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

I. Purification of the Antigen from Infected KB Cells and a Hamster Tumor Cell Line

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The T antigen induced by type 12 adenovirus was purified from KB cells infected in the presence of 10^{-6} M 5-fluoro-2-deoxyuridine to inhibit synthesis of viral capsid antigens. The antigen was purified approximately 200-fold, and the purified product contained only negligible amounts of host-cell contaminants, as judged by the residual radioactivity from ¹⁴C-labeled uninfected cells which had been added to infected cells at the initiation of the purification. Immunoelectrophoresis indicated that the purified T-antigen preparation contained a single antigenic species. The T antigen from a hamster cell line (HT-1) derived from a type 12 adenovirus-induced tumor was purified by the same procedure. The T antigens from the two different sources were shown to be immunologically similar by use of a rabbit antiserum prepared against the purified T antigen from infected KB cells and sera from hamsters bearing tumors induced by type 12 adenovirus.

Type 12 adenovirus can induce three different responses in mammalian cells: (i) productive, lytic infections in permissive cells; (ii) nonproductive, abortive infections in nonpermissive cells; and (iii) malignant transformation induced in vivo in newborn hamsters, rats, and mice or in vitro in embryonic hamster and rat cultures. Synthesis of a new antigen, the "tumor" or T antigen, is induced in affected cells in each instance (12, 14, 20, 22, 23, 24). The antigen, which can be measured by complement fixation, has been demonstrated, by immunofluorescence and electron microscopy, to be localized mainly in nuclei of affected cells; it appears fleck-shaped, fibrous, and in bundles and amorphous masses (15, 20, 22, 23). Genetic information for antigen synthesis seems to reside in the viral deoxyribonucleic acid (DNA; 8), and it is transcribed as an early function, before new viral DNA or virion antigens are produced (7, 23). In fact, T antigen synthesis is independent of DNA synthesis, and it may be made even when 5-fluoro-2-deoxyuridine (5-FUdR) (7, 25) or 1- β -D-arabinofurano-

sylcytosine (arabinosylcytosine; 4, 13) inhibits the production of viral DNA and, therefore, virion antigens (5).

Recent studies imply that cells made malignant with type 12 adenovirus contain new transplantation antigen(s), since hamsters and mice which are injected with virus or X-irradiated tumor cells reject a challenge of tumor cells induced by virus (3, 27, 28). Data are not available on the relationship of the transplantation antigen to the T antigen, the function of the T antigen, or the relationship of the T antigen to transformation of infected cells. Purification and characterization of the T antigen seemed essential for pursuance of these problems.

KB cells infected with adenovirus type 12 in the presence of 5-FUdR, to prevent synthesis of capsid antigens, were utilized for the purification of T antigen. For comparison, T antigen was purified from a hamster cell line, HT-1, derived from a type 12-induced tumor (16). The properties of the purified antigen are described in a companion paper (9).

MATERIALS AND METHODS

Tissue culture. KB cells in monolayer or spinner cultures were employed. Cells were propagated in Eagle's minimal essential medium (MEM) supplemented with 10% calf or human serum, glutamine

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(2 mm in monolayer or 4 mm in spinner cultures), and serine (100 mg/liter) by methods previously described (1, 2, 10).

The tumor cell line, HT-1, induced by type 12 adenovirus (16), was kindly supplied by J. J. Trentin of Baylor University College of Medicine. Monolayer cultures were grown in Eagle's MEM supplemented with 10% calf serum and 2 mM glutamine.

Viruses. Viral seeds were prepared in KB cell monolayer cultures from plaque-purified virus as described previously (7), except that an input multiplicity of 50 to 100 plaque-forming units (PFU)/cell was used and was introduced in 10 ml of maintenance medium (10) for adsorption. An additional 40 ml of maintenance medium was added to the bottles 2 hr after viral infection. Infected cells were scraped from the glass surface and harvested by centrifugation 33 hr after infection. Type 12 virus was purified as described previously (18).

Infectivity assay. Infectious virus was quantitated by the plaque assay previously reported (7), except that the overlay medium was supplemented with 2 mM glutamine.

Sera from tumorous hamsters. The single pool of serum used throughout these studies was obtained from 12 hamsters bearing type 12 adenovirus-induced tumors at the 7th transplant level (purchased from Microbiological Associates, Inc., Bethesda, Md.). The serum had a complement-fixation titer of 1:320 against a hamster tumor extract and a titer of <1:10 against virion antigens.

Rabbit antisera. Antisera containing antibodies directed against the purified T antigen were made in New Zealand white rabbits as follows: 400 μ g of antigen protein in complete Freund's adjuvant was injected into the thigh muscle three times at weekly intervals. After an interval of 5 weeks, three intraperitoneal injections of 400 μ g of T antigen without adjuvant were given at 10-day intervals. The rabbits were bled 10 days after the last injection. The separated sera were heated at 56 C for 30 min and stored at -20 C. Antisera directed against viral structural proteins were prepared by immunization of rabbits with type 5 or 12 adenovirus soluble antigens (7).

Preparation of infected KB cells for purification of T antigen. KB cells from 8-liter spinner cultures (300,000 cells/ml) were sedimented by centrifugation ($180 \times g$ for 15 min) and resuspended to a concentration of 2.4×10^6 cells/ml in "adsorption medium" (Eagle's MEM supplemented with 3% dialyzed chicken serum, 4 mM glutamine, and 100 mg of serine per liter) containing 10^{-6} M 5-FUdR (Hoffman-La Roche, Inc., Nutley, N.J.). Type 12 adenovirus, 50 to 100 PFU/cell, was added to the cell suspension, and the cells were mixed for 2 hr at 37 C. The cells were then centrifuged ($180 \times g$ for 15 min) and transferred to a suspension culture flask containing 8 liters of Eagle's MEM supplemented with 10% calf serum (dialyzed against 15 volumes of saline which was changed daily for 3 days), 4 mM glutamine, serine (100 mg/liter), and 10^{-6} M 5-FUdR. At 22 to 24 hr after infection, cells were harvested by centrifugation ($180 \times g$ for 10 min) and washed once in phosphate-buffered saline (PBS). The cell pellets were stored at -20 C until used for

antigen purification. Cells from 20 liters of infected cultures were used for each antigen purification.

Radioactive labeling of normal host cell macromolecules. For radioactive labeling, 200 μ c of uniformly labeled 14 C-amino acid mixture (New England Nuclear Corp., Boston, Mass.) was added to a 250-ml suspension culture of KB cells, 150,000 cells/ml. A 250-ml amount of fresh medium was added 48 hr later, and the cells were harvested 96 hr after the addition of the isotope. At the time of harvest, the cell count was 550,000 to 650,000 cells/ml. The total radioactivity in the trichloroacetic acid-insoluble fraction of the culture was 35×10^6 to 45×10^6 counts/min.

Complement-fixation assay. The microtechnique of Sever (26) was employed as described previously (7); 1.5 exact units of complement and 6 to 8 units of antibody were used. To achieve a greater accuracy in the complement-fixation titrations, two series of dilutions were used: 1:2, 1:4, etc., and 1:3, 1:6, etc. The end point was taken as the average of the highest antigen dilutions which completely fixed complement in each titration.

Radioisotope counting. Samples from the various steps of the purification procedure were stored frozen. For assay, each was thawed and sonically treated twice for 15 sec to obtain uniform dispersion; 70 μ liters was then placed on a 3 MM filter disc and allowed to dry. The discs were immersed for 15 min in cold 5% trichloroacetic acid, washed once in cold 5% trichloroacetic acid and once in cold acetone, dried at room temperature, and placed in scintillation vials containing 5 ml of scintillation solution composed of 6 g of 2,5-diphenyloxazole (PPO) and 0.5 g of dimethyl 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 1 liter of toluene. Samples were counted in the Packard Tri-Carb liquid scintillation spectrometer.

Ultrasonic treatment. An M.S.E. ultrasonic disintegrator (Instrumentation Associates, New York, N.Y.) was used. Samples were cooled in a bath of alcohol-salt-ice mixture during sonic treatment.

Immunoelectrophoresis. The technique used for immunoelectrophoresis was that of Kohn (17), as modified by Grunbaum and Dong (11), with the use of Sepharose III cellulose polyacetate strips (Gelman Instrument Co., Ann Arbor, Mich). The 2.5×17.2 cm strips were wet with 0.025 M phosphate buffer (pH 7.5). The same buffer was used for electrophoresis. A 10- μ liter amount of the pure T antigen preparation was placed at the center of the strip, half-way between the electrodes. Electrophoreses were carried out with 200 v current for 40 min. The strips were then placed on 4×12 cm glass slides, and 2×120 mm Whatman no. 1 filter paper strips to which the anti-serum had been applied were placed on both edges of the cellulose polyacetate strip. Diffusion was allowed to proceed for 48 hr at room temperature in a moist sealed chamber. The electrophoresed strips were washed for 30 min successively in PBS and in water. The strips were stained in 0.5% Ponceau S in 5% trichloroacetic acid for 5 min. The stained strips were decolorized by repeated washing in 5% acetic acid.

Chemical determinations. Protein was estimated by the method of Lowry et al. (21), with bovine serum albumin as the standard. The activities of nucleases

present in the purified T antigen preparation were assayed with extracted, labeled KB cell DNA and ribonucleic acid (RNA) as substrates. The release of trichloroacetic acid-soluble radioactivity as a function of the time of the reaction was measured. The activities of the nucleases in the T antigen preparation were estimated by comparison with curves obtained with known concentrations of crystalline pancreatic deoxyribonuclease and ribonuclease.

RESULTS

Procedure for the purification of T antigen. Cells infected in the presence of 5-FUDR produce T antigen but do not synthesize virion antigens (7). The main problem posed, therefore, in the purification

was to separate the T antigen from the large amount of host macromolecules. To estimate the amount of impurities at each step of the purification procedure, uninfected KB cells which had been highly labeled for four generations with a ^{14}C -amino acid mixture (and therefore isotope was incorporated into proteins and nucleic acids) were mixed with the infected cells prior to purification. At each step of the purification, samples were removed, and the quantity of host contaminants and the "specific activity" of the antigen were estimated by assaying the radioactivity, the protein content, and the complement-fixation titer.

The four phases of the purification procedure are summarized in Fig. 1; a detailed description follows.

Solubilization. Infected cells (6×10^9) were mixed with uninfected KB cells prelabeled with ^{14}C amino acids (3×10^8) and suspended in 350 ml of 0.025 M phosphate buffer, pH 7.2 (18×10^6 cells/ml). The cell suspension was sonically treated eight times for 30 sec each. To reduce the viscosity and to remove nucleic acids, ribonuclease (crystallized from ethyl alcohol; Worthington Biochemical Corp., Freehold, N.J.) and pancreatic deoxyribonuclease (once crystallized, Worthington Biochemical Corp.), were added to the sonic extract to make a final concentration of $20 \mu\text{g}/\text{ml}$ for each. MgCl_2 (1 M) was added to give a final concentration of 10^{-3} M. The suspension was incubated for 1.5 hr at 37 C with occasional vigorous shaking. To chelate the Mg^{++} , ethylenediaminetetraacetate (EDTA, 0.15 M, pH 7.2) was added to obtain a final concentration of 3×10^{-3} M. After 15 min at 25 C, 5% deoxycholate (special enzyme grade; Mann Research Laboratories, New York, N.Y.) was added to give a final concentration of 0.25%. The preparation was adjusted to pH 7.5 with 1 N NaOH and the suspension was held for 30 min at 25 C with occasional shaking.

Extraction phase. After addition of 1.5 volumes of Freon-113 (E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.) were added, and the preparation was mixed vigorously by hand for 5 min. The mixture was centrifuged for 15 min at $600 \times g$ in an International refrigerated centrifuge. The aqueous phase was saved, and the procedure was repeated two additional times. The pH was adjusted to 7.5 and the preparation was centrifuged at $92,000 \times g$ for 3 hr in the 40 or 50 rotor of a Spinco preparative ultracentrifuge. Cold 1 M MgCl_2 was added slowly to the chilled supernatant fluid with vigorous mixing to make a final concentration of 0.1 M Mg^{++} . Magnesium precipitated the deoxycholate and the T antigen which was complexed to it. After 45 min at 0 to 4

FIG. 1. Summary of the purification scheme.

C, the precipitate was collected by centrifugation at $27,000 \times g$ for 20 min at 0 C. The precipitate was resuspended in 0.05 M phosphate buffer (pH 7.5) to two-sevenths of the original volume and dispersed by vigorous pipetting. Sonic treatment for 15 sec, repeated five times, aided solubilization. The preparation was brought to room temperature, mixed with 1 volume of a mixture containing equal proportions of chloroform, ether, and CCl_4 , and shaken gently by hand for 8 min. The two phases were separated by centrifugation at $1,100 \times g$ in an International refrigerated centrifuge for 30 min. The aqueous layer was collected. This step resulted in a relatively large loss of T antigen, but it was essential to remove contaminating lipoproteins (if greater recoveries are desired, at the expense of some loss in purity, shaking for 3 to 5 min will suffice).

Differential precipitation. To remove desoxycholate and to precipitate differentially T antigen and some host proteins cold (0 C) acetone was added slowly with vigorous stirring to the chilled preparation to make a final acetone concentration of 30%. The precipitate, formed after standing for 45 min at 0 C, was removed by centrifugation at $27,000 \times g$ for 20 min at 4 C. The supernatant fluid was brought to a final concentration of 65% acetone, as described above, and the precipitate (present after 45 min) was resuspended in 40 ml of 0.01 M buffer (pH 7.5). Sonic treatment four times for 15 sec each, was used if the solubilization was not complete. The preparation was stirred at 0 to 4 C, and an equal volume of cold saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7.5) was added slowly. After 45 min, the precipitate was collected by centrifugation and resuspended in 30 ml of 0.01 M phosphate buffer (pH 7.5). Sonic treatment aided solubilization of the precipitate. All purification steps from the precipitation by Mg^{++} to this stage were performed on the same day.

Column chromatography. The partially purified preparation was dialyzed in the cold overnight against 100 volumes of 0.003 M phosphate buffer (pH 7.5), and added to a hydroxyl apatite (Bio-gel HT; Bio-Rad Laboratories, Richmond, Calif.) column (19), 1.5 cm $\times$ 6.5 cm, which was packed under about 2 psi of pressure. Prior to packing, the gel was washed in 0.003 M phosphate buffer (pH 7.5) until it was equilibrated to this pH. The preparation was added to the column at a rate of 0.5 ml/min with a peristaltic pump, and the loaded column was washed with 80 ml of 0.003 M phosphate buffer at a rate of 2 ml/min. Elution was carried out in a linear gradient from 0.003 M phosphate buffer pH 7.5, to 0.2 M phosphate buffer, pH 7.5 (80 ml for each buffer). Fractions, (6 ml) were collected at a rate of 0.3 ml/min.

After 100 ml of eluate had been collected, the column was washed with 40 ml of 0.5 M phosphate buffer, pH 7.5. Results of the optical density measurements at 280 m μ and the complement-fixation activity of each fraction in a chromatogram from one of the purifications are summarized in Fig. 2. Tubes with the highest complement-fixation activity (generally three fractions) were pooled. To concentrate the purified antigen, pooled fractions were dialyzed overnight against 200 volumes of 0.003 M phosphate buffer (pH 7.5) at 4 C and added to a second hydroxyl apatite column (1 $\times$ 4 cm). After loading, the column was washed with 80 ml of 0.003 M phosphate buffer and eluted slowly with 0.1 M phosphate buffer (pH 7.5). Fractions (1.5 ml) were collected, and the tubes with the highest optical density were pooled.

The results of a representative purification are summarized in Tables 1 and 2. Yields of 4 to 5% were generally obtained, and the antigen was purified approximately 200-fold. From the number of counts remaining in the final purified preparation, it was ascertained that the amount of host contaminants left was negligible. The purified preparation had a protein content of 32 $\mu\text{g}/\text{ml}$ and a corresponding complement-fixation titer of 1:96. The starting preparation, which had a protein content of 8.83 mg/ml and a titer of 1:128, contained, therefore, by proportion, ap-

FIG. 2. Purification of the T antigen on a hydroxyl apatite column. The material was eluted with a linear gradient from 0.003 to 0.2 M phosphate buffer at pH 7.5. The complement-fixation titer of the T antigen was assayed with sera from hamsters bearing tumors induced by type 12 adenovirus.

TABLE 1. *Summary of the results of purification (chemical determinations)*

Fraction	Vol (ml)	Total protein content (mg)	Protein (% of original)	Counts per min per ml	Total counts/min ($\times 10^{-3}$)	Counts/min (% of original)
Original homogenate.....	350	3,090	—	114,870	40,200	—
Deoxycholate.....	394	3,100	—	97,120	38,250	96.0
Freon.....	307	1,439	46.5	59,040	18,150	45.2
High speed supernatant ^a ...	295	861	27.8	34,050	10,020	24.9
Mg ⁺⁺ precipitate.....	90	119	3.8	19,120	1,720	4.27
Organic solvents.....	90	16.4	0.5	2,390	215	0.53
Acetone, 65%.....	40	7.7	0.2	2,554	102	0.25
(NH ₄) ₂ SO ₄	30	ND ^b	ND ^b	2,624	79	0.196
Hydroxyl apatite.....	18	0.6	0.02	38	0.68	0.0017

^a The supernatant fluid after centrifugation at $92,000 \times g$ for 3 hr.^b Not done because of interference by the (NH₄)₂SO₄.TABLE 2. *Summary of the results of purification (immunological determinations)*

Fraction	CF titer	Cumulative CF ^a	Yield (% of original)	Specific activity ^b	Fold purification ^c
Original homogenate.....	1:128	44,750	—	14	—
Desoxycholate.....	1:128	—	—	—	—
Freon.....	1:128	39,300	88	27	2
High-speed supernatant fluid ^d	1:64	18,900	42	22	1.5
Mg ⁺⁺ precipitate.....	1:128	11,500	26	97	7
Organic solvents.....	1:32	2,880	6	176	12
Acetone, 65%.....	1:64	2,560	6	332	23
(NH ₄) ₂ SO ₄	1:64	1,920	4	—	—
Hydroxyl apatite.....	1:96	1,728	4	3,000	208

^a Complement-fixation (CF) titer $\times$ volume (in ml).^b CF titer per milligram of protein.^c Specific activity of the fraction/specific activity of the original homogenate.^d The supernatant fluid after centrifugation at $92,000 \times g$ for 3 hr.

proximately 40 $\mu\text{g/ml}$ of T antigen (less than 0.5% of all proteins in the crude preparation). This value is maximal, since it assumes that the purified preparation did not contain inactivated antigen molecules.

The nucleases which were added during purification were unlabeled. Their contents in the purified preparation were therefore determined. No deoxyribonuclease activity was detectable. The ribonuclease content of the purified preparation was less than 0.2 $\mu\text{g/ml}$.

Comments on the purification procedure. More than 75% of the T antigen was lost when the fluorocarbon Freon 113 (6) was employed to extract host-cell lipoproteins and lipopolysaccharides immediately after disruption of the cells either by sonic treatment or by freezing and thawing. However, the antigen was almost completely recovered after freon extraction when, prior to fluorocarbon extraction, deoxycholate was employed to solubilize the lipid-containing membranes in the cell homogenate. These data suggest that the T antigen was closely associated

with the cell membranes. It was also observed that the T antigen was precipitated quantitatively when the deoxycholate was precipitated as the magnesium salt, in the acid form (below pH 6.5 to 6.8), or as a streptomycin-deoxycholate complex. These results further indicate that the T antigen is lipophylic, and imply that it may combine with the apolar nucleus of deoxycholate.

The precipitation of the T antigen-deoxycholate complex by magnesium was employed as an additional purification step which not only reduced markedly the quantity of contaminating host proteins but also reduced further the possibility that virion antigens might contaminate the final T antigen preparation. The viral structural proteins do not complex with deoxycholate, and hence they were not precipitated with the magnesium-deoxycholate salt. The results of an experiment summarized in Table 3 illustrate this point. Cells were infected in the absence of FUDR to permit synthesis of the virion proteins as well as the T antigen. The cells were harvested 33 hr after infection, and the purification procedure de-

TABLE 3. *Separation of the T antigen from virion antigens by magnesium-deoxycholate precipitation*

Fraction	Complement-fixation titer with	
	Anti-5 serum	Hamster sera
Original preparation ^a	1:512	1:64
Mg-deoxycholate supernatant fluid.....	1:512	<1:2
Three times concentrated Mg deoxycholate precipitate ^b	1:32	1:128

^a A preparation containing virion and T antigens after the high-speed centrifugation step (see Table 1). The suspension was prepared from cells infected in the absence of 5-FUdR.

^b The precipitate was resuspended in one-third the original volume.

scribed was carried out through the precipitation of deoxycholate by Mg^{++} . The virion hexon and penton antigens were assayed with type 5 antiserum, and the T antigen was measured with a serum from hamsters bearing tumors. The T antigen was recovered quantitatively in the precipitate, whereas only about 6% of the virion antigens measured were precipitated.

Immunoelectrophoresis of purified T antigen. To determine whether the preparation of T antigen consisted of a mixture of antigens or whether it contained a single antigenic species, the purified material was analyzed by immunoelectrophoresis. The antiserum employed was obtained from rabbits immunized with purified T antigen. The results showed the presence of a single precipitin band, and indicate that only a single species of antigen was present in sufficient quantity to be detectable.

Purification of T antigen from a continuous cell line (HT-1) derived from a type 12-induced hamster tumor. During the purification procedure described, the T antigen was assayed immunologically by use of sera from hamsters bearing tumors induced by type 12 adenovirus. Hence, the T antigen synthesized in infected KB cells was immunologically related to the antigen produced in tumors. A further immunological relationship of the T antigens made in different cells was studied by purifying the T antigen from a continuous cell line (HT-1) derived in vitro from a tumor induced by type 12 adenovirus. The purification methods employed were identical to those described above, except that the final steps in purification, chromatography on hydroxyl apatite columns, were not carried out because of the small amount of material present. The results of the purification procedure (Tables 4 and 5) imply that the T

antigens from both sources were chemically similar, since they reacted similarly to the purification methods used (compare with Tables 1 and 2).

The T antigens from infected KB cells and the tumor cell line were also shown to be closely related immunologically (Table 6). The data summarized in Table 6 further indicate that the antibodies directed against purified T antigen did not cross-react with a mixture of partially purified soluble virion antigens, even when as many as 512 complement-fixing units were used in the complement-fixation titration. Furthermore, when purified type 12 adenovirus particles were disrupted by six cycles of freezing and thawing (Ginsberg, *unpublished data*), cross-reactivity was not observed between T antigen and the antigens contained in the virions. The conditions employed for freezing and thawing the purified virions did not reduce the immunological reactivity of a control preparation of purified T antigen. These data furnish strong confirmation that the T antigen is distinct from and not related to the virion antigens (7), and they also imply that the T antigen is not immunologically related to a possible internal protein.

Purification of T antigen from adenovirus-induced hamster tumors (kindly supplied by J. J. Trentin) was also attempted. The relative quantity of antigen obtained in homogenates (i.e., complement-fixing titer/mg of protein) was too low, however, to permit extensive purification.

DISCUSSION

T antigen, obtained from cells infected with type 12 adenovirus, was purified by use of a series of steps consisting of solubilization, extractions with organic solvents, differential precipitation, and column chromatography. This procedure yielded a highly purified product which contained only a single species of antigen detectable by

TABLE 4. *Summary of the purification of T antigen from the HT-1 cell line (chemical determinations)*

Fraction	Vol (ml)	Total protein (mg)	Protein (% of original)
Original homogenate....	55	347	—
Deoxycholate.....	60	362	—
Freon.....	51	152	44
High speed supernatant ^a ..	48	79	23
Mg^{++} precipitate.....	9	16	4.5
Organic solvents.....	8.5	8	2.3
Acetone, 65%.....	3	0.5	0.14
$(NH_4)_2SO_4$	2	0.3 ^b	0.09

^a The supernatant fluid after centrifugation at $92,000 \times g$.

^b Lowry determination done after dialysis.

TABLE 5. Summary of the purification of T antigen from the HT-1 cell line (immunological determinations)

Fraction	CF titer	Cumulative CF titer ^a	Yield (% of original)	Specific activity ^b	Fold purification ^c
Original homogenate.....	1:16	880	—	2.5	1
Deoxycholate.....	1:24 ^d	1,440 ^d	164 ^d	4	1.5
Freon.....	1:24	1,224	139	8	3
High-speed supernatant fluid ^e	1:24	1,152	131	15	6
Mg ⁺⁺ precipitate.....	1:64	576	65	37	15
Organic solvents.....	1:32	272	31	34	13
Acetone, 65%.....	1:48	144	16	215	116
(NH ₄) ₂ SO ₄	1:64	128	15	427	167

^a Complement-fixation (CF) titer × volume (ml).^b CF titer per milligram of protein.^c Specific activity of the fraction/specific activity of the original homogenate.^d The increase in titer is probably due to better solubilization by desoxycholate.^e The supernatant fluid after centrifugation at 92,000 × g for 3 hr.

TABLE 6. Immunological relationship of the T antigen from infected KB cells to T antigen from a continuous cell line derived from type 12-induced hamster tumor, and to type 12 virion antigens

Antigens assayed	CF titer	
	Hamster serum ^a	Rabbit anti-T serum ^b
T from infected KB cells.....	1:128	1:256
T, continuous cell line (HT-1).....	1:16	1:16
Soluble virion antigens ^c	<1:2	<1:2
Disrupted purified virus ^d	<1:2	<1:2

^a From tumor-bearing hamsters; 8 units of antibody.^b Rabbits immunized with purified T antigen from infected KB cells; 6 units of antibody.^c Mixture of partially purified soluble antigens from infected KB cells; 512 complement-fixing units (measured with type 12 rabbit antiserum).^d Purified type 12 virions were disrupted by six cycles of freezing and thawing; 2,048 complement-fixing units (measured with type 12 rabbit antiserum).

immuno-electrophoresis and sedimentation in sucrose gradients, and only a single molecular species of protein detectable by analytical ultracentrifugation (9).

It has been reported that a second nonvirion antigen, a group antigen, can be detected by immunofluorescence in cells infected with a variety of oncogenic and nononcogenic adenoviruses by use of sera from hamsters bearing tumors induced by type 12 adenovirus (4, 22, 25). If this group antigen has physical and chemical characteristics distinctly different from those of the T antigen purified, it would have been discarded, since it cannot be detected by the complement-fixation reaction. Hollinshead and Huebner (13) reported

a heat-labile and a heat-stable species of type 12 T antigen in a partially purified preparation. The purified T antigen described in this communication is heat-labile (9). It is possible that the heat stability of the antigen described by Hollinshead and Huebner (13) may be attributed to protection conferred on some of the T antigen by contaminating host materials or that the characteristics of the heat stable T antigen were unlike those of the heat labile antigen, and therefore it was lost during the purification procedure employed.

To obtain highly purified T antigen, it was essential that complete solubilization of the cell membranes be accomplished, and that lipid and lipid-containing polymers be extracted with organic solvents. These data, as well as the finding that the T antigen avidly combines with deoxycholate, imply that the protein is lipophilic and that it associates with, and probably aggregates on, the cellular membranes. The demonstration that the T antigen is concentrated in unusual flecks (22) and bundles of fibers or granular material (15) is consistent with the suggestion that the antigen accumulates on membranous structures in type 12 adenovirus-induced tumors or infected cells. However, there is also the possibility that the flecks and fibers represent end-to-end aggregation products of free antigen molecules.

The chemical and physical characteristics of purified T antigen will be described in the accompanying paper (9).

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