Detection of Biologically Active Adenovirions Unable to Plaque in Human Cells

JANET S. BUTEL, JOSEPH L. MELNICK, AND FRED RAPP

Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas

Received for publication 16 March 1966

ABSTRACT

BUTEL, JANET S. (Baylor University College of Medicine, Houston, Tex.), JOSEPH L. MELNICK, AND FRED RAPP. Detection of biologically active adenovirions unable to plaque in human cells. J. Bacteriol. 92:433–438. 1966.—Plaque formation in green monkey kidney (GMK) cells by a defective simian virus 40-adenovirus 7 “hybrid” population (PARA-adenovirus 7) was enhanced by the addition of excess adenovirions. Adenovirus types 2, 7, and 12 were capable of providing enhancement, although none of these viruses gives rise to plaques in simian cells in the absence of PARA (particle aiding replication of adenovirus). Near maximal enhancement of the PARA plaque titer on simian cells was obtained with input multiplicities ranging from 0.02 to 0.14 plaque-forming units (PFU) of helper adenovirus per GMK cell. The PFU of helper adenoviruses tested (types 2, 7, and 12) were measured in the most sensitive assay system, human kidney cells. This input corresponded to three to nine helper virus particles per GMK cell. The majority of particles capable of enhancing plaque formation by PARA banded at a density of 1.34 in CsCl. Adenoviruses inactivated by heat or ultraviolet light were not capable of enhancing plaque formation by PARA. Highest titers were obtained when PARA and helper adenovirus were inoculated simultaneously. Inoculation of the helper adenovirus 24 hr prior to the inoculation of PARA resulted in the formation of only 50% as many plaques, and no enhanced plaques developed when the adenovirus preceded PARA by 48 hr. Conversely, the addition of adenovirus 48 hr after the inoculation of PARA initiated 56% as many plaques as simultaneous inoculation; 4% of the enhanced plaques still formed when helper virus was added as late as 5 days after inoculation of PARA. These results suggest that adenovirus particles unable to plaque on human or monkey kidney cells are nevertheless capable of interacting with PARA in simian cells, thereby facilitating replication of both particles.

A simian virus 40 (SV40)-adenovirus type 7 “hybrid” population has been described (11, 22, 24) which can induce the synthesis of SV40 tumor (T) antigen, but cannot induce cells to produce SV40 viral (V) antigen. The SV40 T antigen synthesized in response to infection with the “hybrid” is immunologically indistinguishable from that present in SV40-transformed cells (1, 8, 16, 19) or from that synthesized during the early stages of the SV40 cytolytic cycle (10, 18, 21, 26). All biological activities of the “hybrid” population are inhibited by neutralization of the virus with adenovirus type-specific antiserum (11, 20, 22, 24). [“Hybrid” is used in the sense that it is a stable virus population which possesses determinants of two distinct parental types but the coat protein of only one parent (the adenovirus) on all virions in the population. The term does not imply a direct interaction of the nucleic acids of the parental types.]

The interaction of two particles in the “hybrid” is required to initiate plaque formation in green monkey kidney cells (3, 25). One of the particles is an adenovirus; the second, termed PARA (20), is a genetically defective SV40 component enclosed in an adenovirus capsid. Neither PARA nor adenovirus in the hybrid population can replicate in green monkey kidney cells in the absence of the other (4). The “hybrid” virus should therefore be more correctly referred to as a PARA-adenovirus population. The mutual dependence between PARA and adenoviruses for growth and plaque formation in simian cells is believed to represent a form of complementation (3, 4).

Addition of a heterotypic adenovirus to simian
cells infected with PARA-7 (PARA coated with adenovirus type 7 protein) results in an antigenic shift of the capsid protein of PARA from that of adenovirus 7 to that of the heterotypic adenovirus (4, 20, 23). The term "transcapsidation" has been proposed to describe this conversion (20).

In the course of these studies, it was observed that the addition of excess amounts of adenovirus to a PARA-adenovirus 7 population enhanced the PARA plaque titer in simian cells by as much as 200-fold (4, 20). This report will describe some of the novel quantitative characteristics of this enhancement phenomenon. In particular, it will show that many adenovirus particles unable to plaque on human cells are capable of interacting with PARA in simian cells to initiate plaque formation.

**Materials and Methods**

**Cells.** Primary cultures of African green monkey kidney (GMK) cells (Cercopithecus aethiops) were grown in lactalbumin hydrolysate (M-H) medium containing 2% calf serum and were maintained in serum-free M-E medium (14).

Human embryonic kidney (HEK) cells were grown in M-H supplemented with 10% fetal bovine serum (FBS) and were maintained in M-H with 2% FBS.

**Viruses.** The history of the SV40-adenovirus type 7 "hybrid," stock SP2, has been previously described (11, 22, 24). It was used after two additional passages in GMK cells. It will be referred to as PARA-adenovirus 7. This virus stock contained infectious adenovirus 7 and PARA-7.

Adenovirus type 7 employed in this study was derived from the SP2 stock by Boyeé et al. (2). It was plaque-purified three times in HEK cells and found to be free from detectable SV40 determinants. Adenovirus type 2 was a recent human isolate supplied by M. Benyesh-Melnick and used after four passages in KB cells. Adenovirus type 12, originally obtained from R. J. Huebner, had undergone numerous passages in KB cells. It was passed once in HEK cells and then once again in KB cells. Both viruses were tested and found to be free from detectable SV40 determinants.

All virus stocks were prepared in cells growing in 16-oz bottles. Viruses were harvested by disrupting the cells into the medium with two cycles of quick-freezing and thawing, followed by treatment in a sonic oscillator. Cell debris was removed by high-speed centrifugation and the supernatant fluids were dispensed in 1-ml portions in glass ampoules. The ampoules were sealed, the contents were quick-frozen, and the ampoules were stored at −90 C.

**Antisera.** Rabbits were used for the preparation of neutralizing antisera against the types 2, 7, and 12 adenoviruses described above. The animals received 1 intramuscular injection of 2 ml of undiluted virus fluids weekly for 3 weeks, a fourth inoculation 2 weeks later, and were bled out 2 weeks after the final inoculation.

**Virus assays.** Plaque assays for adenoviruses in HEK cells and for PARA in GMK cells have been described in detail (2, 4, 6, 20). Briefly, PARA titrations were routinely performed by co-infecting GMK monolayers in 60-mm plates with a dilution of helper adenovirus which was not cytotoxic but yet provided a multiplicity of infection of about 1 plaque-forming unit (PFU)/cell, the PFU having been determined in human kidney cells. The helper virus and the PARA being assayed were allowed to adsorb simultaneously for 60 to 90 min at 37 C, at which time an agar overlay was applied. The overlay consisted of Eagle's medium, 10% FBS, 1% agar, and 0.23% sodium bicarbonate. A second overlay containing a 1:20,000 dilution of neutral red was applied 1 week later. Plaques were visible the following day, but maximal titers generally were not reached until the 4th day after the second overlay was applied. Adenoviruses were titrated in HEK cells with the use of the same overlay. All assays employed two or three plates per dilution.

**Inactivation techniques.** Heat inactivation was accomplished by placing small portions of the virus suspensions in stoppered, thin-walled glass tubes, and heating for 45 min in a water bath at 50 C. Ultraviolet (UV) light inactivation was carried out with a West亲情? Sterilamp 615 T3. Virus suspensions were sonicated and treated prior to exposure to UV to dissociate aggregates. Amounts of 1 ml of the sonically treated suspensions were placed in 60-mm plastic petri dishes and irradiated for 5 min. The tops of the plates were removed, and the contents were swirled continuously during the exposure period. All work with UV-irradiated virus was carried out in a darkened room to exclude photoreactivation.

**Particle counts.** The pseudoreplication and staining methods described by Smith and Melnick (27) were used to prepare specimens for viewing with a Hitachi HU-11B electron microscope.

**Density gradient centrifugation.** Adenovirus was banded in a preformed gradient of cesium chloride (CsCl) by centrifugation at 30,000 rev/min for 18 hr at 4 C in a Spincro model L2 centrifuge with an SW-39L2 rotor. Samples were collected in droplets from the bottom of the tube, the refractive indices of the fractions were determined in a Bausch and Lomb refractometer, and these values were converted to buoyant density.

**Results**

**Enhancement by adenoviruses of plaque formation by PARA in simian cells.** Many human adenoviruses do not replicate in GMK cells in the absence of SV40 (4, 5, 6, 17). When high concentrations of such adenoviruses are added to monolayers of GMK cells, the plaque titer of the PARA-adenovirus 7 stock is enhanced by as much as 200-fold (4, 20). In recent experiments with the virus stocks used for the present work, the PARA-adenovirus 7 titer increased from 10^4.4 to 10^4.4 and 10^4.6 PFU/ml in the presence of additional adenovirus type 7 or type 2, respectively. Adenovirus type 12 enhanced the titer of the PARA-adenovirus 7 stock from 10^4.4 to 10^5.3 PFU/ml.
Thus, it was confirmed that heterotypic adenovirus types 2 and 12 enhanced PARA-adenovirus 7 plaque titers as effectively as the homologous type 7.

Enhancement as a function of the concentration of helper adenovirus. Various dilutions of each helper adenovirus were inoculated onto replicate GMK monolayers, and the degree of enhancement of the PARA plaque titer was calculated. Close to maximal enhancement, over 20 times, was already obtained with multiplicities of infection ranging from 0.02 to 0.14 PFU of helper virus per cell (Fig. 1); the PFU values again, by necessity, had to be determined in HEK cells. These values were obtained with preparations of adenovirus types 2 and 12, respectively. Adenovirus type 7 yielded an intermediate value of 0.09 PFU per cell.

Particle counts were performed on the adenovirus preparations (electron microscopy was performed by Robert Rongey). The number of particles per PFU varied from 32 to 217 (Table 1). Based on these particle counts, essentially maximal enhancement was obtained in the narrow range of an input of three to nine particles of helper virus per cell (Fig. 2). The minimal number of particles required for near maximal enhancement by the different helper viruses tested were specifically: three in the case of adenovirus 7; four with adenovirus 2; and nine particles per cell with adenovirus 12.

The helper adenovirus type 7 was centrifuged in cesium chloride, and fractions at different densities were analyzed for ability to enhance plaque formation by PARA in GMK cells. As seen in Fig. 3, most of the activity was with virus having a buoyant density of 1.34; it is this virus that carries the bulk of adenovirus infectivity as measured in HEK cells. It should be noted that none of the helper adenovirus stocks contained the recently described adeno-satellite virus (13).

Effect on enhancement of time of addition of helper adenovirus. Routinely, the PARA-adenovirus 7 and helper adenovirus were inoculated onto GMK monolayers and allowed to adsorb simultaneously. Experiments were carried out to

![FIG. 1. Effect of concentration of plaque-forming units of helper adenovirus on plaque formation by PARA in simian cells.](image1)

**Table 1. Particles to plaque-forming units (PFU) in the helper adenovirus preparations**

<table>
<thead>
<tr>
<th>Helper virus</th>
<th>Particle count (particles/ml)</th>
<th>Titer (PFU/ml)</th>
<th>Particles per PFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus type 7</td>
<td>$5.5 \times 10^9$</td>
<td>$1.7 \times 10^8$</td>
<td>32</td>
</tr>
<tr>
<td>Adenovirus type 12</td>
<td>$1.9 \times 10^9$</td>
<td>$2.8 \times 10^7$</td>
<td>68</td>
</tr>
<tr>
<td>Adenovirus type 2</td>
<td>$7.6 \times 10^9$</td>
<td>$3.5 \times 10^7$</td>
<td>217</td>
</tr>
</tbody>
</table>

![FIG. 2. Effect of concentration of particles of helper adenovirus on plaque formation by PARA in simian cells.](image2)

![FIG. 3. Ability of adenovirus particles with various densities to enhance plaque formation by PARA in simian cells.](image3)
determine the optimal time of addition of the helper virus. GMK monolayers were inoculated with helper adenovirus either before or after inoculation with PARA. PARA was inoculated by use of a 10⁻⁴ dilution of the PARA-adenovirus 7 stock, as at this dilution no plaques formed in the absence of enhancement by an adenovirus. Inoculated monolayers were held under fluid medium until the second virus was added, at which time an agar overlay was applied. Control monolayers were inoculated with both PARA and adenovirus each day to control any variation in cell susceptibility due to aging. As can be seen in Fig. 4 (left side), only 50% as many enhanced plaques were obtained when the adenovirus was inoculated 24 hr before PARA as compared with titers obtained when the viruses were inoculated together. No enhanced plaques were formed when the helper adenovirus was inoculated 48 hr before PARA.

Titters dropped less rapidly when adenovirus was inoculated after PARA: 56% of the enhanced plaques developed when the adenovirus was inoculated 48 hr after inoculation of PARA; 4% of the enhanced plaques still formed when helper adenovirus was added as late as 5 days after PARA (Fig. 4, right side). Similar results were obtained in two additional tests.

Inactivation of ability of adenovirus to enhance plaque formation by PARA. As expected, the infectivity of helper adenoviruses was inactivated by treatment with either heat or UV light. These inactivated adenoviruses were no longer capable of enhancing plaque formation in GMK cells by PARA (Table 2). The untreated adenovirus type 7 had an initial titer of 10⁻² PFU/ml on HEK cells, and enhanced plaque formation by the PARA-adenovirus population 25-fold. After treatment with heat or UV light, the adenovirus 7 titer was reduced to 10⁻⁴ and 10⁻⁴.⁷ PFU/ml, respectively. These preparations, even though not completely inactivated, were not able to enhance plaque formation by PARA-adenovirus 7. As can also be seen in Table 2, very similar results were obtained with adenovirus type 2.

**DISCUSSION**

Results presented previously (3, 4, 20) and in this paper show that the addition of high concentrations of noncytopathic adenovirions enhances plaque formation in simian cells by a defective SV40-adenovirus population (termed PARA-adenovirus). The enhancement is possible because PARA is unable to replicate unless a coinfecting adenovirus is present to provide the coat protein for PARA (4, 20, 23). Enhancement is not restricted to the homologous adenovirus type 7 but can also be demonstrated with the heterotypic types 2, 4, 5, and 12 (4, 20, 23) and with types 3, 6, 16, and 21 (Rapp, unpublished data). It is not surprising that heterotypic adenoviruses are capable of interacting with PARA since it has been shown that complete SV40 will react in adenovirus-infected cells and potentiate the replication of adenovirus types 2, 5, 7, and 12 in GMK cells (4, 5, 6, 15, 17).

Maximal enhancement is obtained with an input of between 3 and 9 helper virus particles per cell. These values hold even for populations in which only one of every 217 particles plaques as an infective unit on HEK cells, the most sensitive bioassay system known for human adenoviruses. Essentially maximal enhancement was already obtained with inputs ranging from 0.02 to 0.14 PFU (as determined in HEK cells) of helper virus per GMK cell; additional adenovirus did not substantially increase titers of the PARA virus.

**TABLE 2. Failure of adenovirus inactivated by heat or ultraviolet light to enhance plaque formation by PARA**

<table>
<thead>
<tr>
<th>Enhancing adenovirus</th>
<th>Treatment of enhancing adenovirus</th>
<th>Titer of enhancing adenovirus on HEK cells (log₁₀ PFU/ml)</th>
<th>Titer of PARA-adenovirus 7 stock on GMK cells (log₁₀ PFU/ml)</th>
<th>Enhancement of PARA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 7</td>
<td>None</td>
<td>8.3</td>
<td>6.3</td>
<td>25-fold</td>
</tr>
<tr>
<td>Type 7</td>
<td>Heat*</td>
<td>4.3</td>
<td>4.8</td>
<td>None</td>
</tr>
<tr>
<td>Type 7</td>
<td>UV³</td>
<td>4.7</td>
<td>4.8</td>
<td>None</td>
</tr>
<tr>
<td>Type 2</td>
<td>None</td>
<td>7.8</td>
<td>6.9</td>
<td>100-fold</td>
</tr>
<tr>
<td>Type 2</td>
<td>Heat*</td>
<td>5.2</td>
<td>4.9</td>
<td>None</td>
</tr>
<tr>
<td>Type 2</td>
<td>UV³</td>
<td>4.6</td>
<td>4.8</td>
<td>None</td>
</tr>
</tbody>
</table>

* For 45 min at 50°C.  
³ Exposed for 5 min.

**FIG. 4. Effect of time of addition of helper adenovirus on plaque formation by PARA in simian cells.**
Adeno-satellite virus (13) was not present in any stocks and could not be an essential factor.

The explanation for the requirement of less than 1 PFU, as measured in HEK cells, of adenovirus per GMK cell to obtain maximal enhancement of PARA is not known. It is possible that many adenovirions in the population are biologically active in regard to certain functions (perhaps enzyme induction) but cannot plaque even on the sensitive HEK cells. This hypothesis is somewhat supported by Wasserman’s (28) observation that adenovirions are not uncoated efficiently in human cells, but the hypothesis requires the unproven assumption that uncoating (but not subsequent replication) of adenoviruses in simian cells is more efficient than in human cells.

An alternate explanation would be that many of these adenovirus particles contain incomplete nucleic acid complements and that PARA is supplying needed adenovirus information. Somewhat analogous are the observations made with two plant viruses (29). The addition of a non-infectious “middle” component (obtained by centrifugation) to the “bottom” component increased the infectivity of the latter. The “top” component which contains no ribonucleic acid (RNA) had no such enhancing effect. It was suggested by the authors that perhaps some of the RNA in the “bottom” component had been rendered noninfectious in such a way as to allow activation by the incomplete RNA from the “middle” component. However, it is not yet known whether PARA contains any adenovirus nucleic acid in addition to the defective portion of SV40 deoxyribonucleic acid (DNA). Experiments are currently in progress to test this hypothesis.

Fenner and Sambrook (7), in reviewing experiments of phenotypic mixing, suggested that some noninfectious particles in a virus stock can contribute to the total virus yield, at least in cells in which an infective particle is multiplying. This conclusion was based on data obtained by Ledinko and Hirst (12) with poliovirus and by Granoff (9) with Newcastle disease virus. Our results are compatible with this suggestion, but the data thus far available do not allow resolution of the alternate hypotheses discussed above. However, it is clear that the system being studied is a more sensitive measure for infectivity of adenoviruses than the plaque assay in HEK cells, hitherto regarded as the most sensitive system for adenoviruses.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants CA-04600 from the National Cancer Institute and AI 05382 and 5 T1 AI 74 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED


