# Isolation of T Antigen from Solid Hamster Tumors Induced by Adenovirus Type 31 (33652)

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The induction of the nonvirion T or "tumor" antigen by adenovirus in mammalian cells occurs in all three types of virus–cell interaction: lytic, abortive, and malignant transformation (1–3). The antigen is synthesized early after infection and can be detected serologically by complement fixation (CF), immunofluorescence, or electron microscopy (1, 2, 4). Studies performed to date suggest that there may be differences between the T antigens isolated from infected cells and those isolated from transformed cells, although the two are thus far serologically indistinguishable (5, 6). Indeed, many workers distinguish between the two taxonomically, calling the antigen isolated from in fected cells "T" and that from transformed cells "tumor" (5, 7). Further, Green, Huebner, and co-workers distinguish among several T and tumor antigen components (6–8). Despite these differences in T antigens isolated from different sources, relatively few studies have been done on antigen isolated from *in vivo* solid tumors (5). Tumors induced by adenovirus type 31 provide an excellent source for studying T antigen, since they have an almost 100 "take" in infected Syrian hamster weanlings and grow very rapidly, often reaching 60 g in 8 weeks.

Early studies revealed that, in contrast to adenovirus types 12 and 18, and SV-40 where T antigen is primarily intranuclear (2, 4, 6, 7), adenovirus-type-31 T antigen is polydisperse and associated with a spectrum of particles having a wide range of sedimentation coefficients (9). Since earlier work (10) indicated that the antigen was bound to particulate fractions, we attempted, in addition to isolation, to ascertain the effectiveness of methods of H-2 antigen isolation as applied to T antigen (11, 12).

*Materials and Methods. Tissue source.* Random-bred Syrian hamsters injected subcutaneously with an adenovirus-31-induced hamster sarcoma (originally produced at Flow Laboratories, Inc., Rockville, Md.) were obtained from Lakeview Hamster Colony and sacrificed when the tumor had been carried for 8 weeks. The tumors were stored at −20° until ready for use.

*Antisera.* The tumor-bearing animals were bled at the time of sacrifice. The blood was pooled, and the serum was separated, decomplemented, and titered against adenovirus types 31 and 12 antigens obtained from Flow Laboratories. No cross-reactivity was detected by the CF procedure between the adenovirus-31 tumor line used and either Flow Laboratories' spontaneous hamster tumor antigen or SV-40 tumor antigen.

*Complement fixation.* The Laboratory Branch CF method of Casey, modified for microtechnique (13), was used. Complement and hemolysin were obtained from Texas Biological, Fort Worth. Antigen titers were expressed as the reciprocal of the highest dilution of antigen giving 75–100% fixation of complement in the presence of four units of antisera.

*Protein.* Total protein content of the samples was assayed on the Technicon AutoAnalyzer (Technicon Instruments Corp., Ardsley, N. Y.) by using Lowry's method as modified by Elrod (14). The specific activity of the sample was determined by multiplying the reciprocal of its CF titer by 40, to

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give the equivalent CF per milliliter, and dividing by protein concentration in milligrams per milliliter, thus giving the CF activity per milligram of protein.

*Enzymatic digestion.* Twice-purified papain was obtained from Mann Research Laboratories, New York, and was activated with 0.01 *M* ethylenediaminetetraacetic acid and 0.005 *M* cysteine. The papain, at a concentration of 0.2 mg/ml of protein suspension, was added to a tumor homogenate diluted to a protein concentration of 12 mg/ml (15). The reaction was carried out in the cold and then inhibited by the addition of *p*-chloromercuribenzonate to a final concentration of 0.001 *M*.

*Acrylamide gel electrophoresis.* In addition to standard cylindrical disc electrophoresis, we employed the vertical flat-bed electrophoresis apparatus of Allen and Moore (16), with a 6.5% acrylamide running gel and a 4% stacking gel. Between 10 and 30 mg of protein were loaded per gel and run for 3 hr at 150 mA at 2°. After completion, a small vertical strip was cut from the gel and stained. According to the distribution of bands, the remainder of the gel was sectioned, homogenized briefly in Miller-Golder (MG) buffer (pH 7.3, $\mu = 0.2$) (see Ref. 17) and eluted overnight in buffer, with occasional stirring. The mixture was centrifuged to remove the acrylamide, and the supernatants were pressure-filtered to a retentate volume of 6–10 $\mu$ in an Amicon model 50 ultrafiltration cell through a "Diaflo" UM-1 (10,000 mol. wt. cutoff) membrane (Amicon Corp., Lexington, Mass.).

*Extraction procedure.* Pooled frozen tumors were washed and homogenized to a 20% suspension in MG buffer in a Sorvall Omnimixer. After three cycles of freezing to −70°, thawing, and homogenizing, the homogenate was centrifuged in an IEC-PR2, head 259, at 2000 rpm for 25 min at 2° to remove the heavy debris, nuclei, and remaining whole cells. The supernatant was then centrifuged in a No. 30 rotor at 30,000 rpm to $\Omega^2 t = 10^{11}$ at 2° (10). The supernatant (S-2) had a CF activity of approximately 40 units. The pellet was resuspended to a protein concentration of 12 mg/ml (with a CF titer of 128–256) and incubated overnight (16 hr) with cysteine-activated papain (0.2 mg/ml at 0°. After the papain digestion was inhibited with *p*-chloromercuribenzonate, the suspension was again centrifuged (in a No. 50 rotor at 50,000 rpm at 2°) to $\Omega^2 t = 10^{11}$; this time the supernatant was saved and the pellet was discarded. Titration after centrifugation indicated that one-half of the CF titer of the suspension was solubilized. The supernatant was then maintained at 0° and cold acetone was added, with stirring, to a concentration of 37% (v/v). This mixture was allowed to stand for 10 min, then centrifuged at 2000 rpm for 30 min. The pellet was discarded, acetone was added to the supernatant to bring the concentration to 67% (v/v), and the centrifugation was repeated. The pellet was collected, and dissolved in MG buffer. Saturated ammonium sulfate solution was added at 0°, with stirring, to a final concentration of 50% and, after 10 min, the mixture was centrifuged in a No. 30 rotor for 28 min at 21,000 rpm at 0°. The pellet was collected, washed with 50% (w/v) ammonium sulfate, dissolved in MG buffer and dialyzed against the buffer overnight. The retentate was then concentrated through a Diaflo UM-1 membrane in order that at least 20 mg of protein could be applied to the vertical flat-bed acrylamide gel for electrophoresis. After the electrophoretic pattern had been determined and the proteins eluted from the gel sections, each gel section was assayed for total protein and CF activity.

*Results. Early experiments.* We first attempted to solubilize the T antigen by nonenzymatic methods. Alkaline extraction (10) and anaerobic autolysis at 37° (12) had no effect; with more drastic treatment (pH above 9.0, or autolysis for more than 8 hr), all CF reactivity was destroyed. Several detergents, including sodium deoxycholate, Triton X-100, and Triton X-114 (18), were also used ineffectually.

*Papain digestion.* Nathenson and Shimada (15) found that the maximum effect of crude papain on H-2 antigen occurred after a 1-hr treatment, and that after 8 hr all H-2 activi-

TABLE I. Papain Digestion of Pellet from $10^{11}$ $\omega^2t$ Centrifugation.[a]

| Time (hr) of exposure | | CF | Protein (mg/ml) |
|---|---|---|---|
| Expt. 1 | | | |
| Start | supt | 2 | 0.75 |
| | susp | 32 | 6.88 |
| 1 | supt | 32 | 4.15 |
| | susp | 128 | 8.00 |
| 2 | supt | 64 | 4.55 |
| | susp | 128 | 8.75 |
| 3 | supt | 128 | 5.00 |
| | susp | 64 | 9.00 |
| 4 | supt | 128 | 5.15 |
| | susp | 256 | 9.13 |
| Expt. 2 | | | |
| 3 | supt | 32 | 5.30 |
| | susp | 256 | 9.50 |
| 4 | supt | 64 | 5.30 |
| | susp | 256 | 8.00 |
| 6 | supt | 128 | 5.40 |
| | susp | 256 | 10.00 |
| 16 | supt | 128 | 6.20 |
| | susp | 256 | 8.50 |

[a] Abbrev.: CF = complement fixation titer; susp = suspension of pellet in Miller–Golder buffer, after treatment with papain for noted time; supt = supernatant derived, after digestion of suspension with papain, by again centrifuging to $10^{11}$ $\omega^2t$. Original protein concentration: 10 mg/ml; papain concentration: 0.2 mg/ml.

ty was destroyed. Our experiments gave variable results. Table I shows the results of two separate experiments in which the homogenate was exposed to papain for different time periods. Increasing the length of exposure up to 16 hr did not cause destruction of CF activity; rather, the amount of activity solubilized slowly increased, until 50% of the total activity was in the supernatant. If, however, as shown in Table II, this suspension was divided into two fractions, different results were obtained. The C represents all material originally insoluble at $10^{11}$ $\omega^2t$. Fraction A is that part of C which is pelleted at $5 \times 10^9$ $\omega^2t$; fraction B represents the rest of C. Fraction A, like C, resists papain; B, however, is very susceptible to papain action: its activity is significantly diminished by 1 hr of treatment. Furthermore, the S-2 supernatant described above is so sensitive that its CF activity is destroyed by a 15-min exposure to papain.

*Acetone and ammonium sulfate precipitation.* These precipitation steps were designed to increase the specific activity of the preparation; and as shown in Table III, acetone precipitation increases specific activity 3- to 6-fold, whereas treatment with ammonium sulfate concentrates the activity another 3-fold. This gives a specific activity of about 9000, compared with 500–1000 for the papain supernatant. Precipitating first with 40% and then with 67% acetone (40 → 67) removes half the protein without decreasing the CF titer.

*Vertical flat-bed electrophoresis.* The ammonium sulfate pellet still contains 12 protein bands on acrylamide-gel disc electrophoresis. We, therefore, employed the larger slab electrophoresis to separate these bands

TABLE II. Papain Digestion of Pellet and Supernatant from $10^{11}$ $\omega^2t$ Centrifugation.[a]

| Sample | | CF | Protein (mg/ml) |
|---|---|---|---|
| A. $5 \times 10^9$ $\omega^2t$ pellet | | 64 | 8.75 |
| B. $10^{11}$ pellet from A supernatant | | 128 | 5.38 |
| C. Direct $10^{11}$ pellet | | 128 | 6.50 |
| A. Papain × 3 hr: | supt | 64 | 4.16 |
| | susp | 128 | 7.40 |
| × 16 hr: | supt | 64 | 4.65 |
| | susp | 128 | — |
| B. Papain × 1 hr: | supt | 16 | 4.80 |
| | susp | 64 | 6.60 |
| × 6 hr: | supt | 32 | 5.00 |
| | susp | 32 | 6.00 |
| C. Papain × 3 hr: | supt | 32 | 6.62 |
| | susp | 128 | 11.30 |
| × 16 hr: | supt | 128 | 6.75 |
| | susp | 256 | — |
| S-2. $10^{11}$ supernatant | | 32 | |
| Papain × 15 min | | 0 | |

[a] *Symbols:* CF, susp, supt = same as in Table I; A = all material sedimented at $5 \times 10^9$ $\omega^2t$; B = material in supernatant at $5 \times 10^9$ $\omega^2t$, but sedimented at $10^{11}$ $\omega^2t$; C = A + B; and S-2 = all material in supernatant at $10^{11}$ $\omega^2t$.

TABLE III. Acetone and Ammonium Sulfate Precipitation of T Antigen.

| Acetone (%) (v/v) | CF | Protein (mg/ml) | Specific activity |
|---|---|---|---|
| Starting sample | 128 | 10.20 | 501 |
| 0 | 0 | 0.075 | |
| 30 | 2 | 0.25 | |
| 35 | 8 | 0.51 | |
| 40 | 32 | 0.60 | |
| 45 | 32 | 0.95 | |
| 50 | 32 | 1.63 | |
| 55 | 64 | 2.46 | |
| 60 | 64 | 2.79 | |
| 63 | 64 | 2.96 | |
| 66 | 128 | 3.04 | 1648 |
| 69 | 128 | 3.25 | 1575 |
| 72 | 64 | 2.95 | |
| 75 | 64 | 2.95 | |
| 80 | 128 | 3.35 | |
| 40 → 67[a] | 128 | 1.60 | 3200 |
| Ammonium sulfate precipitation | | | |
| (a) Starting sample (papain supt) | 128 | 5.63 | 1140 |
| (b) 40% acetone: pellet | 32 | 4.05 | |
| (c) 67% acetone: pellet (from b supt) | 512 | 7.50 | 3200 |
| (d) 50% $(NH_4)_2SO_4$: supt (from c) | 16 | 1.30 | |
| (e) 50% $(NH_4)_2SO_4$: pellet (from c) | 256 | 1.50 | 9067 |

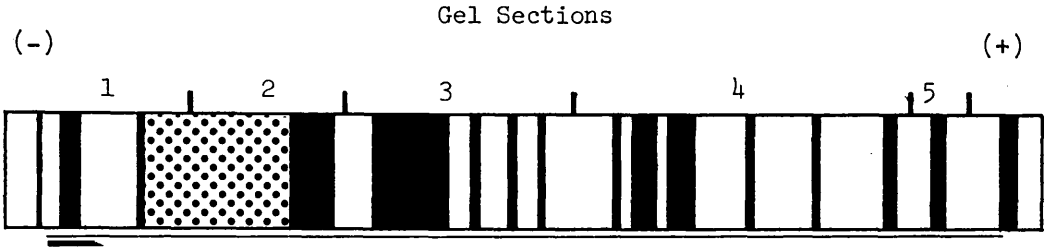
[a] 40 → 67 = supernatant from 40% acetone precipitation with more acetone added to raise concentration to 67%.

on a scale large enough to recover each band individually. In Table IV, numbers 1–5 represent the individual sections cut from the gel. Although there was slight CF activity present in all fractions tested, the greatest activity was in secton 5, which represents a single detectable protein band, with a specific activity of 55,652. Both in the slab and in cylindrical disc electrophoresis, T antigen moved slightly faster than albumin. Unfortunately, some of the total activity was lost in the gel slab.

*Properties of T antigen eluted from section 5.* The isoelectric point of the antigen was determined by isoelectric precipitation to be near pH 4.5. Half of the CF activity was destroyed by heating to 56° for 5 min, but the remaining activity was stable to 1 hr at 56°. It was stable at —70° for at least 3 months, and titer was not lost by intermittent thawing and re-freezing during this period. The activity was not destroyed, at a protein concentration of 2 mg/ml, by exposure to RNase, 50 μg; trypsin, 50 μg; chymopapain, 0.2 mg; or papain, 0.1 mg/ml for 4 hr at 20°. The sedimentation rate of the major peak was 2.93 S in the Beckman model E ultracentrifuge. The T antigen did not cross-react with adenovirus-31 virion antisera.

*Discussion.* Although T antigens have been isolated from several sources, few studies to date have been made on antigen derived from solid tumor. Gilden (5) found that his "tumor" antigen was serologically identical (on Ouchterlony) to the "T" antigen he isolated from tissue-culture-transformed cells, but had a molecular weight of 125,000, compared to 50,000 for T, and had low electrophoretic mobility, compared to the varying mobilities and 7 components he found for T. Tockstein *et al.* (7) compared T antigen from tissue-culture lytically infected cells with tumor antigen from transformed cells. Both are nuclear and detected by CF, but the lytic antigen concentration peaks at 20 hr, the tumor antigen at 60 hr; T is polydisperse, on sucrose density gradient centrifugation, and has a sedimentation coefficient of 4.8, where-

TABLE IV. Vertical Flat-Bed Acrylamide-Gel Electrophoresis.[a]




| Gel section | CF titer | Protein (mg/ml) | Specific activity |
|---|---|---|---|
| SS | >1280 | 7.25 | 14,124 |
| 1 | 80 | 0.75 | 4267 |
| 2 | 40 | 0.50 | 3200 |
| 3 | 40 | 0.75 | 2133 |
| 4 | 80 | 0.68 | 4706 |
| 5 | 320 | 0.23 | 55,652 |

[a] Symbols: (—) = cathode; (+) = anode; SS = starting sample. Fractions 1–5 are the individual sections into which the slab is cut; the diagram shows how the slab stains. Fraction 5 contains a single distinguishable band.

as tumor antigen forms a single broad component. Kalnins *et al.* (4) found fibers in the nuclei of lytically infected cells and in the cytoplasm of adenovirus-12-infected neoplastic cells which reacted with ferritin-labeled anti-T antibodies. Hollinshead and Huebner found both heat-stable and heat-labile T antigen components in adenovirus-12-infected cells that were inhibited with cytosine arabinoside. They have described several adenovirus-12-induced cellular antigens in their *in vitro* system (6, 8). We have succeeded in isolating a single T antigen from adenovirus 31 *in vivo* solid tumors, which is similar to that found by Gilead and Ginsberg in adenovirus-12-infected KB cells (19, 20). The antigen is small, with a sedimentation coefficient of 2.93, and forms a single fast-moving band in acrylamide-gel electrophoresis. Unlike the Gilead–Ginsberg antigen, however, it is partly heat stable, with one-half of the titer stable to 56° for 1 hr. Also, it is found primarily in the cytoplasm membrane fraction rather than in the nucleus. It is, however, much more resistant to papain than the membrane-bound H-2 antigen, and it is not solubilized by autolysis at 37° (12, 15).

We do not believe, however, that this is the only T antigen to be found in these tumors. The starting pellet itself contains two fractions of T, one highly susceptible to papain treatment, and probably destroyed during the extraction procedure, and one resistant to papain. It may be that the resistant moiety is merely more tightly membrane-bound than the susceptible component, or they may be different antigen fractions. Both react to the same antiserum. Furthermore, the originally soluble component (S-2) also contains T antigen activity, which is soluble at $10^{11}$ $\omega^2 t$, is destroyed by a 15-min exposure to papain, had a sedimentation coefficient of 18 and travels very slowly in acrylamide gel, like a macroglobulin (Candler and Jainchill, unpublished data). The fact that T antigen was found in all five sections of the acrylamide gel described here indicates the existence of more than one molecular species of T antigen. As with the other T antigens, the functions, as well as the number, of these substances are now known. Our solid tumor antigen is predominately cytoplasmic, whereas all other T's isolated to date are primarily intranuclear, with the exception of those detected by electron microscopy by Kalnins *et al.* (4), either in induced tumors or in cell lines derived from such tumors.

*Summary.* Nonvirion T antigen was isolated from adenovirus-type-31 solid tumors by a series of differential extractions, includ-

ing overnight digestion with papain, centrifugation, acetone and ammonium sulfate precipitation, and acrylamide-gel electrophoresis on a vertical flat-bed apparatus which enabled us to isolate individual bands of protein. The antigen is membrane-associated, has a sedimentation coefficient of 2.93, is partially heat labile, stable to freeze–thaw, and travels rapidly in acrylamide gel. It is probably not the only T antigen moiety present in these tumors.

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