Characterization of an Enterovirus Associated with Acute Infectious Lymphocytosis

By C. GROSE AND M. S. HORWITZ

Departments of Microbiology and Immunology, Cell Biology, and Pediatrics, Albert Einstein College of Medicine, Bronx, New York 10461, U.S.A.

(Accepted 29 October 1975)

SUMMARY

An enterovirus (EVU-I6) previously isolated from children with acute infectious lymphocytosis has been further characterized. The EVU-I6 virus sediments as a 135S particle in sucrose gradients, has a density of 1.335 g/ml in CsCl, contains 4 polypeptides and has a single stranded RNA genome sedimenting at 35S. These structural features as well as the presence of a virus-related particle, the procapsid, are similar to those of other enteroviruses. However, the largest polypeptide of EVU-I6 is 49000 daltons, which is considerably larger than the corresponding polypeptide from poliovirus; the sizes of the other three viral polypeptides were similar in both viruses. Attempts to induce lymphocytosis by the inoculation of EVU-I6 into various animals, including immunologically aberrant ‘nude’ mice, were unsuccessful.

INTRODUCTION

The isolation and preliminary characterization of an apparently new enterovirus from children with acute infectious lymphocytosis (AIL) has been described (Horwitz & Moore, 1968). AIL is a mild childhood disease characterized by a marked but completely reversible increase of small lymphocytes of normal appearance in the peripheral blood. Although a viral aetiology has long been suspected, there has been no consistent association of the disease with any particular virus. In 1965 during an outbreak of AIL in a children's institution, an enterovirus, designated EVU-I6, was isolated from 20 patients and their contacts. Although EVU-I6 closely resembled the Coxsackie A viruses, it was not neutralized by any of the enteroviral antisera then available at the Centre for Disease Control.

The virus had been isolated and grown in the HEp-2 continuous cell line; however, it also caused a cytopathogenic effect in primary human embryonic kidney, HeLa and KB cells. EVU-I6 did not cause c.p.e. in either primary monkey kidney tissue or human fibroblast lines. In an earlier study (Horwitz & Moore, 1968) the virus was found to be resistant to ether, fully infectious at pH 2.9, replicate in the presence of BUdR in the light, and have a diam. of 29 nm by electron microscopy. When injected into suckling mice, EVU-16 caused both microscopic myositis of the tongue, and skeletal muscle lesions characteristic of the Coxsackie A group. In this paper we describe the further biochemical characterization of this enterovirus (EVU-16).
C. GROSE AND M. S. HORWITZ

METHODS

Origin of cells and virus. The stock virus for these experiments was prepared in HeLa cell monolayers from an isolate of EVU-I6 which was plaque-purified three times on HEp-2 cells. Suspension cultures of HeLa and KB cells which were used for individual experiments have been described previously (Horwitz, 1971).

Plaque assay for infectivity. To determine the virus plaque forming titre, infected HeLa cell monolayers were overlaid with nutrient agar containing MEM with 25 mM-magnesium chloride, 5% foetal calf serum (FCS) and 0.9% Difco Noble Agar (Williams, 1970). The plates were incubated for 4 d and then overlaid with 2 ml of a neutral red solution (0.015%). The plaques were counted 3 to 4 h later.

Conditions of infection. Infection was achieved by resuspending either HeLa or KB cells at a concentration of $5 \times 10^6$ cells/ml in a stock virus solution at an input multiplicity of 3 p.f.u./cell. After 30 min at 37 °C, the cells were centrifuged at 600g and the supernatant fluid discarded. The infected cells were resuspended at $1 \times 10^6$ cells/ml of MEM containing 5% FCS and 5 µg/ml of actinomycin D. After an additional 60 min incubation, 15 µ Ci of [14C]-uridine were added to a 3 ml portion of the culture which had been removed to monitor the course of the infection. Duplicate samples of 0.25 ml were assayed hourly for trichloroacetic acid (TCA)-precipitable material. The plateau of uridine incorporation occurred between 5 and 6 h post infection (p.i.).

Purification of virus. KB or HeLa cells were infected in suspension culture and radioactively labelled either with uridine or amino acids. At approx. 5 h p.i. the cells were centrifuged at 600g and the virus released either by sonication of the whole cells or by preparation of cytoplasm with the use of 0.5% Nonidet P-40 (NP40) detergent in 2 ml NP40 buffer (Borun, Scharff & Robbins, 1967). The extract was placed on a 34 ml linear gradient of 15 to 30% sucrose (w/v) in reticulocyte standard buffer (RSB: 0.01 M-NaCl, 0.0015 M-MgCl$_2$, 0.01 M-tris, pH 7.4). After being centrifuged at 25000 rev/min for 2 h in the SW27 rotor of the Spinco ultracentrifuge, the fractions containing the virus peak (135S) from each gradient were pooled. The samples were reduced in volume to 3.5 ml and then dialysed against 0.01 M-tris buffer, pH 7.4. 1.733 g of CsCl were added and the density of the solution was adjusted to 1.32 g/ml. Equilibrium sedimentation was carried out for 36 h at 37000 rev/min at 4 °C in the type 40 rotor (Spinco). The tube was punctured at the bottom and 51 fractions were collected. The fractions were analysed for radioactivity and for density by refractive index (Ifft, Voet & Vinograd, 1961).

Isolation of viral RNA. Infected cytoplasm or purified virus was analysed for RNA by adding sodium dodecyl sulphate (SDS) to a concentration of 1% and heating the sample to 60 °C for 2 min. The RNA was sedimented through 34 ml, 15 to 30% linear (w/w) sucrose gradients prepared in NETS buffer (0.1 M-NaCl, 0.001 M-EDTA, 0.01 M-tris, pH 7.4, 0.2% SDS) at 21000 rev/min for 17 h at 23 °C in an SW 27 rotor (Hecht & Summers, 1970). Ribonuclease resistance was determined by the addition of pancreatic RNase (10 µg/ml) for 1 h at 37 °C in the presence of 0.3 M-NaCl (Best, Evans & Bishop, 1972).

Analysis of viral proteins. Infected cells were labelled with [35S]-L-methionine or with a mixture of [14C]-L-amino acids (RPH, reconstituted protein hydrolysate) and virus purified by gradient sedimentation. The fractions containing virus were combined, reduced in volume by evaporation and dialysed against 0.01 M-tris, 0.15 M-NaCl (pH 8). The proteins were precipitated by adding an equal volume of 20% TCA; if a visible precipitate was not observed, 50 to 100 µg of bovine serum albumin was also added. The precipitate was centrifuged and washed with cold acetone, dried and dissolved at 45 °C in tris-HCl.
Fig. 1. Sucrose gradient sedimentation of the cytoplasmic extract of EVU-16-infected cells showing the distribution of infectivity and [14C]-uridine. EVU-16 infected KB cells (3 × 10⁶) were labelled in 30 ml with 15 μCi [14C]-uridine in the presence of 5 μg/ml of actinomycin. At 5 h p.i. the cells were pelleted and resuspended in buffer with 0.5 % NP40. The cytoplasmic preparation (2 ml) was centrifuged through a 34 ml 15 to 30 % RSB-sucrose gradient at 25000 rev/min for 2 h. From each of the 30 fractions, 0.15 ml was removed to assay TCA-precipitable radioactivity. Another 0.1 ml was removed from alternate fractions to determine the virus titre. ---, E₄₅₀; ○—○, radioactivity; ○—○, p.f.u./ml.

Materials. The radioisotopes [2-14C]-uridine (50 mCi/mmol) and [35S]-L-methionine (90 Ci/mmol) were obtained from New England Nuclear, Boston, MA, and [14C]-reconstituted protein hydrolysate (RPH) from Schwarz/Mann, Orangeburg, N.Y. The following chemicals were purchased: ribonuclease (General Biochemicals, Chagrin Falls, OH), actinomycin D (Merck, Sharp and Dohme, Rahway, N.J.), caesium chloride (Harshaw Chemical Co., Solon, OH), 2, 5-diphenyloxazole (PPO; Packard Co., Downers Grove, IL), and dimethyl sulphoxide (DMSO); Fisher Scientific Co., Fair Lawn, N.J.). The radioactively labelled poliovirus preparations were a gift of Dr Ronald Lundquist.
C. GROSE AND M. S. HORWITZ

Fig. 2. Sedimentation analysis of RNA from EVU-16 infected cells. EVU-16 infected cells, labelled with 30 μCi of [14C]-uridine from 0.5 to 5 h p.i., were lysed with NP40 detergent. The cytoplasm (2 ml) was treated with SDS, heated to 60 °C for 2 min, and then centrifuged on a 15 to 30% NETS-sucrose gradient. Acid precipitable radioactivity was determined from 0.1 ml samples of each fraction. The extinction tracing demonstrates the ribosomal 28S and 18S RNAs at fractions 9 and 17 respectively from which the values of 35S and 19S were determined for the two EVU-16 RNA peaks. ---, E$_{260}$; O--O, [14C].

RESULTS

Properties of the virion

Cytoplasmic extracts from EVU-16-infected cells which were radioactively labelled with uridine were sedimented in a sucrose gradient (Fig. 1). The gradient was fractionated and assayed for optical density, radioactivity and infectivity. A peak of radioactivity co-sedimented with the infectivity and corresponded to a small shoulder in fraction 13 of the E$_{260}$ tracing. By using the 74S ribosomal peak in fraction 20 as a reference point, a sedimentation value of 135S was estimated. The results were identical if the cytoplasmic extracts were treated with 0.7% sodium deoxycholate (DOC) prior to centrifuging (Penman, Becker & Darnell, 1964). Samples from the 135S region from the sucrose gradients were centrifuged to equilibrium in caesium chloride gradients. The peak of radioactivity corresponded to a density of 1.335 g/ml.

Viral RNA

The incorporation of [14C]-uridine into viral RNA was studied by infecting cells in the presence of 5 μg/ml actinomycin D, which does not inhibit viral RNA synthesis or assembly of picornaviruses (Baltimore, Girard & Darnell, 1966). Cytoplasmic extracts in which the RNA had been liberated from the virion by adding SDS and heating to 60 °C were sedimented through sucrose gradients and analysed for extinction and radioactivity. Two peaks of uridine radioactivity were observed at 35S and 19S (Fig. 2). These peaks were not present in similarly treated uninfected cells. The radioactivity was assayed for RNase resistance; the 35S peak showed a 98% loss of TCA-precipitable counts after the addition of pancreatic RNase, whereas the 19S peak lost only 50% after similar treatment.
An enterovirus associated with lymphocytosis

Fig. 3. Demonstration of a procapsid of EVU-16 in infected cells. $6 \times 10^6$ HeLa cells were infected with $100 \mu$l of virus suspension and resuspended in MEM with $1/20$ the usual concentration of methionine and 5% dialysed FCS. The infected cells were labelled with $100 \mu$Ci of $[^{35}S]$-methionine from 3.5 to 5.5 h.p.i. Cytoplasm was prepared with NP40 detergent and 10 ml samples centrifuged on three 26 ml pre-formed CsCl gradients (density 1.2 to 1.4) at 24,000 rev/min for 15 h at 5°C in an SW 27 rotor. The fractions were assayed for radioactivity and two peaks were demonstrated on each gradient at approximate densities of 1.33 and 1.30. The fractions containing the radioactivity peak of lower density in the pre-formed CsCl gradient were dialysed and divided into two portions, one of which was treated with 1% SDS, prior to centrifuging of both portions through RSB-sucrose gradients for 2 h. The two gradients were fractionated and a small sample from each fraction assayed for radioactivity. The ribosomal 74S marker was found between fractions 17 and 18, and the EVU-16 procapsid was in fraction 18. ○—○, SDS-treated sample; •—•, untreated sample.

Effect of guanidine on viral RNA synthesis

Guanidine, which has been shown to prevent the synthesis of poliovirus RNA (Summers, Maizel & Darnell, 1965), was shown to act in a similar manner on EVU-16. A concentration of 3 mM guanidine prevented the incorporation of $[^{14}C]$-uridine into the RNA of infected cultures. After removal of guanidine, there was an accelerated synthesis of RNA, which reached a peak in less than 4 h following reversal of the inhibition.

EVU-16 procapsid

When cytoplasm was extracted from infected cells radioactively labelled with methionine, a particle with a sedimentation coefficient of 73S was observed in addition to the virion peak at 135S. Although the sedimentation of the virion was not altered by 1% SDS, the 73S ‘top component’ was dissociated by the addition of this detergent (Fig. 3). A ‘top component’ with similar properties has been demonstrated in poliovirus infected cells and represents empty capsids (Maizel, Phillips & Summers, 1967), also called ‘procapsids’ (Jacobson & Baltimore, 1968).

Viral proteins

The protein composition of virion and procapsid was measured on particles which had been purified both on CsCl and on sucrose gradients. Fig. 4 compares $[^{35}S]$-methionine-labelled EVU-16 virion and procapsid with poliovirion. Three of the four EVU-16 virion
Fig. 4. Polyacrylamide gel electrophoresis of EVU-I6 polypeptides ([35S]-methionine-labelled). EVU-I6 virus and procapsid were isolated on pre-formed CsCl gradients as described in Fig. 3 and further purified on 15 to 30% RSB-sucrose gradients. The single radioactive peak from each gradient was collected, the proteins were prepared for electrophoresis and electrophoresed with poliovirion as a marker through 12.5% acrylamide slab gels for 2 h at 100 V (Maizel, 1971). The autoradiogram was exposed for 2 weeks. (Exposure for 8 weeks did not demonstrate any additional proteins). The poliovirion used in this gel contained minor contaminants (4% of total radioactivity, as determined by a scanning densitometer, Transidyne Corp., Ann Arbor, MI).

proteins are shown and their mol. wt. calculated by comparison with the mobility of poliovirus proteins in SDS-polyacrylamide gels (Shapiro, Vinuela & Maizel, 1967). The mol. wt. of poliovirus proteins are 35000 (VP1), 28000 (VP2), and 23 to 24000 (VP3); (Maizel & Summers, 1968; Jacobson, Asso & Baltimore, 1970). EVU-I6 VP1 is 49000, VP2 is 27000 and VP3 is 22000. The EVU-I6 procapsid contains VP1 and VP3, a small amount of VP2 and an additional protein migrating just slower than VP1. This large polypeptide is referred to as VP0 using the nomenclature of poliovirus (Jacobson & Baltimore, 1968). No VP4 was found in EVU-16 virion labelled with [35S]-methionine.

When EVU-16 was labelled with a mixture of [14C]-amino acids and all the polypeptides accentuated by incorporating PPO into the gels before autoradiography, VP4 was easily visualized (Fig. 5). VP4 in the [35S]-methionine-labelled poliovirus could also be detected
An enterovirus associated with lymphocytosis

An enterovirus associated with lymphocytosis

An enterovirus associated with lymphocytosis

Fig. 5. Polyacrylamide gel electrophoresis of EVU-16 polypeptides ([14C]-protein hydrolysate-labelled). HeLa cells (6 x 10⁷) were infected with EVU-16 and labelled with 250 μCi of [14C]-protein hydrolysate from 2 to 5 h p.i. The virus and procapsid, purified on sucrose and pre-formed CsCl gradients as described in Fig. 3 were electrophoresed on 12:5 % polyacrylamide slab gels for 2 h at 100 V. The gels were prepared for fluorography by using diphenyloxazole (PPO) as described.

but VP₄ was absent from the EVU-16 procapsid even after heavily loading the gels with proteins. The mol. wt. of EVU-16 VP₄ was comparable to that of poliovirus VP₄ (4 to 6000; Maizel & Summers, 1968; Jacobson et al. 1970).

DISCUSSION

The enterovirus EVU-16 was isolated during an outbreak of acute infectious lymphocytosis. Initial studies placed the virus within the Coxsackie A group (Horwitz & Moore, 1968). Further characterization described in this paper has elucidated both similarities with and
differences from the most extensively studied enterovirus, poliovirus. The EVU-16 virion sediments at about 135S and is stable in SDS. Like poliovirus and the representatives of two other major subgroups of the picornaviruses, encephalomyocarditis virus and rhinovirus-1A, EVU-16 virion has four polypeptides, with a total mol. wt. of approx. 100000 (Butterworth, 1973). Although the largest virion polypeptide of the other three picornavirus subgroups is between 34 to 35000, the VP1 in EVU-16 is 49000 as determined by electrophoresis with poliovirus markers in acrylamide gels. The VP2 and VP3 of EVU-16 are 27000 and 22000 respectively; these proteins are slightly smaller than their counterparts (28000 and 23000) in poliovirus. The VP4 polypeptides of the two viruses comigrate.

Another similarity is an EVU-16 ‘top component’ of the same sedimentation value (73S) and same basic structure as described by Maizel et al. (1967) for poliovirus. The capsid lacks VP4, contains VP2 in reduced amounts and includes a larger polypeptide. This polypeptide in poliovirus infected cells is designated VPo and is the precursor to VP2 and VP4. Because of its probable role as a precursor in the formation of poliovirion, Jacobson & Baltimore (1968) substituted the name ‘procapsid’ for top component. In contrast to earlier studies they found only VPo, VP1 and VP3 but no VP2 and VP4 in the procapsid. We did not isolate any particle equivalent to the poliovirus intermediate, the provirion, which consists of RNA in a capsid of polypeptides VPo, VP1 and VP3, sediments at 125S and is unstable in SDS and CsCl (Fernandez-Tomas & Baltimore, 1973). Moreover, no evidence of an EVU-16 particle sedimenting faster than 135S was ever found by either RNA-labelling or protein-labelling with [35S]-methionine or [14C]-reconstituted protein hydrolysate. The EVU-16 virus-specific RNA which sediments at 35S in sucrose gradients is characteristic of enterovirus single-stranded RNA; as in poliovirus-infected cells, the broader peak at 19S probably represents partially double-stranded RNA which may contain both replicating intermediate as well as replicating form (Baltimore, 1968).

Although the EVU-16 agent was related by serological and epidemiological techniques to AIL, we have been unsuccessful in reproducing the lymphocytosis in experimental animals (Horwitz & Moore, 1968). Since mice infected with this virus were previously shown to have lymphocytic proliferation within skeletal muscle, we investigated the possibility of inducing the haematological abnormalities in immunologically deficient mice. Young adult ‘nude’ mice which have genetic defects in thymus-dependent immunity (Pantelouris, 1968) were infected with sucrose gradient purified-virus by the intraperitoneal and intracerebral routes. These animals failed to show either signs of illness or lymphocytosis. Thus the association between the enterovirus EVU-16 and lymphocytosis remains circumstantial.

This work was supported by grants AI-00405 and CA-11512 from the National Institutes of Health.

REFERENCES

An enterovirus associated with lymphocytosis


(Received 26 August 1975)