Immunofluorescent Studies of Antigens Induced by Adenovirus Type 12 (35446)

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Intranuclear virus-specific antigens are found in tumors induced by adenovirus type 12. Similar antigens have also been found in human cells infected with that virus. These antigens are synthesized early in the infective cycle and have been termed "T" antigens. The present report utilizes immunofluorescence (IF) to study the synthesis of both viral and T antigens in HeLa cells acutely infected with adenovirus type 12 and exemplifies the diversity of the antibody responses of hamsters bearing adenovirus type 12 tumors.

Materials and Methods. Cell cultures. HeLa cells were carried as monolayers in prescription bottles. The growth medium consisted of Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS). This medium also contained the following antibiotics per ml: 100 units of penicillin G; 100 μg of streptomycin; 10 μg of tetracycline; and 2 μg of fungizone. Maintenance medium consisted of MEM supplemented with 2% FCS. The cells were grown on coverslips in Leighton tubes for immunofluorescent studies and in culture tubes for serological and infectivity studies.

Viruses. The prototype (Huie) strain of adenovirus type 12 was propagated in HeLa cells. The cells were harvested when 75% of the cells showed cytopathic effects (CPE). The virus was released by freezing and thawing 4 times. The cell debris was removed by low-speed centrifugation and the supernatant was stored at $-20°$. This crude virus preparation was used for infection of cell cultures or purified by equilibrium centrifugation in CsCl gradients (1). The average titer of crude virus preparations was approximately $10^{2.5}$ $TCID_{50}$/0.1 ml.

Antisera and antigens. Guinea pigs were immunized with the gradient purified virus using two subcutaneous injections. The first injection contained purified virus and equal amounts of complete Freund's adjuvant. Incomplete Freund's adjuvant was used in the second injection given 1 week later. The animals were bled 2 weeks after the last injection. These sera were used as a source of antibody to viral antigens. Antisera (TBHS) to T antigens were obtained from hamsters bearing either transplanted or virus-induced tumors. Tumor antigens were prepared from adeno 12 tumor homogenates (2).

Fluorescent conjugates. For the direct IF methods, the guinea pig antiadeno 12 globulin and a pool of globulin from hamsters bearing transplanted adeno 12 tumors were conjugated with fluorescein–isothiocyanate (Nutritional Biochemical Corporation No. 9405). Methods of conjugation were similar to those reported by Nairn (3). For the indirect IF methods, commercially prepared fluorecein-conjugated rabbit antiguinea pig (IgG) globulin (Microbiological Associates, Inc., No. 51-796) and fluorescein-conjugated rabbit antihamster (IgG) globulin (Microbiological Associates, Inc., No. 51-795) were used.

Infection of cultures. When monolayers of cells were almost confluent, the culture tubes were fed with 1 ml of maintenance medium to which was added 0.1 ml of crude virus. The cells were incubated for 2 hr at 37°, washed twice in Hanks' balanced salt solution (BSS), and then fed with 1 ml of maintenance medium. Infected cultures were incubated at either 25° or 37°. Certain cultures were incubated at 37° with medium containing 100 μg/ml of 5-fluorodeoxyuridine (FUDR) or 10, 20, and 40 μg/ml of cytosine arabinoside (CA). To test for cross reac-

tions between other adenovirus types, cell cultures were infected with adenovirus types 2, 3, 5, and 7A.

Immunofluorescent techniques. The coverslips were washed 3 times with Hanks' BSS, allowed to air dry at room temperature, fixed in precooled acetone for 10 min at —20° and allowed to air dry again. The fixed coverslips were stained immediately or stored at —20° until used. For the indirect IF methods, the coverslips were covered with a 1:10 dilution of antisera (diluted with PBS, pH 7.6) and incubated in a moist chamber for 30 min at 37°. The antisera were removed and the coverslips were rinsed in PBS, soaked first in PBS for 10 min, then in distilled water and allowed to air dry. Next the antiglobulin conjugate was applied to the coverslips and incubated for 30 min at room temperature, followed by the rinse, soak, rinse cycle. The procedure for the direct method was similar to the indirect method; however, only the conjugated antisera was used. Differences in results between direct and indirect methods were not significant; therefore, indirect methods were used except where stated otherwise. After air drying, all coverslips were mounted in buffered glycerol (90% glycerol and 10% PBS, pH 7.6) and examined under a Zeiss fluorescent microscope.

The controls for the immunofluorescent techniques consisted of infected and uninfected cells which were stained with negative and positive sera, respectively.

Complement fixation tests. Complement fixation (CF) tests were done by the microtechnique (4), employing 1.5–2.0 units of complement.

Results. HeLa cells infected with adenovirus type 12 were examined using direct and indirect immunofluorescent techniques. The time of appearance of the antigens depended upon the multiplicity of infection and the physiological state of cells. Four types of fluorescence were observed in this study. The first were the T antigens (Fig. 1A) which were detected at 6 hr after viral adsorption, and were readily demonstrable by both the

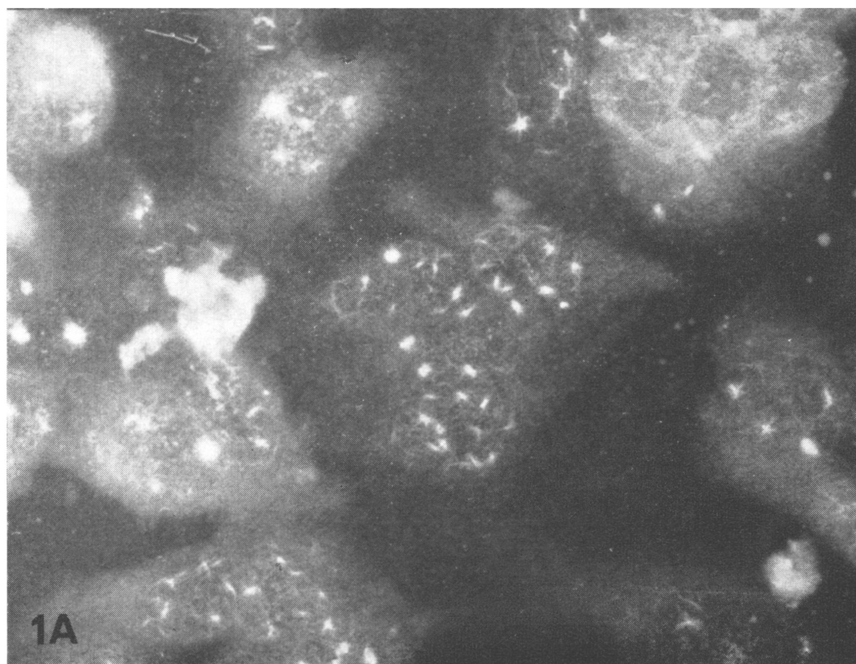
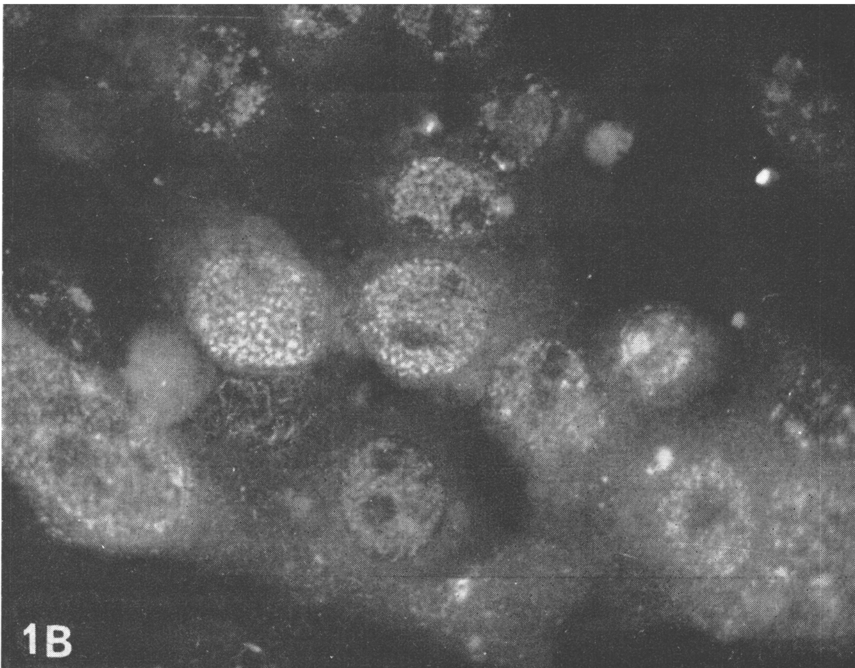
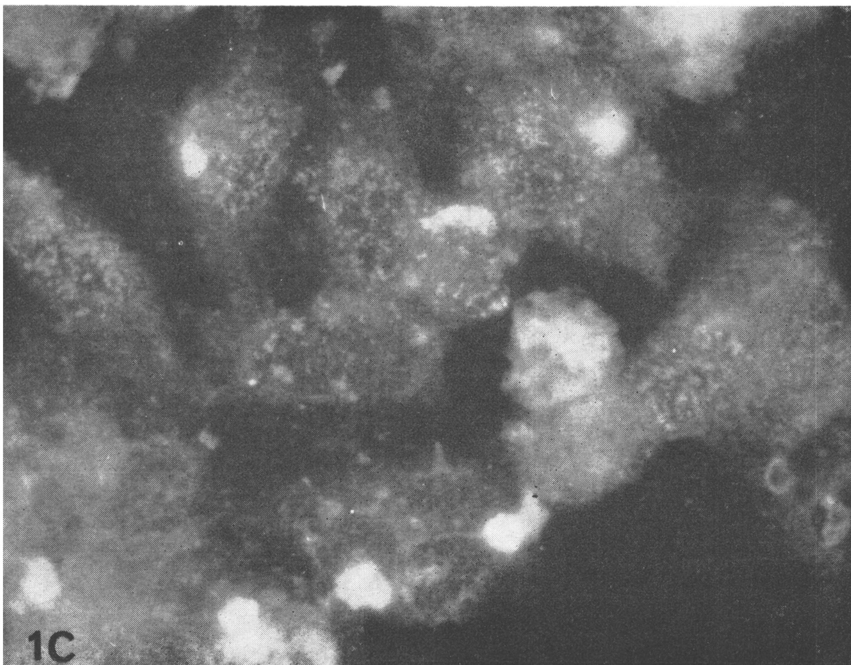
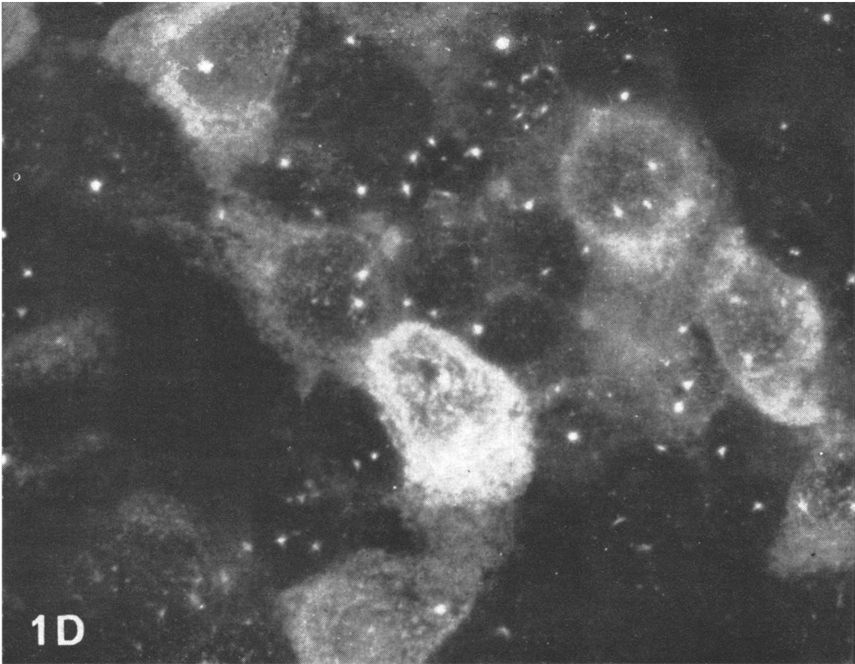


FIG. 1. HeLa cells infected with adenovirus type 12: (A) fluorescent flecks (FF); (B) fluorescent dots and fluorescent flecks (FD, FF); (C) fluorescent viral antigens (FV); (D) fluorescent membranes and fluorescent flecks (FM, FF); and (E) control.



direct and indirect immunofluorescent methods using tumor-bearing hamster sera. These antigens were observed primarily within the nuclei of infected cells, but were also seen in the cytoplasm. Different patterns of fluorescence were observed, *i.e.,* nuclear and cytoplasmic flecks, slender filamentous-like threads, and "stars" which were grouped together and termed fluorescent flecks (FF) (5). The number of cells with this type of fluorescence as well as the intensity of fluorescence increased until 24 hr postinfec-





tion. A decrease in intensity was noted by 72 hr.

A second type of fluorescence, termed fluorescent dots (FD) (5), is shown in Fig. 1B. They were first noted 16–18 hr after viral infection and were demonstrable using certain TBHS. The FD were found only in the nuclei of infected cells and were morphologically distinct from FF. The former were more uniform and frequently appeared in the form of "doughnuts" with unstained centers or were confluent throughout the nuclei. The

TABLE I. Antibodies[a] of Tumor-Bearing Hamsters to Antigens in Acutely Infected Human Cells.[b]

Origin of tumor	No. tested	Positive FF No.	FF (%)	FD No.	FD (%)	FM No.	FM (%)
Primary (virus induced)	9	9	100	6	67	1	11
Transplanted	20	19	95	8	40	7	35
Transplanted (pool)	2	2	100	1	50	0	0
No tumor[c]	1	1	100	0	0	1	100
Total	32	31	97	15	47	9	28

[a] Determined by indirect immunofluorescence.
[b] FF, Fluorescent flecks; FD, fluorescent dots; and FM, fluorescent membrane.
[c] Immunized with tumor cells.

number of cells with FD and the intensity of fluorescence were maximal by 48 hr. Less intense fluorescence was apparent at 72 hr.

The viral antigens (FV) were first detected within the nuclei of infected cells at 18–20 hr postviral infection (Fig. 1C). They were demonstrable by direct staining using guinea pig antiadeno 12 conjugated antisera. FV antigens were distinct from FF and FD. The viral antigens first appeared in the nucleus in the form of small fluorescent dots, gradually becoming larger fluorescent granules. Eventually the nuclei became filled with large amounts of brilliantly stained viral antigens, accompanied by the typical adenovirus CPE.

The fourth antigen, termed fluorescent membranes (FM) (Fig. 1D), was not found at 24 hr after viral infection but could be demonstrated by 48 hr. The number of cells exhibiting this type of fluorescence increased up to 72 hr. Only a limited number of TBHS reacted with these antigens. The fluorescence appeared as very fine dots at the cell membrane and in the adjacent cytoplasm. No specific fluorescence was seen in the control preparations (Fig. 1E), although some faint autofluorescence was noted.

Twenty-nine individual TBHS, two pools of TBHS from transplanted recipients, and one serum from a hamster which had been immunized with tumor cells were assayed for antibodies to antigens in HeLa cells 48 hr after infection with adeno 12. Of the 32 sera tested by IF (Table I), 31 had antibodies to FF, 15 had antibodies to FD, and 9 reacted with FM. Antibodies to FM were found almost exclusively in the transplanted TBHS. One serum from a hamster bearing a primary tumor as well as the serum from the hamster which was immunized with tumor cells reacted with this antigen. Sera which were positive for T antigen by IF were also positive by CF with the exception of one serum from an animal bearing a transplanted tumor. Five of the 32 sera tested reacted with viral antigens by CF. These five sera also reacted with FD antigen.

The maintenance of cells infected with adeno 12 at 25° had a marked effect on the synthesis of antigens (Table II). FF were detected but reached maximum fluorescence later than at 37°; however, neither FD nor viral antigens were observed up to 5 days postinfection. Typical adenovirus cytopathology was also inhibited. When infected cultures, which were incubated at 25° for 3 days, were transferred to 37°, both FD and

TABLE II. Effect of Temperature on the Synthesis of Antigens.[a]

Temp (°)	Tumor (T) antigens FF	Tumor (T) antigens CF	Viral antigens FV	Viral antigens CF	Other FD	Other Infectivity
25	+	+	—	—	—	—
37	+	+	+	+	+	+

[a] FF, Fluorescent flecks; CF, complement fixation; FV, fluorescent viral antigens; and FD, fluorescent dots.

TABLE III. Effect of Antimetabolites on the Synthesis of Antigens.[a]

Treatment	Tumor (T) antigens		Viral antigens		Other		
	FF	CF	FV	CF	FD	FM	Infectivity
FUDR	+	+	−	−	−	−	−
CA	+	+	+	+	+	ND	+
Control	+	+	+	+	+	+	+

[a] FF, Fluorescent flecks; CF, complement fixation; FV, fluorescent viral antigens; FD, fluorescent dots; FM, fluorescent membranes; FUDR, 5-fluorodeoxyuridine; CA, cytosine arabinoside; and ND, not done.

viral antigens were rapidly synthesized, followed by typical adenovirus CPE.

The effects of certain DNA inhibitors are shown in Table III. FUDR at 100 μg/ml of medium inhibited the synthesis of FD, FM, and FV antigens when tested 48 hr postinfection. When FUDR was added to the infected cell cultures prior to 10 hr postinfection, the synthesis of FD and FV was completely inhibited. If added later only partial inhibition of these antigens was obtained. Cytosine arabinoside (CA) did not affect antigen synthesis in this study.

Cross reactions between TBHS and antigens induced by other adenovirus types were noted. Table IV shows some of the reactions obtained when HeLa cells infected with adenovirus types 2, 3, 5, and 7 were covered with adeno 12 TBHS and stained with conjugated antihamster globulin. FD were found in adenovirus 3- and 7-infected cells but not in cells infected with types 2 and 5. FF were found in all infected cells, but to a lesser extent in cells infected with types 2 and 5. With adeno 5 very few cells with fluorescence were found. FM were found in adeno 2- and adeno 5-infected cells. FM were especially prominent in cells infected with Ad 2, with the fluorescence appearing heavier in areas and not confluent with the entire membrane.

TABLE IV. Reactivity of Sera from Tumor-Bearing Hamsters with Cells Infected by Various Adenoviruses Types.[a]

Adenovirus type	FF	FD	FM
12	+	+	+
3	+	+	−
7	+	+	−
2	±	−	+
5	±	−	±

[a] FF, Fluorescent flecks; FD, fluorescent dots; and FM, fluorescent membranes.

Discussion. The present results show 3 distinct antibody specificities present in TBHS which were detectable by immunofluorescence. These antibodies reacted with antigens in acutely infected HeLa cells. The antigens detected were morphologically distinct from structural viral antigens demonstrable using guinea pig antiadeno 12 antisera.

Most of the TBHS had antibodies to FF. The FF antigens had been found previously in various cell types by a number of other investigators (5–10). The various forms of FF antigen observed could represent different stages of development.

The FD antigens appeared shortly before viral antigens, reached maximum levels of fluorescence by 48 hr, and then diminished, while structural viral antigens appeared to increase with time. FD were morphologically distinct from viral antigens and were not detected with antisera to purified virus. The FD antigens were described by Shimojo *et al.* (5). They stated that FD may be a virion antigen since FD were not blocked by the narrow anti-T serum but were blocked by the anti-C serum. Yet FD must be distinct from C antigen because FD were not stained by the anti-C conjugate. Hayashi and Russell (10) also demonstrated antigens associated with adenovirus type 5 infection which are similar to FD.

In the present study, only half of the TBHS tested had antibodies to FD. All TBHS which reacted with viral antigens by CF had antibodies to FD, but some TBHS

which did not react with viral antigens also had antibodies to FD. Antibody reactivity to FD was found in the sera of hamsters bearing either transplanted or virus-induced tumors, indicating the persistence of the antigen in tumor cells following transplantation.

FD were inhibited by growth at 25° and in the presence of FUDR. FD may represent a precursor or enzyme required for the synthesis of viral antigens. An alternative explanation may be that it represents the internal protein, since the presence of proteins within the capsid has been clearly demonstrated (11).

Berman and Rowe (12) reported the presence of an antibody in TBHS to a viral nonstructural antigen present in crude virus. This antigen was distinct from T and fiber antigens and could be detected with certain hyperimmune antiadeno 12 sera. Tockstein *et al.* (13) demonstrated several antigenic components in the nuclear extracts of adeno 12-infected KB cells and adeno 12-transformed hamster embryo cells. One of these could be the FD antigen. In the present studies, FD were not found in the infected human cells in the presence of FUDR. Since FD is synthesized prior to synthesis of structural viral antigens and is inhibited by FUDR, FD probably represents a late function coded by progeny viral genomes.

The synthesis of FM antigen occurred after the synthesis of FF, FD, and FV antigens. FUDR also inhibited the formation of FM, indicating that FM may be a function of progeny viral geomes or could represent the synthesis of rapidly accumulating late proteins which develop prior to cell destruction. Only 9 out of 32 sera tested (1:10 dilution) had antibodies to FM. These antibodies appear to be present primarily in sera from hamsters bearing transplanted tumors. It is therefore possible that transplantation enhances the development of antibody to the cytoplasmic antigen. Since the transplantation antigens are thought to be present at the cell surface, FM may represent a transplantation antigen. The presence of surface antigens detectible by fluorescence on adeno 12 tumor cells has recently been reported (14).

Cross reactivity between T antigens induced by various adenovirus types has been reported (6, 8, 10). The greater cross reactivity between TBHS and antigens induced by types 3 and 7 was noted previously by the above investigators. The significance of FD antigen synthesis by these two immunotypes and not by types 2 and 5 under the present conditions awaits further experimentation. If FD antigen is the internal protein, the time after infection may be critical as sequential time studies were not done with these types. The results discussed here also showed that hamsters bearing either primary or transplanted adeno 12 tumors have individual antibody responses to the developing tumors. Whether or not the humoral antibody response represents an adequate picture of the antigens present in the tumor is yet to be determined.

Summary. Antigens synthesized in HeLa cells infected with adenovirus type 12 were studied by direct and indirect immunofluorescence. Sera used to detect these antigens were antiviral guinea pig sera and sera from hamsters bearing adenovirus type 12 tumors. Most of the hamster sera reacted with T antigens; however, less than 50% of these sera reacted with an exclusively intranuclear antigen and an antigen found at the cell membrane. The latter two antigens were not synthesized in the presence of FUDR or when infected cells were maintained at 25° thereby requiring the synthesis of DNA.

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