Properties of Adeno-associated Virus (Type 1) Replicated in Rodent Cells by Murine Adenovirus

(Accepted 24 June 1980)

SUMMARY

We report for the first time the replication of infectious adeno-associated virus type 1 (AAV-1) in rodent cells [primary mouse kidney (PMK) and mouse L929 cells] using murine adenovirus (MAV) as a helper virus and also the production of AAV-1 virus antigen by herpes simplex virus type 1 (HSV-1) with its temperature-sensitive mutant ts 200 in mouse neuroblastoma (NB) cells. The infectious AAV virions produced by MAV on L cells had a buoyant density of 1.41 g/ml in caesium chloride gradients.

The adeno-associated satellite viruses are unconditionally defective members of the parvovirus group. As a result they cannot replicate unless the host cells are co-infected with a helper adenovirus (Atchison et al. 1965; Hoggan et al. 1966; Parks et al. 1967). Several ts mutants of adenovirus types 31 and 12 have been studied (Handa et al. 1976; Mayor et al. 1977) and shown to complement adeno-associated virus (AAV) antigen as well as virus production in appropriate cells. Weber et al. (1975) have isolated numerous ts mutants of adenovirus type 2 (Ad2). One of them, ts 4, has been shown to complement AAV antigen and infectious virion production in HEP2, KB and HEK cells (B. V. Lipps & H. D. Mayor, unpublished data).

Adenoviruses have been classified into different subgroups depending upon their natural hosts: human, simian, murine, avian, bovine, canine, etc. Besides human and simian adenoviruses, avian adenovirus or CELO virus has been shown to complement AAV replication in appropriate cells (Ishibashi & Ito, 1971).

To our knowledge, replication of AAV has not been achieved in rodent cells. In fact, there is a report that rodent cells will not support AAV functions. Handa et al. (1978b) observed that AAV-1 does not replicate in either rat cells co-infected with human adenovirus or rodent cells transformed by adenovirus. Many autonomous parvoviruses have been shown to cause foetal infections in animals, resulting in still births, malformations and embryonic disease (Cartwright & Huck, 1967; Cartwright et al. 1969). Johnson (1969) and Marimoto et al. (1972) have isolated parvoviruses from aborted and stillborn pigs. Although autonomous parvoviruses are reported to be capable of passing the placental barrier (Toolan, 1960), to date there are no definitive data that the defective parvoviruses can also do so. Despite its requirement for adenovirus functions for the production of infectious particles, AAV has been isolated from human embryo kidney cells in the absence of demonstrable adenovirus (Hoggan, 1971). AAV remains in a carrier state in the serially passaged latently infected Detroit-6 cells and can be rescued by using Ad2 (Berns et al. 1975). Handa et al. (1978a) were able to induce AAV-1 antigen and infectious virus by adenovirus type 31 superinfection, even after 45 and 39 passages in KB13 and KB302, clones, respectively.

Primary mouse kidney (PMK) monolayer cultures were prepared from the kidneys of 8 to 10 day-old suckling Swiss mice. The kidneys were monodispersed with 0.25% trypsin at room temperature and washed once with complete minimal essential medium (MEM) containing 10% foetal calf serum (FCS) (Flow Laboratories, Rockville, Md., U.S.A.). The
cells were resuspended in the medium and seeded in bottles or on 12 mm glass coverslips in Linbro trays. After 3 to 4 days the medium was removed and fresh medium was added. Complete monolayers were formed after 7 to 10 days. HEp2 cells (human epidermoid carcinoma of the larynx), mouse L929 cells and mouse neuroblastoma cells (NB) were purchased from the American Type Culture Collection and grown in MEM containing 10% FCS.

Murine adenovirus (MAV) was obtained from Dr Janet Hartley through Dr Arwin Diwan, NIH and was grown in L cells. Its infectivity titres in PMK and L cells were $10^6$ and $10^6$ TCID$_{50}$/ml, respectively. AAV-1 and Ad2 were prepared as described by Parks et al. (1967). Herpes simplex virus type 1 (HSV-1), strain KOS and its temperature-sensitive mutant $ts$ 200 were prepared as described by Drake et al. (1974).

Monolayer cultures of mouse L cells grown in Petri dishes 60 mm in diam. were co-infected with AAV-1 and MAV at a multiplicity of about 10 fluorescent infectivity units/cell and 2 TCID$_{50}$/cell, respectively. After 48 h when the infected cells began to show c.p.e. they were fixed in osmium tetroxide, processed and sectioned before examination in a Siemens 1A electron microscope. Infected fluids were also examined for virus particles following negative staining with 2% phosphotungstic acid at pH 7. AAV infectivity was determined using a modification of the immunofluorescence (IF) assay described by Ito et al. (1967) using HEp2 cells grown on coverslips and Ad2 free from AAV as a helper virus.

AAV-1 antigen production was detected in PMK and L cells co-infected with AAV-1 and MAV, whereas NB were infected with AAV-1 and HSV-1 or $ts$ 200. Cells were grown on 12 mm coverslips which were fixed at different time intervals with acetone and then stained to demonstrate IF-positive antigens using anti-AAV-1 guinea-pig serum (NIH) and fluorescein-conjugated goat anti-guinea-pig y-globulin heavy chains (Cappel). AAV-specific IF antigens appeared in the nucleus of infected cells after 24 h in the case of PMK and 36 to 48 h in L cells. IF-positive cells in PMK cultures appeared to stain more brilliantly than infected L cells (Fig. 1).

Monolayer cultures of PMK and L cells in 4 oz bottles were co-infected with AAV-1 and MAV. After 1 h adsorption at 37°C the excess inoculum was drained off and 3 ml of medium (MEM containing 2% FCS) was added to each bottle. After advanced c.p.e. were seen the cultures were frozen and thawed three times, sonicated and centrifuged to eliminate cell debris. These preparations were shown to contain AAV-1 and MAV by electron microscopic examination. The mixture containing AAV-1 and MAV was inactivated at 56°C for 20 min to inactivate MAV and was used for further passage using fresh MAV as helper. In this manner five serial passages were made. AAV-1 replication during five passages was assayed by the IF test in HEp2 cells using Ad2 as a helper virus. Table 1(a) indicates the results of IF titres achieved by AAV-1 in PMK and L cells. The replication of AAV-1 is more efficient in PMK cells than in L cells.

Mouse NB cells were co-infected with AAV-1 and HSV-1 or its temperature-sensitive mutant $ts$ 200 as helper viruses. After adsorption for 1 h at 37°C the cultures were washed once with medium, then incubated in fresh MEM containing 2% FCS. The cultures co-infected with AAV-1 and HSV were incubated at 37°C, whereas those infected with AAV-1 and $ts$ 200 at 34°C. The coverslips were stained for IF antigen at 24, 48, 72 and 96 h p.i. Abundant IF-positive cells were seen 24 h p.i. using HSV as a helper virus and at 48 h p.i. when HSV mutant $ts$ 200 was used as a helper virus.

To study the replication of AAV-1 in murine cells, monolayer cultures of PMK and L cells in 4 oz bottles were co-infected with AAV-1 and MAV. After adsorption at 37°C for 1 h the monolayers were washed twice with fresh medium. The cultures were then incubated with 3 ml of medium. At different time intervals (6, 12, 18, 24, 36, 48, 72, 96 and 120 h p.i.) two cultures were frozen and thawed three times, sonicated and centrifuged at low speed.
Fig. 1. IF-positive PMK cells co-infected with AAV-1 and MAV 48 h p.i.

Table 1. Immunofluorescence antigen titres of AAV-1 in (a) serial passages and (b) growth cycles in PMK and L cells using MAV as helper*

<table>
<thead>
<tr>
<th>(a) No. of passage†</th>
<th>( \log_{10} \text{I.F.U./ml} ) in PMK cells</th>
<th>( \log_{10} \text{I.F.U./ml} ) in L cells</th>
<th>(b) Time of harvest (h p.i.)‡</th>
<th>( \log_{10} \text{I.F.U./ml} ) in PMK cells</th>
<th>( \log_{10} \text{I.F.U./ml} ) in L cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.4</td>
<td>3.6</td>
<td>6</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>3.8</td>
<td>3.8</td>
<td>12</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>5.5</td>
<td>4.0</td>
<td>18</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>4</td>
<td>5.3</td>
<td>4.0</td>
<td>24</td>
<td>3.4</td>
<td>3.6</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>4.4</td>
<td>36</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72</td>
<td>4.3</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96</td>
<td>4.5</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>120</td>
<td>4.4</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*PMK and L cell monolayers were co-infected with AAV-1 and MAV. Titres are expressed in fluorescent infectivity units (\( \log_{10} \text{I.F.U./ml} \)).
† For (a), after extensive c.p.e. (4+) at 37 °C, the cells were harvested by freezing and thawing, and sonicated. The supernatants, after light centrifugation, were used for assaying AAV-1 antigen by the IF test using HEp2 cells and Ad2 free from AAV as a helper virus.
‡ For (b), at 6 to 120 h p.i., the cells were harvested by freezing and thawing. The virus in the supernatants was collected and assayed for AAV-1 fluorescent antigen in HEp2 cells using Ad2 as a helper virus.

The supernatants were inactivated at 56 °C for 20 min before titrating for AAV-1 activity by the IF assay. Table 1(b) shows the results of replication of AAV-1 from 6 h in both PMK cells and L cells. The titres obtained in PMK cells were consistently higher than those found in L cells.

To prepare purified AAV-1, monolayer cultures of L cells grown in 16 oz bottles were co-infected with AAV-1 and MAV. When the cells showed advanced c.p.e. they were harvested and processed as described previously (Young & Mayor, 1979). Fractions from CsCl gradients having densities between 1.39 and 1.45 g/ml were pooled, dialysed against 1 x SSC and rebanded on CsCl gradients. Those preparations which gave a single band of
AAV-1 were collected by side puncture. This purified virus was also examined in the electron microscope and showed abundant homogeneous AAV particles. To determine the buoyant density of AAV-1 produced using MAV as a helper, an identical procedure was used except that the cells co-infected with AAV-1 and MAV were labelled with ³H-protein hydrolysate (Schwarz-Mann, Orangeburg, N.Y., U.S.A.; 5 µCi/ml) 6 h p.i. Following centrifugation at 100,000g for 18 h the fractions were collected by bottom puncture and counted by liquid scintillation in a Beckman LS-250 counter. The density of each fraction was estimated by measurement of its refractive index. The peak of radioactivity coincided with a buoyant density for AAV-1 of 1.41 g/ml, a value in agreement with that obtained when other adenoviruses were used as helper viruses (Hoggan et al. 1973).

AAV is a defective virus which cannot replicate fully unless cells are co-infected with adenovirus as a helper virus (Atchison et al. 1965; Hoggan et al. 1966; Parks et al. 1967). However, AAV IF and CF (complement fixing) antigens and DNA can be detected following co-infection with AAV and HSV and its ts mutants (Drake et al. 1974; Mayor & Young, 1978). IF antigen production has been reported in human, simian and chicken cells using adenoviruses as helper viruses (Atchison et al. 1965; Ito et al. 1967; Ishibashi & Ito, 1971). To our knowledge production of infectious AAV or its structural antigens has not been reported previously in rodent cells. We have been able to demonstrate AAV IF antigen, as well as infectious virion production, in PMK and L cells using MAV as a helper virus. Furthermore, we have succeeded in demonstrating AAV IF antigen production in mouse neuroblastoma cells using HSV-1 and its mutant ts 200.

Although human adenovirus types 7 and 31 do not appear to replicate in stationary cultures of hamster cells and adenovirus type 2 replicates very poorly (Green, 1970), Mayor & Ratner (1972) have demonstrated production of infectious AAV in these cells. The growth of AAV is dependent on two factors, a host-range factor and adenovirus-induced helper functions. It seems that the inability to demonstrate replication of AAV in rat cells (Handa et al. 1977) using human adenovirus as a helper may be a reflection of inefficient adenovirus-induced helper functions.

To date, there are no definitive data that defective parvoviruses can cross the placental barrier, although such transmission has been demonstrated for certain autonomous viruses (Toolan, 1960). We believe that the findings in this paper will prove useful in detecting AAV or its IF antigens in experimentally infected mice. The system could be used in the study of transplacental crossing of AAV in pregnant rodents and its subsequent fate in the newborn.

This work was supported by grant CA 14618 from the National Cancer Institute and a grant from the Cockrell Foundation.

Department of Microbiology and Immunology
Baylor College of Medicine
Houston, Texas 77030 U.S.A.

REFERENCES


(Received 14 March 1980)