Adeno-Associated Virus Studies Employing A Fluorescent Focus Assay Technique. (32231)

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Adeno-associated viruses constitute a newly recognized group of small, defective DNA viruses which require adenovirus for replication(1-8). Until recently the only useful methods available for detecting or quantitating these viruses involved some combination of complement fixation (CF) or electron microscopy(1-8). The present report describes the development and use of a fluorescent focus (FF) assay technique for the study of human adeno-associated virus (AAV) type 1. This technique gives sufficient assay precision to permit an evaluation of the relative abilities of various adenoviruses as helpers as well as a more quantitative examination of AAV as a defective virus.

Materials and methods. Most of the viruses, cells, media and antisera used in the present study were those we used in a previous study of AAV(6). The fluorescent focus assay technique is described in detail in another accompanying report(9). Briefly, the viruses we used were: adenovirus prototypes 1, 3, 4 and 12, and another strain of adenovirus 4 which was contaminated with AAV type 1(6). It was this AAV strain which we used for the present study. Adenovirus type 2 (Benyesh-Melnick) is one of the strains we used in a study of adenovirus capsid structure(10). Human epithelial cells (strain KB) were used to grow large lots of virus. Human amnion cells (strain FL) were employed for fluorescence microscopy. The indirect Coons reaction was used for visualizing infected cells, *viz.*, hyper-immune rabbit sera, followed by fluorescein conjugated anti-rabbit γ globulin. Anti-AAV serum was prepared in rabbits as described before(6). Serum had been absorbed twice with adenovirus 12 infected KB cell lysates and clarified by centrifugation for ½ hour at 12,000 $\times$ *g*. This serum gave brilliant fluorescence with cells that were doubly infected with adeno and AAV but gave no visible fluorescence with normal or adenovirus 12 infected cells (see *Results* section, Fig. 1, right). Anti-adenovirus 12 serum was absorbed twice with normal KB cell lysates and clarified as above. This serum gave no visible fluorescence with uninfected cells. FF counts were made at 100 $\times$ magnification using a grid inserted into the ocular of the fluorescence microscope. The area counted within the grid space was about ⅛ the total microscopic field. AAV suspensions containing adenovirus 4 were routinely heated at 60°C for 5 minutes before experimentation. This reduced the concentration of infectious adenovirus without significantly decreasing the infectivity of AAV (4,6). Such heat inactivated suspensions produced no detectable adenovirus FF when inoculated undiluted. When mixed with adenoviruses, inoculated upon human amnion cells, incubated, fixed and stained, however, AAV fluorescent foci could be visualized. Such interaction of various AAV-adenovirus combinations with human amnion cells is the subject of this paper.

Results. Preliminary experiments with adenoviruses 1, 2, 4 and 6 indicated that these viruses cause brilliant immune fluorescence in cells and obvious cytopathic effects within 24 hours following inoculation, although little or no secondary spread is observable by fluorescence microscopy. Longer incubation periods resulted in a progressive secondary spread of virus to neighboring cells and loss of cells by sloughing. Adenovirus 12 produced cytopathic effects much more slowly, however, requiring about 48 hours before gross cytopathic effects were noticeable. Maximum brilliance of infected cells in the fluorescent microscope occurred about 40 hours after infection. Little or no secondary infection of neighboring cells was observable by fluorescence microscopy at 48 hours. For these reasons cells inoculated with adenoviruses 1, 2, 4 and 6 were fixed at about 24 hours and those inoculated with adenovirus 12 at 40-48 hours.

TABLE I. Efficiency of Various Adenoviruses as "Helpers" for AAV.

	Avg No. of fluorescing foci/field counted when cells treated as follows:			
	Inoculated with adenovirus only	Inoculated with adenovirus, then superinfected with AAV		% Cells fluorescing with anti-adenovirus which fluoresced with anti-AAV
Adenovirus type	Stained with anti-adenovirus*	Stained with anti-adenovirus*	Stained with anti-AAV	
1	6.0	1.7	.7	12
2	8.9	5.2	1.2	14
4	4.7	2.1	.6	13
6	27	14	5.3	20
12	53	—	25	47

* Anti-adenovirus 12, which stained cells with other adenovirus types equally well.

Efficiency with which various adenovirus types serve as "helpers" for AAV in the FF assay. An effort was made to study the relative abilities of various adenovirus types to "help" AAV when employed in the FF assay. Various adenovirus types (1, 2, 4, 6 and 12) were serially diluted and adsorbed for 1 hour so as to give an average of 5-50 adenovirus FF/microscopic field (infection of more than 20% of cells with adenoviruses 1, 2, 4 and 6 was avoided because of the tendency of grossly infected cell sheets to slough). Following infection with adenoviruses, inocula were removed and equal amounts of AAV were added to all cell cultures. AAV was allowed to adsorb for 1 hour, inocula were removed, and Eagle's medium replaced. In addition, cell monolayers were infected with identical amounts of (a) adenovirus *without* AAV and (b) with AAV without adenovirus. All monolayers were incubated at 36°C until fixed and stained for fluorescence microscopy. Results obtained from one such experiment are shown in Table I. It can be seen that monolayers infected with adenovirus *only* showed more adenovirus FF/field, on the average, than identical cultures superinfected with AAV (compare columns 2 and 3). It appears that adenoviruses 1, 2, 4 and 6 were poor "helpers" in this system compared with adenovirus 12. On the basis of FF assays, only 12-20% of cells infected with adenovirus types 1, 2, 4 and 6 synthesized sufficient AAV to be recognized by this method, whereas 47% of adenovirus 12 infected cells produced recognizable AAV (see column 5). Cells inoculated with AAV alone showed no FF.

Fig. 1 shows AAV FF on the left and cells infected with adenovirus 12 only (no AAV) on the right (both cell monolayers were stained with rabbit anti-AAV serum, followed by fluorescein conjugated anti-rabbit γ globulin).

Efficiency of adenovirus 12 as a "helper" for AAV. In order to verify the conclusions drawn from the above and other similar experiments, the role of adenovirus 12 as a "helper" was studied further. Cells were infected with serial dilutions of adenovirus 12, then super-infected with identical amounts of AAV. The results are shown in Table II. It can be seen that 10^{-1} and 10^{-2} dilutions of adenovirus 12 gave a proportionate dose-response relationship when cells were stained for adenovirus (indicating that the undiluted adenovirus used in this experiment was in excess and gave a multiplicity of about 4). Monolayers infected with adenovirus *only* showed more adenovirus FF/field than replica cultures super-infected with AAV (compare columns 2 and 3, Table II), as was seen in the experiment described in Table I. The fraction of adenovirus infected cells "helping" AAV in this experiment was 38-48%, averaging 41% (compare columns 2 and 4, Table II). This is also similar to the result obtained for adenovirus 12 in the previous experiment (see Table I). The largest number of AAV FF/field in this experiment (78) was obtained when most of the cells were infected with adenovirus 12 (see columns 2 and 4, Table II).

Cell-virus interactions using various adenovirus 12-AAV combinations. An AAV suspension was serially diluted, then appropriate

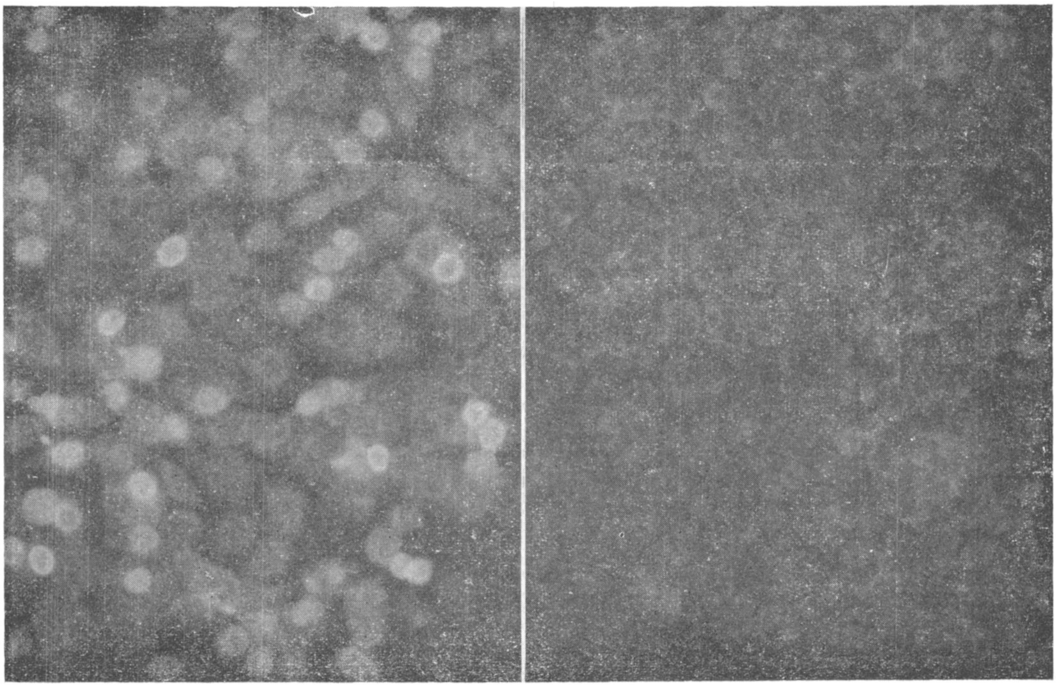

FIG. 1. Left: Cells doubly infected with adenovirus and AAV, stained by the indirect method with rabbit anti-AAV serum and fluorescein conjugated anti-rabbit γ globulin. Right: Cells infected with adenovirus only, stained as above. Photographs were taken on Kodachrome X film, using the same lighting and exposure times. Reversed prints of these transparencies are shown here.

dilutions inoculated on HA cells. Following adsorption for 1 hour, inocula were removed and equal amounts of adenovirus 12 added to all dishes (effective multiplicity of adenovirus infection in all cultures was about 4). Inoculum was again removed after adsorption for 1 hour, medium replaced and all cultures incubated for about 44 hours. Cells were fixed, stained and FF counted in the usual way. The results are shown in Table III. It can be seen that cell monolayers inoculated with AAV dilutions of less than 1:625 gave about the same number of FF/field (33-40, see column 2). Beginning at 1:625, however, there was an almost exactly proportional decrease in AAV FF with increased dilutions of the inoculum. It is concluded that high multiplicities of AAV (up to 400) resulted in no detectable increase or depression in the numbers of fluorescing foci produced on cell monolayers. The infectious virus concentration of this particular AAV suspension, as measured by this technique, was calculated to be 1.0×10^8/ml.

An aliquot of the same AAV suspension used in the above experiment was diluted 1:10, then mixed in equal volumes with adenovirus 12. This mixture was serially

TABLE II. Efficiency of Adenovirus 12 as a "Helper" for AAV.

	Avg No. of fluorescing foci/field counted when cells treated as follows:			
	Inoculated with adenovirus only	Inoculated with adenovirus, then superinfected with AAV		% Cells fluorescing with anti-adenovirus which fluoresced with anti-AAV
Adenovirus dilution	Stained with anti-adenovirus	Stained with anti-adenovirus	Stained with anti-AAV	
Undiluted	163	51	78	48
10^{-1}	82	27	31	38
10^{-2}	8	4	3	38

TABLE III. Titration of AAV Using Adenovirus 12 as a "Helper."

Virus dilution	No. of fluorescing foci/field counted when cells stained with anti-AAV
Undiluted	39
1:5	40
1:25	37
1:125	33
1:625	25
1:3125	4.9
1:15,625	0.9

diluted, inoculated on cell monolayers, virus adsorbed for 1 hour, inocula removed, medium replaced and cells incubated for about 44 hours. In addition, adenovirus 12 *only* was serially diluted, cells inoculated and these cultures handled in the same way. Cells were fixed, stained and FF counted as before. The results of this experiment are shown in Table IV. It can be seen that the average number of AAV FF/field, after the 1:3 dilution, decreased out of proportion to the dilutions of the AAV inocula (Table IV, Part A). This is in contrast to the number of adenovirus FF counted on monolayers inoculated with adenovirus only (Table IV, Part B). Adenovirus FF decreased in almost exact proportion to virus dilutions. The results of Parts A and B of this experiment are shown graphically in Fig. 2, in which the average number of FF/field were plotted against the relative concentrations of the two viruses. The dose-response curve in the region observed for adenovirus (B) approximates a straight line going through the origin whereas the curve for AAV (A), under these experimental conditions, is sigmoid in shape and has a very low initial slope.

Discussion. AAV did not produce an in-

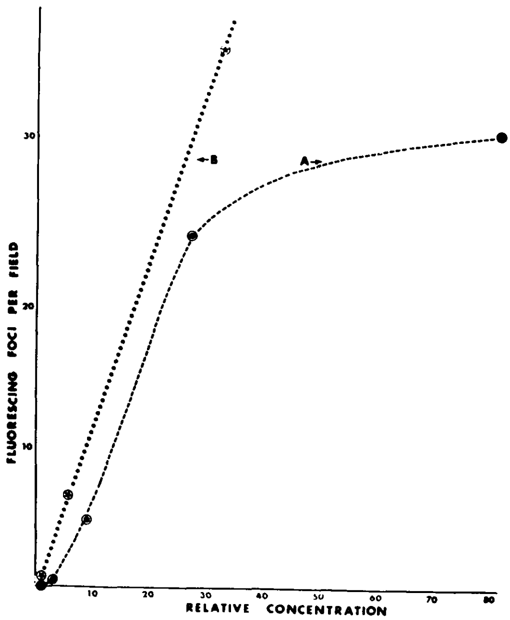

FIG. 2. Dose-response curves for: (A) a suspension containing a mixture of adenovirus 12 and AAV, cells stained for AAV; and (B) a suspension containing adenovirus 12 *only*, cells stained for adenovirus.

fection in cells recognizable by immunofluorescence unless cells were also infected with an adenovirus "helper." This confirms earlier observations with electron microscopy and CF (3,4,6,7). Experiments similar to those described in Tables I-III indicate that adenovirus 12 is a good choice as a helper for AAV, in contrast to Types 1, 2, 4 and 6,

TABLE IV. Dose-Response Study of an AAV-Adenovirus Mixture (Part A) and Adenovirus Only (Part B).

Part A: Adenovirus + AAV		Part B: Adenovirus only	
Dilution of AAV-adenovirus mixture*	Avg No. of fluorescing foci/field counted when cells stained with anti-AAV	Dilution of adenovirus only	Avg No. of fluorescing foci/field counted when cells stained with anti-adenovirus
Undiluted	32	1:5	36
1:3	25	1:25	6.3
1:9	4.7	1:125	1
1:27	.4		
1:81	<.02		

* Equal volumes of AAV and adenovirus were mixed; therefore the final dilution of adenovirus was 2-fold greater than listed; the final dilution of AAV was 40-fold greater than listed (see text).

for the following reasons: 1) cytopathic development is slow in adenovirus 12 infected cells. Such cells show bright immunofluorescence before significant rounding or sloughing takes place, making microscopic examination relatively easy and dependable. 2) A high proportion of adenovirus 12 infected cells produced AAV FF, compared with cells infected with other adenovirus types (Tables I and II). These findings are consistent with our experience in obtaining large yields of AAV (determined by electron microscopy) in adenovirus 12 infected cells. This appears to be true also for prototype adenovirus 18, a slow growing adenovirus serologically very similar to adenovirus 12, which we found was contaminated with AAV type 2(6).

Super-infection of adenovirus infected cells with AAV resulted in a substantial decrease in the number of cells staining for adenovirus (Tables I and II). It appears, therefore, that production of AAV depresses the ability of doubly infected cells to produce adenovirus, especially adenoviruses 1, 2, 4 and 6. This is consistent with previous observations made by several workers(4-8), who used other AAV strains and techniques other than fluorescence microscopy. Perhaps the work of Atchison *et al*(5) with a simian AAV is particularly pertinent. They found that thin sections through doubly infected cells usually contained mainly AAV particles or mainly adenovirus particles, but rarely substantial amounts of both within the same cell. Our results with immune fluorescence confirm these findings.

Dose-response experiments with AAV, in which an excess of adenovirus was present in all cultures so as to give maximum AAV FF, showed strict proportionality between numbers of FF observed and dilutions of AAV inoculated. Similar experiments, in which serial dilutions of adenovirus-AAV mixtures were simply inoculated, have given results consistent with a two-hit requirement for AAV synthesis in cells. This confirms in a more precise quantitative way the conclusion drawn by us and others in earlier work with various AAV's, *viz.*, that adenovirus infection of a cell is required for AAV synthesis(3-8).

The AAV suspension titrated in the experiment described in Table III gave about one FF per gridded space at 100 $\times$ magnification when the virus dilution was over 10^{-4}. Since the gridded space accounted for only about 1/8 the total area observed at this magnification (see *Materials and methods*), a 10^{-5} dilution of the virus would have given about one FF/entire field. This particular AAV suspension titrated only 1:8 as CF antigen and could be diluted only 1:100 before recognition by electron microscopy became difficult. It is estimated, therefore, that the FF assay method employed in this work is at least 1,000 times more sensitive than electron microscopy and 10,000 times more sensitive than complement fixation for detecting AAV. It is practical to use a low magnification scanning lens in order to find rare FF. Closer scrutiny of an occasional focus can then be done at higher magnifications. In this way, assuming little or no non-specific fluorescence in controls, the sensitivity of the FF assay is greater than the estimates given above. At this time it appears to be the method of choice for examining adenovirus stocks for ***known*** AAV's, (for which antisera are available). However, electron microscopy still remains an important method for detecting AAV types for which antisera have not been prepared.

Summary. AAV antigens could not be recognized in cells by immuno-fluorescence unless the cells had been infected with adenoviruses. Of the adenoviruses studied, (types 1, 2, 4, 6 and 12) type 12 was by far the most efficient helper virus. A measurable fraction of adenovirus infected cells failed to produce recognizable AAV antigen(s). AAV super-infection suppressed the number of adenovirus infected cells producing recognizable adenovirus antigens. The FF assay method employed was at least 1,000 times more sensitive than electron microscopy and 10,000 times more sensitive than complement fixation for detecting AAV.

Addendum. During the preparation of this manuscript Dr. David Hoggan kindly provided us with a manuscript, which he and others had submitted for publication(11), describing a quantitative immunofluorescence procedure for titrating AAV. They found that about 16% of adenovirus 7 infected cells developed AAV antigen, which is similar to our experience with adenoviruses 1, 2, 4 and 6.

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Fluorescent Focus Assay of Viruses on Cell Monolayers in Plastic Petri Plates. (32232)

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Fluorescence microscopy and fluorescent focus (FF) assay techniques have been used extensively in the study of viruses and the quantitation of viral infectivity(1-7). All of the methods reported to date, of which we are aware, employed glass cover-slips upon which cell monolayers were grown. Viruses were inoculated upon these cover-slips and each cover-slip put through a series of washings, a fixation, various treatments to reveal viral antigens, and a final mounting on a glass slide. We have found that these manipulations are tedious and usually involve significant risks in breaking the delicate cover-slips bearing infected cells. The present report describes a technique which permits cell growth, inoculation, fixation, staining and examination of fluorescing cells directly in plastic Petri plates.

Materials and methods. Plastic Petri dishes, 35 × 15 mm were obtained from Falcon Plastics Co., Los Angeles, Calif. Human amnion (HA, strain FL) cells were grown in 32-oz. bottles, dispersed by trypsinization, counted in a hemocytometer and mixed with Eagle's No. 2 plus 5% fetal bovine serum to give a concentration of 2×10^5 cells/ml. Two ml of this suspension were added to each plate. Plates were incubated undisturbed for 24 hours at 36°C under 5% CO_2. It was important to have an even distribution of cells in the plates.

Viruses. Adenoviruses 1, 2 and 12 and adeno-associated virus (AAV) type 1 were the same ones used in other studies(8,9). Reovirus type 1(10) and its specific antiserum (prepared in rabbits by injection of density gradient purified particles) were also used.

Inoculation of plates. Medium was removed and 0.50 ml virus added to each dish. Plates were rocked every 10 minutes (at room temperature) to insure complete coverage of the cell monolayers. Inoculum was removed by aspiration at the end of one hour and 2.5 ml of fresh media added to each plate. Plates were reincubated for 24 to 48 hours, depending upon the virus used(9).

Fixation. Medium was aspirated and 2 ml of 0.85% saline added to each plate. Each plate was washed in this manner 4 times, for a total wash time of 3-4 minutes per plate. The fixative, which consisted of 95 volumes of absolute methyl alcohol and 5 volumes of 0.85% saline, was added and the cells allowed to fix for 4 minutes. Fixative was removed, 2 ml of 0.1 M phosphate buffer, pH 7.1, was added and the buffer allowed to remain for