Immunofluorescence of Adenovirus Type 7 T-Antigen(s) and Virion Antigens in Infected KB Cells[1] (34877)

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Mammalian cells transformed by the oncogenic adenoviruses produce virus-specific antigens that are distinct from the surface antigens of the virion. These T-antigens are also produced early in the infectious cycle but their exact function during infection or tumor formation is currently unknown. Because of the frequency of human infection with adenovirus type 7, a member of the weakly oncogenic group of adenoviruses, we have studied the production of tumor and virion antigens in infected KB cells by the fluorescent antibody technique.

Materials and Methods. Cells. A line of KB cells adapted to growth in spinner culture or monolayer was used for the growth curve and immunofluorescent staining. The cells were grown at 37° in a humidified atmosphere containing 5% carbon dioxide.

Virus. The Pinckney strain of human adenovirus type 7 was grown in KB cells, purified by density-gradient centrifugation in rubidium chloride (1), and dialyzed against 0.01 *M* Tris buffer (pH 8.3). This preparation had a titer of 1.8×10^{11} PFU/ml on human embryonic kidney cells.

Media. Eagle's minimal essential medium (MEM) supplemented with 2% fetal bovine serum (Flow Laboratories) and twice the required amount of arginine was used for infected cells. Where required, 5-fluorouridine deoxyriboside (FUDR)[2] in a concentration of 50 μg/ml or thymidine,[3] 300 μg/ml were added.

Procedure. Approximately 2×10^6 KB cells were seeded onto 60-mm petri plates (Falcon Plastics), some containing coverslips. On the next day the confluent cell monolayers were inoculated with adenovirus type 7 at a multiplicity of 130 PFU/cell in 0.5 ml MEM and incubated for 2 hr with frequent rocking. After adsorption the virus inoculum was removed, and the cell monolayers were washed three times with 1 ml of MEM and the appropriate media were added. Samples were taken at intervals for virus assay and immunofluorescence microscopy. At 12 hr, the medium containing FUDR was removed from some of the plates and fresh medium containing thymidine was added.

Virus assay. At intervals, cells were scraped into the medium and stored at −20° until assayed. The samples were frozen and thawed three times, and 0.2 ml of the several dilutions in EM was inoculated in triplicate onto confluent monolayers of human embryonic kidney cells (third-passage culture) in plastic petri dishes. The plates were incubated in a humidified CO_2 incubator with periodic rocking for an adsorption period of 2 hr. The monolayers were then overlaid with 5 ml of MEM containing 5% fetal bovine serum, twice the required amount of arginine, and 0.6% ionagar. The plates were fed 2 ml of the above medium every 3 days. After 10 days of incubation neutral red in a final concentration of 1:80,000 was added and plaque counts were made on the following day.

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[3] Nutritional Biochemicals Corp., Cleveland, Ohio.

Antisera. A serum pool from hamsters bearing tumors produced by adenovirus type 7 transformed cells was obtained from Flow Laboratories and had a complement-fixing (CF) titer of greater than 1:80 against the adenovirus type 7 T-antigen but did not react with the adenovirus group antigen.

Serum from a rabbit immunized with adenovirus type 7-infected tissue culture fluids was treated with ammonium sulfate (50% saturated), and the gamma globulins obtained were conjugated with fluorescein isothiocyanate. The conjugate stained virus-producing cells but did not stain the T-antigen (2).

Fluorescence microscopy. Coverslip cultures taken at intervals were washed in phosphate-buffered saline (PBS, pH 7.2), dipped in distilled water and fixed in acetone at room temperature for 5 min. The cells on the coverslip were air-dried and stored at —20° until used. For indirect FA determinations, the intermediate serum was applied to the coverslips in humidified chambers for 1 hr at 37° or overnight at 4°. After washing three times in PBS, goat anti-hamster gamma globulin conjugated with fluorescein isothiocyanate (2) was added for 1 hr at 37°. The coverslips were then washed, mounted in buffered glycerol, and the cells were viewed in a Zeiss fluorescence microscope.

A similar staining and washing procedure was carried out in the direct FA test for viral antigens using only the fluorescein-conjugated rabbit antiserum.

When normal hamster serum or serum from hamsters bearing tumors induced by SV-40 was used as the intermediate serum, in the indirect procedure, no fluorescence was observed. Uninfected cells showed no fluorescence with the above procedures.

Results. Growth curve. The monolayers were infected at a multiplicity of 130 PFU/-cell. After the 2-hr adsorption period, the inoculum was removed and the monolayers were washed three times leaving an adsorbed multiplicity of 11 PFU/cell. The infectivity declined over the next 10 hr followed by the appearance of new virus at 12 hr as shown in Fig. 1. The infectivity increased sharply during the next 6 hr and then leveled off

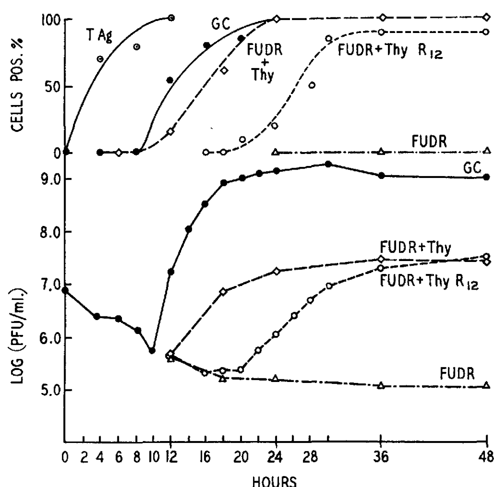

FIG. 1. Antigen production and growth curves in KB cells infected with adenovirus type 7. Upper. Percentage of cells showing immunofluorescence. Lower. Growth curves. Symbols: ●, GC. Growth curve in the absence of inhibition. △, FUDR. Growth curve in the presence of 5-fluorouridine deoxyriboside (FUDR) 50 μg/ml. Open diamond, FUDR + Thy. Growth curve in the presence of FUDR (50 μg/ml) and thymidine (Thy) 300 μg/ml. ○, FUDR + Thy R_{12}. Growth curve in the presence of FUDR (50 μg/ml) from 0–12 hr; at 12 hr the media containing FUDR was removed and fresh media containing Thy (300 μg/ml) was added. ⊙, T-antigen (T-Ag) production in absence of inhibitor (the time of development and morphology were similar for all four growth curves, see text).

until 48 hr when the last assay was done. The maximum titer reached was 1.8×10^9 PFU/ml which represents a virus yield of 3000 PFU/cell.

The development of cytopathology during the experiment was somewhat difficult to observe because the cells tended to round up from the effects of the inoculum. However, typical adenoviral cytopathology was present at 20 hr and was complete by 48 hr.

T-antigen(s). The T-antigen first appeared at 4 hr as small discrete dots and flecks in the nucleus of a majority of the cells (Fig. 2a). At 8 hr the antigen had extended out from these areas to form stellate configurations and linear flecks often located near the periphery of the nucleus similar to the numbers of a clock (Fig. 2b). By 12 hr the length of the antigen had increased further to form

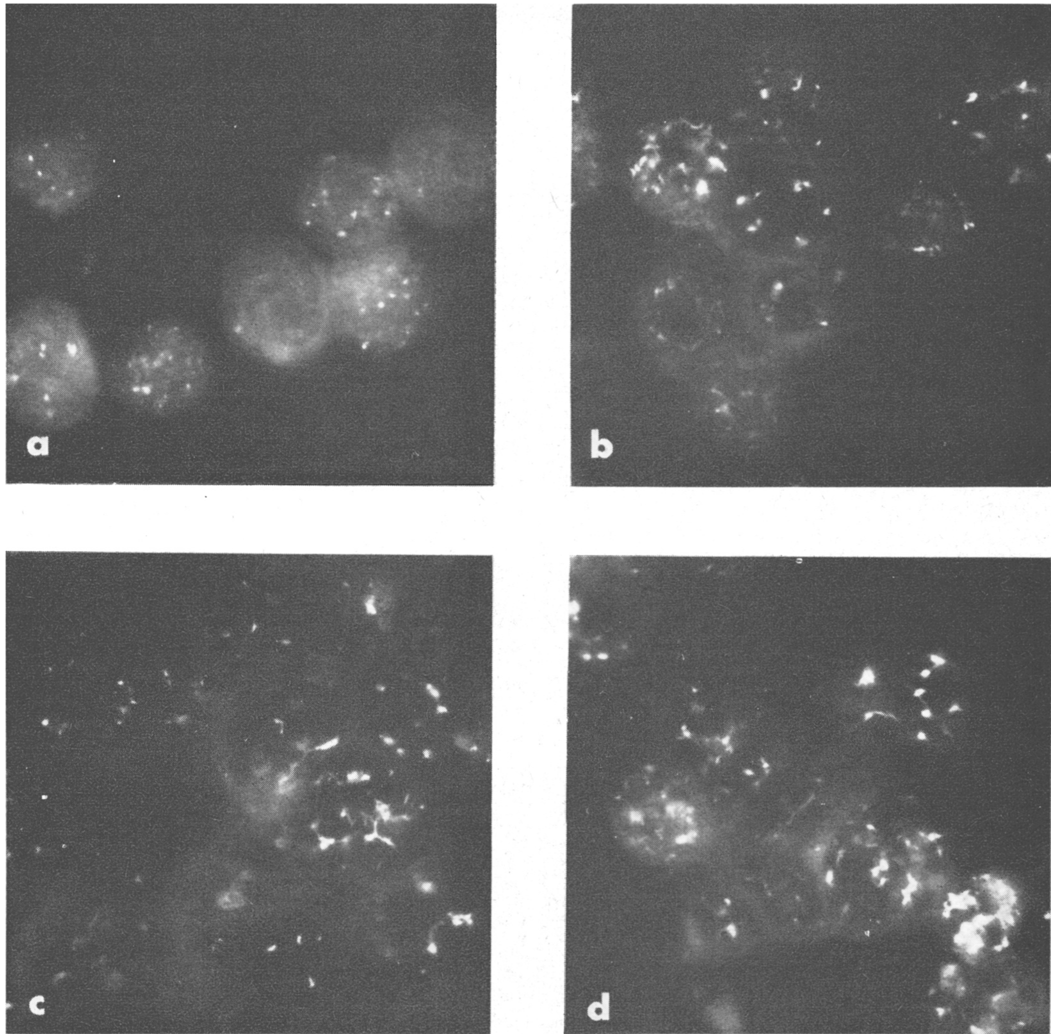

FIG. 2. Development of adeno 7 neoantigen in infected KB cells stained with tumor-bearing hamster serum by the indirect procedure: (a) 4 hr after the adsorption period; (b) 8 hr; (c) 12 hr; (d) 24 hr [during production of new virus] (×500).

long branching filaments (Fig. 2c). Nearly every cell was positive at 12 hr although the T-antigen could be seen in different stages of development in different cells indicating some degree of asynchrony. There was no decline in the number of positive cells at 24 hr after infection but the long filaments tended to coalesce, and faint diffuse staining associated with the production of new virus was also observed (Fig. 2d).

Viral antigens. Immediately after the adsorption period viral antigens were detected in the cytoplasm of the infected cells (Fig. 3a). This staining of the inoculum decreased in intensity with time but was present as late as 36 hr. Characteristic nuclear staining of new virus began at 12 hr (Fig. 3b). The entire nucleus, with the exception of the nucleoli, stained brightly. The number of positive cells increased until nearly every cell was positive at 24 hr. With time the viral antigens appeared to coalesce into globules and to extend into the cytoplasm (Fig. 3c).

Effect of FUDR. In cultures in which

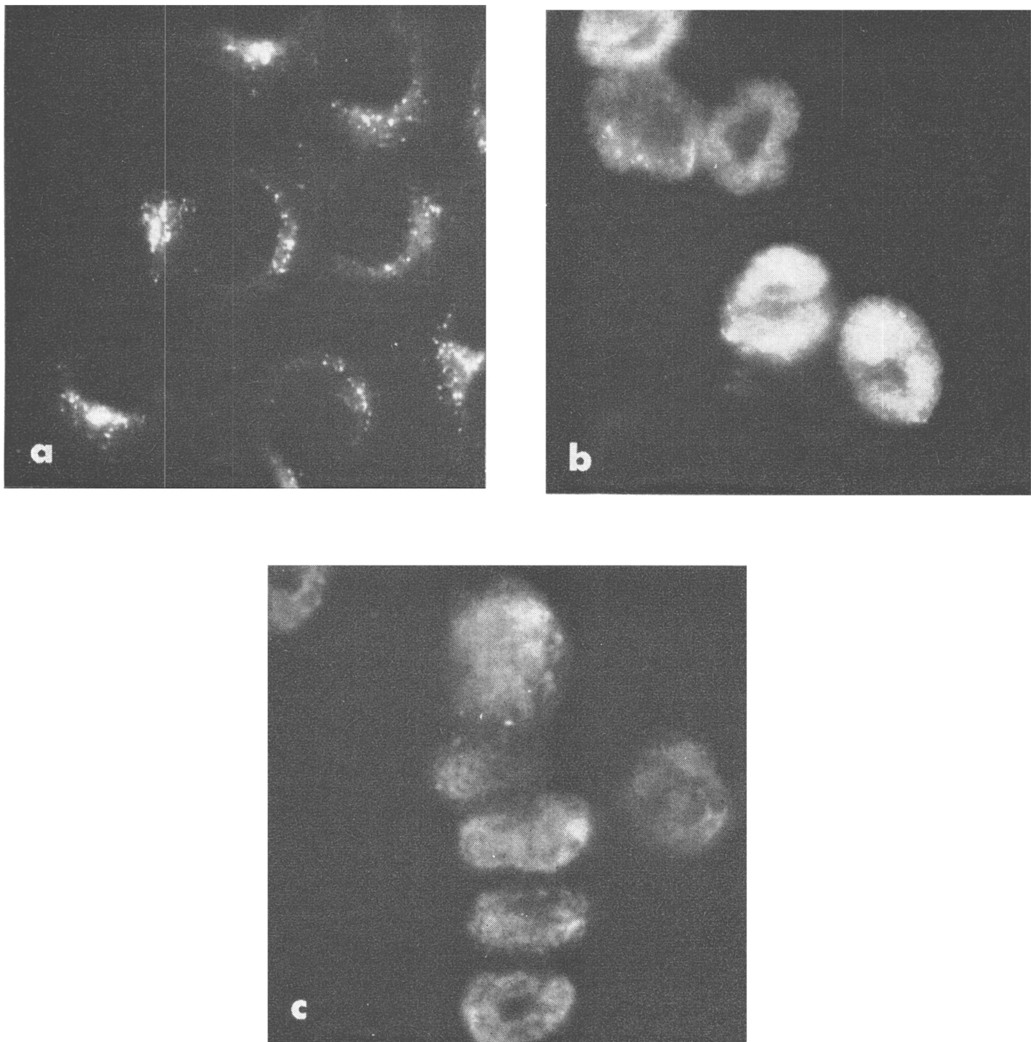

FIG. 3. Infected KB cells stained for viral antigens by the direct procedure with conjugated rabbit antiserum to adenovirus type 7: (a) immediately after the adsorption period showing inoculum in the cytoplasm; (b) cells taken at 12 hr after infection; (c) 24 hr (×500).

FUDR was added to the medium after the adsorption period, the infectivity declined steadily (Fig. 1). For the first 18 hr the infectivity decreased at a rate comparable to the rate of decrease during the eclipse period of the growth curve when virus is being uncoated. The decline leveled off after 18 hr but no increase in titer occurred.

The production of T-antigens was unaffected by the presence of FUDR, and the development of long filaments from small dots and flecks occurred as described for the growth curve except that the eventual coalescence and faint diffuse pattern was not observed. Hence, the T-antigen (T-Ag) curve shown in the upper part of Fig. 1 applies to all four growth curves shown.

Viral antigens from the inoculum were also initially present as dots in the cytoplasm in the cultures with FUDR (Fig. 3a), but in addition nuclear staining characteristic of productive infection appeared only in a rare (less than 0.1%) cell at 36 and 48 hr.

Thymidine reversal. The FUDR-inhibited

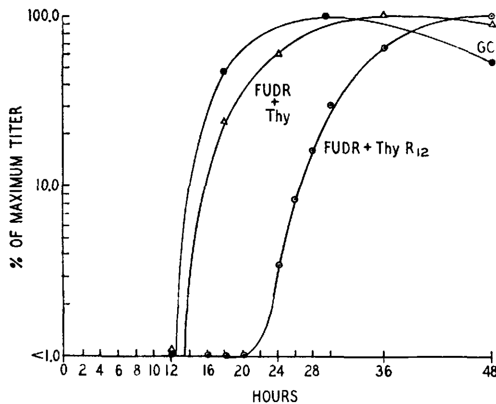

FIG. 4. Development of new infectivity in KB cells infected with adeno 7. Symbols: ●, GC. Growth curve in the absence of inhibitors. Δ, FUDR + Thy. Growth curve in the presence of 5-fluorouridine deoxyriboside FUDR (50 μg/ml) and thymidine (Thy) 300 μg/ml. ⊙, FUDR + Thy R_{12}. Growth curve in the presence of FUDR (50 μg/ml) from 0–12 hr; at 12 hr the media containing FUDR was removed and fresh media containing Thy (300 μg/ml) was added.

cultures to which thymidine was added showed the production of new virus; however, the final titer was one-thirtieth of that attained by the uninhibited cultures. The appearance of virion antigens occurred with the increase in infectivity whether thymidine was added immediately after the adsorption period or at 12 hr (Fig. 1). When thymidine was added immediately after the adsorption period the virion antigens and new infectivity were produced at the same time as for the uninhibited cultures. When thymidine was added at 12 hr after the adsorption period, however, there was a 6- to 8-hr delay in the appearance of virion antigens and the rise in infectivity (Figs. 1 and 4).

Discussion. The fluorescent antibody technique has provided evidence that the conditions for a one-step growth curve have been met in the present experiments (3). The observation that all cells had synthesized the T-antigen by 12 hr indicates that every cell was infected during the initial adsorption period. By 24 hr after infection nearly all cells showed the presence of virion antigens indicating their ability to produce virus.

Despite one-step growth conditions, asynchrony has been observed both in viral antigen synthesis (4) and in the appearance of adenovirus in infected cells examined with the electron microscope (5). In the present study, asynchrony in the development of the T-antigen was observed at 8 and 12 hr where different individual cells showed the whole spectrum of T-antigen morphology from tiny flecks to long filaments. This finding suggests that the asynchrony observed later in virion production could result from variation in the time necessary to complete events immediately after adsorption. Other factors that could be involved are the point in the mitotic cycle at which the cells is infected and the varying multiplicity of infection of individual cells.

Immediately after the adsorption period the inoculum was visualized in the cytoplasm of nearly all of the cells but no fluorescence was present in the nucleus. This pattern remained the same with only a slight decrease in intensity throughout the eclipse period indicating that the virion antigens persist during the process of uncoating.

The appearance of the T-antigen at 4 hr after infection correlates with the early protein synthesis that precedes viral DNA synthesis (6). Because of this temporal association, the T-antigen has been suspected of having enzyme activity necessary for DNA synthesis. However, purified preparations of the T-antigen from adenovirus type 12 infected cells could not be shown to possess thymidine kinase, DNA or RNA polymerase, DNase or RNase activities (7).

It has been shown for adenovirus type 2 that the elcipse period is functionally divided into two intervals (8, 9). The first is from 0–7 hr, at which time the T-antigen and proteins necessary for DA synthesis are made. These events do not involve DNA synthesis and occur in the presence of FUDR. New viral DNA synthesis begins at about 7 hr (8) and a period of 6–7 hr during which DNA and protein synthesis occur must elapse before an increase in infectivity is seen. Adenovirus type 7 follows this pattern as is shown by the 7-hr delay after thymidine reversal of FUDR-inhibited cultures before new virus is detected (Figs. 1 and 4). In the uninhibited cultures, viral antigens are detected by the

FA technique at 9–10 hr and mature virus appears at 12 hr after the adsorption period.

In cultures inhibited by FUDR a rare cell shows the production of virion antigens. This may be due to the production of surface antigens in the absence of DNA synthesis or to the failure of FUDR to block DNA synthesis in these cells.

When cultures inhibited by FUDR were reversed immediately or at 12 hr, the final titer was about one-thirtieth of the uninhibited cultures. This is in contrast to the comparable or even higher virus yields that have been observed after thymidine reversal of adenovirus type 2-infected culture (8). Part but not all of this discrepancy can be accounted for by the minimal loss of cells from the FUDR-treated cultures that occurred during these experiments. Other factors involved in the lower yield obtained here may be the use of a higher concentration of inhibitor, the difference in culture conditions (monolayer vs suspension), or the difference in adenovirus types (type 2 vs type 7).

Adenovirus type 7, a member of the weakly oncogenic group of adenoviruses, is similar to the nononcogenic adenovirus types 2 and 5 in the time of development of early virus specific antigens (T-antigen), virion antigens, and mature virus (4, 8, 10). This is in contrast to the highly oncogenic type 12 in which the production of the T-antigen does not begin until 6 hr and a majority of the cells are not positive until 12 hr after infection (11). The appearance of virion antigens and new virus is also more rapid in infection with type 7 than in infection with type 12. New infectious virus begins to appear between 10 and 12 hr and increaeses until about 20 hr whereas in type 12 infection new virus does not appear until 24 hr and the maximum titer does not occur until 48 hr. Although a different human cell line was used in the two experiments, the final titer attained in the adeno 7-infected cells was 100-fold higher than for adeno 12.

The morphology and development of the T-antigens is similar for both types 7 and 12. Only intranuclear staining is observed which begins as tiny dots and flecks. The staining material then extends out from these points to form stellate configurations and rosettes. Later in the eclipse period long branching filaments are formed and finally, when new virus is being produced, the T-antigen coalesces into globules in a faintly staining nuclear background.

These investigations have shown the similarity between the growth kinetics of adenovirus type 7 and those of the "nononcogenic" adenoviruses. The morphology of the T-antigen induced by adenovirus 7 during the lytic cycle is similar to that of the highly oncogenic type 12 when studied by the fluorescent antibody technique.

Summary. The production of adenovirus type 7 T-antigen(s) and virion antigens in infected KB cells has been detected by immunofluorescence and correlated with the one-step growth curve. The T-antigen first appears at 4 hr as small discrete intranuclear dots and flecks which extend with time to form stellate configurations and eventually long branching filaments by 12 hr after infection. Virion antigens appear at 9–10 hr and new infectious virus is detected by 12 hr. The appearance and morphology of the T-antigen is unaffected by the presence of FUDR; however, the synthesis of virion antigens is delayed until about 7 hr after reversal of the inhibition with thymidine. The growth kinetics of adenovirus 7, a member of the weakly oncogenic group of human adenoviruses, are similar to those of the "nononcogenic" types 2 and 5 in contrast to the longer time required for the growth of the highly oncogenic adenovirus 12.

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