Inhibition of Adenovirus Transformation *in Vitro* by AAV-1[1] (36397)

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(Introduced by W. McD. Hammon)

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Adeno-associated viruses decrease the yield of helper adenovirus in cell cultures infected with both viruses (1–3). Inhibition of the adenovirus productive cycle cannot be attributed to an interferon-like material (2) and can be abolished by UV-irradiation (3) or by heating of the adeno-associated virus at 65° (4).

Adeno-associated virus type 1 (AAV-1) inhibits transformation of hamster embryo cells by adenovirus type 12 (Ad12) or simian adenovirus SA7 (5), and Ad12 oncogenicity *in vivo* (6). Further insight into the mechanism of adenovirus tumorigenesis might be attained by determining the nature of the inhibition of adenovirus expression by AAV-1 in the nonproductive cycle (transformation). This report presents data from experiments designed to quantitate the inhibition of adenovirus transformation by AAV-1.

Materials and Methods. Viruses and cell cultures. The source and passage history of Ad12, SA7, and SV11, and the methods for preparation of cell cultures, virus assays and transformation of hamster embryo cells have been described (7–9). The Mont strain of human adenovirus type 1 (Ad1) (10) was passaged several times in human embryonic kidney cells. Two additional passages were made in primary hamster embryo cell cultures. All virus stocks used in these experiments were titrated by the plaque method either in BSC-1 cells (SA7 and SV11), or in secondary human kidney cells (Ad12 and Ad1).

Preparation of AAV-1. Stocks of AAV-1 (11) were prepared by inoculating LLC-MK_2 cell cultures, contained in 100 mm plastic petri dishes, with a mixture of SV15 and AAV-1. Following a 2-hour adsorption period, 5 ml of Eagle's medium without serum were added to each plate of inoculated cells. Cells and fluid were harvested when cytopathic effects were maximal, usually between 4 and 6 days following inoculation. Cell debris and whole cells were removed by centrifugation of the harvested medium at 10,000 rpm for 10 to 20 min. The cell pellet was then extracted with 0.5% sodium deoxycholate or an equal volume of genetron. The extracts, following separation from cell debris by centrifugation, were added to the supernatant fluids from the first centrifugation. Most AAV stocks were freed of infective helper adenovirus by filtration through stacked Millipore filters (50, 220, 450, 650 nm) or in a few instances by heating concentrated pools to 60° for 10 to 15 min (1).

Concentration of individual AAV pools was accomplished by one of the following methods: (i) virus from the combined supernatant fluids was precipitated with 33% methanol (final concentration); (ii) virus was sedimented by centrifugation at 25,000 rpm in the SW25 rotor of the Spinco Model L ultracentrifuge; (iii) virus was banded in CsCl at a density of 1.37 g/ml. In each instance, attempts were made to concentrate the AAV 20- to 30-fold.

AAV titrations were done by the plaque inhibition technique (2) using SV11, SV15, SV34, or Ad1 as challenge viruses. The titers

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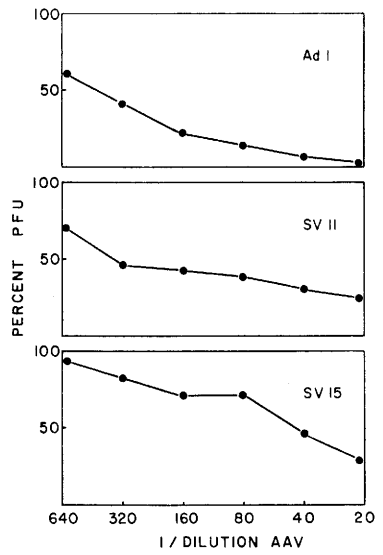

FIG. 1. Plaque inhibition of 3 adenoviruses by AAV-1. At each dilution of AAV-1, the number of adenovirus plaques was normalized to 100% for the controls without AAV.

of individual pools were expressed as adenovirus plaque inhibiting units (PIU) per 0.1 ml (2). A computer was used to calculate AAV titers by a modification of the method of Seligman and Mickey (12); input to the computer consisted of the adenovirus plaque count from each assay dish.

Inhibition of transformation. One-tenth milliliter of serially diluted AAV was added to 60 mm dishes of hamster embryo cell cultures (HEC) followed immediately by 0.2 to 0.3 ml of the appropriate adenovirus suspension. Adsorption was for 3 to 4 hr, after which cells were transferred as previously described (7). After approximately 12 to 14 days, all plates were overlayed with 0.5% agar in Eagle's medium containing 0.1 m*M* calcium (7). Final focus counts were made macroscopically 3 to 4 weeks after virus additions.

Results. Inhibition of Ad1 in hamster cells by AAV-1. Before attempting to inhibit adenovirus transformation of hamster cells by AAV, it was first necessary to determine the sensitivity of hamster cells to infection by AAV-1. This was done by comparing the inhibition of Ad1 plaque formation in hamster cells to that obtained in monkey cells using SV11 or SV15 as challenge viruses. Figure 1 shows that Ad1, which undergoes a complete replicative cycle in hamster cells, is inhibited by AAV-1. The reduction of Ad1 plaque number in hamster embryo cells was greater at low dilutions of AAV than the reduction in plaques in LLC-MK_2 cells using SV15 or SV11 as challenge viruses. In experiments designed to quantitate the inhibition of adenovirus transformation, the amount of AAV used was based upon the titer obtained in HEC against Ad1.

It is not immediately obvious, but the assay used here is quantal. The apparent paradox of having plaques occur in a quantal assay arises because the test units in this case are individual cells containing or not containing an infectious AAV particle when challenged with an adenovirus particle. Each cell in a culture is analogous to a test tube of cells in the usual end-point dilution titration and the 50% response level occurs at a multiplicity of 0.693 inhibiting units of AAV/cell.

It is appropriate to apply the Weibull transform rather than a probit transform to quantal data (13). Percentage response data transformed this way fall on a straight line with unit slope when plotted against the logarithm of the virus multiplicity. If the efficiency for inhibition of transformation differs from that for inhibition of plaque formation, or if there is a different efficiency for inhibition of different adenoviruses, the data still generate straight lines with unit slope but the lines are shifted horizontally on the abscissa (which represents virus multiplicity).

Shortley and Wilkins (13) discussed other characteristics of the Weibull transform. With a homogeneous test population, the 1-hit line has a slope of 1; a slope greater than 1 implies cooperative action (more than one hit). Heterogeneity of host response produces a multicomponent curve; a fit of such data by a straight line has a slope less than 1.

The Weibull transform was further modified by Dr. Howard A. Schneider (personal communication) to yield a unit called a Weibit (Weibit $= \log_{10} [-\log_e F] + 5.159175$, where F is the response as percentage of controls), analogous to the probit so

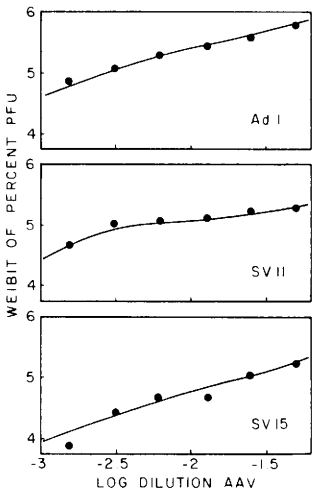

FIG. 2. Data of Fig. 1 plotted under the assumption that a single infectious AAV is sufficient to inhibit an adenovirus from initiating a plaque. The curves were fitted according to the method of Shortley and Wilkins (13) using the parameters shown in Table I.

that the 50% dilution end point occurs at Weibit 5.0 and negative values of the Weibull transform are eliminated. The Weibit plot for quantal response is thus analogous to a probit plot for data from bioassay of low-virulence materials.

If the host population responds heterogeneously, a single-hit curve can be fit to the data according to the equation

$$\text{Weibit } F = 1 - \gamma_1 e^{-.693\beta m/\delta_1} - \gamma_2 e^{-.693\beta m/\delta_2} \quad \text{Eq. (1)}$$

where γ_1 is the fraction of hosts with one degree of sensitivity, γ_2 is the fraction of hosts with a uniform but lower sensitivity ($\gamma_1 + \gamma_2 = 1$), δ_1 and δ_2 are the multiplicities necessary for 50% response for each group, and β is the efficiency of AAV-1 inhibition in that system. The fitting procedure is by trial and error, rather than least squares.

Data of Fig. 1 have been plotted again in Fig. 2 but with the Weibit transform of percentage inhibition of adenovirus plaque count as a function of multiplicity of added AAV-1. With each adenovirus, the slope is less than unity. The implications are that a single AAV virion is sufficient to inhibit a cell from undergoing the cytocidal response to adenovirus and that the cells respond heterogeneously. By assuming that there are two host-cell populations with respect to sensitivity for each adenovirus and that one AAV virion is sufficient to inhibit, as suggested by Shortley and Wilkins (13), reasonably good fits to the data points can be obtained with Eq. (1), as indicated by the curves in Fig. 2. The parameters used to fit the 3 curves are in Table I.

Inhibition of adenovirus transformation by AAV. The data presented in Table II show the results of representative experiments with SA7, SV11, or Ad12 and AAV-1. When SA7 was used as the challenge virus, 5 to 6 PIU of AAV/cell were insufficient to cause a 50% reduction of transformed cell foci. However, an input multiplicity of 2 to 3 PIU/cell was sufficient to cause 50% inhibition when either SV11 or Ad12 was used as challenge virus.

The data in Table II represent experiments done with two separate pools of AAV, one of which was concentrated by CsCl banding (Ad12 experiment) and the second by methyl alcohol precipitation (SA7 and SV11 experiments). To minimize the possible variation that could arise in using different pools of AAV, other experiments were performed in which, in a single experiment, aliquots of a single preparation of AAV and a single lot of cultures were used for all assays. The results of one such experiment are shown in Fig. 3. About 1.5 to 2 PIU of AAV/cell appear to be necessary to achieve a 50% reduction in SV11 or Ad12 transformed cell foci, whereas approximately 7 to 8 PIU/cell are required to cause a 50% reduction in SA7 foci. Inhibition by AAV-1 of Ad12 and SV11 transformation appear to be similar; the data for SA7, however, show a rise above control levels at log multiplicities from about —0.6 to 0 (Fig. 3). This rise also has been seen in several other such assays.

The data for inhibition of transformation from Table II and Fig. 3 have been plotted in Fig. 4 according to the 1-hit model. Here, as with inhibition of adenovirus plaque formation, straight lines fitted to the data by the method of least-squares have slopes less than 1 for Ad12 (slope = 0.813) and SV11 (slope = 0.745). The difference between the slopes of the lines for these two viruses is not

TABLE I. Parameters for Fitting Data of Fig. 1 By Eq. (1) Assuming Two Host-Cell Populations.[a]

Virus	γ_1	γ_2	δ_1	δ_2
Ad1	0.788	0.212	0.00198	0.0126
SV11	0.541	0.459	0.00168	0.0501
SV15	0.207	0.793	0.00300	0.0316

[a] Calculations are according to the method of Shortley and Wilkins (13).

significant; however, both slopes are significantly less than unity ($p < .025$), implying heterogeneity of host-cell response.

Parameters were therefore found to fit curves to the data according to the method of Shortley and Wilkins (13) as was done above for plaque inhibition. In this case, both Ad12 and SV11 were fit with the same curve (where $\gamma_1 = 0.457$, $\gamma_2 = 0.543$, $\delta_1 = 0.693$, and $\delta_2 = 6.31$) as shown in Fig. 4. Because a good fit is obtained when $\delta_1 = 0.693$, β appears to be equal to 1; if so, inhibition of Ad12 and SV11 transformation by AAV is as efficient as plaque inhibition in the population of sensitive host cells.

The SA7 data require separate comment. First, only the data points with foci less than 100% of control could be coverted to Weibits. As is evident in Fig. 4, the four data points with SA7 are shifted relative to the comparable portions of the data with the other two viruses, indicating that more AAV is required to produce inhibition to the same level as with the other two viruses. The slope of the least-squares line (slope = 1.332) is significantly greater than 1 ($p < .025$). This means either that in this experiment a 1 in 40 chance event has occurred with the data points falling this far from a line with unit slope or that cooperative action of AAV is necessary to inhibit transformation by SA7.

Discussion. Inhibition of adenovirus replication by adeno-associated viruses has been well documented (see 1st ¶). However, prior to the reports from this laboratory (5) and that of Kirschstein,

TABLE II. Inhibition of Adenovirus Transformation of Hamster Embryo Cells by AAV–1.

	Control			With AAV–1[d]			
Virus	PFU added	*M* per[a] cell	No. of foci[b]	PIU of AAV added	*M* per[c] cell	No. of foci	Percentage of control
SA7	2.2×10^7	4.4	652	2.8×10^7	5.6	384	59
				1.4×10^7	2.8	489	75
				7.0×10^6	1.4	633	97
				3.5×10^6	0.7	696	100
SV11	5.0×10^6	1.0	567	2.8×10^7	5.6	207	36
				1.4×10^7	2.8	237	42
				7.0×10^6	1.4	291	51
				3.5×10^6	0.7	495	87
				1.8×10^6	0.4	615	100
Ad12	1.2×10^8	18.1	244	2.0×10^7	3.0	84	34
				1.0×10^7	1.5	144	59
				5.0×10^6	0.8	164	67
				2.5×10^6	0.4	272	100

[a] Number of adenovirus PFU per hamster cell.

[b] Total foci arising from four inoculated plates.

[c] Number of adenovirus plaque inhibiting units per hamster cell as determined by titration in HEC against Ad1.

[d] One pool of AAV–1 was used in the experiment with SA7 and SV11, and a second pool was used with Ad12.

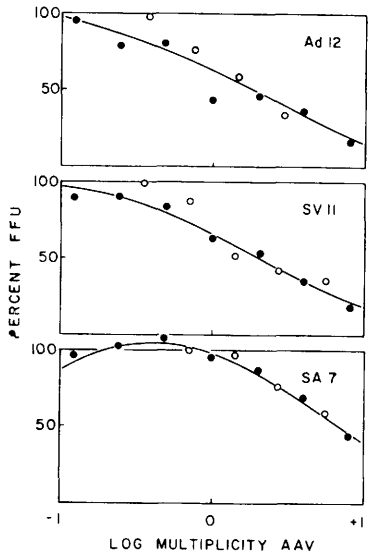

FIG. 3. Inhibition by AAV-1 of transformation by 3 adenoviruses. At each dilution of AAV-1, the number of adenoviral-induced foci of transformed cells was normalized to 100% for the controls without AAV. In each, the closed circles are data obtained using a single pool of AAV-1 to inhibit the adenovirus transformation; the 3 adenoviruses were tested at the same time in the same lot of hamster embryo cell cultures. (○) data (Table II) obtained using different pools of AAV-1; the 3 assays were not done simultaneously. The curve drawn in each is only to show the trend in the points. Multiplicity is defined as input of AAV PIU/cell.

Smith, and Peters (6), the only known mechanism whereby AAV might interfere with adenovirus tumorigenesis was through the reduction of the infective titer of adenovirus in virus stocks contaminated with AAV.

The *in vivo* studies by the latter group showed that the incidence of tumors among animals inoculated with mixtures of Ad12 and AAV was reduced 2- to 8-fold depending upon the titer of AAV present in the inoculum, and therefore the inhibition of tumor formation did not result simply from having lowered titers of adenovirus in the inoculum. However, the method of assay did not allow for a quantitative comparison between the inhibition of adenovirus replication and inhibition of tumorigenicity.

Experiments reported here indicate that inhibition of adenovirus transformation by AAV is an intracellular phenomenon in hamster embryo cells and as such is similar to inhibition of the infective cycle of adenovirus. Inhibition of transformation differs from inhibition of plaque formation in that it does not result from inhibition of adenovirus replication in the hamster cells, as the adenoviruses used here for transformation do not replicate in these cells.

Higher multiplicities of AAV, however, are required to inhibit transformation than to inhibit plaque formation. For example, with SV11 and Ad12, a multiplicity of about 1.5 to 2.5 PIU/cell is needed for a 50% inhibition of transformation. Inhibition of SA7 transformation in different experiments required 5 to 7 PIU of AAV/cell to achieve comparable levels of inhibition. This difference in the multiplicity of AAV necessary to inhibit SA7 and Ad12 or SV11 transformation cannot be attributed to differences in the adenovirus challenge dose, since the number of SA7 PFU added was approximately 4-fold less than, and 4.5-fold more than, that of Ad12 and SV11, respectively (Table II). If the dose of adenovirus is considered in terms of the number of focus-forming units (FFU) added (7, 8), then the effective challenge dose was essentially the same for SA7 and SV11 and approximately 2-fold less for Ad12.

The response data, analyzed by the method of Shortley and Wilkins (13), support fully a one-hit relationship between AAV and inhibition of infection by Ad1, SV11, and SV15. The observed heterogeneity of host-cell response can be explained in several ways.

Heterogeneity intrinsic to the cells, or resulting from their being in different stages of the mitotic cycle, is certainly possible, but if so, the response to all three virus combinations should be the same since all infections were performed simultaneously with the different viruses. Another possible reason for differing heterogeneity is that the different adenoviruses may begin their replicative cycles at different times after infection so that some virus–cell complexes may become refractory to AAV inhibition earlier with one adenovirus than with another. Thus, perhaps Ad1-infected cells reach a refractory stage much sooner than SV11-infected cells, which

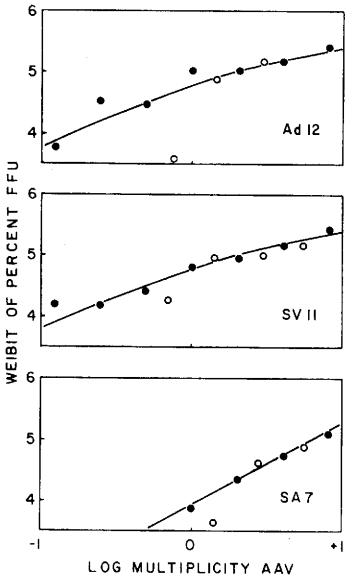

FIG. 4. Data of Fig. 3 plotted as the Weibit of the observed percentage of foci on the ordinate against the log of the multiplicity of AAV. The curves were fitted using the parameters given in the text for Ad12 and SV11; the SA7 data are fitted with the least-squares straight line.

in turn is faster than SV15-infected cells. The parameters of Table I indicate that 78.8% of cells infected with Ad1 are able to respond by forming a plaque at a higher multiplicity of AAV than SV15, with only 21% of cells in the group more able to form plaques with a given multiplicity of AAV. The different values for δ_1 could be due either to a different β for each adenovirus or to experimental variation. Nevertheless, there is nothing to suggest cooperative action between AAV particles to inhibit the adenovirus productive cycle; the data are fully consistent with the hypothesis that one AAV virion is sufficient to inhibit the adenoviral cytocidal effect in an infected cell.

Inhibition of transformation induced by Ad12 and SV11 also appears to be one-hit with respect to AAV, and the inhibition appears to be as efficient as inhibition of plaque formation in the susceptible cell population. The fact that a slightly higher multiplicity of AAV is necessary to reduce the focus yield to 50% than to reduce the plaque yield to 50% is apparently due to having different proportions of susceptible cells in the two assays. In the transformation assay with Ad12 and SV11, the fraction of cells susceptible to transformation is lower (7); also the cells destined to transform may reach a refractory state later than cells destined to produce virus in the plaque assay. Either a different mechanism exists so that the refractory state for transformation may not be reached until the cell next synthesizes its DNA or there could be an inherent difference in the cell types. It also seems possible that each virus has a different "target cell" and these cells could be in different proportions in the cultures.

Data for SA7 are interpreted either of two ways. An anomalous result could have occurred in the experiment reported, with a 1 in 40 chance event having taken place, or more than one AAV may be necessary to inhibit SA7 transformation. Reproducibility of the response data in the many other experiments done at different times and with different SA7 and AAV pools argues against an anomalous result and suggests instead that the possible need for cooperative action may be real.

In Fig. 3, the percentage of foci observed with SA7 rises to more than the controls when an AAV multiplicity of less than 1 per cell is used. This rise has been observed repeatedly. Our data and this analysis do not explain this rise. One possible reason, however, subject to test by further experiments, is that 1 PIU of AAV/cell increases the probability of transformation while more than 1 per cell decreases the probability.

Inhibition of adenovirus tumorigenesis has also been demonstrated in the case of another member of the Parvovirus group. Toolan and Ledinko (14) have shown that the incidence of tumors in hamsters following injection of Ad12 could be reduced from 67% to 28% by co-infection with H-1 virus. As does AAV (15), H-1 virus effectively inhibits infectious center formation by adenovirus (16). The quantitative relationship between H-1 and Ad12 for inhibition of transformation has not yet been investigated *in vitro*.

Summary. To obtain information on inhibition of adenovirus transformation by AAV,

cultures of hamster embryo cells were inoculated simultaneously with constant amounts of simian adenoviruses SA7 or SV11, or with human adenovirus type 12 and with varying multiplicities of AAV-1. Induction of transformed cell foci by SV11 or Ad12 was inhibited by more than 80% when 9 to 10 plaque inhibiting units (PIU)/cell of AAV-1 were added. The amount of AAV-1 necessary to reduce transformed cell foci by 50% ranged from 1.5 to 2.5 PIU/cell for SV11 and Ad12 to 5 to 7 PIU/cell for SA7. Analysis of the dose–response data indicates that 1 unit of AAV-1/cell is sufficient to inhibit transformation by Ad12 and SV11. The relative resistance of SA7 transformation to inhibition seems best explained as a requirement for more than 1 inhibiting unit/cell of AAV-1.

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