltons) and P-4 (9,000 daltons). Further, addition of ZnCl₂ to infected cultures inhibited virus plaque development and interfered with post-translational cleavage.

Acute hemorrhagic conjunctivitis (AHC) has been epidemic throughout much of the West African and East Asian coastal regions since 1969 (4, 8). The disease is a painful transient punctate epithelial keratitis that persists for about a week; occasionally limbar radioculomyelopathy and secondary infections have been associated with AHC (8). The etiological agent of AHC recently has been recognized as a new entity for the human enterovirus group (13) and designated enterovirus type 70 (E-70).

Previously we reported some biological, biophysical, and antigenic characteristics of four E-70 strains (3) and showed that they have many echovirus-like properties and are antigenically distinguishable from other enterovirus and rhinovirus types. We have extended our study in this report to examine virion and intracellular proteins by high-resolution discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (disc SDS-PAGE). Further, we have observed that zinc chloride, when added to overlay medium for plaque assay of virus, gives concomitant reductions in virus titer and plaque size. Pulse labeling experiments with E-70-infected cell cultures indicated that these reductions resulted from inhibition of the post-translational cleavages necessary for virus maturation. In this regard, the maturation of E-70 and its inhibition by zinc chloride was similar to other picornaviruses observed by Butterworth and Korant (2) and Korant et al. (9); for AHC this might be important therapeutically.

E-70 (strain R6) was propagated in monolayer cultures of human rhabdomyosarcoma (RD) cells. The RD cells were a gift from R. McAllister. Conditions for virus growth at 35 C in roller bottle cultures, medium for radiolabeling virus proteins, and the plaque assay for infectious virus have been described (3). Purification of virus from clarified infectious cell culture fluid required concentration of the virus by centrifugation for 16 h at 22,000 x g in a Beckman type 19 rotor. Virus pellets were resuspended in 0.01 M Tris buffer (pH 7.1) and centrifuged through a 40% (wt/vol) sucrose cushion (2 ml) at 195,000 x g for 3 h (SW41 rotor). These virus pellets were resuspended in 0.1 M Tris buffer (pH 7.1) and layered onto a preformed gradient (15) of CaCl₂ (p = 1.25 to 1.40 g/ml) in 0.1 M Tris buffer (pH 7.1). After centrifugation at 100,000 x g for 15 h at 20 C (SW41 rotor), the visible virus band (p = 1.34 g/ml) was collected and diluted with 0.1 M Tris buffer (pH 7.1); the virus was sedimented through a sucrose cushion as described above. The virus was purified further by collecting only the 150S sedimenting particles after velocity centrifugation in 15 to 45% (wt/vol) sucrose gradients (7). Poliovirus type 1 (Mahoney) grown in human amnion (FL) cell cultures was purified similarly.

The virion proteins of differently labeled preparations of E-70 and poliovirus were compared by co-electrophoresis in 15% disc SDS-gels (10, 14). When the virus proteins were separated by electrophoresis for 5 h at 3 mA (Fig. 1A), only three proteins were resolved for E-70, whereas the four characteristic structural proteins of poliovirus (VP-1, -2, -3, and -4) were separated. However, when electrophoresis was for 14.5 h at 2.5 mA (Fig. 1B), a fourth protein was resolved for E-70 (with these conditions the smallest protein of E-70 and poliovirus migrated off the gel). It was curious that the relative migrations of poliovirus VP-1 and E-70 protein 1 and VP-3 and protein 3 changed with the electrophoresis conditions; the reason for this protein behavior was not determined. The molecular weight of E-70 protein 4 was estimated to be 9,000 from electropherograms similar to Fig. 1A. The molecular weights for protein 1, 2, and 3 (35,000, 28,000, and 27,000,
Fig. 1. Co-electrophoresis of enterovirus type 70 and poliovirus type 1 proteins by disc SDS-PAGE. Purified preparations of $^{14}$C-amino acid-labeled (●), E-70 and $^{3}$H-amino acid-labeled (○) poliovirus were mixed. After being dissociated at 100°C for 3 min in a buffer with 2.5% SDS and 5% 2-mercaptoethanol, the proteins were separated by disc SDS-PAGE (10, 14), using a 3.6% stacking gel and a 15% resolving gel. Electrophoresis at a constant current of 3 mA/gel for 5 h was used for (A); 2.5 mA/gel for 14.5 h was used for (B). After electrophoresis, each gel was sliced into 1-mm fractions, and the distribution of labeled proteins in the gel was determined by liquid scintillation spectrometry. The positions of the poliovirus proteins (VP-1, -2, -3, and -4) have been indicated.

respectively) were derived from electrophoreograms similar to Fig. 1B because of the resolution with this method. The reported molecular weights for enteroviruses (1) were used for this comparative determination. In experiments with lower gel concentrations, the disc SDS-PAGE system and the continuous SDS-phosphate PAGE system (11, 14) resolved E-70 proteins into three distinct species. When the disc SDS-resolving gel concentration was increased
to 15% and the electrophoresis was extended, a fourth E-70 protein was observed. Electrophorograms similar to Fig. 1A that resolve three E-70 proteins were also found for four other E-70 strains (R. Kono, personal communication; S. Yamazaki, K. Natori, and R. Kono, Abstr. 3rd Int. Cong. Virol. C189, p. 238, 1975).

The incorporation of heavy metals, particularly Zn\(^{2+}\), into the overlay media for plaque assay was found by Korant and co-workers (2, 9) to inhibit the growth of several different picornaviruses (i.e., human rhinoviruses, encephalomyocarditis virus, and poliovirus). When ZnCl\(_2\) was added to the overlay medium for plaque assay of E-70 in RD cells, virus growth was restricted (Table 1). Approximately 90% plaque reduction occurred with 0.08 mM ZnCl\(_2\). Concomitant with the reduction in titer, a decrease in plaque size from 3 mm to ≤1 mm was observed. Concentrations of ZnCl\(_2\) greater than 0.08 mM were cytotoxic in the plaque assay.

<table>
<thead>
<tr>
<th>ZnCl(_2) (mM)</th>
<th>Virus titer(^a) (PFU/ml \times 10(^9))</th>
<th>Plaque size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td>0.008</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td>0.040</td>
<td>1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>0.080</td>
<td>0.1</td>
<td>≤1.0</td>
</tr>
<tr>
<td>0.100</td>
<td>Toxic</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Confluent monolayer cultures of RD cells in 35-mm petri plates were infected with 0.1 ml from 10-fold dilutions of E-70. After 1 h for virus adsorption, the inoculum was removed and the cells were washed twice with medium. Zinc chloride was added from a 0.1 M stock solution to the liquid overlay media to give the indicated concentration. Each monolayer was overlaid with 4 ml of minimal essential medium containing 1.5% agarose. Plates were incubated at 35°C for 3 days in a humidified atmosphere of 5% CO\(_2\).

To determine the antiviral action of Zn\(^{2+}\) on the replication of E-70, the intracellular proteins synthesized at 4 to 5 h postinfection in E-70-infected RD cells with actinomycin D were examined by disc SDS-PAGE after pulse labeling the cells in the presence or absence of ZnCl\(_2\) (data not shown). Without ZnCl\(_2\), a number of peaks were resolved reminiscent of those in poliovirus-infected cells; the sum of their molecular weights exceeded by several times the theoretical coding capacity of virion RNA, whose molecular weight has been estimated at 2.5 × 10\(^6\) (17). In this regard the protein synthesis mechanism during E-70 replication seemed analogous to the post-translational cleavage mechanism established for other picornaviruses (5, 6, 12, 16). With ZnCl\(_2\), which is known to inhibit cleavages in picornavirus-infected cells (2, 9), many peaks were not apparent; a few large protein peaks were found to indicate a similar inhibition. The mechanism of cleavage inhibition by Zn\(^{2+}\), whether it is inhibiting a viral or cellular function, is not yet understood.

Since AHC is usually localized externally and systemic complications have been infrequently reported, the early topical treatment of E-70-derived conjunctivitis with ophthalmic preparations that contain Zn\(^{2+}\) might be therapeutically useful.

**LITERATURE CITED**