Characterization of the Tumorlike (T) Antigen Induced by Type 12 Adenovirus¹

II. Physical and Chemical Properties

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T antigen, purified from KB cells infected with type 12 adenovirus, was characterized by chemical and physical methods. The immunologically active molecule had an ultraviolet-absorption spectrum characteristic of a protein, and was partially inactivated (i.e., approximately 85%) by trypsin (final concentration of 150 $\mu\text{g/ml}$). Centrifugation analyses indicated that the purified antigen was a single molecular species with an average sedimentation coefficient of 2.40S (measured by zonal centrifugation in sucrose gradients) and an $S_{20,w}$ of 2.58 determined by analytical centrifugation. The antigen was inactivated at temperatures above 40 C, it was stable between pH 5.7 and 8.8, and it had an isoelectric point of approximately 5.0, as determined by electrophoresis on cellulose polyacetate membranes.

Studies of crude preparations of the T antigen from tumors induced by type 12 adenovirus or from cells infected with this virus indicated that the antigen is heat-labile (11, 13) and that it is smaller than the viral capsid proteins (7).

A procedure developed for the purification of the type 12 T antigen yielded a purified product with negligible amounts of detectable host contamination, and the methods employed suggested that the antigen is a protein which is highly lipophilic (9). Utilization of purified antigen has permitted further study of its chemical and physical properties. The chemical nature of the antigen, its sedimentation coefficient, its thermal and pH stabilities, and its isoelectric point will be reported in this communication.

MATERIALS AND METHODS

The methods for cell cultures, viral propagation, T antigen preparation, complement-fixation assay, immunoelectrophoresis, and protein determination were identical to those described in the accompanying paper (9).

Ultraviolet-absorption spectrum of the purified T antigen. The ultraviolet-absorption spectrum of the purified antigen was determined in a Zeiss spectrophotometer with the use of a quartz cell with a 10-mm

light path. A solution of 200 μg of antigen per ml in 0.1 M phosphate buffer (pH 7.5) was employed.

Sucrose density gradient centrifugation. The procedure of Martin and Ames (15) was employed with the use of a gradient of 5 to 20% in 0.05 M phosphate buffer (pH 7.5). A purified ³H-labeled equine γG anti-p-amino-phenyl- β -lactoside antibody (14), which was labeled as described by Haber (10), was used as a marker. Samples were centrifuged at 41,000 rev/min for 17 hr in a SW-50 rotor at 8 C. Fractions of 3 drops were collected through a hole in the bottom of the tube, and each fraction was analyzed by complement-fixation titrations and radioactivity assay. Amounts of 40 μl of each fraction were put on Whatman 3 MM filter discs and treated and counted as described previously (9).

Analytical ultracentrifugation. The analytical ultracentrifugation analyses utilized a modified optical system of the Beckman-Spinco analytical ultracentrifuge (19), and the sedimenting material was measured by ultraviolet absorption at 235 $m\mu$. The sedimentation coefficient of the purified T antigen, in 0.1 M phosphate buffer (pH 7.5) was determined on preparations containing 120 to 150 $\mu\text{g/ml}$ with an optical density at 235 $m\mu$ (OD_{235}) of approximately 0.6. The sedimentation was carried out for 144 min at 50,740 or 59,780 rev/min at 20 C, with the use of a 30-mm Epon-aluminum centerpiece. Photographs were taken at 8-min intervals with 235- $m\mu$ light on Kodak 111-0 film. Densitometer tracings of the photographs were made with a modified Beckman RB Analytrol.

RESULTS

Chemical properties of the antigen. The results of a representative ultraviolet-absorption spec-

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FIG. 1. Ultraviolet-absorption spectrum of the purified T antigen. A purified preparation containing 200 $\mu\text{g}/\text{ml}$ in 0.1 M phosphate buffer (pH 7.5) was analyzed in a Zeiss spectrophotometer.

TABLE 1. Effect of tryptic digestion on the antigenic activity of T antigen

Sample ^a	Original titer	Titer after incubation
T antigen.....	1:32	1:32
T antigen + enzyme.....	1:32	1:4

^a Samples (0.8 ml) of purified T antigen were mixed with 0.2 ml of 0.1 M phosphate buffer or with 0.1 ml of trypsin (1.5 mg/ml). After incubation for 1 hr at 37 C, enzymatic digestion was terminated by the addition of 0.1 ml of soy bean inhibitor (1.5 mg/ml).

trum of the T antigen are presented in Fig. 1. The spectrum, which has a maximum at 278 $\text{m}\mu$ and a minimum of 250 $\text{m}\mu$, is characteristic of a protein. It was not practicable to measure nucleic acids directly because of the relatively small amounts of each preparation available, but the spectral properties suggest that the antigen did not contain significant amounts of nucleic acids.

The antigenic activity of the purified T antigen was reduced at least 85% by tryptic digestion (150 $\mu\text{g}/\text{ml}$ of reaction mixture) for 1 hr at 37 C (Table 1). It should be noted, however, that a relatively high concentration of enzyme (twice

recrystallized; Nutritional Biochemicals Corp., Cleveland, Ohio) was required for the partial inactivation. The digestion was terminated by the addition of soy bean inhibitor (five times recrystallized; Nutritional Biochemicals Corp.).

Because of the relatively small amounts of purified antigen obtained, it was not possible to determine whether the antigen contained non-proteinic fractions, such as lipids or polysaccharides, and whether such fractions are essential to the antigenic conformation.

Determination of the sedimentation coefficient of the T antigen. Because of the relatively small quantities of T antigen available, its sedimentation in the analytical ultracentrifuge could not be measured by schlieren optics or by the usual ultraviolet-absorption optics. The sedimenting material was, therefore, analyzed at 235 $\text{m}\mu$ with the use of a special monochromator (19) through the kindness of J. Rosenbloom of the Philadelphia General Hospital. A composite drawing of tracings of pictures made at 32-min intervals during a typical centrifugation at 50,740 rev/min is shown in Fig. 2. The protein sedimented as a single component with an average $S_{20,w}$ of 2.58 (mean of four different preparations).

To determine whether the macromolecule measured by analytical ultracentrifugation was in fact the T antigen, the purified preparation was also analyzed by zonal centrifugation in a sucrose gradient, and the antigen was detected by complement-fixation titrations. Purified T antigen, 0.3 ml, was mixed with 15 μl of a ^3H -labeled γG marker and centrifuged through a linear sucrose gradient. The results of a representative experiment (shown in Fig. 3) demonstrate that the antigen sedimented as a single symmetrical peak. In four experiments, the T antigen had sedimentation coefficients ranging from 2.2 to 2.6S, as calculated by use of the 6.6S γG marker. These

FIG. 2. Analytical ultracentrifugation of purified T antigen. A composite drawing of tracings of photographs taken at 32-min intervals after attaining a rotational speed of 50,740 rev/min. The sedimenting peak was measured with ultraviolet optics at 235 $\text{m}\mu$.

FIG. 3. Estimation of the sedimentation coefficient of the T antigen by centrifugation in a linear sucrose gradient. A 15- μ l amount of a ^3H -labeled γ_G immunoglobulin, which served as a marker of known sedimentation coefficient, was mixed with 0.3 ml of purified T antigen, and the mixture was analyzed in a 5 to 20% sucrose gradient. The gradients were centrifuged for 17 hr at 41,000 rev/min in a SW-50 rotor.

FIG. 4. Determination of the thermal stability of the T antigen. Samples of purified or partially purified T antigen preparations were heated for 30 min at the temperatures indicated. The complement-fixation titers of the residual antigen were assayed with sera from tumor-bearing hamsters.

data correspond well to the $S_{20,w}$ of 2.58 obtained with analytical ultracentrifugation, and indicate that the macromolecules measured by both methods were identical.

Thermal sensitivity of the T antigen. Purified T antigen or a preparation of T antigen which was partially purified by Freon extraction and high-speed centrifugation (9) was employed to determine the stability of the antigen to heat. Samples (0.5 ml) were placed in tubes or sealed ampoules, heated at various temperatures for 30 min, and then plunged into ice. The results of a typical experiment (Fig. 4) indicate that the antigen was markedly thermosensitive. T antigen was only stable at temperatures of 40°C or below, unlike the virion antigens which resist heating at 56°C for 30 min. The purified T antigen, unprotected by contaminants, had a faster inactivation rate than the relatively crude preparation.

pH stability of the T antigen. To characterize the T antigen by the pH range in which it is stable, 0.35-ml samples of a purified preparation were placed in small-diameter dialysis bags and dia-

lyzed for 20 hr at 4°C against 1,800 ml of 0.05 M buffers of various pH values. Phosphate buffers were used to obtain values from pH 5 to 8.8. For pH 9.8, a glycine-NaOH buffer was employed. The antigen was assayed by complement-fixation titrations with sera from tumor-bearing hamsters. It was unnecessary to restore the dialyzed samples to pH 7.5 before assay because antigen in the first well was diluted 1:6 in the Veronal buffer employed. The results obtained (Fig. 5) demonstrate that the antigen was stable between pH 5.7 and 8.8.

Isoelectric point of the T antigen. To determine the isoelectric point of purified T antigen with the small quantities available, immunoelectrophoresis was utilized with cellulose polyacetate strips because the antigen did not interact with this supporting medium and only a few micrograms of protein could be detected. T antigen preparations containing 200 $\mu\text{g}/\text{ml}$ were dialyzed to the desired pH; buffer of the same characteristics was employed in the electrophoresis chamber. The technique employed was identical to that described in

FIG. 5. Determination of the pH stability of the T antigen. Samples of a purified T antigen preparation containing 200 $\mu\text{g/ml}$ were dialyzed against 0.05 M buffers of various pH values for 20 hr at 4 C. The complement-fixation titers were assayed with hamster sera.

the accompanying paper (9). Electrophoresis was carried out with 200 v for 40 min except at those pH values at which the rate of migration was too great. The values in such cases were extrapolated to 40-min values. After staining, the distance from the point of application to the center of the precipitin arc was measured. The results obtained are summarized in Fig. 6. The isoelectric point, approximated by extrapolating to the point of zero migration, was between pH 4.5 and 5.5. The value obtained for the isoelectric point can only be considered to be an estimation for the following reasons: (i) the precipitin arcs obtained were relatively long and their centers could not be established with great accuracy; (ii) although cellulose polyacetate is considered to be inert and, unlike agar, exhibits very little electroosmotic flow (2), the possibility of some effect on mobility cannot be ruled out; and (iii) it could not be ascertained with certainty that the T antigen was completely devoid of traces of contaminants, e.g., nucleic acids or deoxycholate, which could influence the electrophoretic migration.

DISCUSSION

Type 12 adenovirus induces synthesis of a macromolecule, the T antigen, which is apparently

lipophylic, entirely or at least predominantly composed of protein, labile at temperatures above 40 C, stable in the range of pH 5.8 to 8.8, and an acidic protein with an isoelectric point between pH 4.5 and 5.5. The antigen is a relatively small molecule with an $S_{20,w}$ of 2.58. The small size of the T antigen molecule is probably not due to depolymerization or cleavage caused by the purification method, since crude T antigen preparations (produced by deoxycholate solubilization and Freon extraction) have similar sedimentation characteristics. Tavitian and co-workers reported (22) a sedimentation coefficient of 7.6 to 7.8S (measured by rate zonal centrifugation in a linear sucrose gradient) for a T antigen obtained from transplanted tumors induced by type 12 adenovirus. The size of the antigen was measured after partial purification by sedimentation from the crude homogenate and a single precipitation in half-saturated $(\text{NH}_4)_2\text{SO}_4$. No estimation was made of the degree of purification attained or the

FIG. 6. Electrophoretic migration of the T antigen as a function of pH. Samples of a purified T antigen preparation were dialyzed against 0.05 M buffers of various pH values. The various preparations were studied by immunoelectrophoresis by use of the dialysis buffers in the electrophoresis chamber. Rabbit serum prepared against purified T antigen was used for the immunological precipitation. The distances migrated are plotted as a function of pH.

quantity of host structures associated with the T antigen. The discrepancy in the size of the T antigen purified from infected KB cells and that extracted from transplanted tumors may be explained by the following. (i) The transplanted tumor antigen was relatively crude and therefore probably complexed with host macromolecules (e.g., lipoproteins); hence, the sedimentation coefficient reported (22) may be an overestimate. (ii) The transplanted tumor antigen was assayed with antiserum from hamsters immunized with tumor fragments (22); the antigen studied may therefore have been different from the T antigen purified from infected KB cells and measured with serum from tumor-bearing hamsters.

By many characteristics, the T antigens induced by type 12 adenovirus and simian virus 40 (SV40) are similar: (i) they are synthesized early in the multiplication cycle before production of viral deoxyribonucleic acid (DNA) and capsid proteins (5, 6, 7, 18, 20); (ii) they are synthesized in the absence of DNA synthesis (4, 6, 7, 12, 17, 20); (iii) they are dependent upon *de novo* protein synthesis for their production (5; Gilead and Ginsberg, *unpublished data*); (iv) they are heat-labile proteins (6, 11, 13); and (v) they both depend for their synthesis upon a portion of the viral genome which is considerably smaller than the size of the genome essential for the manufacture of infectious virions (1, 8). There may, however, be a marked difference in the size of the two antigens: whereas the T antigen induced by type 12 adenovirus is relatively small, having an $S_{20,w}$ of 2.58, the SV40-induced T antigen has been reported to be approximately 18S (6). This marked difference in size may have been influenced by the fact that the size of the SV40 antigen was determined with a crude preparation on linear sucrose gradients; Gilden et al. consider it possible that the material measured may represent a complex of aggregated antigen molecules and host materials rather than the actual size of the SV40 antigen molecule.

Extensive interest has been shown in the T antigen since its discovery by Huebner et al. (13), but its role remains undefined. Nonetheless, several possible functions may be proposed. (i) The continued presence of the T antigen in virus-induced tumor cells, as well as in virus-transformed cells, suggests the possibility that it may serve as an "inducer" of malignancy, or perhaps as a derepressor of normal cell regulatory controls. (ii) The synthesis of the T antigen before biosynthesis of viral DNA and in the absence of DNA synthesis (4, 7, 12, 20) by definition implies that it is an "early protein" in the cycle of viral replication. This consideration suggests that the T antigen may be a virus-induced enzyme neces-

sary for synthesis of viral DNA. (iii) The demonstration that the T antigen appears as an "early" virus-induced protein also suggests that it may serve as an internal protein of the virion (16). (iv) The T antigen may serve as a transplantation antigen which is present in virus-transformed cells (3, 21, 23). (v) Finally, the possibility exists that the T antigen is an opportune marker of a functioning portion of the viral genome in tumor cells as well as in virus-infected cells, but that the antigen does not perform a function either in oncogenesis or in the synthesis of virus.

A consideration of the characteristics of the T antigen, as described in this and the accompanying paper (9), permits the conclusion that the T antigen does not serve as an internal virion protein. First, the T antigen is an acidic protein, which implies that it would be unsuitable to combine with viral DNA to function as an internal protein. Second, the T antigen cannot be detected in disrupted purified virions (9). It should be possible to investigate the other hypotheses concerning the biological role of the T antigen now that highly purified preparations of the protein can be obtained.

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