THE PRESENCE OF ANTIBODIES IN HUMAN SERUM TO EARLY (T) ADENOVIRUSANTIGENS*

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The discovery by Huebner et al.\textsuperscript{1, 2} that hamsters bearing noninfectious tumors induced by adenoviruses develop antibodies against certain virus-specific antigens has led to the recognition of a new class of virus-induced antigens, the T antigens. By means of complement fixation (CF) and fluorescent antibody (FA) tests with the sera of tumor-bearing hamsters, it has been shown that T antigens are present in both tumors and acutely infected cells, and are produced by all human adenoviruses tested.\textsuperscript{1-7} They differ from the adenovirus structural antigens (referred to here as V antigens) previously defined with convalescent and hyperimmune antisera in a number of respects: (1) the T antigens form prior to viral DNA replication in the acutely infected cell, and their formation is unaffected by treatment of the cells with 5-fluorodeoxyuridine (FUDR) or cytosine arabinoside;\textsuperscript{6, 8, 9} (2) they do not appear to be incorporated into the mature virus particle; (3) they are generally heat-labile; and (4) they have characteristic morphologic appearances in the FA test, appearing as flecks or dots, primarily in the nucleus in the acutely infected cell. Recent evidence indicates that the T antigens constitute a complex group of antigens, differing from one another in morphology, breadth of reaction with heterologous types, and probably in degree of reactivity in different serologic tests.\textsuperscript{7, 10}

In view of the formation of T antigens in acutely infected cells, it appeared possible that antibody responses to T antigens might be detected following acute adenovirus infections in man. However, intensive testing by the CF procedure has yielded negative results.\textsuperscript{11} Since we have found the FA procedure often to be more sensitive than the CF test for detection of antibody, we applied this procedure to detection of T antibody responses in humans.

It is the purpose of this paper to report that by use of the indirect FA technique, antibodies are readily detectable in human sera to adenovirus T antigens present in acutely infected cells and in certain hamster tumor cells. Sera from children acutely infected with adenovirus types 1, 3, and 7 were studied and found frequently to have antibody rises to these antigens. In addition a small percentage of serums from adult patients with and without cancer have antibodies which react with Ad. 12 T antigens.

Materials and Methods.—Patients: Sera from children with acute respiratory tract infections were supplied by Drs. R. H. Parrott, H. W. Kim, and R. M. Chanock. The sera were selected from a study of acute respiratory disease during 1958-1966 at the D. C. Children’s Hospital. The acute phase serum was taken at the initial clinic visit, and the convalescent serum about 3 weeks later. Eighteen patients with Ad. 1, 3, or 7 infection were studied; in all cases the infections were diagnosed by virus isolation accompanied by fourfold or greater rise in adenovirus group CF antibody. The patients ranged in age from 3 to 124 months, with various illnesses including exudative and nonexudative pharyngitis, bronchitis, and pneumonia. Control sera were from 11 patients in the same age range with pharyngitis, bronchitis, bronchiolitis, or croup, with virus isolation and serologic response to parainfluenza 1, 2, 3, or Coxsackie B-5 virus; none of these patients showed CF antibody rise to adenovirus.
Sera from adults were collected at the George Washington University Medical Center and the D.C. General Hospital (with the assistance of Dr. Louis Alpert under NCI contract PH43-64-515) during 1961–1964. The sera used in the present studies were selected from a group of patients living in the vicinity of Washington, D.C., and carefully matched for age, race, and sex; the patients in this group were evenly distributed among three diagnostic categories—those having nonneoplastic diseases or those having histologically diagnosed tumors of either the gastrointestinal or upper respiratory tract.

Viruses: The prototype strains of Ad. 1, 3, and 12, and the E46- strain of Ad. 7 were used. These strains have been maintained by passage in KB or human embryonic kidney (HEK) cell cultures; the pools used in the present study were demonstrated to be free of adeno-associated viruses, types 1-4.

FA antigens: Two types of immunofluorescent antigen preparations were used for study of antibody responses to antigens formed during adenovirus infection of HEK cells. “T antigen coverslips” consisted of cells infected in the presence of FUDR, while “V antigen coverslips” were cells from the same batch infected without FUDR; the latter demonstrated both T and V antigens.

Primary cultures of HEK obtained from Flow Laboratories, Rockville, Md., were trypsinized and seeded onto glass coverslips as previously described. Maintenance medium was Eagle’s basal medium No. 2 with 2% heated (56°C for 30 min) Agamma calf serum (Hyland Laboratories, Los Angeles), antibiotics, and 2 mM glutamine. One hour before virus inoculation the cultures receiving FUDR were rinsed twice with Eagle’s basal medium and refed with maintenance medium containing 10–4 M FUDR (obtained from the Drug Development Branch of the National Cancer Institute); the Agamma calf serum used in the FUDR-treated cultures had been dialyzed against phosphate-buffered saline pH 7.2.

The cultures were inoculated with 10–60 plaque-forming units of adenovirus per cell, and the coverslips were fixed in cold acetone 15–24 hr later. The slips were air-dried and stored at −20°C for periods no longer than 6 weeks. Uninfected control coverslips with and without FUDR were harvested at the same time.

Each antigen lot was tested for the presence of T and V antigens by FA staining with tumor hamster serum and hyperimmune rabbit antiserum, respectively.

Reference antisera: For standardization of Ad. 3, 7, and 12 T antigen coverslips a serum pool from Ad. 12 tumor-bearing hamsters was used. For Ad. 1 the reference serum was from a hamster bearing a virus-free transplanted tumor originally induced by the Ad. 1–SV40 “hybrid” virus; this serum had no CF or neutralizing antibody to Ad. 1, but had a titer of 1:80 in FA against Ad. 1 T antigen coverslips. Rabbit antisera used for standardizing viral antigen coverslips were typing antisera prepared against the prototype strains and having neutralizing antibody titers of 1:320 or greater. T antigen coverslips gave 15–90% of cells positive when stained with the hamster serum and less than 0.01–0.1% positive with the rabbit serum, while V antigen coverslips gave 5–50% staining with the rabbit serum. An occasional batch of HEK cells contained a cytoplasmic antigen which reacted intensely in FA with certain hamster sera; such lots were not used in the present studies.

FA procedure: All FA tests were done by the indirect method as described previously. Human sera were tested unheated and without absorption; they were diluted in phosphate-buffered saline pH 7.4. Screening was done with a 1:5 serum dilution. Rabbit antisera were heated at 56°C for 30 min, absorbed with HEK or KB cells, and used at 1:10 dilution.

Fluorescein-conjugated horse antihuman-globulin (lot 311) and sheep antirabbit-globulin (lot 409) were obtained from Progressive Laboratories, Baltimore, Maryland. The conjugates were used unabsorbed at final dilutions of 1:8 and 1:10, respectively. Coverslips were counterstained with Lissamine rhodamine conjugated bovine serum albumin prepared by the method of Smith.

Coverslips were examined on an American Optical microscope using high-intensity ultraviolet illumination provided by a Spencer Fluorolume illuminator, model 645.

Results.—FA staining of T and V antigen coverslips with paired sera from acute adenovirus infections: Initial FA screening done with 1:5 dilutions of the convalescent sera from the 18 children with acute adenovirus infections revealed that the majority stained T antigen coverslips of the homologous adenovirus type.
TABLE 1

**Patterns of T Antibody Response in Children with Acute Adenovirus Infections**

<table>
<thead>
<tr>
<th>Virus isolated</th>
<th>No. patients tested</th>
<th>No. Patients with Viral antibody rise by</th>
<th>No. Patients with Indicated Pattern of T Antibody Response as Detected by FA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CF*</td>
<td>FA†</td>
</tr>
<tr>
<td>Ad. 1</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Ad. 3</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Ad. 7</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Parainfluenza or Coxsackie B</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Titrated in CF against 4-8 units Ad. 2 standard viral antigen.
† Fourfold or greater rise against homologous antigen.
‡ Fourfold or greater increase in antibody titer or conversion from negative acute to positive convalescent serum at 1–5 dilution.

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TABLE 2

**Titers of Adenovirus T and V Antibody in Human Serums**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Diagnosis and virus isolated</th>
<th>Serum</th>
<th>Antigens induced by</th>
<th>Unheated</th>
<th>Heated†</th>
<th>Nuclear</th>
<th>Cytoplasmic</th>
<th>V Antigen Coverslip</th>
<th>Staining morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. J.</td>
<td>9 mos.</td>
<td>Infectious mononucleosis, Ad. 1</td>
<td>Acute</td>
<td>&lt;8</td>
<td>Ad. 1</td>
<td>10</td>
<td>NT</td>
<td>Dots &amp; balls</td>
<td>10</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Convalescent</td>
<td>16</td>
<td>160</td>
<td>&lt;10</td>
<td>Dots &amp; balls</td>
<td>160</td>
<td>160</td>
<td>Diffuse granular &amp; aggregates</td>
</tr>
<tr>
<td>K. M.</td>
<td>4 mos.</td>
<td>Exudative pharyngitis, Ad. 3</td>
<td>Acute</td>
<td>32</td>
<td>Ad. 3</td>
<td>10</td>
<td>NT</td>
<td>Flecks</td>
<td>160</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Convalescent</td>
<td>128</td>
<td>40‡</td>
<td>&lt;5</td>
<td>Flecks</td>
<td>640</td>
<td>160</td>
<td>Diffuse granular &amp; aggregates</td>
</tr>
<tr>
<td>A. I.</td>
<td>124 mos.</td>
<td>Pharyngitis, bronchitis, Ad. 7</td>
<td>Acute</td>
<td>32</td>
<td>Ad. 7</td>
<td>&lt;10</td>
<td>NT</td>
<td>Flecks</td>
<td>10</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Convalescent</td>
<td>128</td>
<td>40‡</td>
<td>&lt;5</td>
<td>Flecks</td>
<td>160</td>
<td>160</td>
<td>Diffuse granular &amp; aggregates</td>
</tr>
<tr>
<td>H. C.</td>
<td>60 yr</td>
<td>Carcinoma, tonsil</td>
<td>Single serum</td>
<td>&lt;10</td>
<td>Ad. 12</td>
<td>20‡</td>
<td>&lt;5</td>
<td>Flecks</td>
<td>20</td>
<td>NT</td>
</tr>
<tr>
<td>H. S.</td>
<td>61 yr</td>
<td>Carcinoma, stomach</td>
<td>Single serum</td>
<td>&lt;10</td>
<td>Ad. 12</td>
<td>20</td>
<td>&lt;5</td>
<td>Dots &amp; Flecks</td>
<td>80</td>
<td>NT</td>
</tr>
<tr>
<td>M. K.</td>
<td>67 yr</td>
<td>Carcinoma, palate</td>
<td>Single serum</td>
<td>10</td>
<td>Ad. 12</td>
<td>&gt;80</td>
<td>&lt;5</td>
<td>Flecks</td>
<td>&gt;80</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Titrated in CF against 4-8 units Ad. 2 standard viral antigen.
† Heated at 56°C for 30 min. NT = not tested.
‡ Reaction blocked by absorption with Ad. 7 hamster tumor extract CF pool 4. K. M. also has a 1–5 titers against Ad. 3 hamster tumor cells.
§ Reaction blocked by absorption with Ad. 12 hamster tumor extract.
Titrations of the paired sera by the FA procedure were then carried out, with results as shown in Table 1; the results on selected patients are shown in more detail in Table 2.

Eleven of the 18 paired sera demonstrated significant rises in FA antibody titer to the T antigen coverslips, four showing conversion from negative to positive (the convalescent serum generally showing low titer) and seven showing fourfold or greater increase in titer above that of a positive acute phase serum. Five patients showed comparable titers in both sera, while two had no detectable antibody in either serum. All 18 showed antibody response when titrated against homologous V antigen coverslips (Table 1).

The morphologic patterns produced by the children's sera on the T and V antigen coverslips were quite distinct and in general closely resembled the staining patterns seen with homologous adenovirus tumored hamster and hyperimmune rabbit antisera. The convalescent serum from Ad. 1 patients consistently stained numerous, tiny, bright, intranuclear "dots" and larger "balls" in Ad. 1 T antigen coverslips, while the Ad. 3 and Ad. 7 convalescent sera stained short, thin, intranuclear flecks in homologous T antigen preparations, flecks which were similar to but somewhat shorter than those described for Ad. 12 T antigen staining. The reactions with the adenovirus V antigens in HEK were similar in each system. Entire nuclei were either filled with a brilliant homogenous stain, or contained either large, amorphous, crystalline aggregates or smooth circular or lobulated discs and were similar in appearance to those previously described by Boyer et al. In addition, many cells with positive nuclei also showed uniform cytoplasmic staining of lower intensity.

Of the 11 pairs of sera from control patients with other viral-induced respiratory illnesses, none showed demonstrable rises by FA to Ad. 1, 3, 7, or 12 T antigen coverslips. Both sera from four of these patients, however, had comparable antibody titers to Ad. 1 and/or Ad. 12 T and V antigen preparations. Each of these four patients had prior adenovirus infection as indicated by the presence in both serum specimens of comparable levels of group-specific CF antibody.

None of the 18 serum pairs from the adenovirus-infected children nor the four pairs from the adenovirus-positive controls showed comparable staining of un inoculated HEK cells with or without FUDR, or HEK cells infected with SV40 or herpes simplex virus in the presence of FUDR. Most of the sera when tested at dilutions of 1:5 or lower against uninfected cells gave dull, finely granular, nuclear staining often confined to the center of the nucleus, which tended to obscure the specific reactions in low-titer sera.

Studies on sera from adult patients: As a preliminary screen of cancer patients, sera of 80 persons with tumors of the gastrointestinal and upper respiratory tracts and 40 matched control patients without tumors were tested at 1:5 dilution against Ad. 12 T antigen coverslips prepared in African green monkey kidney cells (AGMK) with FUDR. Three of the sera from patients with cancer stained fine flecks characteristic of those stained by Ad. 12 tumored hamster sera; with two of the sera the flecks were both nuclear and cytoplasmic, while with the third serum the flecks were almost exclusively nuclear. The titers of the three sera against T and V coverslips prepared in HEK are shown in Table 2; the sera did not stain FUDR-treated HEK cells infected with Ad. 1, 3, 7, or SV40, or un inoculated HEK cells with or without FUDR. When tested on Ad. 12 transformed hamster cells, all three sera gave
bright staining of one or two large cytoplasmic flecks in a moderate number of cells; these flecks were indistinguishable from a type of fleck seen on staining with Ad. 12 tumored hamster sera. These sera did not stain normal or SV40-transformed hamster cells. Of 40 sera from patients without cancer, one gave comparable staining on both the Ad. 12 T antigen and transformed cell preparations.

Four of the 80 cancer sera, including one of those (M. K.) reacting with Ad. 12, gave dot-type nuclear staining of 100% of unoinoculated HEK cells. In the case of M. K. the nonspecific staining was seen only with low serum dilutions and in infected cells was obscured by the more brilliant flecks. None of the 40 control sera showed this nonspecific pattern.

Characterization of the reactive antigens: The above findings established that the antigens in the T antigen coverslips which were stained by human sera had two of the characteristic features of T antigens as defined with tumored hamster sera. That is, they had the characteristic dot or fleck morphology and they formed efficiently in the presence of FUDR.

Additional characterization was carried out with the selected sera in Table 2. Cytosine arabinoside (10 μg/ml) did not inhibit formation of the FA-stainable antigens in HEK. Also, the antigens formed efficiently in FUDR-treated AGMK cells; in AGMK cells, even without FUDR, adenovirus infection is abortive, T antigens forming efficiently and later structural antigens forming very inefficiently.18

Perhaps the best parameter with which to distinguish T from V antigens is their time of appearance in infected cells. To determine the time course of the development of the Ad. T and V antigens stained by human convalescent sera as compared with those detected by tumored hamster and hyperimmune rabbit sera, sequential studies on antigen development were done in HEK with and without FUDR. Coverslips were harvested at four-hour intervals from the time of inoculation to 24 hours and stained with eight or more units of human,hamster, and rabbit antibody. The children's sera shown in Table 2 were tested against the sequential harvests of the homologous adenovirus type, and the three cancer sera against Ad. 12. With each virus type, in the FUDR-treated cells the antigens reacting with human and hamster sera followed the same time curve, while in the cells without FUDR the antigens with viral morphology, detectable with the rabbit and human sera, appeared four to eight hours later. It should be noted that the human sera also stained early, dot- or fleck-shaped antigens in the cells without FUDR. A typical set of curves is shown in Figure 1.

In contrast to viral structural antigens, certain of the adenovirus T antigens are labile to heating at 56°C for 30 min.14 To test for heat lability of the FA antigens, T and V antigen coverslips were immersed for 30 min in buffered

![Figure 1](image-url)

**Fig. 1.**—Sequential studies on the development of Ad. 3 T and V antigens in HEK. Ag = antigen; HS = hamster serum; RS = rabbit serum.
saline pH 7.4 at 56°C, then rinsed once in room temperature saline, once in distilled water, and stained with serial dilutions of the human sera (Table 2). In all instances the sera failed to react with heated T antigen coverslips, while against the heated V antigen coverslips the reaction was unchanged or decreased only fourfold. With hamster sera, heating abolished staining with Ad. 1, but did not affect the staining of Ad. 3, 7, or 12 by the pool of Ad. 12 tumored hamster sera used in these experiments.

To attempt to relate the human antibodies to the hamster tumor systems, two approaches were used: staining of homologous hamster tumor or transformed cells and blocking tests with hamster tumor extracts. As mentioned above, the three Ad. 12-reactive sera gave positive staining of one morphologic type of antigen in Ad. 12 transformed cells; the Ad. 3 patient K. M. (Table 2) stained nuclear flecks in Ad. 3 tumor cells grown in tissue culture, while the Ad. 1 and Ad. 7 patients did not stain homologous hamster tumor cells. Blocking tests were performed by mixing two to four FA units of serum with equal volumes of SV40, Ad. 7 (Gomen strain) or Ad. 12 (Huie strain) hamster tumor extracts or with 20-fold concentrated suspensions of HEK infected with homologous adenovirus in the presence of FUDR. Mixtures were incubated at 37°C for 30 min prior to staining coverslips. A serum was considered blocked by the absorbing antigen if there was obliteration or marked reduction in per cent staining and intensity compared with an aliquot absorbed with SV40 or uninoculated HEK suspension. Staining of homologous T antigen coverslips by the serum from Ad. 3 patient, K. M., was successfully blocked by absorbing the serum with the Ad. 7 hamster tumor extract; also, the staining of homologous T antigen coverslips by the serum from the Ad. 7 patient, A. I., was blocked by absorbing with either the Ad. 7 or the Ad. 12 hamster tumor extract. Previous studies have demonstrated sharing of T antigens between Ad. 3 and 7 in CF2 and among Ad. 3, 7, and 12 in FA tests. Staining by these two sera was likewise blocked by the concentrated HEK suspensions infected with homologous adenovirus. Staining by one serum of the cancer patients was blocked by adsorption with Ad. 12 hamster tumor extract but the two other sera gave negative or equivocal results.

Discussion.—Although the original studies of hamster tumors indicated that induction of antibody to the T antigens was dependent on the chronic, massive antigenic stimulus provided by a tumor, more recent studies have demonstrated that this is not the case. Sabin has reported that monkeys infected with SV40 develop T antibody detectable by CF. We have been able to induce T antibody detectable by FA by immunization of hamsters with cell-free extracts of adenovirus-induced tumors, and have found that rabbits and monkeys immunized with SV40 regularly develop T antibody detectable by FA.

Thus it is not surprising that infected humans develop antibody to certain of the early antigens of the infecting adenovirus. It should be emphasized that the children studied here had respiratory illness associated with, and probably caused by, adenovirus infection. Whether subclinical infection, with presumably less antigenic stimulation, is as frequently productive of antibody rise to these antigens remains to be determined.

The finding that some of the human sera stained adenovirus transformed or hamster tumor cells and the ability of hamster tumor extract to block three of the
five sera tested suggest that some, but not all, of the antigens detected with the various human sera are immunologically similar to those produced in hamster tumors.

The chief importance of the present work lies in the potential use of the FA method for seroepidemiologic evaluation of the possible role of adenoviruses in the etiology of human cancer. If adenoviruses play a significant role, this should be detectable as excess frequency of T antibody in patients with certain types of malignancy as compared with suitable controls. Obviously, as with any serological test, the finding of T antibody in serum of a cancer patient, as reported here, is in itself of no etiologic significance.

For seroepidemiologic studies proper preparation and standardization of T antigen coverslips becomes essential. Preliminary results suggest that to obtain readily detectable percentages of T positive cells, coverslip cultures must be infected with high multiplicities (10–60 PFU per cell) of virus. Because of the prevalence of FA-stainable viral antibody in the general population or possible variation among batches of tissue culture, each lot of T antigens must not only be shown to be reactive with a T positive serum but must have a very low percentage staining with a potent, specific antiviral serum. Preliminary data suggest that the use of cells from heterologous species and/or harvesting coverslips at earlier time points prior to the development of detectable viral antigens may help surmount some of the problems in the preparation of specific antigens. In addition, these two procedures have the advantage of giving coverslips with less cytopathogenicity.

A major problem presently envisioned in the interpretation of serologic surveys is the breadth of reactivity seen in some of the human responses; preliminary results suggest that human responses to heterologous adenovirus types may be broader and of different patterns than those of tumor-bearing hamsters. In view of the marked differences between oncogenicity of various adenovirus subgroups, a subgroup-specific or type-specific antibody test might be most desirable for cancer etiology studies.

Another aspect of the human T antibody response which must be clarified is the persistence of the homologous and heterologous antibodies. The adenovirus hamster tumor model suggests that the antibody might be of very short duration, since hamsters generally show a rapid fall in CF antibody to T antigens following excision of the tumor. The frequent finding of antibody in the acute phase serum speaks against this possibility. It is conceivable that the children with preexisting antibody represent those who harbor an adenovirus in their adenoids or tonsils.

In addition to seroepidemiologic studies, the FA test for T antibody may be important in selecting patients in attempting to demonstrate adenovirus genetic material in tumor tissue.

The present studies also indicate that the indirect FA test is highly sensitive for detection of antibodies to the late viral antigens, and may have usefulness as a diagnostic tool.

Summary.—Of 18 children infected with adenovirus types 1, 3, or 7, 16 had antibodies detectable by indirect immunofluorescent techniques to T antigens produced in acutely infected cells by homologous adenoviruses. Eleven of the children had demonstrable antibody rises to the T antigens. Some of the T antigens reacting
with the sera from patients infected with Ad. 3 and Ad. 7 are immunologically related to antigens present in the homologous hamster tumor system.

In addition, sera from three patients with cancer and one patient with a chronic nonneoplastic disease from a matched group of 120 adults contained antibody to Ad. 12 T antigens present in both acutely infected cultures and in Ad. 12 transformed hamster embryo cells. The ability to detect such antibodies by immunofluorescence may provide a useful method for epidemiological studies to determine the possible role of adenoviruses in human cancer.

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