Characterization of a Tumorlike Antigen in Type 12 and Type 18 Adenovirus-Infected Cells

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ABSTRACT

GILEAD, ZVEE (University of Pennsylvania, Philadelphia), AND HAROLD S. GINSBERG. Characterization of a tumor-like antigen in type 12 and type 18 adenovirus-infected cells. J. Bacteriol. 90:120-125. 1965.—An antigen that reacts with antibody from type 12 adenovirus tumor-bearing hamsters was identified in extracts of KB cells infected with type 12 or 18 adenovirus. In contrast, viral structural proteins separated by chromatography on diethylaminoethyl-cellulose did not react with the sera from tumorous hamsters. The tumorlike (T) antigen in infected cells was found to be smaller than the viral structural antigens and, therefore, could be separated from them by centrifugation in a linear sucrose gradient. Investigation of the production of the T antigen in virus-infected cells further distinguished it from viral structural proteins by the following properties: (i) the T antigen was first detected 3 to 4 hr after infection, whereas viral antigens were synthesized 17 to 20 hr after infection; and (ii) the T antigen was produced when deoxyribonucleic acid (DNA) biosynthesis was inhibited by 5-fluorodeoxyuridine (10⁻⁶ M), but viral proteins were not synthesized in the absence of viral DNA replication.

A unique antigen, but not infectious virus, is present in type 12 and 18 adenovirus-induced tumors or in vitro transformed cells, and in tissue culture cell lines derived from tumors. The tumor-like (T) antigen is also present in cultured cells infected with type 12 and 18 adenoviruses (Huebner, Rowe, and Lane, 1962; Huebner et al., 1963; McBride and Wiener, 1964; Gerber, 1964; Kitamura et al., 1964). The novel antigen is distinguished by its reaction with sera from tumor-bearing hamsters and from rabbits immunized with homogenates of cells infected with either oncogenic adenovirus.

The structural proteins of the adenoviruses studied consist of polygonal, hollow capsomeres and fiberlike structures. The polygonal capsomere, the predominant virion protein, is identical with the group, cross-reactive “A” or “L” soluble antigen; the fiber, a minor virion protein, corresponds to the type-specific “C” or “E” soluble antigen (Pereira et al., 1959; Wilcox and Ginsberg, 1961, 1963; Wilcox, Ginsberg, and Anderson, 1963). These antigens will be referred to as polygon and fiber antigens, respectively. The objective of this investigation was to purify the T antigen from infected cells, to study its properties, and to determine its relationship to the known viral structural proteins. During the course of these studies, the conflicting reports were made that the viral fiber (“C” or “E”) antigen was immunologically and chromatographically similar to the T antigen (Huebner et al., 1964), and that a T antigen, distinct from the viral antigens, could be detected in the cytoplasm of infected cells by immunofluorescent techniques (Pope and Rowe, 1964).

The data to be reported in this communication indicate that in type 12 or 18 adenovirus-infected cells the T antigen is distinct from the viral structural proteins, that the T antigen is synthesized before the viral antigens, and that the production of T antigen does not require deoxyribonucleic acid (DNA) synthesis.

MATERIALS AND METHODS

Tissue culture. Monolayers or spinner cultures of KB cells were employed. Cells were propagated in Eagle’s minimal essential medium (MEM) supplemented with 10% calf or human serum by methods previously described (Ginsberg et al., 1955; Eagle, 1950).

Viruses. Type 12 adenovirus, strain Huie, was kindly supplied by J. Trentin, and type 18 virus, strain D.C., was obtained from Robert Huebner. Both serotypes were plaque-purified twice and propagated in KB cultures. To prepare seed virus pools and for the experiments to be described, cells were infected with a viral multiplicity of 5 to 10 plaque-forming units (PFU) per cell and were...
harvested after 40 hr at 36 C. Infected cells were concentrated 10-fold, and were disrupted by six cycles of freezing and thawing to release virus. Type 5 virus was prepared as previously described (Wilcox and Ginsberg, 1961). The clarified viral suspension was stored at -28 C.

Infecitivity assay. Infectious virus was quantitated by a plaque assay using 10 ml of an overlay consisting of 0.85% Difco purified agar, Eagle's MEM modified to contain the usual concentration of amino acids, 0.4 the usual concentration of vitamins, and twice the concentration of Earles' balanced salts (Earle, 1950). The medium was supplemented with 0.3% glutamine, 0.5% lactalbumin hydrolysate, 1% peptone, and 15% chicken serum. Because plaques produced by adenovirus type 12 and 18 could not be counted until 21 days and 18 days, respectively, after infection, it was necessary to introduce additional overlays of 4 ml each on days 9 and 13 for type 18 virus, and 3 ml each on days 8, 13, and 18 for type 12 virus. To the last overlay was added neutral red solution to make a final dilution of 1:50,000.

Preparation of antisera. Rabbits were immunized with concentrated extracts of KB cells infected with type 5, 12, or 18 virus. The rabbits received four injections of 10 ml each according to the following schedule: day 0, intravenous injection; days 10, 20, 21, and 50, intraperitoneal injections. Rabbits were bled 10 days after the last injection; the separated sera were heated at 56 C for 30 min, and were stored at -28 C. Before use, sera were adsorbed with a concentrated suspension of disrupted KB cells to remove antibodies to host antigens, and were clarified by two cycles of centrifugation: 3440 X g for 15 min, and 87,000 X g for 2 hr. The efficiency of adsorption was tested by complement-fixation titrations with extracts of uninfected KB cells as antigen.

Tumor-bearing hamsters' sera. Three serum pools, each containing sera from 8 to 13 hamsters, were generously supplied by J. Trentin. The sera were obtained from hamsters bearing transplantable type 12 adenovirus tumors. The tumor on each hamster had been passed 16 to 18 times at the time of bleeding. The sera were heated at 56 C for 30 min, and were clarified by centrifugation at 87,000 X g for 30 min.

Complement fixation. The microtechnique was employed as described by Sever (1962). Antigen titers are expressed as the highest dilution that completely fixed 2 full units of guinea pig complement in the presence of 4 to 8 units of antibody.

Purification of virus. Virus was purified as reported previously (Wilcox and Ginsberg, 1963) for type 5 adenovirus, except that the final equilibrium density centrifugation was made in a preformed linear CaCl2 gradient with a density from 1.2 to 1.4 g/ml. The partially purified and concentrated virus was layered carefully on top of the linear CaCl2 gradient, and the mixture was centrifuged in the SW 39 rotor at 34,000 rev/min for 4 hr in the model L preparative ultracentrifuge.

Separation of antigens from type 12 or 18 virus-infected cells. Infected cells were washed with Hanks' balanced salt solution, concentrated approximately 10-fold, and disrupted by freezing and thawing six times or by adding 0.25% sodium deoxycholate. The suspension was treated with 2 equal volumes of Freon 113 two times to remove host cell impurities, and was centrifuged 87,000 X g for 30 min to remove viral particles. The partially purified cell extract was employed to identify the virus-specific antigens present.

Diethylaminoethyl (DEAE)-cellulose chromatography. Chromatography was carried out as previously described (Wilcox and Ginsberg, 1961), except that the elution of antigens was accomplished with use of a linear gradient from 0 to 0.25 M NaCl in 0.05 M phosphate buffer (pH 7.5).

Sucrose density gradient centrifugation. The procedure was that of Martin and Ames (1961), with a linear gradient of 5 to 20% sucrose in 0.01 M phosphate buffer (pH 7.5). Centrifugations were carried out in the Spinco model L ultracentrifuge with use of the SW 39 or SW 25 swinging bucket rotor at 35,000 rev/min for 18 hr or 24,000 rev/min for 30 hr, respectively.

Chemical determinations. Incorporation of tritiated deoxyctydine (specific activity 1.1 c/mmole, purchased from Schwarz Bio Research Inc., Orangeburg, N. Y.) into DNA was measured as described by Kit and Dubbs (1962). DNA was measured by the Burton (1958) technique, with calf thymus DNA as a standard. Protein was estimated by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

Results

Soluble antigens in cells infected with type 12 or 18 adenovirus. To separate and identify the soluble antigens in cells infected with oncogenic adenoviruses, types 12 and 18, extracts were chromatographed on DEAE-cellulose, and the eluates were assayed by complement fixation to use of types 5, 12, and 18 rabbit specific antisera. Two soluble antigens, identified as the viral polygon and fiber antigens, were present in extracts of type 12 and 18 virus-infected cells. The chromatographic characteristics were similar to those described by Heubner and co-workers (1964) for type 12 antigens. When concentrated suspensions of the purified soluble antigens from type 12 or 18 virus were assayed with sera from tumor-bearing hamsters, neither the polygon nor the fiber antigen could be detected. But when the hamster sera were used with crude extracts of type 12 or 18 virus-infected cells, antigen titers of 1:16 to 1:32 were observed (Table 1).

Separation of the antigens by sucrose density gradient centrifugation. The above data suggested that a third antigen, similar to the hamster tumor antigen, was present in type 12 or 18 virus infected cells but was not eluted efficiently from DEAE-cellulose under the conditions employed.
TABLE 1. Immunological relationship of tumorlike antigen to viral structural proteins

<table>
<thead>
<tr>
<th>Antigen assayed</th>
<th>Complement fixation with</th>
<th>Type 12 rabbit antisera</th>
<th>Tumorous hamster serum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude type 12 antigens†</td>
<td>1:256 1:16-1:32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 12 polygon antigen‡</td>
<td>1:1,024 &lt;1:2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 12 fiber antigen‡</td>
<td>1:512 &lt;1:2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* From hamsters bearing type 12 transplanted tumors.
† Prepared by freezing and thawing type 12 infected cells 48 hr after infection.
‡ Purified by chromatography on a DEAE-cellulose column, and concentrated by lyophilization.

Attempts were made to separate the T antigen from the viral antigens by zonal centrifugation in a linear sucrose gradient. The results obtained with an extract of type 12 virus-infected cells are presented graphically in Fig. 1. Three antigen bands were detected. The cross-reactive polygon antigen, which reacts with type 12 and 18 rabbit antisera but not with hamster serum, was the heaviest molecule. An antigen that reacted with antibodies present in the hamster serum, as well as in type 12 and 18 antisera, was detected near the top of the gradient. The fiber antigen, which reacts only with homologous type 12 antisera, had intermediate sedimenting characteristics.

Time of appearance of the T antigen in type 12 and 18 adenovirus-infected cells. The relationship of the T antigen to the viral structural proteins was investigated further by determining the time each was synthesized. Spinner cultures of KB cells were infected with type 12 virus at a multiplicity of 30 to 50 PFU per cell, and samples were removed at intervals after a 2-hr adsorption period. The infected cells were washed, diluted to a concentration of $4 \times 10^4$ to $6 \times 10^6$/ml, and disrupted by sonic treatment in an MSE ultrasonic disintegrator at 0 C. The results of a representative experiment, summarized in Fig. 2, demonstrate that an antigen that reacted with sera from tumorous hamsters was first detected 3 hr after infection and increased until about 15 hr after addition of virus. The same antigen was measured with type 12 or 18 rabbit antisera but not with antiserum directed against type 5 adenovirus. In contrast, viral structural antigens, assayed with type 5 antiserum as well as with types 12 and 18 antisera, were not detectable until 17 to 20 hr after infection. Infectious virus was first produced about 20 hr after infection. The antigen that reacted with hamster sera was not detectable in type 5 adenovirus-infected cells or in uninfected KB cells. Similar results were obtained in cells infected with either type 18 adenovirus or with highly purified type 12 virus. These data

Fig. 1. Separation of the T antigen and the viral structural proteins by centrifugation in a linear sucrose gradient. An extract of type 18 virus-infected cells was layered on a linear 5 to 50% sucrose gradient and was centrifuged in the SW 28 rotor for 30 hr at 24,000 rev/min. The antigens were identified by complement fixation with rabbit specific antisera and sera from tumor-bearing hamsters.

Fig. 2. Kinetics of synthesis of intracellular virus, viral structural proteins, and the T antigen in type 12 virus-infected cells (2a). Spinner cultures of KB cells were infected with 30 to 50 PFU per cell, and samples were taken at intervals after a 2-hr adsorption period. Washed cells made to a concentration of $4 \times 10^4$ to $6 \times 10^6$ cells/milliliter were sonic treated and assayed for infectious virus and complement-fixing antigens. A type 8 virus-infected culture was assayed similarly (2b).
indicated that the T antigen was produced before viral structural antigens and added further evidence implying that the T antigen was distinct from the viral subunits.

**Effect of inhibiting viral DNA synthesis on production of the T antigen.** Biosynthesis of adenovirus protein subunits requires prior biosynthesis of viral DNA (Flanagan and Ginsberg, 1962). The finding that the T antigen appeared 14 to 17 hr before viral structural proteins, and before detectable replication of viral DNA, appeared to distinguish sharply the two sets of antigens. These data also suggested that viral DNA synthesis was not essential for the production of T antigen. This postulate was tested directly by inhibiting DNA synthesis with 5-fluorodeoxyuridine (FUdR; Cohen et al., 1958; Hartmann and Heidelberger, 1961). Experiments were done as described above except that infected cells were suspended in Eagle's medium supplemented with dialyzed chicken serum, and one infected culture received 10^-6 M FUdR. DNA synthesis was measured by the incorporation of tritiated deoxythymidine. FUdR completely inhibited synthesis of DNA and infectious virus and completely suppressed detectable biosynthesis of viral antigens (Fig. 3). In striking contrast, the T antigen was synthesized, as measured with sera from tumor-bearing hamsters and with type 12 and type 18 rabbit antisera. Similar to the experimental results summarized in Fig. 2, the T antigen was first detected by 4 to 6 hr after viral infection.

**Relative size of the T antigen synthesized in the presence of FUdR.** To characterize further the T antigen synthesized in type 12 virus-infected cells in the presence of 10^-4 M FUdR, its relative size was estimated by centrifugation in a linear sucrose gradient. The cell extract was prepared 20 hr after infection, concentrated fivefold by lyophilization, and centrifuged in a 5 to 20% linear sucrose gradient. For comparison, the fiber antigen, purified by chromatography on a DEAE-cellulose column, was centrifuged concurrently in a second gradient. The results of a representative experiment, presented in Fig. 4, demonstrate that the antigen synthesized in the presence of FUdR sedimented more slowly than the viral fiber antigen, and that neither the polygon nor the fiber antigen could be detected in cells infected in the presence of FUdR. The data confirm the findings summarized in Fig. 1, indicating that the T antigen was lighter than the viral antigens.

![Graph](http://jb.asm.org/ADENOVIRUS-TUMOR-ANTIGEN_123.png)
DISCUSSION

Tumors initiated by oncogenic adenoviruses contain at least one distinctive antigen, but infectious virus does not persist. The so-called tumor or "T" antigen is also found in cell lines derived from hamster tumors, in cells transformed in vitro by type 12 virus, and in cultured cells infected with type 12 or 18 adenovirus. Pope and Rowe (1964), using immunofluorescent techniques, demonstrated that the antigen in the hamster tumors or "transformed" lines appeared as unique cytoplasmic flecks and was distinct from viral antigens. The data presented above demonstrate that an antigen distinct from the viral structural proteins is synthesized in type 12 and 18 adenovirus-infected cells. When identified with sera from tumor-bearing hamsters, the antigen in infected cells and in tumors appear related.

The T antigen of infected cells is distinguished from the viral subunits, the polygon (A or L) antigen, and the fiber (C or E) antigen, by the following characteristics: (i) it is immunologically distinct; (ii) it is synthesized many hours prior to the viral structural proteins; (iii) its biosynthesis does not require replication of viral DNA; and (iv) it is smaller than the virion antigens. Some of the properties of the T antigen listed suggest that it may be a macromolecule, perhaps an "early protein" required in the sequential biosynthesis of the virion (Cohen, 1961). However, a relationship of the T antigen with viral replication or tumorogenesis is uncertain and perforce will remain so until its nature and function are defined.

SVφ virus also induces tumors in newborn hamsters, and similar to adenovirus-induced tumors, a new tumor antigen but not infectious virus is present (Huebner et al., 1963; Black et al., 1963). The SVφ tumor antigen is also synthesized in virus-infected cells in culture and appears within 8 to 12 hr after infection, many hours before the appearance of the viral antigen (Sabin and Koch, 1964; Rapp et al., 1964).

It is tempting to postulate that the information for synthesis of the T antigen is contained initially in the DNA of the tumorigenic adenovirus and becomes integrated in the genome of the tumor cells. This notion is consistent with the available data. Alternatively, and not contrary to existing evidence, are the possibilities that (i) the information could have been carried into the cell within the adenovirus particle but not as a part of the viral genome; or (ii) the biosynthesis of the tumor antigen and, indeed, even the tumor production, could result from a virus-induced inheritable alteration of the cell, i.e., a mutation.

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ADDENDUM

Since this manuscript was prepared for publication two additional papers have been published that directly bear upon the data described in the present communication. Hoggan et al. (Proc. Natl. Acad. Sci. U.S. 53:12, 1963) confirmed earlier findings that a tumorlike antigen was present in cells infected with type 12 or 18 adenovirus. In addition, they showed that the tumorlike antigen appeared considerably before the viral antigens. Rapp and co-workers (Science 147:625, 1965), studying infections with SVφ virus, reported that the tumor antigen was synthesized in the presence of cytosine arabinoside, an inhibitor of DNA biosynthesis.

LITERATURE CITED


