Isolation and Characterization of an Equine Adenovirus

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A viral agent was isolated from lung tissue obtained upon necropsy of an Arabian foal which had exhibited clinical signs of pneumonia. The virus is 75 nm in diameter, cubic in symmetry, and resistant to chloroform and low pH (3.0). It contains deoxyribonucleic acid and has a buoyant density of 1.31 g/cm³ in cesium chloride.

These findings indicate that the virus is a member of the adenovirus group.

Respiratory disease is a common and often serious condition affecting equine foals. Numerous agents have been associated with such conditions in the young equine (12, 16, 17). A pneumonia in newborn and young Arabian foals characterized by the presence of intranuclear inclusion bodies in the bronchial and bronchiolar epithelial cells has been previously described (8, 11, 20). Adenoviruses have been isolated from Arabian foals suffering from respiratory diseases (11, 20) as well as from clinically normal foals (6, 14). This report concerns the isolation and characterization of an adenovirus from pneumatic lung tissue of an Arabian foal in California.

MATERIALS AND METHODS

Cells and virus. An equine dermis cell line (ECID) provided by A. J. Hackett (Cell Culture Div. of the Naval Biomedical Lab., Oakland, Calif.) was used to propagate the viral isolate. Equine dermis cells were grown on Falcon plastic flasks in Eagle minimal essential medium containing 5% fetal calf serum, 2,000 U of penicillin per ml and 200 μg of streptomycin per ml. Other cell lines used in this investigation were Madin Darby bovine kidney and pig kidney cells.

The viruses used in this study were infectious bovine rhinotracheitis virus (IBRV), vesicular stomatitis virus (VSV), New Jersey serotype, and the adenovirus of equine origin isolated in our laboratory. The designation equine adenovirus (EAdV) is for convenience only, since its host range has not been established.

Isolation of the equine adenovirus. Lung tissues taken upon necropsy of a 3-month-old Arabian foal were homogenized with the aid of a mortar and pestle. A 10% (wt/vol) homogenate was obtained by the addition of Eagle medium. The homogenate was centrifuged at 6,000 × g for 15 min and the resulting supernatant fluid was passed through a 0.45-μm membrane filter. The filtrate was inoculated onto 2-day-old ECID cells and incubated at 37 C for 7 days. Cells were then scraped from the glass and frozen and thawed three times before being used to inoculate subsequent passages. After several passages, cytopathic effect was consistently observable at 2 to 3 days post-infection.

ECID cells from passages 20 to 30 were found to be the most sensitive to cytopathic effects, and only cells of those passage numbers were used for all experiments. Likewise, the addition of arginine at a concentration of 1.5 mM/ml enhanced cytopathic effects and was used throughout all experiments.

Growth and assay of viral isolate. Confluent monolayers of ECID cells were washed once with Eagle medium and the viral inoculum was allowed to adsorb for 1 h at 37 C before the addition of Eagle medium containing 5% fetal calf serum, 60 μg of tylocin per ml, and 1.5 mM arginine (EFTA medium). After an incubation period of 5 days at 37 C, infected cells were scraped into the medium, frozen and thawed three times, and centrifuged at 6,000 × g for 15 min. The supernatant fluid was stored at −70 C and passed three times before it was used as viral stock.

The titer of infectious virus was determined by the microtitration procedure (18). Briefly, serial log₂ dilutions of the viral sample were made and 0.05 ml of each dilution was transferred to flat-bottom wells constructed in plastic microplates (Model IS-FB-96, Belco Glass, Inc., Vineland, N.J.). Eight replicates were made per dilution and 0.05 ml of ECID cells (8,000 to 10,000 cells/well) suspended in EFTA medium was added to each well; the plates were covered with sealing tape and incubated at 37 C. Microplate cultures were observed for evidence of cytopathic effect with the aid of an inverted microscope. Infectivity titers, recorded after 8 days of incubation, were calculated by the method of Kärber (9).

Chloroform sensitivity. The procedure used
to determine sensitivity to chloroform treatment was that described by Feldman and Wang (4).

**Nucleic acid determination.** Confluent monolayers of ECID cells were infected with 1.0 ml (10^{-4.4} mean tissue culture infective doses per ml) of stock virus. At 2 days postinfection, 5-bromo-2'-deoxyuridine (BUDR) was added to four cultures at a final concentration of 40 μg/ml. On the 3rd day postinfection, thymidine at a final concentration of 80 μg/ml was added to two of the cultures. Untreated infected cultures served as controls. Cultures were frozen on the 5th day and duplicate samples were pooled for viral titration.

A representative virus for each nucleic acid type was also tested. Cultures of Madin Darby bovine kidney cells infected with IBRV and pig kidney cell cultures inoculated with VSV were treated immediately after viral adsorption with BUDR at a level of 100 μg/ml and incubated for 24 h at 37 °C. The titers of IBRV and VSV were determined by the plaque technique (3).

**Buoyant density determination.** At 1 day postinfection 5.0 μCi of 14C-thymidine (50.8 mCi/mmol; Schwartz/Mann) was added to each infected culture. Virus was harvested from these cultures 5 days postinfection by the procedures described previously for propagating unlabeled viral stocks. Labeled and unlabeled viral stocks were pooled and centrifuged at 78,400 × g for 90 min at 4 °C. The resulting pellet was suspended in 0.01 M tris(hydroxymethyl)aminomethane hydrochloride, 0.001 M ethylenediamine-tetraacetic acid, and 0.1 M NaCl (pH 7.4; TES buffer) and extracted twice with chloroform for 15 min at 4 °C. The extract was then layered onto a preformed CsCl density gradient, 20 to 40% (wt/wt) in TES buffer and centrifuged at 153,000 × g in a Spinco SW41 rotor at 4 °C for 20 h. Fractions were collected from a hole punctured in the bottom of the tube and acid-precipitable radioactivity was determined by the method of Parks et al. (13). Samples were counted in a Mark II liquid scintillation spectrometer. Density gradient fractions were also examined by electron microscopy.

**Electron microscopy.** Virus was concentrated by centrifugation at 78,400 × g for 90 min at 4 °C and suspended in TES buffer. Samples from viral concentrate or from density gradient fractions were placed onto Formvar-coated copper grids and allowed to stand for 15 min. Excess fluid was removed with a piece of filter paper and the grids were stained with 1.5% potassium phosphotungstate (pH 7.4) for 2 min. Grids were examined with an AEI EM 6B electron microscope.

**RESULTS**

**Morphology.** Negatively stained EAdV particles exhibited cubic symmetry and did not possess an envelope (Fig. 1). The average diameter of EAdV (estimated by measuring 50 particles) was 75 nm, a value which is within the size range reported for adenoviruses (15).

**Nucleic acid type.** The replication of IBRV and EAdV was found to be inhibited by BUDR, whereas that of VSV was not impaired. Furthermore, BUDR inhibition of EAdV replication was reversed by the addition of thymidine. The results shown in Table 1 offer presumptive evidence indicating that the genome of EAdV is deoxyribonucleic acid (DNA) in nature. It is important to

![Fig. 1. Morphology of an equine adenovirus showing cubic symmetry.](http://iai.asm.org/)

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note that inhibition of replication by BUdR does not eliminate the possibility that EAdV is a ribonucleic acid (RNA) virus which depends upon DNA synthesis for the production of progeny virus. However, no cubically symmetrical, non-enveloped RNA virus has been shown to be inhibited by BUdR. The fact that substantial amounts of 14C-thymidine is incorporated into particles that have a density of 1.31 g/cm³ lends additional support to a contention that the nature of the nucleic acid is DNA.

**Chloroform sensitivity.** The effect of chloroform treatment on EAdV infectivity is illustrated in Table 2. The finding shows that this virus does not contain essential lipids.

**Resistance to low pH.** When the pH of the viral suspension was adjusted to 3.0 by the addition of sodium citrate-citric acid buffer and held at room temperature for 1 h, there was no appreciable loss in viral titer (Table 3), indicating that EAdV is acid stable.

**Buoyant density.** A representative density gradient profile is shown in Fig. 2. The peak of

| Table 1. Effect of 5-bromo-2'-deoxyuridine (BUdR) on viral replication |
|---------------------------------|-----------------|-----------------|-----------------|
| Treatment                        | EAdV (TCID₅₀/ml) | IBRV (PFU/ml)   | VSV (PFU/ml)    |
| Control                          | 5.0 x 10⁴        | 5.5 x 10⁸       | 3.5 x 10⁸       |
| BUdR                             | 5.0 x 10²        | 1.4 x 10⁸       | 3.2 x 10⁸       |
| BUdR plus thymidine              | 4.2 x 10⁴        | ND              | ND              |

* ND, Not done.

![Fig. 2. Distribution of 14C-thymidine radioactivity from fractions of a preformed cesium chloride density gradient.](http://iai.asm.org/)
radioactivity was found at a density of 1.31 g/cm³. Large numbers of adenovirus particles were observed when peak fractions were examined by electron microscopy. These particles appeared singly and were found to be free of membranous material.

**DISCUSSION**

A viral agent has been isolated from lung tissue taken upon necropsy of a 3-month-old Arabian foal which exhibited clinical signs of pneumonia. The isolate, propagated in equine dermis cells, was examined by electron microscopy and found to (i) be free of an envelope, (ii) possess cubic symmetry, and (iii) have a diameter of 75 nm. These morphological characteristics are distinctive to members of the adenovirus group (1, 15).

Biological and physiochemical studies indicated that the isolate (i) was resistant to chloroform, (ii) contained DNA as its genetic material, and (iii) was resistant to inactivation at low pH (3.0). Such findings furnish additional evidence supporting the placement of our isolate into the adenovirus group.

The isolate was also shown to have a buoyant density of 1.31 g/cm³ in cesium chloride. Although this value is less than that of 1.34 g/cm³ reported by Green and Pina (5) for the type 2 human adenovirus, or that of 1.35 g/cm³ described by Carmichael (2) for infectious canine hepatitis virus, variation in the buoyant density of human adenovirus types has been demonstrated (5). This variability among individual types appears to be due to differences in their DNA content and base composition (5). These parameters were not determined for our isolate, but they may account for the lower buoyant density of the equine adenovirus isolated in our laboratory.

Adenoviruses have been isolated from numerous mammalian species. However, for many of these species only a few instances of disease have been attributable to natural adenovirus infection (1, 10). Adenovirus isolations have been made from various tissues of apparently normal animals (1, 7, 10), suggesting that they can be maintained as inapparent or subclinical infections. Due to the ubiquitous nature of adenoviruses, caution should be exercised when assessing their role in a particular disease. A possible epidemiological relationship between the viral isolate and the occurrence of pneumonia in Arabian foals is currently under investigation in our laboratory.

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**LITERATURE CITED**