Fate of Adenovirus Types 2 and 12 in Infected Serial Cultures of Non-primate Origin

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SUMMARY
In RHF-1 cells infected with either adenovirus 2 or 12, the formation of infectious virus and antigens decreased with each successive passage of cells until the virus was ultimately eliminated from the cultures. These cultures then emerged into a new phase in which some virus-induced proteins were present in at least a small proportion of cells. Adenovirus 2 fibre antigen persisted throughout the 15th subculture, whereas adenovirus 12 early (T) and late (fibre) antigens were carried throughout the 30th subculture over a period of 600 days. Virus-free but antigen-containing cells may therefore have possessed at least a portion of the virus genome. Shortly after the disappearance of virus, distinct multilayered foci of cells emerged in both lines. This phenomenon became a characteristic feature of the cultures only in the adenovirus 12 line.

INTRODUCTION
Efforts to establish persistent adenovirus infection of human cells in vitro have been previously described (Ginsberg, 1958; Van Hoosier et al. 1964); the success of these systems depended upon the presence of anti-virus antisera and other factors in the medium. Similar mechanisms operating within these cultures may be responsible for the continued survival of both virus and cells. In another system, the interaction between adenovirus 12 and a line of human embryonic lung cells led to a gradual disappearance of virus after prolonged serial cultivation (Sultanian & Freeman, 1966). However, by an unknown mechanism, cell transformation occurred in these cultures.

No reports have as yet appeared regarding persistent adenovirus infection in non-primate cells. A line of rabbit heart fibroblasts (RHF-1) is capable of supporting a slow but productive adenovirus infection (Ankudas & Khoobyarian, 1962). We thought that a model of this sort might pinpoint differences in the behaviour of an oncogenic and a non-oncogenic adenovirus. Although the experiments performed did not produce evidence for persistent infection, they did reveal that both type 2 and 12 adenoviruses can ultimately be eliminated from the continuous serial cultures with the retention of some virus antigens.

METHODS
Viruses and cells. The prototype strains of adenovirus types 2 and 12 (strain HUE) were propagated frequently in Hep-2 or KB cells before use. Stock cultures of rabbit heart cell line, RHF-1 (Khoobyarian & Palmer, 1961) and primary or secondary human embryonic

Kidney cells were routinely grown in medium 199 + 8% calf serum + 0.1% lactalbumin hydrolysate. All RHF-1 cells used in these studies were first subcultured at least twice in N-16 medium (Puck, Cieciura & Robinson, 1958) supplemented with 8% calf serum before use.

Infection of cultures. Monolayer cultures of RHF-1 cells grown in N-16 medium in 3 oz. prescription bottles (2 to 3 × 10⁶ cells/bottle) were inoculated with known concentration of either type 2 or 12 adenovirus and incubated at 37°C for 6 hr. After virus adsorption, the monolayers were washed twice in N-16 medium and overlaid with fresh growth medium (10 ml./bottle) and incubated at 37°C for 4 to 6 days. During this time the medium was changed at least twice before the first subcultures were made. Samples of packed cells and medium taken from the infected lines at various subcultures were stored at -20°C until assayed for infectious virus and virus antigens.

Infectivity assays. Primary or secondary monolayer cultures of human embryonic kidney cells in tubes were inoculated with 1 ml. of 0.5 log. dilutions of the test fluids, using 4 tubes/dilution, and incubated at 37°C for 20 to 28 days. The highest dilution of virus giving 1 + CPE or a single plaque was considered positive. The infectivity titres (TCD₅₀/ml.) were determined by the method of Reed & Muench (1938). Cultures negative for infectious virus were handled in two ways. Materials from the inoculated cell cultures (medium and cell extracts) were blindly passaged at least once in fresh cells which were observed for signs of virus damage; and intact cells were added to cells and the cultures observed for virus damage over a period of 21 days. The cultures were considered negative when a combination of these procedures failed to reveal the presence of virus.

Antiserum preparations. Antisera to virus were produced in adult New Zealand white rabbits as follows: an initial intravenous injection of 3 to 5 ml. of adenovirus type 1, 2 or 12 preparation was followed by intraperitoneal injection of 5 ml. at weekly intervals for a total of 8 to 10 weeks. Adenovirus materials for inoculations were derived from extracts of infected Hep-2 cells which had been either homogenized with fluorocarbon or precipitated with methanol (30%) and resuspended in phosphate-buffered saline (PBS), pH 7.2. Antisera against the adenovirus structural antigens (hexon and fibre) were prepared by injecting adult rabbits with three or four intramuscular doses of three times chromatographed antigen material in Freund's complete adjuvant at weekly intervals. The last intramuscular injection was followed 1 week later by one intravenous or intraperitoneal injection without adjuvant. All sera were collected 10 to 14 days after the final injection, inactivated at 56°C for 30 min. and stored at -70°C in small volumes. Sera conjugated with fluorescein isothiocyanate, from hamsters bearing tumours induced by adenovirus type 12, were bought.

Haemagglutination tests. Haemagglutination tests for adenovirus particles or their structural antigens were performed according to the methods described by Pereira & de Figueiredo (1962) and Schmidt, King & Lennette (1965). Adenovirus type 1 rabbit antiserum diluted 1/100 to 1/200 was used as a source of heterotypic serum.

Complement-fixation tests. Complement-fixation tests (CF) were performed in tubes by the method of Hilleman & Werner (1954), except that the volumes of reagents were doubled. All antisera were absorbed on Hep-2 and RHF-1 cell monolayers for one hr each at 37°C before use. Two full units of complement and four to eight units of antibody were used. CF titres were expressed as the highest dilution of antigen giving complete fixation.

Chromatographic separation of virus antigens. The adenovirus types 2 and 12 structural antigens were separated from the infected cells on DEAE-cellulose columns according to the methods described by Klemperer & Pereira (1959), and Hollinshead & Huebner (1966). The cell extracts were ultracentrifuged three times each at 105,000 g for 90 min. to remove
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virus particles. Following this, the supernatant fluids were vacuum-dialysed against starting buffer (0.01 M-phosphate buffer, pH 6.8 to 7.2) and then applied to columns of DEAE-cellulose or DEAE Sephadex A-50 equilibrated with the same buffer. The antigens were eluted by stepwise gradients from 0 to 0.70 M-NaCl in starting buffer. The eluates were examined for antigenic content by complement-fixation and haemagglutination tests using antisera to virus particles and partially purified antigens.

Preparation of antisera for immunofluorescent staining. All antisera were precipitated with 34 to 50% saturated ammonium sulphate overnight in the cold. The precipitated globulins were dialysed extensively against PBS (pH 7.2); the protein content of the globulins was adjusted to about 1 g./100 ml. and then conjugated with fluorescein isothiocyanate. To remove non-specific elements, the labelled globulins were individually absorbed on acetone-fixed Hep-2 and RHF-I cell monolayers for 1 hr each at 37°C. After appropriate testing, the stock preparations of the labelled globulins were diluted in such a way (four to eight units of staining titre) that they would, when applied, ensure the detection of antigens.

Immunofluorescent test. The coverslip cultures of normal and infected lines were rinsed three times in PBS (pH 7.0), air dried and fixed with acetone for 5 to 10 min. at room temperature. Coverslips were cut into two equal parts, and the cells were flooded with the desired antisera. The cells plus conjugate were allowed to react overnight or for 2 hr in a humidified chamber at 37°C, when they were washed three times with PBS, and then mounted in either 1/10 dilution of glycerine in PBS or in Unimount (Brunswick Co.). A Zeiss fluorescence microscope was used for observations and photography. At least 1000 cells/coverslip (five to ten fields/coverslip) were examined on duplicate coverslips. The percentage of positive cells was determined by dividing the average number of fluorescent cells/field by the average total number of cells/field.

RESULTS

General description

To devise conditions for establishing a long-term serial culture of adenovirus-infected cells, a series of preliminary experiments was performed. For each adenovirus type, replicate cultures of actively growing RHF-1 cells were exposed to a wide range of virus multiplicity (5 to 80 TCD50/cell) at 37°C until 50 to 75% of the cell population showed virus changes, or until more than 80% of them contained virus antigens, as determined by immunofluorescence. At this juncture, a large number of first subcultures were initiated. Briefly, cultures infected with either virus type at a multiplicity of 60 or 80 TCD50/cell could not be maintained in the first passage, since too few cells remained attached to the glass. With 35 to 50 TCD50 of virus/cell, at least 40 to 50% of the infected cells survived the first serial cultivation and virus of the order of 10^6 TCD50/ml was present in the cultures. With lower virus input there was a proportional increase in cell survival and a comparable decrease in virus concentration in the first passage. A set of conditions was established which usually led to the maintenance of infected cells in serial passage. The principal conditions were as follows: (1) actively growing cultures were exposed to type 2 or 12 adenovirus at an input multiplicity of 10 to 50 TCD50/cell; (2) the first subculture was made when not more than 75% of the cell population showed virus cytopathic effects; (3) cells were subcultivated in high concentration (equivalent to at least the level of initial cell concentration) during the first three or four passages to facilitate monolayer formation; and (4) cultures were serially subcultivated every 18 to 20 days.
Detection of infectious virus and virus antigens in serial cultures

Infected lines of RHF-1 cells, initially exposed to one of five different input multiplicities of either type 2 or 12 adenovirus (5, 10, 20, 25, 35 and 50 TCD50/cell), were examined at each passage for the presence of infectious virus and virus antigens. Regardless of the initial multiplicity of virus used, adenovirus type 12 titres declined sharply during the first 3 subcultures and less dramatically during the following four passages (Fig. 1). There was also a gradual decrease in the amounts of hexon and fibre antigens through the first seven serial passages (Fig. 2). Beginning with the 8th passage and continuing until the 30th, the cultures were devoid of infectious virus but contained small but detectable quantities of type 12 fibre antigen. With the exception of samples taken from the first subcultures of the lines initially exposed to 35 or 50 TCD50 of virus/cell, no penton-reacting materials could be detected in HA tests in the subsequent subcultures.

A similar phenomenon was evident in the lines infected with adenovirus 2 (Fig. 3). No infectious virus was detectable beyond the sixth passage but the next nine subcultures contained detectable fibre antigen (Fig. 4). Beyond the 15th passage, the cultures were devoid of demonstrable fibre antigen. Penton antigen, on the other hand, was detected in the first four subcultures; but its presence could not be confirmed in the same or the subsequent samples when such samples were frozen and thawed a few times. For this reason, penton antigen titres were not included in Fig. 4.

In view of the similarities observed between the two adenovirus models, the subcultures containing infectious virus were designated as Phase I cultures, while those in which infectious virus was undetectable but in which virus antigen persisted throughout several generations were referred to as Phase II cultures.
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Chromatographic separation of structural antigens from serial cultures

Chromatographic studies were made to confirm the identity of the adenovirus structural antigens in cells of Phase I and Phase II cultures. Subculture materials of various passage levels from both infected lines (initially exposed to 50 TCD50/cell) were subjected to chromatographic separation on DEAE-cellulose columns. Representative materials from the first and third passage levels of adenovirus 2 Phase I cultures and of the sixth, ninth and fourteenth subcultures of Phase II cultures were analysed in detail. Because there were similarities in elution profiles between first and third and between sixth and ninth and fourteenth subculture materials, the chromatographic profiles and the CF values of only the first and ninth subculture materials are shown in Fig. 5. Similarly, the fractionation results of materials from the first and ninth passage levels of adenovirus 12 line, representing Phase I and Phase II cultures respectively, are presented in Fig. 6.

Two major peaks were found in materials from Phase I cultures of the adenovirus 2 line (Fig. 5). The first peak eluting between 0.05 and 0.075M-NaCl reacted specifically to a high titre in CF test with antisera to fibre antigen (panel a). The second peak eluting between the 0.15 and 0.30M-NaCl range reacted equally well with hexon antisera (panel b). Although penton antigen, as shown by HA test, was present in the 0.20M-NaCl fractions, the fractions eluting at 0.30M-NaCl contained traces of this antigen during the first cycle. However, there was no detectable penton antigen in the rechromatographed 0.30M-NaCl fractions.

On the other hand, fractions of materials from Phase II cultures showed one predominant peak which eluted between 0.025 and 0.074M-NaCl and which contained only fibre antigen (panel c).
Materials from Phase I cultures of the adenovirus 12 line showed two peaks (Fig. 6). The fractions eluting between 0.025 and 0.075 M-NaCl contained hexon antigen (panel a); those eluting between 0.125 and 0.175 M-NaCl had fibre antigen (panel b). The fractions separated from Phase II cultures of the adenovirus 12 line reacted only with type 12 anti-fibre antisera (panel d). Occasionally, separation of materials from Phase II cultures of the both infected lines resulted in minor peaks which failed to react specifically with antisera to respective adenovirus types or to their structural antigen. Whether or not this finding had any significance in these models was not investigated.

**Fig. 5**
Fractionation of adenovirus 2-infected cell pack samples, taken from serially passaged lines, by rechromatography on DEAE-cellulose columns using stepwise elution with increasing concentrations of NaCl. a and b, samples taken from Phase I cultures. c and d, samples taken from Phase II cultures. ——, $E_{260}$; ●●●, hexons; ○—○, fibres.

**Fig. 6**
Fractionation of adenovirus 12-infected cell pack samples, taken from serially passaged lines, by rechromatography on DEAE-cellulose columns using stepwise elution with increasing concentrations of NaCl. a and b, samples taken from Phase I cultures. c and d, samples taken from Phase II cultures. ——, $E_{260}$; ●●●, hexons; ○—○, fibres.

**Immunofluorescence of virus antigens in serial cultures**

To measure the proportion of cells synthesizing virus antigens, cells from various passages of both infected lines (initially exposed to 50 TCD50/cell of virus) were stained with specific fluorescein-labelled globulins. Since the number of cells containing visible amounts of each virus antigen was, in the main, greater at 20 days of incubation than earlier, only the results of the 20 day analysis are presented in Fig. 7. In these experiments, the first five and seven passage series of adenovirus 2- and 12-infected lines, respectively, constituted the Phase I cultures. The percentage of cells containing adenovirus type 2 or 12 hexon and fibre antigens was steadily reduced in the passage series of Phase I cultures (panel a, b). While only adenovirus 2 fibre antigen persisted in several passages of Phase II cultures, its presence was no longer visible beyond the 15th passage (panel a). On the other hand, the proportion of cells containing adenovirus 12 fibre antigen remained relatively constant (20 to 25%) throughout the passage series of Phase II cultures, including the 30th subculture (panel b). There was no
distinct difference in the staining characteristics of hexon and fibre antigens. Both antigens appeared as evenly distributed granules in the nuclei, although there was an apparent reduction in the amounts of these antigens in cells of higher passages.

A reduction from 85 to 30% in early or T-antigen-containing cells was also apparent in the passage series of adenovirus 12 Phase I cultures (panel b). It was interesting to note that early antigen was clearly visible in more than 30% of cells of Phase II cultures. Neither the adenovirus 2-infected line nor the control cells showed positive staining with T-reacting antiserum.

Morphological changes in Phase II cultures

Both infected lines were regularly examined for evidence of changes in cell morphology. Initially, infection of cells with adenovirus 12 led to cytopathic changes. When such cells were serially passed, CPE became less evident and the cultures began to look normal until the sixth or seventh passage level. At this juncture, aggregated masses of cells surrounded by fibroblast-like cells began to appear. The number of these masses increased during the subsequent passages until the cultures became heavily populated with numerous individual foci of multilayered cells (Fig. 8a). No such changes were noted in the uninfected control cells (Fig. 8b). In subcultures 9 to 30 these foci became a constant feature of the cultures. Following trypsinization, the newly seeded cells first grew into confluent monolayers then formed into new foci within the first 5 to 10 days. New lines established in the same manner frequently showed similar morphological changes.

In the adenovirus 2-infected line, the morphological alterations were first observed in the fifth subculture. During the nine subsequent passages, nests of multilayered cells were seen scattered throughout the individual cultures. However, such changes were rarely observed in the 15th to the 25th passages.
Fig. 8 (a). Foci of multilayered cells surrounded by fibroblast-like cells in the eighth serial passage of adenovirus 12-infected line 160 days after infection with 50 TCD50/cell. Unstained, ×20. (b). Uninfected cells at the tenth serial passage level. Unstained, ×20.
DISCUSSION

Two important findings resulted from these studies. One was the evidence that by continuous passaging of the adenovirus-infected cells, the infectious virus could be eliminated from the cultures. The other was the evidence for continuous persistence of some virus antigens in cells of virus-free cultures.

The observation that adenovirus 2 fibre antigen was not detectable beyond the 15th passage may be attributed to the loss of the virus genome during cell passaging or to a marked decrease in the amount of visible antigen within the cells. On the other hand, adenovirus 12 early (T) and late (fibre) antigens were clearly visible in a small number of cells throughout all passage levels over a period of 600 days. The observation that two distinct antigens were indeed present in these cells was considered valid in view of the fact that early antigens were detected in immunofluorescence tests with T-reacting hamster serum, while fibre antigen was stained with rabbit antisera specific for this antigen.

The production of T, or T and fibre antigens have also been reported in other adenovirus-cell systems (Schlesinger, 1969). However, the role of these antigens, particularly in transformed cells or in animal tumours, is not yet known. By the same token, we have no definitive evidence to indicate that the observed changes in cell morphology were primarily the functions of these antigens. Further studies concerned with this problem will be reported in a separate communication (W. D. Winters and N. Khoobyarian, in preparation).

Although our present studies were not directly concerned with the mechanism of persistence of virus antigens, certain possibilities regarding the nature of this phenomenon will be considered. The entire virus genome may be present in a small proportion of cells but the only active regions of the genome are those which specify the production of early (T) and late (fibre) antigens. If this is so, it may be possible to induce such cells to synthesize infectious virus as well as other virus proteins. However, attempts to recover infectious virus from high passage cells by the co-cultivation technique were unsuccessful, although other, possibly more sensitive methods were not tried. In the same vein, various techniques used in inducing infectious virus in transformed and tumour cells have thus far proved unsuccessful (Landau et al. 1966; Larson, Gosnell & Hillman, 1966; Kusano & Yamane, 1967; Burns & Black, 1969). Perhaps not all but only a segment of the virus genome responsible for the transcription and translation of the antigens is integrated into the cell genome. This may well be so, since not all virus-specific proteins were present in these cells. This hypothesis has already been subjected to experimental tests by Fujinaga & Green (1966) and their results show that only a small fraction of the virus genome is preferentially transcribed in adenovirus 12 transformed cells. Finally, infectious virus in undetectable amounts might be merely carried along with the cell and thus elicit the production of some virus-specific proteins.

The choice between the first and second alternatives is a difficult one, although in our opinion the second hypothesis is more probable. Much direct evidence will have to be obtained to distinguish between these two alternatives. The third alternative is least likely because it is difficult to accept the fact that residual virus could have survived the conditions of the experiment.

A virus + cell model of this sort in which some virus proteins, but not infectious virus, are present may have useful application in studies concerned with the mechanism of genetic transmission of the virus DNA.
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REFERENCES


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