Demonstration of Intranuclear T-Antigen by Antisera to Purified Adenovirus 7[1] (34136)

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Neoplastic transformation of mammalian cells by the oncogenic adenoviruses results in the production of intranuclear virus-specific antigens. Antisera to these tumor antigens (T-antigens) can be produced by animals bearing tumors or by immunization with transformed cells or tumor extracts. The usual diagnostic antisera, however, react with the surface antigens of the adenovirion but are not known to react with the T-antigen or viral antigens produced early in the infective cycle. The present report describes the production of intranuclear T-antigen in primary hamster kidney cells and the reaction of this antigen with antisera to purified adenovirus type 7. The possibility that the T-antigen is an internal component of the virion is discussed.

Materials and Methods. Cells. Newborn hamster kidneys were minced and treated for 2 hrs with 0.25% trypsin. The cells were diluted 1:300 by volume with Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and 5 ml were planted in 60-mm plastic petri dishes (Falcon) containing coverslips. The plates were incubated in a humidified atmosphere of 5% CO_2 in air. Confluent monolayers of spindle shaped cells were obtained after 5 days.

A line of KB cells adapted to growth in spinner culture or monolayers was used for the preparation of virus stock and for the demonstration of virus-specific antigens by immunofluorescence.

Virus stock. The Pinckney strain of human adenovirus type 7 was grown in KB cells and purified by density gradient centrifugation in rubidium chloride as described by Green and Piña (1). The virus band was dialyzed for 24 hr against 0.01 *M* Tris buffer (pH 8.3) with 4 changes of the buffer. Serial 10-fold dilutions were made in MEM and 0.2 ml of appropriate dilutions was inoculated in triplicate onto confluent monolayers of human embryonic kidney cells grown in MEM with 10% FBS (third passage culture) in plastic petri dishes. The plates were incubated in the humidified CO_2 incubator with occasional rocking for an adsorption period of 2 hr. The monolayers were then overlaid with 5 ml of MEM containing 5% FBS, twice the required amount of arginine and 0.6% ion-agar. 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. The virus preparation had a titer of 1.8×10^{11} PFU/ml when assayed in this manner.

Antisera. The virus stock was diluted 1:20 with 0.01 *M* Tris (pH 8.3) and was held at 4° for 3 weeks prior to use for immunization of animals. These conditions are known to result in large numbers of disrupted virions (2). The virus suspension was emulsified with an equal volume of complete Freund's adjuvant and 0.5 ml was injected intramuscularly into 4-month-old hamsters. Four weekly injections were given and the animals were bled 10 days after the last injection. This serum is designated S-1356 no. 5.

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

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Serum from a rabbit immunized with adenovirus type 7 infected tissue culture fluids was conjugated with fluorescein isothiocyanate. The conjugate stained virus-producing cells but did not stain the T-antigen (3). Antiserum against hamster gamma globulin was made in a goat. This serum was conjugated with fluorescein isothiocyanate for use in the indirect fluorescent antibody (FA) procedure (3).

Fluorescence microscopy. Hamster kidney or KB cells grown on coverslips were fixed in acetone and stored at —20° until used. For the indirect FA procedure, appropriate dilutions of the intermediate antisera (TBHS or S-1356 no. 5) were allowed to react with the coverslip culture for 1 hr at 37° or overnight at 4°. The cultures were washed 3 times in phosphate buffered saline (PBS) pH 7.2, and reacted with fluorescein-tagged antihamster serum for 1 hr at 37°. After washing in PBS the coverslips were mounted in buffered glycerol and examined in a Zeiss fluorescence microscope. A similar staining and washing procedure was carried out in the direct FA test for viral antigen using only the fluorescein-conjugated rabbit antiserum (3).

When normal hamster serum or serum from hamsters bearing tumors induced by SV-40 was used as the intermediate reactant in the indirect FA procedure, no fluorescence was observed. Uninfected cells did not stain with any of the antisera.

Infection of coverslip cultures. Primary hamster kidney cells were grown on coverslips and after 5 days were infected with adenovirus type 7 at a multiplicity of approximately 100 PFU/cell. After a two hr adsorption period with frequent rocking, Eagle's minimum essential medium, with and without 5-fluorouracil (FU) at a concentration of 50 μg/ml, was added and the cells incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Coverslips were taken at intervals for testing the cells with the different antisera. No cytopathology was observed in the hamster kidney cells. Coverslip cultures of KB cells were infected in a similar manner. After viral adsorption, Eagle's minimum essential medium without FU was added and the cultures were incubated for 24 hr prior to fixing of the coverslips for staining.

TABLE I. Type of Immunofluorescence Observed in KB and Hamster Kidney Cells Infected with Adenovirus Type 7.

Cell type	Antisera: Rabbit antivirion[a]	TBHS[b]	S-1356 no. 5[c]
KB	V[d]	T	V[e]
Hamster kidney	0	T	T

[a] Direct FA procedure.

[b] Tumor-bearing hamster serum, indirect FA procedure.

[c] Hamster antiserum to purified adenovirus type 7, indirect FA procedure.

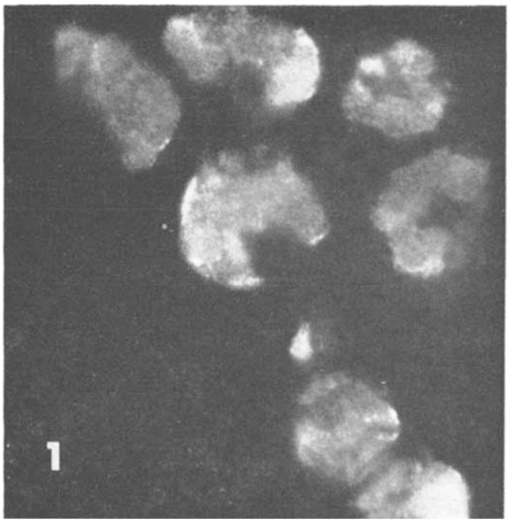
[d] Abbrev.: V = diffuse staining of virus producing cells; T = discrete intranuclear branching filaments characteristic of T-antigen; 0 = no staining.

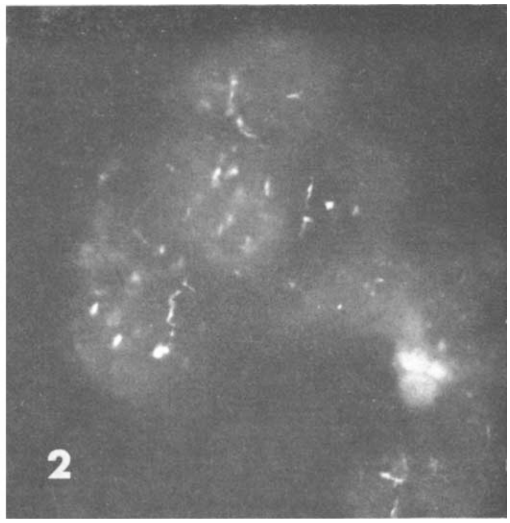
[e] The diffuse staining of productively infected cells obscures the intranuclear flecks characteristic of the T-antigen.

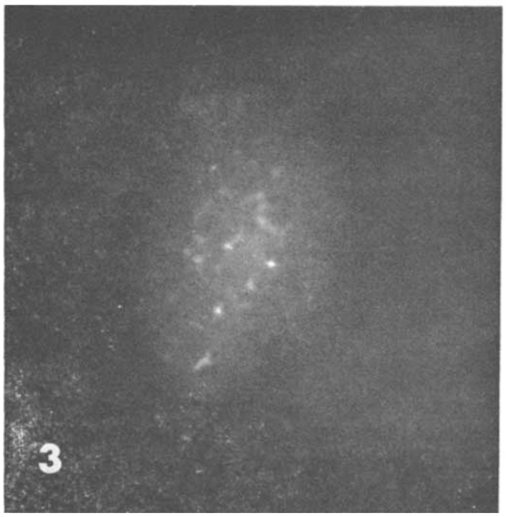
Results. Productively infected KB cells incubated in MEM for 24 hr, stained diffusely in the indirect FA test with antiserum S-1356 no. 5 (Fig. 1). An identical fluorescence pattern was obtained when productively infected KB cells were stained with rabbit antiserum to infected tissue culture fluids by the direct FA procedure. When the infected KB cells were stained by TBHS in the indirect procedure branching intranuclear filaments characteristic of the T-antigen were observed (Fig. 2).

In contrast to the staining characteristic of the virus-producing cells, infected hamster kidney cells when reacted with rabbit antiserum to infected tissue culture fluids in the direct FA procedure showed no fluorescence indicative of virus production. However, the infected hamster kidney cells revealed the presence of rather long branching filaments in the nucleus when stained with serum from tumor-bearing hamsters in the indirect FA procedure (Fig. 3). The same intranuclear structure was visualized when antiserum S-1356 no. 5 was used as the intermediate reactant (Fig. 4). The results of immunofluorescent staining are summarized in Table I.

The number of hamster kidney cells staining at different intervals is shown in Table







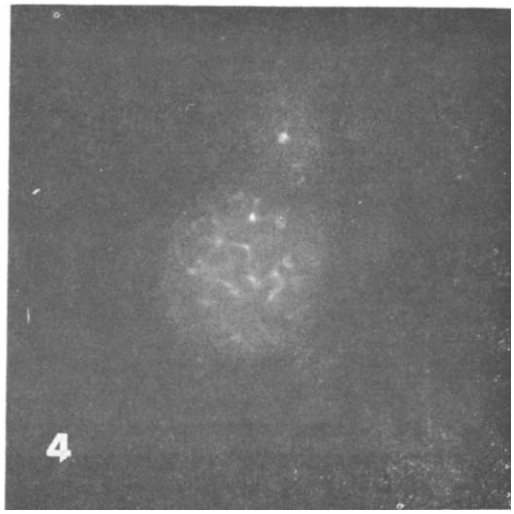


FIG. 1. Appearance of productively infected KB cells after 24 hr when stained with hamster antisera to purified adenovirus type 7 (S-1356 no. 5) by the indirect immunofluorescence technic utilizing fluorescein tagged goat antihamster gamma globulin; original magnification ×500.

FIG. 2. Appearance of productively infected KB cells after 24 hr when reacted with serum from hamsters bearing tumors induced by cells transformed by adenovirus type 7. The cells are stained by the indirect fluorescent antibody technic utilizing fluorescein tagged goat antihamster gamma globulin; original magnification ×500.

FIG. 3. Appearance of newborn hamster kidney cells 24 hr after infection with adenovirus type 7 when reacted with serum from hamsters bearing tumors induced by cells transformed by adenovirus type 7. The cells are stained by the indirect fluorescent antibody procedure utilizing fluorescein tagged goat antihamster gamma globulin; original magnification ×500.

FIG. 4. Appearance of newborn hamster kidney cells 24 hr after infection with adenovirus type 7 when stained with hamster antisera to purified adenovirus type 7 (S-1356 no. 5) by the indirect immunofluorescence technic utilizing fluorescein tagged goat antihamster gamma globulin; original magnification ×500.

TABLE II. Indirect Immunofluorescence of T-Antigen in Primary Hamster Kidney Cells Infected with Adenovirus Type 7.

Intermediate serum and dilution	Medium added after infection	Time				
		24 hr	48 hr	72 hr	6 days	10 days
TBHS[a] (flow pool)	MEM[a]	81[b]	118	70	19	—
1:320	MEM and FU[a]	106	111	62	35	20
	Uninfected		0			0
S-1356 no. 5[c]	MEM	54	62	27	69	—
1:40	MEM and FU	62	60	28	39	22
	Uninfected		0			0

[a] TBHS = tumor-bearing hamster serum; MEM = Eagle's minimum essential medium; FU = 5-fluorouracil, 50 μg/ml.

[b] Five fields containing approximately 200 cells each were counted and the results reported as the number of T-antigen containing cells per 1000 cells.

[c] Hamster antiserum to purified adenovirus type 7.

II. As shown, the T-antigen is produced in the presence of fluorouracil and is detected both by TBHS and S-1356 no. 5. The number of T-antigen positive cells appears to increase to a maximum at about 48 hr and then decrease. Uninfected cells were negative.

Discussion. Adenovirus type 7, a member of the weakly oncogenic group of human adenoviruses, causes an abortive infection in hamster kidney cells with the production of virus specific tumor antigens as has been shown for adenovirus type 12 (4). In addition, the abortive type of infection is characterized by an increase in the activity of thymidine kinase (5, 6) and the induction of transient DNA synthesis (7) but no cytopathic effect or synthesis of new virus. Because of the temporal association between the appearance of the tumor antigens and increased enzyme activity in both abortively and productively infected cells the tumor antigens have been suspected of having such activity. 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 (8).

The increase and decline in the number of cells containing T-antigen observed here appears to coincide with the transient DNA synthesis induced by the abortive infection of hamster cells (7). However, the presence of fluorouracil demonstrates that neither cellular nor viral DNA synthesis is necessary for T-antigen production. Conversely, the DNA synthesis induced by SV-40 has occurred in the presence of interferon while the production of the T-antigen is suppressed (9). If the above findings with SV-40 are generally applicable, it appears that while induced DNA synthesis and production of T-antigen occur simultaneously, these events are not interdependent.

Several different methods have established the presence of an internal nucleoprotein core within the adenovirion (2, 10–13). In contrast to the surface antigens, the protein moiety appears to be produced early in the infective cycle and is not blocked by inhibitors of DNA synthesis (2, 14) features which are shared with the T-antigens of the oncogenic adenoviruses. The internal component can be released from the surface antigens of the virion by treatment with acetone (10), formamide (13), or simply by "aging" in buffer (2). In order to explain the characteristic staining of the T-antigen in abortively infected hamster cells by antisera to purified virus, two alternatives seem reasonable. The internal antigen that is released by aging could stimulate antibodies that react with the T-antigen indicating an antigenic similarity or identity between them. On the other hand, the aged virus preparation used for immunization is infectious and the

T-antigen could be produced *in vivo* with consequent antibody production.

The inoculation of newborn hamsters with either adenovirus type 12 or polyoma virus results in the production of tumors from which continuous cell lines have been derived. These cell lines are capable of inducing progressively growing tumors in hamsters aged 3 months or more and contain the virus specific T-antigen but no viral infectivity or virion antigens can be demonstrated. It has been shown that prior immunization with either homotypic or heterotypic advenoviruses (15) or with polyoma virus (16) can induce resistance to tumor production by the corresponding transformed cell line. Furthermore, extensive cross reactions have been found between the T-antigens produced by heterotypic adenoviruses (human and simian) and serum from hamsters bearing tumors induced by adenovirus type 12 (17). As shown here, antibodies against the T-antigen can be present in animals immunized with virus preparations and may be involved in the observed resistance of such animals to the establishment of tumors by virus transformed cells.

A study of the reaction of tumor-bearing hamster serum with disrupted virions by immunodiffusion and of the staining of T-antigen containing cells by antisera to noninfectious preparations of disrupted virions may help to resolve the question of a relationship between the T-antigen and the internal protein of the adenovirion.

Summary. Adenovirus type 7 was purified by density gradient centrifugation, dialyzed against 0.01 *M* Tris buffer (pH 8.3), and allowed to stand for 3 weeks at 4°. These conditions are known to result in large numbers of disrupted virions. Immunization of hamsters with this preparation produced antisera that reacted both with the surface antigens of the virion and with the T-antigen. Primary hamster kidney cells were infected with adenovirus type 7 at a multiplicity of 100 PFU/cell and intranuclear T-antigen was detected in a proportion of cells which rose to a maximum of about 10% at 48 hr and then declined. Two possibilities to explain the production of antibodies to the T-antigen by hamsters immunized with purified virus are discussed: the internal antigen which is released by the aging of adenovirus in buffer may stimulate antibodies that react with the T-antigen or the T-antigen could be produced *in vivo* with consequent antibody production. Either mechanism may be involved in the inhibition of tumor formation by virus induced tumor cells that is observed in previously immunized animals.

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