Functional Heterogeneity of Virions in Human Adenovirus Types 2 and 12

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Received for publication 9 September 1969

Purified preparations of adenovirus types 2 and 12 were used to infect KB cells at different input multiplicities. The resulting infected cultures were scored for inclusion body formation, production of infectious centers, and cloning efficiency. Both preparations were found to contain some defective particles capable of preventing a cell from cloning but unable to induce inclusion bodies or form plaques. The proportion of such defective particles in adenovirus 12 was about 10 times that in adenovirus 2. At high input multiplicities, the percentage of cells displaying an inclusion body was less than that predicted by the Poisson distribution and reached a maximum of 40 to 60% for adenovirus 2 and 12 to 15% for adenovirus 12. This reduction may be due to interference by large numbers of non-plaque-producing particles infecting each cell. The per cent of cells forming infectious centers was substantially greater for adenovirus 2 than for adenovirus 12 when compared at the same input plaque-forming units, reaching a maximum of 35 to 73% for adenovirus 2 and 5 to 10% for adenovirus 12. The low value for adenovirus 12 may be a result of the same interference phenomenon.

Animal viruses generally have ratios of particles to infectious units greater than unity (18). For the human adenoviruses, these ratios have been shown to vary for different types ranging from 11 to 23,000 (8). Generally, one single virus particle is sufficient to produce a plaque. It is not certain whether this spread of ratios reflects differences in the biological efficiency of plaque formation or different degrees of functional heterogeneity in the virus populations. It has been shown that ultraviolet (UV)-irradiated viruses, not capable of producing a plaque, can still carry out certain viral functions such as tumor induction (4), transformation (11), neoantigen production (5, 16), and chromosome breakages (16, 20). It is of interest to examine whether the non-plaque-producing particles in the adenovirus preparations are capable of carrying out some other viral functions. In this report, we have studied the relative abilities of both adenovirus 2 and adenovirus 12 to form plaques, nuclear inclusions (1), and to prevent host cell cloning (6).

MATERIALS AND METHODS

Cells and virus. Human KB cells were grown as monolayers at 37 C in 32-oz (ca. 900 ml) prescription bottles (Brockway Glass Co., Inc., Parkersburg, W. Va.). The growth medium was Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum. Both adenovirus 2 and the adenovirus 12 (Huie strain), originally obtained from M. Green,

St. Louis University Medical School, St. Louis, Mo., were used throughout this study. Virus stock was prepared by infecting KB cells in suspension and was subsequently purified by the method of Green and Pina (7). The virus band collected from the second cesium chloride gradient was diluted 10-fold with TBS (19) plus 20% glycerol and was stored at -45 C until used. Storage under these conditions produced no significant loss in infectivity. Examination of these preparations under the electron microscope by using negative staining showed complete virions with no detectable adeno-associated virus.

Experimental procedure. To ensure that cells in the same physiological state and under identical infection conditions were used for different viral functions, all assays were carried out with the same infected cultures. Stock cells were scraped off by using a rubber policeman and were dispersed. Cells (10^6) were mixed together with an appropriate amount of virus in 1 ml of MEM without serum in a 12-ml plastic tube. The suspension was rotated in a roller wheel at 37 C for 90 min; then the cells were diluted fivefold with warm MEM plus 5% fetal calf serum and 5% pooled human serum, which was to prevent possible reinfection of cells by progeny virions. In some experiments, dilute type-specific viral antisera (obtained from Grand Island Biological Co., Grand Island, N.Y.) were added. Then samples of cells were removed for determination of cell concentration, infectious centers, and cloning efficiency. The remainder of the cells were further incubated at 37 C for the nuclear inclusion assay. A cell count was also performed at the end of this incubation period.

Assay for infectivity. The infectivity was assayed by plaque formation by the method described by Green et al. (8). Infectious centers were assayed by the method of Green and Daesch with slight modifications (6). After adsorption and antiserum treatment when required, the suspension was diluted 200-fold with medium containing no antiserum. Samples of 0.1 to 0.2 ml containing 100 to 1,000 cells were then added to confluent KB cell sheets grown on 60-mm petri dishes (Falcon Plastic, Los Angeles, Calif.). After the suspension had spread evenly, the cells were overlaid carefully with 1 ml of nutrient agar. When the agar had hardened, an additional 4 ml of nutrient agar was added. These cells were then further treated for plaque production.

Cloning of cells. The technique used is essentially that of Puck and Marcus (15). Infected cells were seeded on 60-mm petri dishes (Falcon Plastic) containing 6×10^4 γ -irradiated (5,000 rads) KB cells as feeders. MEM containing 5% fetal calf serum plus 5% pooled human serum was used as the cloning medium. Under these conditions, the colony count represents the true fraction of cells escaping the lethal action of the virus, since noninfected KB cells plated together with or without heavily infected cultures [10 plaque-forming units (PFU)/cell] showed no difference in colony counts. The number of clones was determined by using $5\times$ magnifications after 7 to 9 days of incubation at 37 C in 5% CO₂ and 95% air. Under these conditions, the efficiency of uninfected cells varied from 50 to 70%.

Formation of inclusions. Cells from suspension culture were sedimented by low-speed centrifugation, treated with 1% sodium citrate, fixed with an acetic acid-alcohol mixture (1:3), and stained with orcein. Usually, 500 to 1,000 cells were examined for the presence of nuclear inclusion bodies.

UV irradiation of virus. Adenovirus was suspended in 1.5 ml of MEM and irradiated in a petri dish (35 mm in diameter) at a distance of 10 cm from a General Electric ultraviolet lamp G8T. During irradiation, the virus suspension was stirred continuously with a magnetic bar and kept cool with cold running water (about 10 C) through a metal plate supporting the petri dish. The incident dose rate under these conditions was 8 μ w/mm², as determined by a UV intensity meter.

RESULTS

Time course of inclusion body formation. At various times after infection, the cells were examined cytologically and found to display various degrees of nuclear change. These signs of infection ranged from the appearance of dense nuclear inclusion bodies, chromatin condensation, and micronuclei in the interphase cells to contraction and pulverization of chromosomes in the metaphase plates. Although at the higher input multiplicities all cells showed various degrees of infection, only those cells displaying a dense nuclear inclusion were scored (Fig. 1).

The percentage of cells displaying an inclusion

body at the various times after infection is an underestimate for this function of the virus because of the multiplication of noninfected cells. Consequently, the following correction was applied to the observed percentage of inclusion bodies (IB): $IB (\text{corrected}) = IB (\text{observed}) \times (\text{cell number at the time of observation} / \text{cell number at the time of infection})$.

The time course of the corrected percentage of inclusions is shown in Fig. 2. The percentage of cells displaying an inclusion was maximal at about 48 hr for adenovirus 2 and 72 hr for adenovirus 12 over the range of input multiplicities employed. The decline with time after 48 hr for the adenovirus 2 curve is probably due to a selective loss of cells displaying dense inclusions, since these cells might be more fragile.

Effect of input multiplicity on viral functions. KB cells were infected with different multiplicities of either adenovirus 2 or adenovirus 12, and the percentage of cells capable of forming clones, producing infectious centers, and forming an inclusion body was determined (Table 1 and Fig. 3).

It can be seen that, for the same input multiplicity, more cells were being prevented from cloning than were forming infectious centers or inclusion bodies. The effect was much more pronounced in the adenovirus 12-infected cultures than in those infected with adenovirus 2. The viral function of clone prevention followed the one-hit kinetics predicted from the Poisson distribution (13). The formation of dense inclusions was consistent with one-hit kinetics for low values of input multiplicity (below 0.1 for adenovirus 12 and 0.5 for adenovirus 2), but with higher multiplicities the percentage of cells with an inclusion was substantially less than that predicted from the Poisson distribution. The percentage of inclusions reached a maximum of 40 to 60% for adenovirus 2 and 10 to 15% for adenovirus 12 for different experiments. It is possible that, at high input multiplicities, the large number of nonplaque particles infecting a cell interferes with inclusion body formation.

As shown in Fig. 3, the percentage of infectious centers was substantially greater for adenovirus 2 than for adenovirus 12 when cells were infected with the same input PFU. For adenovirus 2, it reached a maximum of 35 to 73%, similar to values reported previously (6, 12); however, for adenovirus 12, it was only 5 to 10%. To obtain a more quantitative comparison of the different viral functions, part of the data are replotted in Fig. 4, with the fraction of cells surviving that particular viral function plotted versus input multiplicity. Viral functions consistent with one-

FIG. 1. Photomicrograph showing adenovirus-induced inclusion bodies in KB cells. (a) Inclusion body by adenovirus 2; (b) infected by adenovirus 12, but no dense inclusion; (c) inclusion body by adenovirus 12; (d) noninfected cell.

hit kinetics yielded exponential survival curves with a slope equal to the ratio of the number of particles capable of that function to the input multiplicity in PFU. These ratios are shown in Table 2. The ratio of clone-inhibiting units (CIU) to input PFU had a range of 1.1 to 1.9 for adenovirus 2 and 20 to 85 for adenovirus 12. The ratio of infectious-center units to input PFU was less than 1.0 for both viruses. For low input multiplicities, the ratio of inclusion body units (IBU) to input PFU had a range of 0.6 to 0.85 for adenovirus 2 and 0.8 to 3.7 for adenovirus 12. The spread of these ratios reflects, in part, the variation in adsorption efficiency and uncertainty in the determination of input PFU in different experiments. When these variations are eliminated by taking the ratio of CIU to IBU, the reproducibility is substantially better (Table 2). These ratios of CIU to IBU are not the result of storage, since fresh virus preparations gave similar results.

Inhibition of inclusion body formation by UV-irradiated virus. Since some non-plaque-producing virus particles may interfere with inclusion body formation, the addition of UV-irradiated virus

preparations (containing more non-plaque-producing particles) should enhance the interference phenomenon. Adenovirus 2 was irradiated for 400 sec with UV. With this dose, the ability to form plaques was reduced by a factor of 10^{-5} , but the ability to inhibit clone formation was reduced by only 10^{-2} . KB cells were infected with different amounts of UV-irradiated virus together with a constant number of nonirradiated adenovirus 2. At 48 hr after infection, the cells were examined for the presence of an inclusion body (Table 3). Inclusion body formation by the nonirradiated virus was greatly reduced when a sufficient amount of UV-inactivated virus was added. It is unlikely that this reduction is due to a saturation of receptor sites (14) but may be related to those non-plaque-producing particles which can inhibit cell cloning.

DISCUSSION

Purified preparations of adenovirus 2 and adenovirus 12 contain some defective virus particles capable of preventing a cell from cloning

FIG. 2. Time course of inclusion body formation in adenovirus 2- and adenovirus 12-infected cultures. Symbols: ○, adenovirus 2 infected at 0.2 PFU/cell; ●, adenovirus 12 infected at 0.1 PFU/cell.

but unable to induce inclusion bodies or to form plaques. The proportion of such defective particles in adenovirus 12 was about 10 times that in adenovirus 2. It is of interest to note that the particle to PFU ratios found by Green et al. (8) also indicate that adenovirus 12 contains more

non-plaque-producing particles than adenovirus 2. These results suggest that at least some of the non-plaque-producing particles are capable of clone prevention. This capacity is a viral function since it can be inactivated by UV (Table 3). A

FIG. 3. Effects of input multiplicity of adenovirus types 2 and 12 on the formation of inclusion bodies, production of infectious centers, and inhibition of host cell cloning in KB cells. Symbols: ■, clone prevention; ●, inclusion body formation; ○, infectious centers.

TABLE 1. Effects of input multiplicity of adenovirus types 2 and 12 on the formation of inclusion bodies, production of infectious centers, and inhibition of host cell cloning in KB cells

Virus	Input multiplicity (PFU/cell)	Per cent inclusions (48 or 72 hr)	Final cell concn per ml ^a	Per cent inclusions corrected	Per cent infectious centers	Per cent surviving clones
Adenovirus type 2	0	0	6.90×10^5	0	0	100
	0.09	3.8	5.64×10^5	10.2	5.5	87.5
	0.85	26.8	2.99×10^5	38.0	28.2	19.9
	4.25	21.5	2.41×10^5	21.5	45.3	1.8
	85.0	16.0	1.87×10^5	16.0	73.0	0
Adenovirus type 12	0	0	5.15×10^5	0	0	100
	0.12	8.4	2.18×10^5	10.7	1.3	7.5
	1	6.6	1.80×10^5	7.0	4.1	0
	4	4.6	1.79×10^5	4.8	4.9	0
	16	7.6	1.72×10^5	7.6	5.3	0

^a Initial cell concentration was 2.1×10^5 cells/ml for adenovirus 2 and 1.7×10^5 cells/ml for adenovirus 12.

FIG. 4. Replot of data from Fig. 3 to obtain a quantitative comparison of the different viral functions.

TABLE 2. Relative ratios of virus particles capable of different functions in adenovirus 2 and adenovirus 12

Ratio ^a	Adenovirus 2	Adenovirus 12
CIU/input PFU ^a	1.1-1.9	21-85
IBU/input PFU (low MOI) ^b	0.65-0.85	0.8-3.7
CIU/IBU (low MOI)	1.7-2.4	17.5-26
ICU/input PFU	<1	<1

^a Range for three experiments.

^b Multiplicity of infection.

TABLE 3. Inhibition of inclusion body formation by UV-irradiated adenovirus type 2^a

Nonirradiated virus (PFU/cell)	UV-irradiated virus ^b		Per cent inclusions
	Surviving PFU/cell	Surviving CIU/cell	
2	0	0	47.5
2	2×10^{-5}	2×10^{-2}	49.2
2	2×10^{-4}	2×10^{-1}	37.9
2	2×10^{-3}	2	32.6
2	2×10^{-2}	20	13.1
0	2×10^{-2}	20	13.4

^a KB cells (10^6) were infected with 2×10^6 PFU of nonirradiated adenovirus 2 and different amounts of UV-irradiated adenovirus 2.

^b UV treatment for 400 sec. Surviving fraction: 10^{-5} for PFU; 10^{-2} for CIU.

single particle appears to be sufficient for this function, similar to the situation found for Newcastle disease virus (13). Defective virus particles which possess limited viral functions have

also been found in other animal viruses (10, 17). It is possible that the large ratio of particles to PFU in animal viruses may, in part, be the result of heterogeneity in the virus population. However, the effects of a biological inefficiency in plaque formation cannot be completely ruled out. It is possible that, in a homogeneous population, only a fraction of the virus particles can complete the complex process of plaque formation.

At low input multiplicities, the formation of inclusion bodies by adenoviruses may be used for virus titration as in Shope Fibroma virus (9). The failure of the inclusion body formation to follow "single-hit" kinetics at high input multiplicities may be due to interference by large numbers of non-plaque-producing particles infecting a cell. This is consistent with the observation that adenovirus 12-infected cultures produce a very low maximum percentage of cells containing an inclusion body, since adenovirus 12 has a much higher proportion of defectives. Furthermore, the inhibition of inclusion body formation by UV-irradiated virus lends support to this hypothesis. It is not known whether the 10% maximum infectious centers in adenovirus 12-infected cultures is due to the same interference phenomenon or to a difference in the biological efficiency of infectious center formation. High multiplicities of virus infection have been shown to reduce the yield of total infectious virus (2, 3) and infectious centers (12). The interference may be the result of competition or destruction of the host capacity to support virus growth by the defective particles.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Cancer Institute of Canada and the National Research Council of Canada. A. J. R. was a Research Fellow of the National Cancer Institute of Canada.

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