JOURNAL OF VIROLOGY, Nov. 1996, p. 7498–7509 Vol. 70, No. 11
0022-538X/96/$04.00+0

Evaluation of the Concentration and Bioactivity of Adenovirus Vectors for Gene Therapy

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Received 8 April 1996/Accepted 24 July 1996

Development of adenovirus vectors as potential therapeutic agents for multiple applications of in vivo human gene therapy has resulted in numerous preclinical and clinical studies. However, lack of standardization of the methods for quantifying the physical concentration and functionally active fraction of virions in these studies has often made comparison between various studies difficult or impossible. This study was therefore carried out to define the variables for quantification of the concentration of adenovirus vectors. The methods for evaluation of total virion concentration included electron microscopy and optical absorbance. The methods for evaluation of the concentration of functional virions included detection of gene transfer (transgene transfer and expression) and the plaque assay on 293 cells. Enumeration of total virion concentration by optical absorbance was found to be a precise procedure, but accuracy was dependent on physical disruption of the virion to eliminate artifacts from light scattering and also on a correct value for the extinction coefficient. Both biological assays for enumerating functional virions were highly dependent on the assay conditions and in particular the time of virion adsorption and adsorption volume. Under optimal conditions, the bioactivity of the vector, defined as the fraction of total virions which leads to detected target cell infection, was determined to be 0.10 in the plaque assay and 0.29 in the gene transfer assay. This difference is most likely due to the fact that detection by gene transfer requires only measurement of levels of transgene expression in the infected cell whereas plaque formation is dependent on a series of biological events of much greater complexity. These results show that the exact conditions for determination of infectious virion concentration and bioactivity of recombinant adenovirus vectors are critical and must be standardized for comparability. These observations may be very useful in comparison of data from different preclinical and clinical studies and may also have important implications for how adenovirus vectors can optimally be used in human gene therapy.

Recombinant adenovirus vectors are being developed at multiple centers as gene delivery vehicles in strategies for human gene therapy to treat both hereditary and acquired disorders (4, 29, 34). Cystic fibrosis lung disease is the first disorder for which this in vivo adenoviral vector delivery approach has been used clinically, but other examples are now following (29, 34). The approach for cystic fibrosis has involved in vivo transfer of a normal human cystic fibrosis transmembrane conductance regulator cDNA to airway epithelium in affected individuals. Preclinical studies for this work have been carried out in a large number of laboratories, and human trials using adenovirus gene transfer vectors have been initiated in 10 different clinical centers (5, 14, 22, 32, 36–38, 40, 41).

In the context of adenovirus vectors as potentially therapeutic pharmaceuticals, preclinical and clinical testing requires the use of good manufacturing practice in the preparation and testing of these agents. Determination of the concentration of such vectors with sufficient accuracy and precision is therefore critical to ensure adequate comparability of data obtained in various intrainstitutional and interinstitutional studies as well as to ensure comparability between preclinical and clinical studies. At present, no widely accepted standard method is used for determining adenovirus vector concentration. Rather, a number of different methods, including the plaque assay on 293 cells (9), fluorescent focus assay (25), and methods based on optical absorbance (15, 18), are currently in use. Furthermore, these methods are performed under a variety of conditions, leading to markedly different apparent measured values for vector concentration.

Methods for measuring adenovirus vector concentration can be divided theoretically and practically into biological methods and physical methods. Biological methods rely on infection of cells in culture followed by subsequent events required for detection which depend on some aspect of the biological functionality of the vector, e.g., formation of a visible plaque in a monolayer of cells which permit replication of the vector, or histochemical or immunohistochemical staining of infected target cells expressing a particular vector-associated reporter transgene (30). Physical assays, in contrast, measure the virion concentration by methods which are independent of biological functionality, e.g., direct visualization by electron microscopy (26) or optical absorbance of the virion DNA (15, 18).

The biological assay most widely used for quantification of E1-deleted adenovirus vectors is the plaque assay on 293 cells, which support replication of these vectors (8, 9). Detection of the virion in this assay involves infection of a cell in a cultured monolayer by a virion suspended in the medium above the cells followed by virion replication and spread of infection to nearby cells sufficient to form a visible plaque in the cell monolayer. This process is dependent on the functionality of both the vector and the cell target and also on the conditions under which their interaction is conducted. Thus, a number of variables could determine the outcome of a particular measurement. For example, adenovirus vector infection of cells in culture is known to be rate limited by adsorption of virions to

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cells (19). Since this process has been previously shown for wild-type viruses to be highly dependent on virion diffusion (1–3, 15), one would expect that shorter vector adsorption times could significantly underestimate the determined concentration if insufficient for exhaustive adsorption. It is interesting that many but not all studies have used virion adsorption times of 1.5 h or less and plaque development times of 2 to 3 weeks (11, 15, 28, 30). Another important variable is suggested by the fact that virologists commonly tend to use very small adsorption volumes in titer determinations (11, 15, 16, 27). Because the measured apparent adenovirus vector concentration is dependent on assay conditions not standardized in various laboratories, it is possible that some of the differences observed in some of the preclinical and clinical studies are due to uncertainty in the amount of vector used in each study. The result is that important observations from studies in both animals and humans may be difficult or impossible to compare.

In contrast to biological assays, methods for determining physical adenovirus vector concentration are independent of vector functionality and thus yield values of total concentration or amount of virions both functional and nonfunctional. Previous evaluations have established a correlation between the infectious titer of replicative adenovirus and the calculated number of adenovirus virions determined by optical absorbance, colorimetric methods, or electron microscopy or by weighing lyophilized virus of known titer (11, 15, 18). Similar studies have not been published for replication-deficient, recombinant adenovirus vectors. Because preparations of many animal viruses (including adenovirus) contain particles which appear to be nonfunctional (11, 15, 31), the Food and Drug Administration, in an effort to establish minimum acceptability criteria for use of adenovirus vectors in human clinical trials, has recommended use of adenovirus vectors with a ratio of total virions to infectious virions of ≤ 100. This corresponds to a biologically infectious fraction of $\geq 1\%$. In calculating the ratio of biologically infectious to total virions, most commonly the fraction of infectious virions is measured by using the plaque assay and the total virion concentration is calculated from optical density at 260 nm (OD_{260}), using a previously determined extinction coefficient (18).

Consistent standardized methods to determine the concentration and biological activity of adenovirus vectors are needed to facilitate comparison of historical data and to provide a standard measure for future studies. To this end, the objective of this study was to evaluate methods and conditions for accurately and precisely determining adenovirus vector concentration. Additionally, the methods for evaluating the biological activity of these vectors were evaluated.

MATERIALS AND METHODS

Adenovirus vector. Av1nBg is an E1-, E3-deleted first-generation adenovirus expressing a nucleus-localizing β-galactosidase reporter enzyme (previously referred to as Av1LacZ4 [33, 39]). The construction, purification, amplification on 293 cells, and storage were as previously described (22). Briefly, purification included preparation of cleared viral lysate from Av1nBg-infected cells (36 h after infection at 5 PFU per cell) followed by five cycles of freezing and thawing to release intracellular vector. A clearing centrifugation (8,700 × *g*, 4°C, 5 min) was followed by CsCl step-gradient centrifugation (220,000 × *g*, 20°C, 1 h), then isopycnic density centrifugation (220,000 × *g*, 20°C, 18 h), and finally dialysis into 10 mM Tris (pH 7.4)–1 mM $MgCl_2$–10% (vol/vol) glycerol. Vector was stored in polypropylene vials at −70°C until use to avoid reduction in potency. To initially estimate the concentration of Av1nBg virions, the biological titer was determined by the plaque assay on 293 cells under standard conditions (defined as 1.5-h vector adsorption period, 1-ml adsorption volume, 10^6 293 cells per well in a six-well plate infected 24 h after plating, 2-week plaque development time, and visual inspection for counting plaques) as previously described (19, 22). For Av1nBg lot BB7-7, this plaque titer value was 5×10^{10} PFU ml^{-1}; for lot BB9-6, the plaque titer was 1.5×10^{11} PFU ml^{-1}.

Cells. The 293 cell line is a human embryonic kidney epithelial cell line transformed by the left-hand 11% of the adenovirus type 5 (Ad5) genome (8). 293 cells (ATCC CRL 1573) were obtained at passage 31 from the American Type Culture Collection and were used for up to 17 additional passages but not more. Between these passage numbers, no effect of cell passage number on titer was observed. Cells were cultured in improved modified Eagle's medium (IMEM) supplemented with 10% (vol/vol) heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) containing 2 mM glutamine (IMEM-10), maintained in a humidified 5% CO_2 atmosphere at 37°C, and subcultured every 3 days (22).

Ultrastructural analysis of vector. The adenovirus vector (lot BB7-7) was evaluated morphologically by electron microscopic visualization of negatively stained virions (13) under GLP conditions at Advanced Biotechnologies, Inc. (Columbia, Md.). To accomplish this, concentrated (5×10^{10} PFU/ml) vector stock was thawed and evaluated as concentrated virus or after dilution (1:50) with ultrapure water after routine preparation of grids as previously described (19). After staining in 1% methylamine tungstate, grids were evaluated and photomicrographed with a Hitachi HU-12A electron microscope operated at 75 kV (final magnification, ×120,000).

Quantification of adenovirus vector by electron microscopy and latex sphere dilution. The total physical concentration of virions was determined by the latex sphere dilution method (26). Briefly, 110-nm-diameter latex spheres of known concentration (2×10^{12}/ml; final dilution of 2×10^{10} latex spheres ml^{-1} in vector solution) were mixed with Av1nBg vector (lot BB7-7; final vector dilutions of 1:50, 1:75, and 1:100) in ultrapure water. After negative staining with 1% methylamine tungstate (14), grids were evaluated as described above. The concentration of adenovirus vector virions (v) in the stock vector solution was determined by comparison with the number of latex spheres (LS) counted (LSC) per grid to the number and the number of vector particles counted (VPC) per grid, using the equation

$$\text{adenovirus vector concentration } (v\ \text{ml}^{-1}) = \text{VPC} \times \frac{2 \times 10^{10}\ \text{LS ml}^{-1}}{\text{LSC}} \times d \quad (1)$$

where is d is the dilution factor for the vector sample being evaluated. In each case, counting of virions and latex spheres continued until 1,000 latex spheres had been counted.

Quantification of adenovirus vector by optical absorbance. For evaluation of adenoviral vector concentration by OD_{260}, vector was thawed and diluted 20-fold in virion lysis solution (0.1% sodium dodecyl sulfate [SDS], 10 mM Tris-Cl [pH 7.4], 1 mM EDTA). The sample was incubated for 10 min at 56°C with shaking in a tightly capped Eppendorf microcentrifuge tube. A sample of the solution of disrupted virions was placed in a cuvette, and the OD_{260} was determined. The concentration of adenovirus vector virions was determined by multiplying the absorbance by the appropriate dilution factor and then dividing by the extinction coefficient determined for wild-type adenovirus ($\varepsilon_{260} = 9.09 \times 10^{-13}$ OD ml cm $virion^{-1}$) calculated from the data of Maizel et al. (18). The concentration of vector particles, determined in this way, is reported as optical particle units (OPU) to distinguish it from other methods of determination of virion concentration. This value for the extinction coefficient was based on data of 1.1×10^{12} virions per OD_{260} of wild-type adenovirus (Ad5) and was calculated from measurements of virion total protein and OD_{260} (assuming a molecular mass for Ad5 DNA of 2.3×10^7 Da [12] and also that 87% of the dry weight of Ad5 is protein [7, 10]).

Quantification of adenovirus vector by biological methods. The concentration of adenovirus vector was determined by using two separate biological methods which relied on either detection of cells expressing the transgene (gene transfer assay; measured in gene transfer units [GTU]) or detection of the formation of visible plaques in a monolayer of 293 cells (plaque assay; measured in PFU). Briefly, vector was diluted and adsorbed onto a monolayer of cells under specific conditions, after which the vector-containing adsorption fluid was removed. Subsequent steps depended on which particular detection assay was used. Each quantitative determination of titer consisted of enumeration of plaques or cells with blue nuclei per well from three separate wells.

(i) Adsorption of Av1nBg vector to 293 cells. The adsorption conditions described here are the conditions of our standard plaque assay used throughout this study. Use of conditions other than these standard ones is specifically noted. 293 cells were first plated in six-well plates at 10^6/35-mm-diameter well in IMEM-10 and cultured as described above. Immediately prior to evaluation, a series of vector dilutions was prepared in sterile tubes (labeled 1 to 8) in a laminar flow hood, using aseptic technique. To accomplish this, IMEM-2 (0.99 ml) was added to tube 1 and 0.9 ml of IMEM-2 was added to tubes 2 to 8. Av1nBg vector was thawed at room temperature and vortexed briefly, and a 10-μl aliquot of vector was withdrawn with a Gilson Pipetteman (Rainin Inc., Wobern, Mass.). Care was taken to avoid plunging the pipette tip into the vector solution to avoid unwanted carryover of the vector. This aliquot was transferred to tube 1, again without plunging the tip into the recipient medium. The receiving dilution tube was mixed in a vortex mixer (5 s at setting 5). Starting from tube 1, serial 1:10 dilutions were prepared in tubes 2 to 8. Thus, the dilution factors for tubes 1 to 8 were 10^{-2} to 10^{-9}, respectively. For transduction, medium was removed from the 293 cells and replaced with a mixture of 100 μl of vector-containing medium from tube 8 (unless otherwise indicated) plus 900 μl of IMEM-2. The infected cells were incubated in a humidified, 5% CO_2 atmosphere at 37°C for 90 min

(unless otherwise indicated) with gentle rocking on a mechanical rocker platform (Bellco Glass Co., Vineland, N.J.). The subsequent steps used to complete each type of titer assay following adsorption depended on the particular detection assay used and are described with each procedure (see below).

(ii) Vector titration by gene transfer. When quantification by gene transfer was used following vector adsorption, vector-containing adsorption medium was gently aspirated and either discarded or saved in a new sterile tube. Fresh IMEM-10 (2 ml) was replaced on the cells, and incubation was continued for 16 h in a humidified, 5% CO_2 atmosphere at 37°C to allow for sufficient transgene expression and accumulation of nuclear targeted β-galactosidase in transduced cells. Medium was aspirated, and the cells were fixed (2% formaldehyde, 0.2% glutaraldehyde) and stained with X-Gal stain (50 mM ferricyanide, 50 mM ferrocyanide, 2 mM $MgCl_2$, 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside [X-Gal] per ml) for 3 h at 37°C. Cells were visualized by inverted phase microscopy, and the number of cells with blue-stained nuclei per well was counted as representing the number of successful virion infections. When the supernatant from an initial adsorption was to be evaluated by repeat adsorption, the adsorption medium removed from the initial infection was placed on fresh 293 cells plated at 10^6 per well in a six-well plate. The cells were incubated for 16 or 24 h in a humidified CO_2 incubator at 37°C and analyzed for β-galactosidase expression, and the number of blue-stained nuclei per well was counted as described above. The mean number of cells with blue nuclei per well from three separate wells, each inoculated with 100 μl of stock vector from dilution tube 8 in a final volume of 1 ml (unless indicated otherwise), was determined, and the titer was calculated by multiplying this number by the dilution factor (10^{10}). In experiments with adsorption volumes other than 1 ml, the dilution factor depended on the ratio of diluted stock vector used and the final adsorption volume (stated in this text).

(iii) Vector titration by plaque assay. The plaque titration procedures described here are our standard conditions used throughout this study. Use of conditions other than these is specifically noted. When quantification by plaque assay was used following vector adsorption, vector-containing adsorption medium was gently aspirated and either discarded or saved in a new sterile tube. Cells were then overlaid with minimal essential medium containing 15% FBS, 4 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), 2% (wt/vol) amphotericin B (Fungizone), and 1% SeaPlaque low-melting-point agarose (maintained at 42°C immediately prior to application to prevent solidification). Titer plates were then cultured in a humidified, 5% CO_2 atmosphere at 37°C. After 5, 10, and 13 days following adsorption of vector, the cell monolayers were overlaid with minimal essential medium containing 1% (wt/vol) SeaPlaque agarose. The overlay at day 13 also included neutral red (0.1% [wt/vol]) to enhance plaque visualization. Plaques were counted on day 14 (standard plaque assay) by visual inspection with the naked eye. The mean of the number of plaques from three separate wells, each inoculated with 100 μl of stock vector from dilution tube 8 in a final volume of 1 ml (unless indicated otherwise), was determined, and the titer was calculated by multiplying this value by the dilution factor (10^{10}).

Statistical evaluations. All data are presented as mean ± standard error of the mean. Mathematical modeling evaluations were performed by using SigmaPlot software (version 2.01 for Windows) on an IBM-compatible microcomputer. Regression analysis for nonlinear curve fits of data were done by using the least-squares algorithm of Marquardt (20). The results of determination of nonlinear curve fit parameters were evaluated by computing the coefficient of determination (r^2) for the observed data and the values from the fit equations using the determined parameters. Statistical comparisons were made by using Student's t test.

Theoretical considerations. (i) Physical behavior of small particles in fluid. Viruses, which range in size from 20 nm for parvovirus strains to 200 nm for poxvirus, and similar-size latex beads are not actively motile and must depend on external forces for their transport. Valentine and Allison demonstrated for fowlpox virus and vaccinia virus (approximately 260 by 450 nm) as well as latex spheres (80 to 690 nm in diameter) that their movement in fluid was dependent on random Brownian motion (1). They found that the number of particles which adhere to a surface exposed to culture medium containing particles was a function of adsorption time, particle concentration, depth of the fluid layer, and electrostatic charges on the surface but was independent of mechanical agitation of the fluid (3). They first derived mathematical relationships for the theory of adsorption and attachment of particles to nonbiological surfaces (1) and later extended this to adsorption of viruses to cells in suspension (2) and cell monolayers (3).

(ii) Transduction of cell monolayers by viral vectors. Previous studies of adsorption of viruses to cell monolayers have demonstrated that the infection of cells in culture can be modeled by considering the cell monolayer to be a flat surface covered to a depth of d with fluid (culture medium) containing a suspension of viral particles. If the virions are assumed to be uniform spherical particles, then they are expected to conform to the diffusion equation with a diffusion coefficient D (centimeters per second) given by

$$D = \frac{kT}{6\pi\eta r_v} \tag{2}$$

where k is Boltzmann's constant (1.38×10^{-16} g cm^2 s^{-2} K^{-1} molecule^{-1}), T is the absolute temperature (Kelvin) η is the viscosity of the suspending fluid (centipoise), and r_v is the radius (~35 nm) of the virion v (1). Using these assumptions, Allison and Valentine (1) demonstrated that the theoretical maximum for the fraction of virions arriving at the surface of a cell monolayer from the suspending fluid after a time t is given by

$$F_{\text{arriving}} = 1 - \frac{8}{\pi^2}\left[\sum_{m=0}^{m=\infty} \frac{1}{(2m+1)^2} e^{-\left(\frac{(2m+1)^2\pi^2 Dt}{4d^2}\right)}\right] \tag{3}$$

where D is the diffusion coefficient for the virion given by equation 2, d is the depth of fluid in centimeters, m is an integer from 0 to ∞, and t is the time in seconds. Their studies (1) also showed that the infinite series in equation 3 could be replaced by one of two approximations (which are accurate to <1% for certain values of Dt/d^2). The first of these expressions is given by

$$F_{\text{arriving}} = \frac{1.13\sqrt{Dt}}{d} \tag{4}$$

where all terms are as defined above. This expression was theoretically shown to be useful for values of $t < 0.12\ d^2/D$ when less than 40% of the virions have been adsorbed. A second expression (which uses only the first term of the infinite series of equation 3, i.e., when $m = 0$) is given by

$$F_{\text{arriving}} = 1 - \frac{8}{\pi^2} e^{-\left(\frac{\pi^2 Dt}{4d^2}\right)} \tag{5}$$

where all terms are as defined above. This expression was theoretically shown to be useful for values of $t > 0.12\ d^2/D$ when more than 28% of the particles have been absorbed. It is important to note that for these equations, Allison and Valentine assumed that all collisions of virions with the cell membrane resulted in adsorption and that all such events would be detected (3). They showed that by multiplying these equations through by $C_0 d$, the number of virions adsorbed per unit area (square centimeters) of the cell monolayer could be expressed at a given time t. Thus, for $t < 0.12\ d^2/D$ and when less than 40% of virions have adsorbed,

$$N = 1.13\, C_0 \sqrt{Dt} \tag{6}$$

and for $t > 0.12\ d^2/D$ and when more than 28% of virions have adsorbed,

$$N = C_0 d\left(1 - \frac{8}{\pi^2} e^{-\left(\frac{\pi^2 Dt}{4d^2}\right)}\right) \tag{7}$$

where N is the total number of virions absorbed per unit area at time t, C_0 is the initial concentration of virions (number per unit volume), and all other terms are as defined above.

These theoretical predictions based on Brownian theory agree closely with experimental observations for a variety of small particles, including latex spheres and virus particles (e.g., poxvirus) adsorbing to nonbiological surfaces under conditions in which electrostatic repulsive forces are neutralized (1). However, in radiolabeling studies, the actual rate of virus adsorption to cell monolayers was previously found to be about half of the value calculated on the basis of Brownian theory for a variety of viruses, including fowlpox virus, vaccinia virus, herpes simplex virus, and poliomyelitis virus (3). The quantification of virion adsorption is even more complicated when the method of detection is dependent on biological function of the virion after infection of the cell. Thus, virion concentration is typically substantially lower (underestimated) when enumerated by infection of cells followed by counting of visible plaques in the cell monolayer (3, 17) or expression of a gene carried into the cell by the virion (i.e., a transgene [19]).

The discrepancy between measurements of total or physical virion concentration and biologically functional virion concentration is a function of both the fraction of intrinsically functional virions and the ability to detect such virions. While intrinisic functionality is difficult to measure directly, the ability to detect virions by infection of cells is a function of diffusional virion movement to the cell, adsorption, and all of the subsequent, sequential steps required for detection of the cell infection event (15–17, 23, 24, 35). Therefore, the frequency of ultimately detecting a virion by measuring infection of a cell in the monolayer is the product of consecutive probabilities consisting of the fraction of virions reaching the cell surface in the time allotted multiplied by the fraction of virions successfully passing all of the sequential steps. This relationship can be expressed by

$$F_{\text{detected}} = F_{\text{arriving}} \cdot F_{\text{successful}} \tag{8}$$

where F_{detected} is the fraction of virions detected in the assay, F_{arriving} is the fraction of virions reaching the cell surface within the allotted time (given by equation 3 and approximated by equations 4 and 5) and thus is a parameter relating purely to the kinetics of extracellular virion movement prior to contact with the cell, and $F_{\text{successful}}$ is the fraction of virions reaching the cell surface that ultimately undergo all of the subsequent steps necessary for detection of the infection event. The first term on the right refers to all virions irrespective of their ability to function by successfully infecting the cell. The latter term actually represents two distinct and fundamentally different components: (i) the fraction

of intrinsically functional virions and (ii) the fraction of intrinsically functional virions which after arriving at the cell will undergo all of the subsequent events of infection to be detected in the assay. These subsequent events consist of rolling of the virion on the cell surface (lateral diffusion [16, 35]), attachment to the cognate cell surface receptor (16, 35), entry into the cell (23, 24, 35), translocation to the nucleus, and execution of its genetic program. Each of these steps is associated with a probability of occurrence which is presumably (and in some cases experimentally determined to be) less than 1 (19). When detection of the infection event is based on detection of transgene expression, $F_{\text{successful}}$ is most simple, i.e., dependent on events occurring only within the initially infected cell. In this case, equation 8 can be rewritten to include these additional steps as

$$F_{\text{detected}} = (F_{\text{arriving}})\,(F_{\text{intact}})\,(F_{\text{attaching}} \cdot F_{\text{penetrating}} \cdot F_{\text{intranuclear}} \cdot F_{\text{transgene expression}}) \quad (9)$$

where F_{detected} and F_{arriving} are as defined above and F_{intact} is the fraction of virions which are functionally intact and capable of infecting the cell (i.e., intrinsically functional). This includes virions which are structurally intact, including some but not necessarily all virions with a normal morphologic appearance, as well as those virions which may be morphologically abnormal but still able to achieve gene transfer. $F_{\text{attaching}}$ is the fraction of virions reaching the cell surface that attach to the receptor, $F_{\text{penetrating}}$ is the fraction of attached virions which penetrate the cell membrane and are internalized, $F_{\text{intranuclear}}$ is the fraction of internalized virions which are correctly translocated to the intranuclear compartment, and $F_{\text{transgene expression}}$ is the fraction of intracellular virion genomes which adequately express the transgene so as to permit detection of the infected cell. In this model, the three terms in parentheses on the right side of equation 9 represent (i) the extracellular kinetics or fractional arrival of virions at the cell surface, (ii) the fraction of intrinsically functional virions, and (iii) the fractional detection of functional virions based on events occurring at the cell membrane or within the cell.

Detection of virions by plaque assay is somewhat more complicated because, in addition to the above, this involves sequential infection of many cells through successful completion of multiple rounds of the viral life cycle. Specifically, plaque formation requires efficient intracellular virion replication, numerous rounds of cell lysis, release of infectious virions, and infection of neighboring cells sufficient for formation of a plaque in the cell monolayer which is large enough to be visible. Thus, for the plaque assay, the last term of equation 9 is replaced by another term, F_{plaque}, which represents the fraction of virions present within the nucleus of the initially infected target cell which ultimately results in a detectable plaque in the monolayer.

Substituting into equation 8 the expression developed by Allison and Valentine for the fraction of virions arriving at the cell surface at time t (equation 3) and multiplying through by C_0d, one obtains

$$N_{\text{detected}} = C_0 d \left(1 - \frac{8}{\pi^2}\left[\sum_{m=0}^{m=\infty} \frac{1}{(2m+1)^2}\, e^{-\left(\frac{(2m+1)^2\pi^2 Dt}{4d^2}\right)}\right)\right) F_{\text{successful}} \quad (10)$$

where N_{detected} is the number of virions which should be detected per unit area of the monolayer at time t, the term within parentheses represents the fraction of virions arriving at the cell surface, e is the base of natural logarithms, and all other terms are as defined above. The approximations given by equations 4 and 5 can be similarly rewritten to include the term $F_{\text{successful}}$. Theoretically, this equation should more accurately describe the number of detectable infectious virions by biological assays.

This theoretical treatment provides a mathematical model of adenovirus vector-mediated gene transfer which predicts certain aspects of the transduction kinetics that can be evaluated by fitting the observed data to mathematically defined relationships which can be compared statistically. Movement of the immotile virion via diffusion due to Brownian forces through the extracellular fluid has been emphasized (equations 2 to 7) because theory predicts that the extracellular conditions of transduction should greatly affect the infection rate; i.e., the measured biological titer. Specifically, equations 2 to 7 mathematically describe the extracellular movement of virions and the importance of adsorption time and adsorption fluid height, and they suggest the irrelevance of the multiplicity of infection (for practical adsorption times and independent of the effect on virion concentration). The adenovirus transduction process was also described in terms of steps subsequent to arrival of the virion at the cell surface (equations 8 to 10). This is particularly helpful in understanding the differences in titer values determined by various methods. This rigorous treatment provides a basis for evaluating and comparing titer data produced by the various techniques currently used by different investigators in the gene therapy field.

RESULTS

Evaluation of vector morphology. Electron microscopy of concentrated ($\sim 5 \times 10^{10}$ PFU ml^{-1}) and diluted ($\sim 10^9$ PFU ml^{-1}) adenovirus vector (lot BB7-7) after negative staining revealed typical adenovirus particles of approximately 85 to 100 nm in diameter (Fig. 1). Very little aggregation was present even in the concentrated vector; approximately 97% of the virions were present as singlet virions. Importantly, no cellular debris, nucleic acid, or proteinaceous material was observed in any of the grids ($n = 4$). Morphologically defective particles were less common (ratio of normal to defective, 8:1) and had visibly damaged capsids measuring between 95 and 110 nm in diameter. Whether these virions represented damaged virions present in the original vector stock or virions damaged during the process of evaluation by electron microscopy is uncertain and could not be determined.

Quantification of vector particle concentration by physical methods. (i) Direct visualization by electron microscopy. Adenovirus vector concentration was determined by the latex bead dilution method (26) (Table 1). The titers of vector particles determined at serial dilutions varied linearly with the dilution ($r^2 = 0.9715$). The mean titer was $(2.26 \pm 0.24) \times 10^{11}$ v ml^{-1} ($n = 3$). If one assumes that the fraction of virions which appeared morphologically damaged (11%) is representative of the vector stock and that only morphologically normal-appearing virions are intrinsically functional, then approximately 89% of the stock vector particles should be structurally intact (e.g., $F_{\text{intact}} = 0.89$). Thus, by direct electron microscopic visualization of the particles, the concentration of functional virions in the vector stock can be estimated at 2×10^{11} v ml^{-1}. No attempt was made to control for potential differential adherence to the grid or staining efficiency of virions and latex spheres.

(ii) OD_{260}. Previous studies have reported data regarding the determination of the concentration of wild-type adenovirus by using OD to quantify viral DNA within the adenovirus virion (18, 15). This method relies on use of an extinction coefficient for the virion which has been determined empirically by several methods (15, 18). Optical absorbance measurements can be made on intact virions or after disruption of the virion capsid structure. Theoretically, determination of the optical absorption of virion DNA within intact virions presents two problems. First, measured absorbance values will be confounded by light scattering from intact virion structures due to the Tyndall effect. Second, determination of concentration of a substance by optical absorbance with an extinction coefficient requires that the substance being measured, e.g., vector DNA molecules, be in solution or at least consistently hydrated. In intact virions, the DNA is in a chromatin-like condensed coil structure inside the capsid. To investigate this aspect of the quantification of replication-deficient adenovirus vectors by optical absorbance, aliquots of vector were evaluated in the absence or presence of SDS-mediated capsid disruption. Disruption of capsid structure and solubilization of the virion DNA were evaluated by first subjecting vector preparations to SDS, mild heating, and agitation followed by centrifugation prior to optical absorbance evaluation. As expected, a given concentration of intact virions had a significantly higher apparent absorbance than disrupted virions (0.566 ± 0.009 versus 0.251 ± 0.006; $P < 0.0001$ [Fig. 2]). Furthermore, centrifugation significantly reduced the apparent absorbance of intact virions (0.566 ± 0.009 versus 0.165 ± 0.005; $P < 0.0001$) but did not affect the absorbance of SDS-disrupted virions (0.251 ± 0.006 versus 0.257 ± 0.007; $P = 0.22$ [Fig. 2]). A linear correlation between the vector concentration and the measured optical absorbance of the vector was demonstrated with serially diluted vector subjected to SDS-mediated virion lysis followed by measurement of optical absorbance (dilution—OD_{260}: 1/2—0.1275 ± 0.008; 1/4—0.0827 ± 0.006; 1/8—0.0437 ± 0.002 [$n = 3$ for each dilution]). Using this method with virion lysis, the concentration of Av1nBg lot BB7-7 was $(5.51 \pm 0.13) \times$

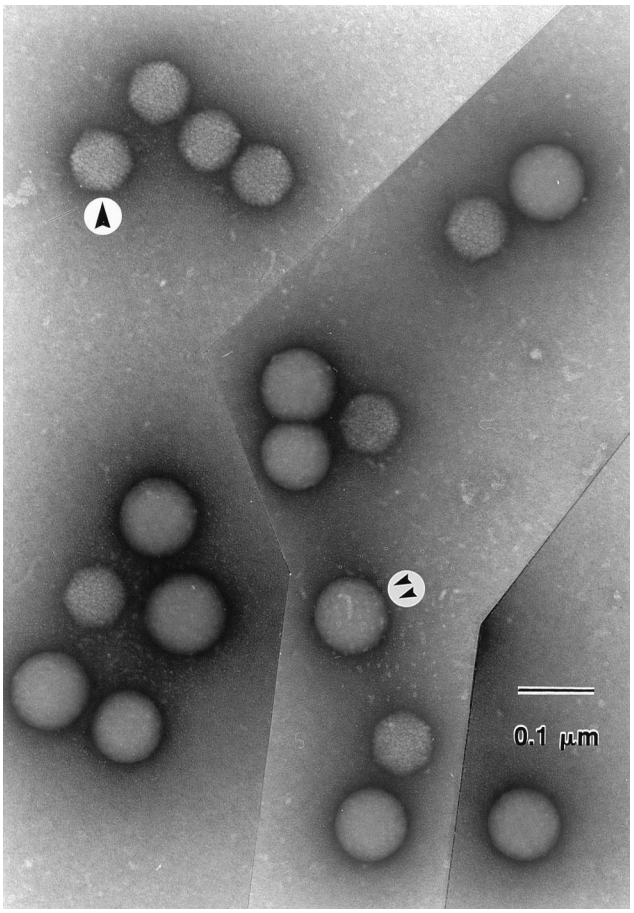

FIG. 1. Electron micrographic evaluation of mixtures of Av1nBg and latex spheres. Av1nBg (arrow) was mixed with 110-nm latex spheres (double arrow) and subjected to electron microscopy as described in the text. Note the absence of free DNA, protein, and cellular debris. All virions pictured appear morphologically intact (final magnification, ×120,000).

10^{12} OPU ml^{-1}, and that of lot BB9-6 was $(6.16 \pm 0.08) \times 10^{12}$ OPU ml^{-1}.

Quantification of adenovirus vector concentration by biological methods. Viruses are most commonly quantified by biological methods which depend on their ability to infect cells. One such approach to quantifying a recombinant, replication-deficient vector expressing the nuclear targeted β-galactosidase reporter transgene is by X-Gal staining and enumeration of cells with blue nuclei (due to the action of β-galactosidase on X-Gal). In order to use this method, however, it was necessary to first prove that a single observed transduction event (one cell with a blue nuclei) was due to infection by only one Av1nBg virion. This was accomplished by evaluating serial dilutions of the vector to demonstrate a linear relationship between the decline in rate of transduction with vector dilution (which can be described by a Poisson distribution [6, 17, 19, 31]). Transduction rate decreased linearly with the concentration of vector over a range from an average of 311 GTU $well^{-1}$ down to an average of 1.6 GTU $well^{-1}$ (Fig. 3A), supporting the one virion-one transduction event relationship (17). A similar experiment was carried out by using plaque assay detection. Seven separate dilution series demonstrated linearity between the decline in rate of plaque formation with vector dilution (Fig. 3B), confirming the one virion-one plaque relationship for Av1nBg (17). From these experiments carried out at the standard adsorption time of 1.5 h, the vector (lot BB9-6) stock had titers of $(2.5 \pm 0.45) \times 10^{11}$ GTU ml^{-1} by gene transfer and $(7.9 \pm 0.57) \times 10^{10}$ PFU ml^{-1} by plaque assay ($n = 7$ for both). These values are in agreement with the value determined by electron microscopy but far less than the value determined by optical absorbance. Thus, plaque assay detection is 3.2-fold less sensitive than the gene transfer assay detection. This means that the ratio of $F_{\text{transgene expression}}$ to F_{plaque} (and also the ratio of $F_{\text{successful}}$ [gene transfer assay] to $F_{\text{successful}}$ [plaque assay]) is 3.2.

Since adenovirus vector-mediated transduction is a diffusion-limited process (19), quantifying vector concentration by methods which require virion-cell interactions between virions suspended in the fluid culture medium and the cell monolayer is expected to be highly dependent on the adsorption time. To evaluate this for Av1nBg, gene transfer and expression was monitored while varying the time of adsorption to cultured 293 cells. As adsorption time increased from 1.5 to 8 h, apparent titer increased significantly ($[1.40 \pm 0.19] \times 10^{11}$ versus $[4.72 \pm 0.84] \times 10^{11}$ GTU ml^{-1}; $P = 0.003$ [Table 2]). Concomitantly, the postadsorption supernatant was also more depleted of virions after longer initial adsorption periods (readsorption titer after initial period of 1.5 versus 8 h, $[3.7 \pm 0.47] \times 10^{11}$ versus $1.0 \pm 0.36 \times 10^{11}$ GTU ml^{-1}; $P = 0.011$ [Table 2]). Even after 8 h of initial adsorption, a large percentage (at least 18% in one experiment [Table 2]) of the vector had not been adsorbed and detected. Thus, at short adsorption times, the observed titer underestimates the true titer because of incomplete adsorption.

To confirm that the underrepresentation of virion concentration observed by using gene transfer was due to incomplete adsorption and not some other aspect of the detection system (i.e., something specific to detection by gene transfer), the experiment was repeated by using plaque assay detection. Further, the adsorption time curve was extended to 40 h in an attempt to maximize adsorption. Adsorption at this very long time was carried out by incubation of the vector-containing medium during two sequential adsorption periods of 16 and 24 h on separate 293 cell monolayers. This sequential approach was used to avoid secondary infections due to intracellular vector replication and infection of nearby cells, which could falsely elevate the measured titer. As expected, the concentration of infectious virions as determined by plaque assay on 293 cells was also highly dependent on the adsorption time (Fig. 4; Table 3). The data obtained appeared to follow the pattern of an exponential rise to a maximum titer with increasing time. To verify this, the data were fit to the most general form of an equation for this type of relationship given by

$$N_{\text{well}}(t) = [x(1 - ye^{-zt}) + w]u \qquad (11)$$

where $N_{\text{well}}(t)$ is the number of plaques per well at time t, t is the time of virion adsorption, and x, y, z, w, and u are constants.

TABLE 1. Determination of adenovirus vector virion concentration by electron microscopic visualization

Vector dilution factor	VPC/grid	LSC/grid	Diluted vector concn (v/ml, 10^9)	Stock vector concn (v/ml, 10^{11})[a]
50	191	1,019	3.75	1.87
75	180	1,071	3.36	2.52
100	121	1,011	2.40	2.40

[a] Determined from columns 2 and 3 by using equation 1. The mean equals $(2.26 \pm 0.24) \times 10^{11}$.

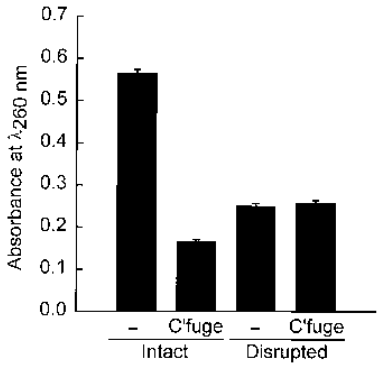

FIG. 2. Evaluation of adenovirus vector concentration by OD_{260} and effect of SDS-mediated disruption of the virion capsid. Av1nBg (lot BB9-6) was diluted to a concentration of 7.5×10^9 PFU ml^{-1} (standard units) and either subjected to SDS-mediated lysis or not followed by brief centrifugation (microcentrifuge, setting 14, 5 min, room temperature) or not prior to measuring the OD_{260} mn as described in the text. Note that centrifugation has a dramatic effect on intact virions but no effect on disrupted virions.

TABLE 2. Evaluation of the effect of adsorption time of the quantification of adenovirus vector titer by gene transfer and expression

Initial adsorption[a]		Repeat adsorption[b]		Vector remaining after initial adsorption[c] (% of total)
Time (h)	Titer (GTU/well, mean ± SEM)	Time (h)	Titer (GTU/well, mean ± SEM)	
1.5	14.0 ± 1.9	16	37.0 ± 4.7	73
4	29.5 ± 5.5	16	16.8 ± 2.7	36
6	31.0 ± 3.8	16	14.8 ± 5.2	32
8	47.2 ± 8.4	16	10.2 ± 3.6	18

[a] The concentration of infectious Av1nBg virions (BB7-7) was determined under standard conditions except for the change in adsorption time as noted.
[b] The concentration of infectious Av1nBg virions in the supernatant recovered following the initial adsorption was determined by incubation of the neat supernatant under standard conditions with fresh 293 cells, using a 16-h adsorption time.
[c] Calculated as [repeat adsorption titer/(initial adsorption titer + repeat adsorption titer)] × 100.

Equation 11 was not derived from the theoretical considerations above but rather was used only to empirically describe the observed data (Fig. 4) with the best fit possible using the least number of constraints. If we set $y = u = 1$ (the usual form of the general equation), then $x + w$ is the titer with units of PFU in well at $t = \infty$ (infinite adsorption time) and z is the rate constant with units of $hour^{-1}$. Fitting $N_{well}(t)$ (equation 11) to the plaque titer data observed at various adsorption times (Fig. 4) yielded values for x, z, and w of 60.21 ± 1.67 PFU $well^{-1}$, 0.115 ± 0.010 h^{-1}, and 0.345 ± 1.0 PFU $well^{-1}$, respectively ($r^2 = 0.996$). Adsorption was not complete even after 16 h, as indicated by a significant titer remaining in the post-initial adsorption supernatant recovered after 16 h of initial adsorption and subsequently evaluated by a second adsorption and titration on fresh 293 cells (Fig. 4, open circle). In fact, inspection of the data shows that even at 24 h of adsorption, not all of the vector had been eliminated from the medium, as indicated by the non-zero slope of the regression curve at that time (Fig. 4). Theoretically (and supported by these data), after a sufficiently long adsorption period, all virions suspended above the cell monolayer will be adsorbed (i.e., $F_{arrival} \rightarrow 1.0$ in the limit as $t \rightarrow \infty$). Thus, extrapolation of $N_{well}(t)$ to infinite adsorption time (Fig. 4) yields an estimate of the expected true plaque titer of 60.2 PFU $well^{-1}$ (6.02×10^{11} PFU ml^{-1} for the stock vector).

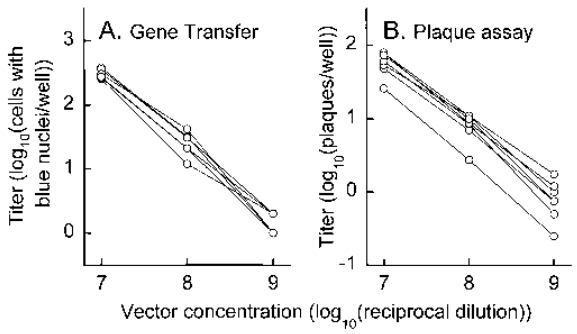

FIG. 3. Evaluation of adenovirus vector transduction at various vector concentrations. Av1nBg was diluted, adsorbed to cells, cultured, and evaluated under standard conditions as described in Materials and Methods. (A) Evaluation using the gene transfer detection assay. Each point is the result of one determination for Av1nBg (lot BB-7; $n = 7$). (B) Evaluation using the plaque assay detection assay. Each point is the mean of four determination for Av1nBg (lots BB9-6, BB9-2, BB9-1, BB8-6, BB8-5, BB7-2, and BB7-1; $n = 7$). For both A and B, note the linear dependence of observed titer on vector concentration demonstrating that a detected transduction event is the result of a single virion infection of each cell (17).

If we set $y = 8/\pi^2$ (a constant of integration), $w = 0$ (no plaques at time $t = 0$), and $u = 1$ ($F_{successful} = 1.0$), then equation 11 has the form of equation 7, the second Allison and Valentine approximation expression describing the number of virions adsorbed onto the cell monolayer per square centimeter at a given time t, assuming that every virion colliding with a cell results in a detected event. Fitting equation 11 to the data (Fig. 4) with these assumptions and the constraints for equation 7, suggested by Allison and Valentine, demonstrated a good fit ($r^2 = 0.962$). This analysis returned values for x and z of 59.99 PFU $well^{-1}$ and 0.1 h^{-1}, respectively. Since $N_{well}(t)$ PFU $well^{-1} = N$ PFU cm^{-2} (at time t) · 9.6 cm^2 $well^{-1}$, by substitution into equation 11, solving for N, and comparison with equation 7, we find that $x = C_0$ PFU $ml^{-1} \cdot d$ cm · 9.6 cm^2 $well^{-1}$. Substituting 59.99 PFU $well^{-1}$ for x and 0.11 cm for d (average fluid height for 1 ml of medium in a 35-mm-diameter well), C_0 is determined to be 62.5 PFU ml^{-1} (6.25×10^{11} PFU ml^{-1} for the stock vector). This theoretical prediction from the Allison and Valentine relationship with the given assumptions listed is accordingly in close agreement with the value determined by empirically fitting the data to a general form of equation 11 with fewer constraints (6.25×10^{11} versus 6.02×10^{11} PFU ml^{-1}, respectively). Interestingly, this value is higher than the value determined by electron microscopy and gene transfer (with a 90-min adsorption) but still less than the value determined by optical absorbance. The latter observation suggests that either not all of the virions are infectious or the calculated number of total virions based on optical absorbance is overestimated.

Comparison of equation 11 (using the constraints and assumptions given in the preceding paragraph fit to the data; Fig. 4) with the Allison and Valentine relationship (equation 7) allows computation of an empirically determined diffusion coefficient D from the rate constant z for the increase in titer with adsorption time; since z was determined to be 0.1 h^{-1} ($\approx 2.78 \times 10^{-3}$ s^{-1}) and $z = \pi^2 D/4\ d^2$, D for the adenovirus vector virion = 1.3×10^{-7} cm^2 s^{-1}. To permit comparison of this value of D with a value predicted from the virion diameter and η, the value of η for IMEM-2 at 37°C was experimentally determined ($n = 3$) to be 0.75 cP using a Ubbelohde viscom-

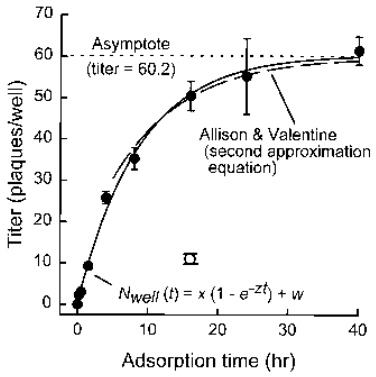

FIG. 4. Evaluation of the effect of increasing adsorption time on the quantification of adenovirus vector titer. Av1nBg (lot BB9-6) was diluted under standard conditions and adsorbed to 293 cells. At the times indicated, vector-containing medium was removed and cells were overlaid in agar and cultured. Plaques were evaluated on day 14 postinfection. Note that the curve has not plateaued even at 24 h of adsorption. The supernatant removed from cells after the 16 h of initial adsorption was readsorbed to fresh cells for an additional 24 h and evaluated subsequently for plaques as described above (open circle). See text for additional details.

eter. This value for D is in reasonably close agreement with the theoretical value (7.0×10^{-8} cm^2 s^{-1}) calculated from equation 2, assuming the virion to be spherical with a capsid radius of 36.5 nm (7) and without consideration of contributions from shape or solvation factors.

On the basis of theoretical considerations, decreasing the depth of the adsorption fluid at constant numbers of virions and target cells (i.e., constant multiplicity of infection) would be expected to increase virion adsorption per unit time and consequently increase the plaque titer. This was confirmed for short adsorption periods (1.5 h) during which the apparent titer was approximately twofold higher when adsorption volume was decreased by a factor of 2 (while maintaining adsorption vessel dimensions; 0.5 versus 1.0 ml; 1.0 ± 0.15 versus 5 ± 1.7 PFU well^{-1}, respectively; $P = 0.108$ [Fig. 5A]). At longer adsorption times (24 h), there was no detectable difference (1.8 ± 0.32 versus 2.0 ± 0.40 PFU well^{-1}; $P = 0.813$ [Fig. 5A]).

TABLE 3. Effect of vector adsorption time on the determination of adenovirus vector concentration and bioactivity on 293 cells

Adsorption time (h)	Titer (PFU/ml)[a]	% of maximum titer[b]	Bioactivity[c]	Particle ratio[d]
0.25	2.25	3.7	0.004	245
1	3.00	5.0	0.005	200
1.5	9.25	15.4	0.017	59
4	25.75	42.8	0.047	21
8	35.25	58.6	0.063	16
16	50.5	83.9	0.092	11
24	55.25	91.8	0.100	10
40	62.25	103	0.113	9
∞	60.21[e]	100	0.109	9

[a] Titer of diluted virus evaluated was determined by using standard conditions except for the change in adsorption time as noted.
[b] Derived by fitting the data in columns 1 and 2 to equation 11 at infinite adsorption time (see text).
[c] Defined by equation 14. The total number of physical particles (virions) was calculated using a value for the adenovirus extinction coefficient of 9.09×10^{13} OD ml cm virion^{-1} determined from the data of Maizel et al. (18).
[d] Defined as the inverse of bioactivity.
[e] Determined by extrapolation of equation 11 at infinite adsorption time (see text).

To evaluate the dependence of the observed plaque titer on the time allowed for formation of plaques, the titer was observed at various times for up to 3 weeks after a standard adsorption (90 min). As implied by the complex events which determine F_{plaque}, the time allowed for plaque formation affected the apparent plaque titer determined at 7 versus 14 days postadsorption (0.67 ± 0.33 versus 5 ± 1.7 plaques well^{-1}, respectively; $P < 0.07$). However, vector titer was not different when determined at 14 versus 21 days (5 ± 1.7 versus 5 ± 1.7 plaques well^{-1}; $P = 1.0$ [Fig. 5B]).

The effect of the presence of serum proteins on virion adsorption was evaluated by carrying out infections in medium containing FBS. Concentrations of FBS from 0 to 10% did not consistently influence the apparent plaque titer (0% FBS, 3.2 ± 0.34 PFU well^{-1}; 2% FBS, 2.2 ± 0.21 PFU well^{-1}; 10% FBS, 2.9 ± 0.19 PFU well^{-1} [data not shown]). Similarly, the density of 293 cells in the monolayer at the time of adsorption also did not significantly influence apparent plaque titer (0.5×10^6 cells per well, 2.6 ± 0.38 PFU well^{-1}; 10^6 cells per well, 2.2 ± 0.21 PFU well^{-1}; 1.5×10^6 cells per well, 2.6 ± 0.11 PFU well^{-1} [data not shown]).

As expected for events following a Poisson distribution, the variability in titer determination was higher when the number of observations of gene transfer units per well was very low (Fig. 5C). To determine the range of mean observations per well which is practically useful for the plaque assay for replication-deficient adenovirus vectors on 293 cells, data were evaluated from 168 separate determinations of titer for 21 different lots of Av1nBg ($n = 8$ determinations per lot). The results were plotted as the standard error of the mean of observations per well (normalized for the mean value) versus the mean number of observations per well (Fig. 5C). These data follow a double exponential decay as given empirically by

$$f(w) = xe^{-yw} + ze^{-qw} \quad (12)$$

where w is the mean number of plaques per well, x is the amplitude of the first exponential, y is the rate constant for the first exponential, z is the amplitude of the second exponential, and q is the rate constant for the second exponential. This general equation was used simply to describe the data in a purely empirical manner. Fitting equation 12 to the data (Fig. 5C) yielded values for x, y, z, and q of 48.48 ± 14.94, 0.359 ± 0.283, 23.38 ± 14.4, and 0.015 ± 0.023. This observation demonstrates that the error markedly increases and thus yields less reliable titer results when fewer than 10 plaques are counted per well.

The foregoing observations regarding the determination of the concentration of functional adenovirus vector virions demonstrates that in conventional assays based on infection of cells in a monolayer, both adsorption time and volume can dramatically influence the observed apparent titer of infectious units. Furthermore, titers determined by the gene transfer method were consistently higher for the same lot of vector than values determined by the plaque assay method. This is consistent with the more complicated biology involved in generation of a visible plaque compared with expression of the transgene. In this context, the relationship between apparent titer and adsorption time and volume were further explored by using the more sensitive gene transfer assay. To more accurately define the relationship with volume, aliquots of Av1nBg (lot BB9-6) were adsorbed ($t = 4$ h) to cells in volumes of medium ranging from 0.1 to 3 ml. The results indicated that the decline in transduction events with increasing adsorption volume (and thus the height of fluid) followed a decreasing sigmoidal curve (Fig. 6A). To verify this, the data (Fig. 6A) were empirically fit to a

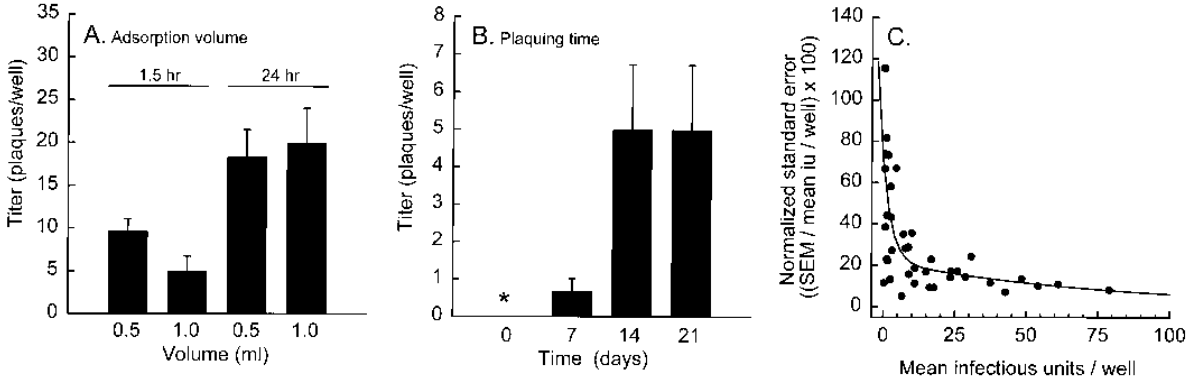

FIG. 5. Evaluation of several variables on the measurement of plaque assay titer. Av1nBg (lot BB7-7) was diluted under standard conditions except that vector was also prepared at twice the concentration in one-half of the volume for the incubations at 0.5-ml adsorption volume (A). (A) Effect of adsorption volume on plaque titer for short (90 min) or prolonged (24 h) adsorption times. (B) Effect of the time between adsorption and enumeration of plaques in the cell monolayer. (C) Effect of the number of detected transduction events counted on normalized error of counting.

general form of an equation for this sigmoidal-type relationship given by

$$N_{\text{well}}(d) = (x(1 - ye^{-\left(\frac{k}{d^2}\right)}) + w)u \quad (13)$$

where, for a given adsorption time (4 h here), $N_{\text{well}}(d)$ is the number of cells with blue nuclei observed for a particular adsorption volume of depth d; x, y, w, and u are as defined for equation 11 and are constants. If we set $w = 0$ and $u = 1$ and fit equation 13 to the data shown in Fig. 6A, we obtained values for x, y, and d of 122.61 GTU well^{-1}, 0.83, and 0.01 cm^2, respectively ($r^2 = 0.9844$). Thus, in the limit as volume approaches zero, the maximum obtainable titer can be determined to be 122.8 GTU well^{-1} (1.23×10^{12} GTU ml^{-1} for the stock Av1nBg vector, lot BB9-6). Inspection of equation 13 reveals that if we set $k = \pi Dt/4$ and $y = 8/\pi^2$, then equation 13 has the form of equation 7, Allison and Valentine's second approximation describing the number of virions arriving at the cell surface in the allotted time at a given fluid height. It is noteworthy that the empirically fit value of y (0.83) is very close to the theoretical expectation ($8/\pi^2 = 0.81$). If we set $y = 0.81$ and refit the data (Fig. 6A) to equation 13, we obtain fit values for x and d of 122.8 GTU well^{-1} and 9.57×10^{-3} cm^2 ($r^2 = 0.9836$). These values are in close agreement with the fit parameters determined under fewer constraints.

Further inspection of the regression curve found by using equation 13 at low adsorption fluid depths shows that little change occurs in the measured titer at volumes less than 0.5 ml. When the plates were evaluated at times of intermittent rocking, the cell monolayer was noted to be visibly dry but the cells appeared viable, and no sloughing had occurred. Subsequent evaluation of cell integrity during enumeration of transduction events confirmed the lack of cell death. To evaluate any possible effect that this might have on titer, titers were determined by using a 0.5-ml adsorption volume and a 4-h adsorption time with either no rocking, intermittent rocking, or continuous rocking on a mechanical platform. Drying occurred with no rocking and intermittent rocking but not with continuous rocking (observed titers of $[7.9 \pm 0.12] \times 10^{11}$, $[9.5 \pm 0.5] \times 10^{11}$, and $[1.7 \pm 0.07] \times 10^{12}$ GTU ml^{-1}, respectively). Rocking had an important effect on the titer outcome; continuous rocking led to a significantly higher titer than intermittent rocking ($P < 0.001$) or no rocking ($P < 0.0001$), while intermittent rocking had a significantly higher titer than no rocking ($P < 0.025$ [Fig. 6B]). This is of practical value because determinations made at lower volumes increase the adsorption but can result in drying of the monolayer, leading to erroneously low titer values.

To determine the most accurate value possible for the vector titer under optimized conditions, evaluations were carried out for various adsorption times in a 0.5-ml adsorption volume with mechanical rocking and detection by the gene transfer method (Fig. 6C). Fitting these data to equation 11 (with $y = u = 1$), we obtain values for x, z, and w of 155.986 GTU well^{-1}, 0.219 h^{-1}, and 4.122 GTU well^{-1}, respectively ($r^2 = 0.994$). Under these conditions, the titer of Av1nBg was determined, by extrapolation to infinite adsorption time, to be 1.6×10^{12} GTU ml^{-1}, which is closer to but still less than the value determined by optical absorbance ($6.16 \pm 0.08 \times 10^{12}$ OPU ml^{-1}).

From these data, it is apparent that even at the reduced adsorption volume of 0.5 ml, adsorption time is a critical factor affecting the ultimate titer determination. Even at this reduced adsorption volume, F_{arriving} increases from 0.2 to 0.97 as adsorption time increases from 1 to 16 h (Table 4). However, at the longer adsorption times, the risk of secondary infections due to vector replication increases and could lead to overestimation of the true titer. From the data above (Fig. 6A), the true titer could also be more accurately measured by using a smaller adsorption volume. At lower volumes, however, drying of the cell monolayer occurs (Fig. 6B) and leads to underrepresentation of the true titer. Thus, it is apparent that accurate measurements of the true titer will be difficult to make in a biological assay. In this context, a focus on obtaining precise determinations of titer by use of consistent assay conditions seems reasonable. Knowledge of the value of F_{detected} for a particular adsorption time (Table 4, for 0.5-ml adsorption volume in a six-well [3.5-mm diameter] dish) can be used to calculate the true titer for a range of plaque assay adsorption times.

Evaluation of adenovirus vector bioactivity. Comparison of titer determined by both physical and biological methods suggested that not every virion of a preparation of adenovirus vector is functional with regard to transfer and expression of the transgene. Hence, adenovirus vector bioactivity is a useful concept, and its measurement can be operationally defined by

$$\text{bioactivity} = \frac{\text{apparently functional adenovirus virions}}{\text{total adenovirus virions}} \quad (14)$$

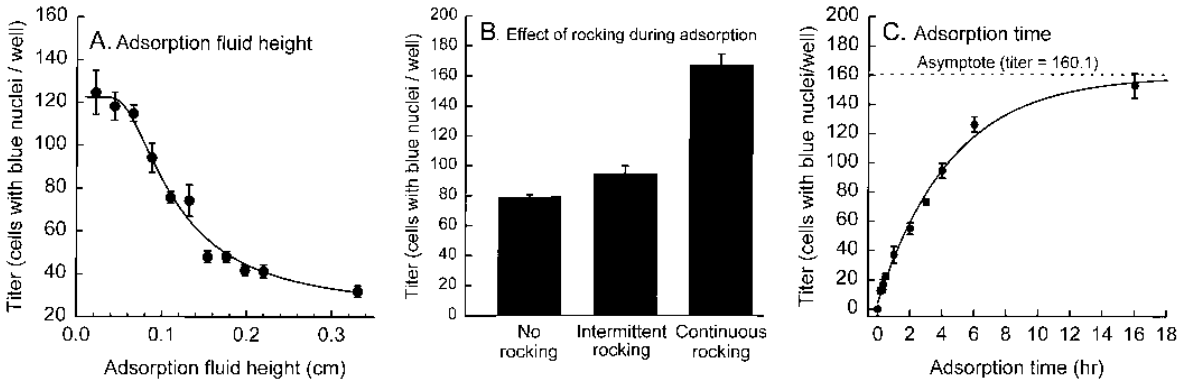

FIG. 6. Evaluation of adenovirus vector titer determined as a function of adsorption volume, motion, and time. (A) Effect of adsorption volume on titer. Av1nBg (lot BB9-6) was diluted under standard conditions, and 100-μl aliquots of stock virus from dilution tube 8 were adsorbed (t = 4 h) to cells in various final volumes (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, and 3.0 ml). The average height of fluid was calculated from the area of the well (9.6 cm^2 per well) and the volume of adsorption fluid used. (B) Effect of rocking the titer plates during vector adsorption on final titer. Av1nBg (lot BB9-6) was diluted under standard conditions, and 100-μl aliquots of stock virus from dilution tube 8 were adsorbed (t = 4 h) to cells in a final volume of 0.5 ml. During adsorption, the plates were either not subjected to any rocking or were rocked by hand every 30 min (intermittent) or continuously on a mechanical rocker platform (setting 3). (C) Evaluation of the effect of adsorption time on the quantification of adenovirus vector titer at low adsorption volume. Av1nBg (lot BB9-6) was diluted under standard conditions, and 100-μl aliquots of stock virus from dilution tube 8 were adsorbed to 293 cells for various times in a final volume of 0.5 ml. At the times indicated, vector-containing medium was removed and cells were cultured, stained, and evaluated as described in Materials and Methods. See text for additional details.

where bioactivity is a unitless value from 0 to 1 representing the fraction of virions able to achieve detectable transfer and expression of the transgene in the target cell under defined conditions. The terms on the right can be expressed as the total measured amounts or as concentration. Here "apparently functional" refers to infectious virions which are intrinsically functional and actually detected, and "total" refers to all virions, i.e., all physical particles, both infectious (i.e., intrinsically functional virions which are detected as well as those which fail to be detected) and noninfectious (intrinsically nonfunctional). Apparently functional or infectious virions can be measured by either the gene transfer assay or the plaque assay; however, the two methods return different values (Table 5). Further, apparently functional virion concentration determined by each of these assays depends significantly on the conditions under which the assay was conducted; i.e., longer adsorption times yield titer increasing values which approach an asymptote. Even when titer results are derived from equations well fitted to the data and extrapolated to infinite adsorption time, these two assays give different titer values, suggesting an inherent difference in the efficiency of detection (Table 5). Similarly, the two assays used to measure total physical virion concentration did not yield similar values for total virion concentration (Table 5).

Using the plaque assay titer to measure the apparent functional adenovirus virion concentration and the OD_{260} to calculate the total adenovirus virion concentration, the bioactivity for lot BB9-6 calculated from equation 14 varies from 0.004 to 0.113, or 28-fold, as the adsorption time increases from 30 min to 40 h (Table 3). The excellent approximation to observed data by a model explicitly limited only by virion diffusion kinetics strongly suggests that postdiffusion processes are not rate limiting for the majority of the transduction events. It does not, however, permit a precise definition of the number of bioactive adenovirus virions, given the possibility that $F_{\text{successful}}$ may be less than 1 as a consequence of the properties of the reporting cell population (i.e., 293 cells). Accordingly, the number of intrinsically bioactive virions determined by the GTU/OPU ratio (e.g., 0.113) merely represents a lower limit for the intrinsic bioactivity of the vector preparation. Looking at this from the opposite perspective, this value corresponds to a ratio of total virions to infectious virions ranging from 245:1 to 9:1 (Table 3). If instead the gene transfer assay is used and combined with a reduced adsorption volume and infectious titer determined at 16 h ($[1.53 \pm 0.08] \times 10^{12}$ GTU ml^{-1}), a bioactivity of 0.28 (total to infectious virion ratio of 3.6:1) is obtained. Determination of infectious virion concentration by extrapolating the fitted equation to infinite adsorption time (1.60×10^{12} GTU ml^{-1} [Fig. 6C]) does not significantly change the bioactivity (0.29, total-to-infectious virion ratio of 3.4:1 [Table 5]).

The foregoing considerations show that the concentration of apparently functional and total virions can be determined in multiple ways which are highly dependent on the assay conditions and that depending on the assay and conditions used, bioactivity will vary over a wide (~56-fold) range from 0.004 to

TABLE 4. Fractional detection of infectious adenovirus vector virions as a function of adsorption time[a]

Adsorption time (h)	F_{detected}[b]
1	0.22
2	0.37
3	0.50
4	0.59
5	0.67
6	0.74
8	0.83
10	0.89
12	0.93
14	0.96
16	0.97
∞	1.00

[a] Data are derived from experiments using an adsorption volume of 0.5 ml in a six-well dish (3.5-cm-diameter well) with slow, continuous mechanical rocking (Fig. 6C).

[b] Calculated from equation 11, using values of $y = u = 1$, $x = 155.986$ GTU $well^{-1}$, $z = 0.219$ h^{-1}, and $w = 4.122$ GTU $well^{-1}$; determined by fitting equation 11 to the data from Fig. 6C (coefficient of determination (r^2 = 0.994) as described in the text. F_{detected} was determined by dividing $N_{\text{well}}(t)$ (for various values of t ranging from 1 to 10^6 h) by N_{well} ($t = 10^6$ h).

TABLE 5. Comparison of several methods for determination of adenovirus vector concentration

Method[a]	Adsorption		Lot	Concn[b] (mean ± SEM)
	Time (h)	Vol (ml)		
Electron microscopy	NA[c]	NA	BB7-7	$(2.3 \pm 0.25) \times 10^{11}$ *v*/ml
Gene transfer	1.5	1	BB7-7	$(2.5 \pm 0.45) \times 10^{11}$ GTU/ml
Plaque assay	1.5	1	BB7-7	$(0.34 \pm 0.9) \times 10^{11}$ PFU/ml
Optical absorption[d]	NA	NA	BB7-7	$(5.51 \pm 0.13) \times 10^{12}$ OPU/ml
Gene transfer	4	0[d]	BB9-6	1.24×10^{12} GTU/ml
Gene transfer	∞[e]	0.5	BB9-6	1.6×10^{12} GTU/ml
Plaque assay	∞[e]	1	BB9-6	6.2×10^{11} PFU/ml
Optical absorption	NA	NA	BB9-6	$(6.16 \pm 0.08) \times 10^{12}$ OPU/ml

[a] Experimental details for each method are described in the text.
[b] Units used for each entry correspond to the method used: *v* as determined by the latex bead dilution method; GTU as determined by counting plates of transduced, X-Gal-stained cells for blue nuclei; PFU as measured on 293 cell monolayers; OPU as determined from the optical absorbance of SDS-disrupted virions together with a value for the adenovirus extinction coefficient of 9.09×10^{-13} OD ml cm virion^{-1} as determined from the data of Maizel et al. (18).
[c] NA, not applicable.
[d] Titer determined by extrapolating equation 13 (fit to data from Fig. 6A) to zero adsorption volume (see text).
[e] Titer determined by extrapolating equation 11 (fit to data from Fig. 6C (gene transfer titer) or Fig. 4 (plaque titer) to infinite adsorption time (see text).

0.29 (particle ratios of 245 down to 3.4). Thus, the most accurate determination of bioactivity will be obtained by separately optimizing detection of infectious and total virions. For measurement of apparently functional virions, gene transfer at low volume and long adsorption time gave the best representation (Fig. 6C; Table 2). By comparison with biological titers, which necessarily must be no greater than physical titers, optical absorbance was more accurate than electron microscopy for total virion concentration (Table 5). Therefore, the best estimate for concentration and bioactivity of Av1nBg can be calculated by using the gene transfer assay (at 0.5-ml adsorption volume, extrapolated to infinite adsorption time) and optical absorbance assay.

DISCUSSION

Lack of standardization of the methods for quantifying adenovirus vectors has hampered otherwise useful comparisons between published preclinical and clinical studies with these vectors. In this study, several methods for determining adenovirus vector concentration and bioactivity were compared and the assay conditions were evaluated. Adsorption time was one of the most important variables in biological assays, and it was established that use of an insufficient virion adsorption time can significantly underestimate the actual titer. Adsorption volume was also found to be very important, with smaller volumes yielding higher estimates of true titer. For the plaque assay on 293 cells, the time allowed for plaque formation was important, but neither the concentration of FBS in the adsorption medium nor the density of target 293 cells was important within a practical range, much as has been previously observed for transduction of human smooth muscle cells (19). Methods for determining the physical concentration of adenovirus virions independent of virion functionality yielded reproducible results, although the use of electron microscopic visualization with the latex bead method of counting (26) gave virion concentrations unexpectedly lower than did optical absorbance methods and lower than did either of the biological methods under optimized assay conditions. Light scattering from intact virions significantly influences the estimation of the virion concentration by optical absorbance as previously described for wild-type adenovirus (18). Thus, optical absorbance should be performed only after virion disruption.

Quantification by physical methods. Electron microscopy showed a high proportion of morphologically normal singlet virions and the absence of observable subcellular organelle, protein, or nucleic acid debris. While the determination of virion concentration by the latex bead method was precise, the observed titer was far lower than expected and in fact less than determined by both biological assays. This result was surprising and contrasts with what might be expected intuitively, i.e., that direct visualization should yield higher observed concentrations than do the less efficient biological methods. However, comparison with the results of both biological assays indicates that the latex bead method was not accurate. There are three reasonable possible explanations for underrepresentative concentrations by electron microscopy: (i) an inaccurate latex sphere standard, (ii) differential adherence of the latex spheres and virions during preparation of the grids for electron microscopy, and (iii) differential negative staining of latex beads and virions prior to electron microscopy (13).

The optical absorbance assay was very precise, although it gave higher values for particle numbers than any of the other methods, including optimized biological methods. The are several possible reasons for this, which fall into three categories: either optical absorbance overestimates virion concentration, biologic titers underestimate virion concentrations, or a large proportion of nonfunctional morphologically damaged particles exists. Direct visualization did not confirm this latter possibility, although a fraction of the morphologically normal-appearing virions may, in fact, be noninfectious. This will be difficult to evaluate. Given the complexities of detecting functional virions by infection of cells, it is likely that the biological assays may not be fully optimally detecting functional particles. However, this conclusion may be reached only after exclusion of other possibilities. There are several important reasons why the virion concentration calculated from the OD_{260} could be overestimated. This would occur if residual cellular debris, e.g., RNA or DNA, were present. However, this is unlikely because direct examination of the virion preparation did not demonstrate such material.

A more likely explanation for the high virion concentrations measured by optical absorbance is that the extinction coefficient used here was not correct. The value of 9.09×10^{-13} OD ml cm virion^{-1} determined from the data for wild-type adenovirus by Maizel et al. (18) was chosen arbitrarily over other different values (15) and either may not be accurate or may not be correct for recombinant adenovirus vectors whose genome is smaller than the wild-type adenovirus genome. Maizel et al. (18) calculated an extinction coefficient for Ad5 from the ratio of protein to OD_{260} on the assumption that the molecular mass

of Ad5 DNA is 2.3×10^7 Da (12) and 87% of the virion dry weight is protein (7, 10). Lawrence and Ginsberg (15) also measured the OD of purified virions, and their data yield a much lower value of 3.03×10^{-11} OD ml cm PFU^{-1} for the extinction coefficient of Ad5. They performed studies similar to those of Maizel et al. but also directly measured adenovirus titer by plaque assay and further directly quantified viral DNA (Burton method) and evaluated for the presence of contaminating RNA (orcinol method). Their plaque assay evaluation used an adsorption time of 10 min with an average fluid height of 0.007 cm; thus, according to equation 4, their data should be corrected for a value of $F_{\text{arriving}} = 0.926$. This value can be further adjusted for the inefficiency of plaque assay based on F_{plaque}. From the observations here, F_{plaque} is threefold less than $F_{\text{transgene expression}}$. If we assume that all vector genomes reaching the nucleus are detected (i.e., $F_{\text{transgene expression}} = 1.0$), then F_{plaque} is ≈0.33. Then, disregarding all other components of $F_{\text{successful}}$ (i.e., set $F_{\text{intact}} = F_{\text{attaching}} = F_{\text{penetrating}} = F_{\text{intranuclear}} = 1$), their extinction coefficient (15) can be corrected to a value of 9.26×10^{-12} OD ml cm virion^{-1}. Because of the assumptions about the remaining components of $F_{\text{successful}}$, the correct extinction coefficient may be slightly higher. Lawrence and Ginsberg found a ratio of particles to PFU of 11:1 for Ad5. Thus, if we take their original value of 3.3×10^{10} PFU per OD_{260} and multiply by this ratio, an extinction coefficient of 2.75×10^{-12} OD ml cm virion^{-1} is obtained. If this value is corrected for $F_{\text{arrival}} = 0.926$ and $F_{\text{plaque}} = 0.33$, a corrected extinction coefficient of 8.42×10^{-13} OD ml cm virion^{-1} is obtained. This adjusted value is very close to, albeit still larger than, the value determined by Maizel et al. (18). The current studies were not intended to reevaluate the accuracy of the adenovirus extinction coefficient, which is beyond the scope of this report, but it is clearly an issue that needs to be addressed in future studies.

Quantification by biological methods. Factors relevant to the efficiency of enumerating adenovirus vector virions by biological assays can be divided roughly into intrinsic properties of the virion and events which occur during the evaluation process itself as described in equations 8 and 9. Biological assays require quantitative target cell infection, which in turn requires that virions, suspended in the fluid above the cell, diffuse to the cell surface, roll or diffuse laterally on the membrane, attach to specific receptors, internalize, and translocate the vector genome to the nucleus. Detection of an infection event by histochemical detection of transgene product function (gene transfer assay) further requires that expression of the transgene must occur and lead to sufficient accumulation of enzymatically active protein for microscopic visualization. Detection of infected cells by formation of plaques in the a cell monolayer (plaque assay) is even more complicated. The steps up to and including nuclear translocation are identical but are followed by and are dependent on execution of the complex adenovirus genetic program leading to virion replication, cell death, breakdown of the cellular membrane, release of infectious virions (adenovirus is not a lytic virus [7]), and infection of neighboring cells. Successive rounds of these events must occur sufficient to result in a visible plaque. The efficiency of this process is further compromised by the fact that when titers of E1-deleted adenovirus vectors are determined on 293 cells, the complex molecular program depends on coordinate expression of interdependent, albeit spatially separated adenoviral genes, i.e., those integrated into the 293 cell chromosomes (E1 genes) and those remaining within the epichromosomal recombinant vector genome.

A fraction of virions are truly nonfunctional, e.g., are defective and not detected because of intrinsic lack of structural or functional integrity (represented by $1 - F_{\text{intact}}$). A fraction of fully functional virions will appear defective as a result of adverse events occurring during detection. Functional virions will not be detected when (i) they attach to dead cells, (ii) they are inactivated during detection, (iii) their otherwise normal growth is terminated by premature growth arrest (plaque assay) or death of the host cell, or (iv) they infect a cell which is already infected by another functional virion. The latter is governed by the Poisson relationship and is minimized at the low multiplicity of infection commonly used. Because of the sensitivity of 293 cells to culture conditions, it is possible that the warm agarose-medium overlay used in the plaque assay causes growth arrest or death of infected cells or may possibly directly inactivate the virion. Given the more numerous steps of increased complexity required in the plaque assay, it is not surprising that plaque assay is less efficient than the gene transfer assay. Because the measurement of truly defective virions are not distinguished from the fraction of undetected, functional virions, optimization of the biological assay conditions is critical for correct assessment of concentration and to avoid underestimating the bioactivity of the vector.

A new set of standard conditions for plaque assay titration of E1-deleted adenovirus vectors on 293 cells can be recommended on the basis of the data presented here. First, the adsorption volume should be minimized to achieve a small average fluid height but not so much that drying of the cell monolayer occurs. A volume of 0.5 ml in a culture dish of 3.5-cm diameter yields an average adsorption fluid height of 0.055 cm, which seems acceptable. Second, the adsorption time should be sufficiently long to maximize virion adsorption but not so long as to disrupt the integrity of the cell monolayer. Six hours is appropriate and allows the entire assay setup to be conveniently done within a day. Third, continuous slow mechanical rocking should be used to ensure adequate bulk fluid distribution and to prevent drying. Fourth, plaque development should be continued for 14 days. Theoretically, these conditions should allow detection of approximately 74% of the infectious virions. However, from the knowledge of the fractional detection of virions for a given adsorption time (Table 4), the true titer can be calculated. One alternative to use of an exhaustive adsorption time is to determine titer at several adsorption times, fit the data to equation 11, and extrapolate to the titer at infinite adsorption time. Another alternative is to use a single adsorption time (e.g., 3 h) and divide the observed titer by the corresponding value of F_{detected} for that adsorption time (e.g., 0.5 [Table 4]).

Pharmacological implications for human gene therapy. Data regarding the bioactivity of potential gene therapeutic agents such as adenovirus vectors are important to provide the means to compare data from various preclinical and clinical studies. For example, bioactivity data make possible comparisons of biological and therapeutic equivalency of similar formulations of a vector in different clinical protocols and also different formulations, i.e., newer generations of adenovirus vectors which harbor additional attenuating genomic deletions. However, bioactivity is determined by assays which involve quantitative infection of cells and thus are relative to cell type and assay conditions. Thus, for bioactivity data to be useful, standards must be set for determination. The observations here suggest that OD_{260} is a very precise and a useful method for determining the physical quantity of virions in an adenovirus preparation. In contrast, determining the concentration of functional virions by biological methods is not completely efficient. While the gene transfer assay used here is more sensitive than the plaque assay, it is specific to the β-galactosidase transgene and will not be useful for comparing vectors express-

ing different transgenes. Hence, the plaque assay will likely continue as the assay of choice for determination of virion functionality. Use of optimal conditions for plaque assay evaluation is thus very important.

Pharmaceutical formulations of a drug can be defined and compared by measures of chemical, biological, and therapeutical equivalency (21). Ultimately, evaluation of therapeutic equivalency (equal therapeutic benefit in a clinical trial) will depend on knowledge about chemical equivalency (similar chemical and physical properties, e.g., bioactivity) and in vivo biological equivalency (similar concentrations of the drug in blood or tissues for a given dose). The bioavailability (21) of an adenovirus vector can usefully be conceptualized as the concentration of transgene achieved in a particular tissue for a given amount of vector administered. Measures of bioavailability will therefore require some means of evaluating amount of gene transfer into the target organ and also of the total number of virions in a particular vector formulation. This obviously parallels the concept developed above regarding in vitro vector bioactivity, and these measures are expected to correlate.

ACKNOWLEDGMENTS

We thank Paul Tolstoshev and Harold Ginsberg for critical reading of the manuscript, Bob Jambou for helpful discussions, and Gerri Smith for editorial assistance and typing of the manuscript.

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