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Comparative Studies on Functions of Human Adenovirus Type 12 and Its Low Oncogenic Mutant Virions

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Physical and biological properties of highly oncogenic human adenovirus type 12 were compared with a low oncogenic mutant (cyt mutant). Parental and cyt mutant virions had very similar density and DNA size. However, the parental strain virion preparations contained a much higher proportion of defective virions (capable of cell killing, but not able to induce T- or V-antigen) than cyt mutant stock. It was also found that cyt mutant had a reduced virus yield in several human cell lines compared with the parental strain.

Viruses of the same serotype having different oncogenic potentials should be useful in understanding the mechanism of viral oncogenesis. Human adenoviruses consisting of 31 types can be broadly classified into three groups: highly oncogenic, weakly oncogenic, and non-oncogenic. Extensive comparative studies on the physical and chemical properties of purified virus preparations have been made (5). Some biological functions, such as induction of cellular DNA synthesis (12) and induction of chromosome aberrations, have also been compared (13). However, the lack of substantial homology in the viral DNA belonging to different groups makes it difficult to determine the viral gene functions essential for oncogenesis (5). Adenovirus type 12 (Ad12) can induce tumors in newborn hamsters and transform cells in vitro (2, 17). Takemori et al. have isolated low oncogenic mutants (cyt mutants) from Ad12 (15). These mutants produce large clear plaques on human embryonic kidney (HEK) cells compared with the small fuzzy-edged plaques produced by the parental type. Cells infected with the mutants show a different cytopathic effect, accompanied by a rapid marked cellular destruction. Yamamoto et al. (20) have independently isolated a similar class of mutants. Although some biological properties have been compared, no studies on the physical properties of virions have been made. We have purified preparations of a cyt mutant, compared their physical and biological properties with those from the parental strain, and found that the parental strain virus stock contains a much higher proportion of defective virions than the cyt mutant stock.

MATERIALS AND METHODS

Cells and viruses. Human KB, HEp-2, and HEK cells were routinely grown as monolayers in 32-oz (ca. 907.2 ml) prescription bottles. The growth medium was Eagle minimal essential medium supplemented with 10% fetal calf serum plus streptomycin and penicillin. Cyt mutant (cyt 129 of Takemori et al. [15]) was obtained from H. F. Stich. The parental strain virus was propagated in KB cells in suspension and was purified by using the methods of Green and Pina (6). For the propagation of cyt mutant, KB cells were infected in suspension but incubated on monolayers to minimize cellular destruction. After 40 h, the infected cells were harvested, and the virus was purified using the same procedure as for the parental virus.

Adsorption. ^{3}H-labeled virions were obtained by adding [^{3}H]thymidine to infected cultures during incubation. HEp-2 cells (10^6) were infected in suspension with the purified ^{3}H-virions in 1 ml of minimal essential medium supplemented with 0.5% fetal calf serum. After 90 min of incubation at 37 C, the cells were sedimented by low-speed centrifugation. A sample of the supernatant was added to nitrocellulose filter. After drying, the radioactivity was determined in 5 ml of toluene containing 0.02 g of Omnifluor (New England Nuclear). After the cells were washed with phosphate-buffered saline, radioactivity associated with them was also determined.

Virus yield analysis. KB, HEp-2, and HEK cells were infected in suspension with about 100 virus particles per cell and incubated on a monolayer at 37 C. At 12 h after infection, [^{3}H]thymidine was added to a final concentration of 10 μCi/ml (specific activity, 4.7 μCi/μg). At 40 or 72 h after infection, cells were harvested and treated with deoxycholate and fluorocarbon (Freon 113). After low-speed centrifugation, the supernatant was analyzed for virus content by using rate zonal centrifugation as described previously (9). Radioactivity sedimented in the position of marker (purified [^{14}C]Ad2 virion) was taken as an estimate of virus yield.

Cloning of cells. The ability to form a clone was used as a criterion for cell survival after infection. HEp-2 cells infected in suspension were seeded on 60-mm Falcon plastic dishes containing minimal essential medium supplemented with 15% fetal calf

serum, 2% vitamin solution, and 2% amino acid mixtures (Gibco). Antiserum against Ad12 virions from immunized rabbits was added (0.3%) to prevent reinfection by progeny viruses. The number of clones was determined after 7 to 8 days of incubation. Under these conditions, the plating efficiency of noninfected cells varied from 90 to 100%.

Immunofluorescence techniques. Infected HEp-2 cells grown on cover slips were washed with phosphate buffered saline, air-dried, and fixed with carbon tetrachloride. The indirect immunofluorescence technique was used to detect the presence of Ad12-specific T-antigen and V-(structural) antigen (10). The source of anti-T antiserum was obtained from hamsters bearing Ad12-induced tumors. The source of antiserum against V-antigen was from rabbits immunized with purified Ad12. Fluorescein-conjugated antisera against hamster and rabbit gamma globulin was purchased from Roboz Surgical Instrument Co.

RESULTS

Growth, purification, and yield of virus from cyt mutant. Takemori et al. originally grew the cyt mutants in HEK cells (15). To facilitate the characterization of the virus, relatively large amounts of purified virus would be necessary. Because of the convenience, we have attempted to cultivate this mutant virus by using KB cells in suspension. However, these attempts were not successful due to early cell lysis. Cell number in a culture infected with the parental Ad12 remained relatively constant for at least 48 h. In contrast, cell number in cultures infected with cyt mutant began to decrease at about 24 h after infection, leading to complete cell lysis by 72 h. This confirms the cytocidal nature of this mutant, as reported previously (15). Cell lysis was avoided by infecting the cells in suspension and subsequently incubating them on monolayer cultures. Cyt mutant virus could then be purified from these cells.

Amount of virus particles produced in KB cells infected with either cyt mutant or the parental strain Ad12 was compared. It can be seen from Table 1 that cells infected with cyt mutant produced only 10 to 20% of that produced by parental virus infection. Cytological observations of the cells showed that all the cells in both cultures were infected. The low virus yield by the mutant may be due to a poor recovery in the purification procedure or production of fewer virions by the infected cells. To distinguish these alternatives, the following method of estimating the total number of virions from infected cells without purification was employed. The intracellular virus was labeled with [^{3}H]thymidine and subsequent quantitation by rate zonal centrifugation after the virus was dissociated from the cells. Sufficient [^{3}H]thymidine (2 μg/ml) was added to ensure its continued availability and to minimize the possible differences in precursor pools in different infected cultures. The amount of radioactivity sedimented at the position of complete virions should give an estimate of the number of virions produced. Figure 1 shows typical profiles of ^{3}H activity from cells infected with either parental or mutant virus. These data confirm the observation that cyt mutant-infected KB cells produce fewer virions.

It is still possible that mutant virions are more easily disrupted by sonication and fluoro-

TABLE 1. *Virions produced by KB cells infected with Ad12 parental strain or cyt mutant*

Virus	Virions produced/10^9 cells[a]	
	Passage 1[b]	Passage 2[b, c]
Parental	6.0×10^{12}	9.0×10^{12}
cyt mutant	1.1×10^{12}	9.0×10^{11}

[a] Determined by optical density (OD) at 260 nm (1.0 OD = 3.5×10^{11} virions/ml [8]).

[b] KB cells were infected with 300 virions/cell.

[c] Passage 2 resulted from cells infected with purified virions from Passage 1.

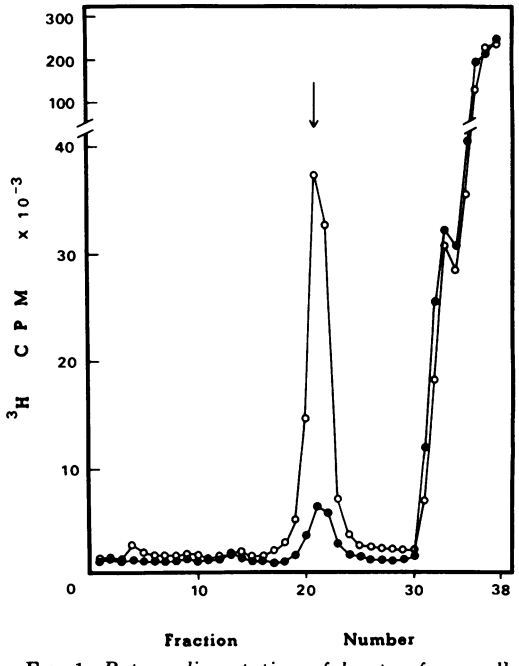

FIG. 1. *Rate sedimentation of lysates from cells infected with Ad12 virions in a 5 to 30% sucrose gradient. Sedimentation is from right to left. Symbols:* (○), *KB cells infected with parental strain of Ad12;* (●), *KB cells infected with cyt mutant. Arrow indicates the position of* [^{14}C]*Ad2 virions as a marker.*

carbon treatment. Deoxycholate treatment instead of sonication has been used for purification of adenovirus type 5 (7). Therefore, various methods of releasing the virus from ^{3}H-labeled cells were compared. The amount of radioactivity sedimenting as complete virions in a sucrose gradient was similar whether the cells were disrupted by (i) deoxycholate, (9×10^3 counts/min), (ii) deoxycholate plus fluorocarbon (10.2×10^3 counts/min), or (iii) sonication plus fluorocarbon treatment (8.3×10^3 counts/min).

Due to the cytocidal nature of the mutant, virions may be released into the medium. The amount of extracellular virus was determined by [^{3}H]thymidine labeling of the infected cells, sedimentation of the extracellular virions onto a CsCl cushion, and subsequent CsCl density equilibrium centrifugation. Table 2 shows that only a small percentage of the total virions were found extracellularly both at 40 and 72 h after infection under our experimental conditions. It can be concluded that KB cells infected with the cyt mutant produced fewer virions than cells infected with the parental strain.

Virus yield from different human cells. It has been reported that some cyt mutants cannot grow in a subline of KB cells (16). Therefore, we examined whether the low yield was due to the degree of permissiveness in our KB cells. KB, HEp-2, and HEK cells were infected with cyt mutant virus and labeled with [^{3}H]thymidine, and the amount of virions was determined by sucrose gradient centrifugation. Different cell lines gave different amounts of radioactivity associated with virions (Table 3); however, there was a similar reduction in virus yield in cyt mutant-infected cultures independent of cell types used. Thus, it appears that this defect is probably a function of the virus.

TABLE 2. *Amount of virions released into the culture medium from infected KB cells*[a]

Time after infection (h)	Total counts/min in virion band $\times 10^{-3}$			
	Parental strain		cyt mutant	
	Cell-associated	In the medium	Cell-associated	In the medium
40	1,520	2	310	17
72	2,200	5	390	27

[a] Amount of virions was determined by [^{3}H]thymidine labeling together with sucrose gradient sedimentation analysis (see Materials and Methods).

TABLE 3. *Amount of virions produced by different human cell lines and HEK cells*[a]

Cell types	Total counts/min in virion band $\times 10^{-3}$		Yield ratio (cyt mutant-parental)
	Parental Ad12	cyt mutant	
KB	102	21	0.21
HEp-2	15	3.9	0.26
HEK	332	77	0.23

[a] Amount of virions was determined as in Table 2.

Properties of the cyt mutant virions. (i) Physical properties. The sedimentation profile of cyt mutant virion DNA in alkaline sucrose gradient was essentially identical to that of the parental virion DNA (data not shown). Density of cyt mutant and parental virions in CsCl is not different (Fig. 2). Under the experimental conditions used, a difference of 10% in DNA size or a difference of 5% in DNA-to-protein ratio could be detected. Thus, the amount of protein and DNA is probably the same for both the parental and mutant virions.

(ii) Synthesis of T- and V-antigens. The time course of development of virus-coded antigens in infected HEp-2 cells was studied by using immunofluorescence. Figure 3 shows that T-antigen was detected about 3 to 4 h after infection, whereas V-antigen was not detected until about 15 h after infection. It can also be seen that the kinetics for the formation of the antigens was similar whether the cells were infected with the parental strain or the mutant. Thus, the cytocidal effect on cells is probably not due to the accelerated development of the mutant compared with the parental strain.

(iii) Functional heterogeneity of the virions. Particle-to-PFU ratio for animal viruses is generally greater than unity. Furthermore, within a virus preparation, some virions are defective in that they are capable of limited viral functions (1, 8, 18, 19). The proportion of defectives in parental virion preparations was compared with that in cyt virion preparations by assaying several viral functions (cell killing, induction of T- and V-antigens in infected cells).

To ensure that cells in the same physiological state and under identical infection conditions were used for different viral functions, all these functions were assayed with cells from the same infected cultures. After HEp-2 cells in suspension were infected with virus, a sample was removed and assayed for cell survival by cloning. The remainder of the culture was then seeded onto cover slips. At 48 h after infection, the cells were prepared for immunofluorescence. The percentage of cells capable of forming clones and producing T- and V-antigen was determined for different virus input multiplici-

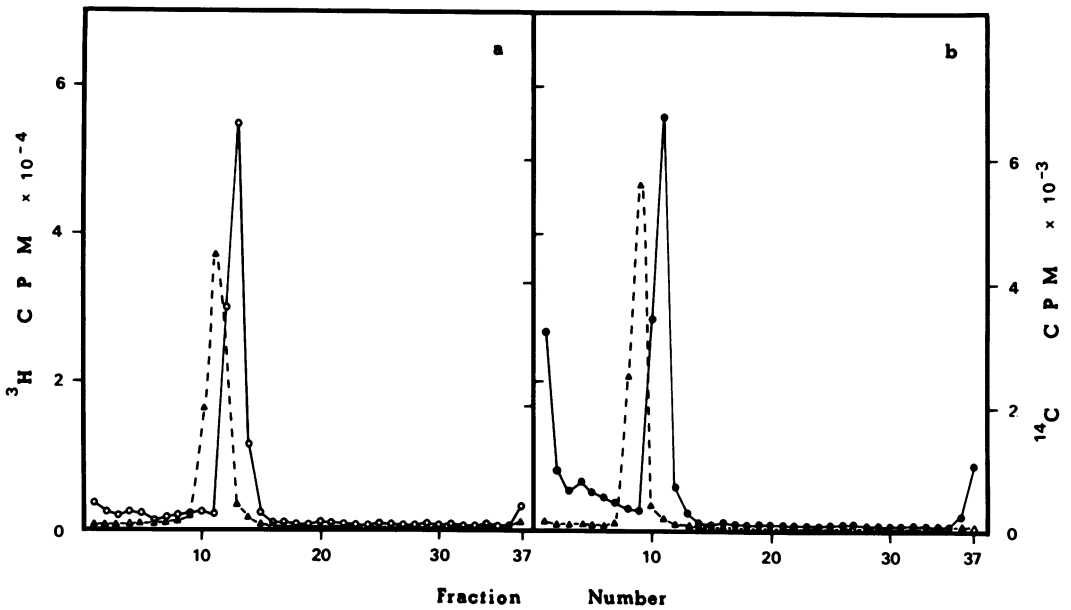

FIG. 2. *Distribution of purified [^{3}H]Ad12 virions in a cesium chloride density gradient. [^{3}H]Ad12 virions were mixed with [^{14}C]Ad2 virions (as marker) and centrifuged at 33,000 rpm for 20 h at 5 C in a 50Ti rotor (Beckman). Radioactivity in the fractions was assayed after trichloroacetic acid precipitation. Sedimentation is from right to left. (a) Parental virions; (b) cyt mutant. Symbols: (○), parental strain; (●), cyt mutant; (Δ), [^{14}C]Ad2 marker.*

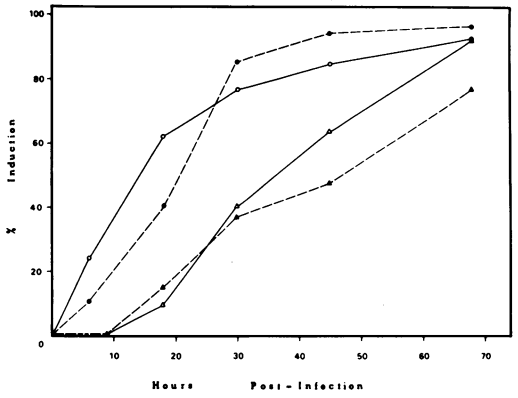

FIG. 3. *Time course of T- and V-antigen synthesis in Ad12-infected HEp-2 cells. Symbols: (●), T-antigen by cyt mutant; (○), T-antigen by parental strain; (▲), V-antigen by cyt mutant; (Δ), V-antigen by parental strain.*

TABLE 4. *Effects of input multiplicity of Ad12 parental strain and cyt mutant on the induction of T- and V-antigens and inhibition of host-cell cloning of HEp-2 cells*

Virus	Input multiplicity (virions/cell)	T-antigen[a] (%)	V-antigen[a] (%)	Surviving clones (%)
Parental strain	0	0	0	100
	5	19.9	23.0	65.8
	25	30.4	56.1	1.98
	50	63.6	74.0	0.55
	100	92.7	89.7	0.086
	500			0.017
cyt	0	0	0	100
	5	6.4	19.8	90.8
	25	40.8	34.8	31.5
	50	70.7	66.1	8.93
	100	87.7	90.6	1.45
	500			0.24

[a] Assayed at 48 h after infection.

ties (Table 4). It can be seen that, for the same input multiplicity, the number of cells positive for T- and V-antigens was less than the number of cells killed. This effect was much more pronounced in cultures infected with parental strain than with cyt mutant. To quantitate the number of input virions necessary for a particular function, the data are shown in Fig. 4, with the fraction of cells surviving that particular viral function plotted against input multiplicity. Survival for various functions follows a single-hit kinetics, as predicted by the Poisson destribution. Table 5 shows the input multiplicity of parental and mutant virus to give 63% positive response (on the average, 1 U per cell) for the various functions. It can be seen that in

both cases there are more virions capable of killing cells than of inducing T- and V-antigens. The parental type contains a much higher proportion of defectives capable of cell killing only.

Adsorption of virus to host cells. It requires four to six times more cyt mutant virions than parental virions to give a 63% positive response for cell killing (Table 5). This difference may be a reflection of differential adsorption efficiencies, alternately, that the virus can adsorb, but insufficient viral genes are expressed to cause cell killing. By using purified radioactively labeled virions, adsorption efficiency of the virus to HEp-2 cells was found to be about 20% under the experimental conditions used (Table 6). It can be seen that there was no difference in adsorption efficiency between cyt mutant and parental virions. Similar results were obtained when KB cells were used instead of HEp-2 cells. These data suggest that a very high proportion of the virions from the parental strain can express the viral function of cell killing. On the other hand, many cyt virions may not express sufficient viral functions to cause cell death.

DISCUSSION

Takemori et al. (15) isolated a number of mutants (cyt mutants) from the highly oncogenic human adenovirus type 12. They produce

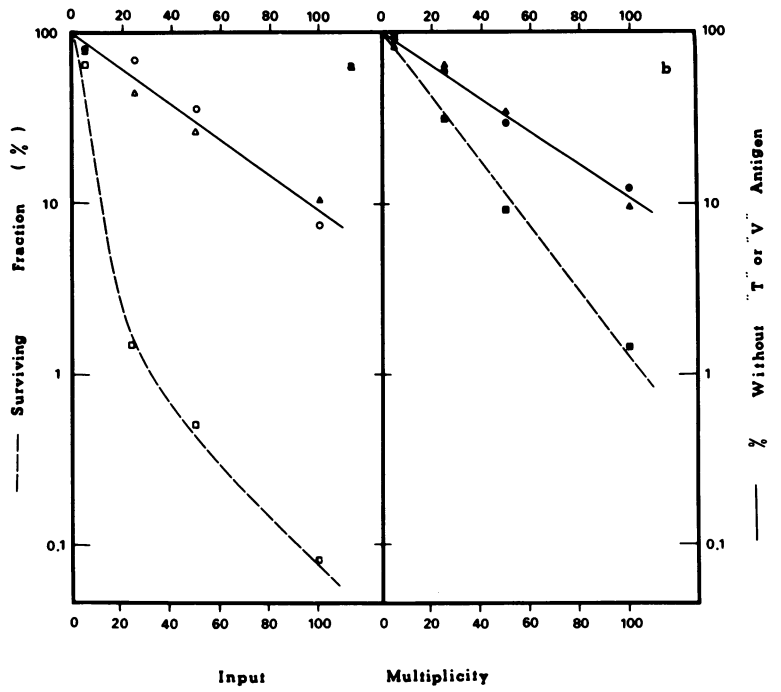

FIG. 4. *Effects of input multiplicity (virions/cell) on cell killing and induction of T- and V-antigens in HEp-2 cells. The ordinate represents the percent of cells not showing that particular viral function. (a) Parental strain; (b) cyt mutant. Symbols:* (■, □), *clone formation;* (●, ○), *T-antigen induction;* (▲, △), *V-antigen induction.*

TABLE 5. *Number of input virions per cell necessary to induce positive response in 63% of the cells in an infected culture of HEp-2 cells*

Virus	Virus preparation no. 1				Virus preparation no. 2			
	Cell killing	T-antigen induction	V-antigen induction	Ratio of V-antigen induction to cell killing	Cell killing	T-antigen induction	V-antigen induction	Ratio of V-antigen induction to cell killing
Parental strain	6	41	40	6.7	5	33	36	7.2
cyt mutant	23	44	45	1.9	30	46	48	1.6

TABLE 6. *Adsorption of [^{3}H]Ad12 parental virions and cyt mutant virions to HEp-2 cells*[a]

Virus	Input multiplicity (virions/cell)	Total counts/min added	Cell-associated counts/min	Counts/min remaining in supernatant	Per cent absorbed
Parental	500	29,613	5,830	24,800	19.7
	100	7,733	1,305	6,610	16.9
cyt mutant	500	8,020	1,275	6,540	15.9
	100	1,840	425	1,475	23.1

[a] Adsorption period was 90 min.

marked cellular destruction of infected human cells. Some of these mutants have low oncogenicity. We have studied the physical and biological properties of cyt mutant virions (cyt 129) and compared them with those of the parental virus. Incubation of KB cells infected with the mutant virus in suspension led to complete cell lysis, in contrast to the behavior of KB cells infected with parental strain. Destruction of KB cells could be prevented by incubating the infected cells on monolayers, suggesting that the cells have increased fragility rather than cell lysis per se. The mutation also leads to a low yield of virus from infected human cells. This reduction in yield is due to extensive degradation of intracellular viral DNA (H. Ezoe and S. Mak, manuscript in preparation).

Purified cyt virions do not show any large difference in density in CsCl or the size of the viral DNA. Thus, the mutation probably does not lead to gross changes in the virus structure. Although both parental and mutant virion populations show sharp bands in CsCl density gradients (see Fig. 2), there are functional differences among the virions. In the purified virus preparations, there are more particles capable of cell killing but not of induction of T- and V-antigens. Similar results were found by Strohl by using crude virus stock of Ad12 on nonpermissive BHK cells (14). He suggested that an infected cell synthesizing T-antigen is likely to be killed. Our data indicate that T-antigen synthesis is not necessary to inhibit a cell from forming a clone. Thus, the viral genes responsible for cell killing are not identical with those for T-antigen synthesis.

Data presented in this report show that there is a greater degree of functional heterogeneity in the parental virus preparations than in the mutant virion preparations. The basis for the formation of these defectives is not known. It is of interest to note that nononcogenic Ad2 also shows a low degree of heterogeneity compared with Ad12 (11). It has been suggested that defective virions have a higher oncogenic potential and transforming ability than nondefective virus (3, 4). Although both the nononcogenic Ad2 and low-oncogenic cyt mutant of Ad12 contain fewer defectives, the exact relationship between the degree of defectiveness of a virus preparation and its oncogenic potential is not known.

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