JOURNAL OF VIROLOGY, Nov. 1969, p. 574-579
Vol. 4, No. 5

Simian Virus 40-Induced T and Tumor Antigens

C. W. POTTER, BRIDGET C. MCLAUGHLIN, AND J. S. OXFORD,
Department of Medical Microbiology, University of Sheffield, England

Received for publication 18 June 1969

Antigen extracts from simian virus 40 (SV40) transplanted hamster tumors were studied by rate-zonal centrifugation. Three species or molecular forms of antigen were demonstrated. The major antigen component corresponded to a molecular weight of 65,000 to 75,000, and two larger species were detectable in smaller quantities. Similar studies were carried out on SV40 virus-induced T antigen from BSC-1 cells. Three antigen components were again detected. Quantitative differences in the expression of "T" and tumor antigen species were reproducibly found.

In the transformation of mammalian cells by oncogenic deoxyribonucleic acid (DNA) viruses, the virus nucleic acid is integrated wholly or in part into the host cell genome (24). The nature and properties of the proteins coded by this incorporated virus DNA are of interest as possible initiators of malignant change. Two virus-specific antigens have been described in virus transformed cells; the transplantation antigen (15) and the tumor antigen (13, 14). Virus-specific tumor antigens, detectable by complement fixation tests, have been described in the cells of tumors induced by adenoviruses (13), simian virus 40 (SV40) (2, 21), polyoma virus (9), and chick embryo lethal orphan (CELO) virus (19).

During cytolytic infection of tissue cultures by oncogenic DNA viruses, virus-specific antigens, serologically indistinguishable from homologous tumor antigens, are induced (3, 10). Huebner (12) has proposed that these latter antigens be termed "T" antigens and that the antigens found in tumor cells be termed the "tumor" antigens. This communication describes the results of rate-zonal centrifugation studies of SV40 tumor antigens and compares the findings with similar studies of SV40 virus-induced "T" antigens.

MATERIALS AND METHODS

Virus. A single pool of SV40 virus prepared from virus obtained from A. J. Girardi, Wistar Institute of Anatomy and Biology, Philadelphia, Pa., was used throughout. Primary monolayer cultures of African green monkey kidney (AGMK) cells were infected with virus (0.01 TCD_{50} per cell), and the cultures were maintained in Eagle's basal medium (EBM) containing 2% inactivated calf serum, 0.88 g of sodium bicarbonate per liter, and antibiotics (100 units of penicillin and 100μg of streptomycin per ml). When cytopathic effect was complete, cultures were frozen and thawed twice (−80 C/22 C) and centrifuged at 3,000 rev/min for 20 min, and the supernatant fluids were stored in 5- to 10-ml volumes at −80 C. The titer of this virus preparation after 21 days of incubation in AGMK cells was $10^{8.3}TCD_{50}$ per ml (20).

Tissue cultures. A hamster tumor cell line, derived from an SV40 virus-induced tumor (catalogue no. TT101) and BSC-1 cells (11), were obtained from Flow Laboratories Inc., Irvine, Scotland, and were grown as monolayer cultures in 20-oz medical flat bottles (600 ml) in EBM containing 10% inactivated calf serum, 10% tryptose phosphate broth, and 0.44 g per liter of sodium bicarbonate and antibiotics. Confluent cultures of BSC-1 cells were maintained in EBM containing 2% calf serum and 0.88 g per liter sodium bicarbonate and antibiotics.

Preparation of tumor antigen. Hamsters, bred by random mating within a closed colony were inoculated subcutaneously at 3 to 4 weeks of age with 10^6 SV40-induced hamster tumor cells (51st to 76th in vitro generation). Palpable tumors detectable 2 to 3 weeks later were excised, freed of blood and adhering tissues, and finely chopped, and fragments were inoculated subcutaneously into hamsters within 72 hr of birth (7). Tumors, approximately 10 to 15 mm in diameter, were removed 8 to 12 days later, finely chopped, washed with Hanks saline, and centrifuged at 140 × *g* for 10 min. The pellet obtained was suspended in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride containing 0.15 M NaCl (*p*H 8.0), briefly homogenized in a Waring Blendor, and frozen and thawed once (−80 C/22 C). After thawing, the tumor homogenates were centrifuged at 16,000 × *g* for 30 min, and the cell debris was discarded. The supernatant fluid, containing the SV40 tumor antigen extracts, was stored in 2- to 3-ml volumes at −80 C.

SV40 tumor antigen extracts were also prepared from confluent tissue cultures of SV40 virus-induced hamster tumor cells (85th to 95th in vitro generation). Cell sheets were washed with two changes of Hanks saline, scraped from the culture vessels, and centrifuged at 55 × *g* for 10 min. The cell pellet was suspended in 0.01 M Tris-saline to give a 20% (v/v) suspension, frozen and thawed (−80 C/22 C), and centrifuged at 16,000 × *g* for 30 min, and the supernatant fluid was stored in 0.5-ml volumes at −80 C.

Preparation of "T" antigen. Monolayer cultures of BSC-1 cells were infected with SV40 virus at a multiplicity of 5 to 10 TCD_{50} per cell. After 1 hr of incubation at 37 C, maintainance medium was added and the infected cultures were incubated at 36 to 37 C for 3 days. The monolayers were then washed with two changes of Hanks saline, and antigen extracts were prepared as described above for tumor cells grown in tissue culture. The extracts contained complement-fixing antigen to titers of 1:64 against four units of SV40 hamster tumor antiserum but no complement-fixing activity against normal hamster serum or serum from hamsters bearing transplanted adenovirus 12 or CELO virus-induced tumors (19). Similarly, SV40 virus-induced tumor antigen extracts contained no complement-fixing activity against adenovirus 12 and CELO virus tumor antiserum or against normal hamster serum.

Serological tests. Serum was collected from hamsters bearing large (>50 mm diameter), transplanted SV40 tumors. The serum pools contained no complement-fixing antibody for SV40 virus but had complement-fixing antibody for SV40 tumor antigen to titers of 1:160 to 1:320. The serum was stored in 0.5-ml volumes at −20 C.

Complement fixation tests were performed by using overnight fixation at 4 C with 2 units of complement (22). In some tests for the presence of antigen in fractions from sucrose gradients, complement fixation tests were carried out by using a modification of the method of Wasserman and Levine (27). Complement was titrated by mixing dilutions of complement in 0.25-ml volumes with equal volumes of Veronal buffer and 4 to 8 complement-fixing units of serum [calculated using the method of Sever (22)] from hamsters bearing transplanted SV40 tumors. After overnight incubation at 4 C, 0.25 ml of 0.2% sensitized sheep cells was added, and the tests were incubated for 1 hr at 37 C. The suspensions were then centrifuged at 140 × *g* for 10 min, and the supernatant fluid was carefully removed and diluted 1:3 with Veronal buffer; the degree of hemolysis was estimated in a spectrophotometer (Unican 600) at 413 nm. From these results, the 50% hemolytic dose of complement in the presence of hamster serum was estimated. In tests for the presence of antigen in fractions, 0.25 ml of each sample was added to an equal volume of 4 to 8 complement-fixing units of hamster serum and 1.2 (50%) units of complement. After overnight incubation at 4 C, complement fixation was estimated as described for the complement titration. The results were expressed as the spectrophotometer reading for the serum control less the test reading.

Linear sucrose gradients. Linear sucrose gradients were prepared with the aid of a mixing device from 5 and 20% (w/v) solutions of sucrose (Analar) in 0.01 M Tris saline. The gradients (5.0-ml volume) were carefully layered with antigen preparation in 0.25-ml volumes. Aldolase (20 μliters) or horse alcohol dehydrogenase (15 μliters) was added to the antigen preparations to provide a marker of known molecular weight. The gradients were centrifuged at 100,000 × *g* at 4 C by using an SW-39 swinging bucket rotor in a Spinco L preparative ultracentrifuge. After centrifugation, 14-drop fractions (approximately 0.2 ml) were collected by bottom puncture, and each fraction was tested for antigen and for enzyme activity.

Enzymes. Aldolase was obtained as an ammonium sulfate precipitate (Worthington Biochemical Corp., Freehold, New Jersey). Prior to use, the enzyme was dialyzed overnight at 4 C against 0.01 Tris-saline. Samples of fractions from linear sucrose gradients were diluted 1 in 5 with distilled water and assayed for aldolase by using the method of Sibley and Lehninger (23). The results were plotted as spectrophotometric readings at 540 nm. Horse alcohol dehydrogenase was obtained from Calbiochem Ltd. and was assayed by the method of Theorell and Bonnichsen (25). The molecular weights of virus-induced antigen(s) were estimated with reference to the position of peak enzyme activity by using the methods of Martin and Ames (18).

Ribonuclease, obtained from B. D. H. Poole, Dorset, England, was added to some antigen extracts to give a final concentration of 50 to 150 μg/ml, and extracts were incubated at 30 C for 2 hr prior to antigen studies by rate-zonal centrifugation.

RESULTS

Tumor antigen extracts from four groups of transplanted SV40 tumors were each analyzed three or more times by rate-zonal centrifugation in 5 to 20% linear sucrose gradients. The findings for a typical test of tumor antigen (diluted to complement fixation titer of 1:16) are shown in Fig. 1. Tumor antigen, present in gradient fractions, was assayed by a modification of the complement fixation technique of Wasserman and Levine (27); this technique was found to be 5- to 10-fold more sensitive in the detection of complement-fixing antigen than the methods of Sever (22). The studies showed that the SV 40

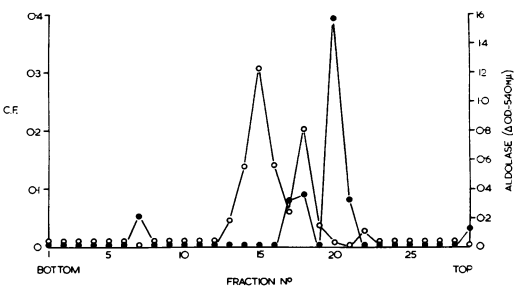

FIG. 1. *Rate-zonal centrifugation of SV40 tumor antigen extracted from hamster tumors. Of SV40 tumor antigen extract, 0.25 ml, containing 20 μliters of aldolase, was layered on a 5 to 20% linear sucrose gradient and centrifuged at 100,000 × g for 12 hr. Fractions were collected by bottom puncture and assayed for aldolase* (○) *and complement-fixing* (*CF*) *activity* [(●) *by using the method of Wasserman and Levine* (*27*)].

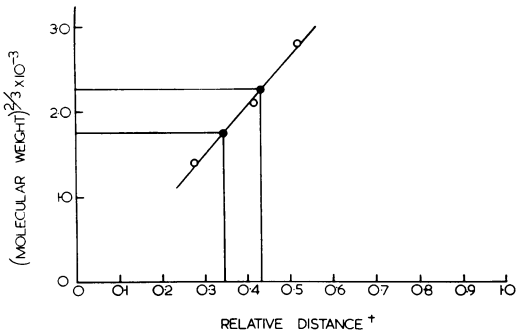

FIG. 2. *Estimation of molecular weight of SV40 tumor antigen(s). Relative distance is ratio of distance moved as number of fractions passed to total length of gradient in number of fractions.*

tumor antigen was detected in three distinct zones.

Molecular weight estimates for SV40 tumor antigen were calculated with reference to the position in the gradients of the enzyme aldolase. The results (Fig. 1) indicate three peaks of aldolase activity. Either during the purification procedures in manufacture or in the procedures described here, the enzyme aldolase, normally occurring as a trimer, depolymerized. The three peaks correspond to the natural trimer, molecular weight 150,000, together with the dimeric and monomeric forms. The positions of all three molecular forms were used in the estimation of molecular weight of tumor antigen (Fig. 2). The majority of the tumor antigen (Fig. 1) was detected in fractions corresponding to an estimated molecular weight of 65,000 to 75,000. The positions of the two further zones of complement-fixing activity corresponded to estimated molecular weights of 110,000 to 120,000 and 280,000 to 350,000. These molecular weight values are given as approximations only, and, in particular, the estimate of molecular weight given for the largest antigen form is most uncertain owing to its position in the gradient and to the large distance between the antigen species and the marker enzyme. However, the estimation was confirmed in sucrose gradients centrifuged for shorter periods of time (4 to 6 hr), when the antigen was found nearer the center of the gradients. Results similar to those in Fig. 1 were obtained for tumor antigen extracted with buffers to which bentonite (0.1 mg/ml) had been added to inhibit ribonuclease activity.

In further experiments, SV40 tumor antigen extracts (complement fixation titer 1:64) from transplanted hamster tumors were centrifuged in 5 to 20% linear sucrose gradients, and the antigen in the gradient fractions was titrated to investigate the relative amounts of tumor antigen in the various peaks by the complement fixation technique of Sever (22). Alcohol dehydrogenase, molecular weight 84,000 (5), was used as a marker in place of aldolase. The majority of the antigen was identified in fractions corresponding to a molecular weight of 65,000 to 75,000 (Fig. 3). This corresponds to the major peak of activity given in Fig. 1. A possible second and larger antigen species was suggested by the shoulder of complement-fixing activity in fractions 14 to 16 (Fig. 3) and corresponds to the antigen species of estimated molecular weight of 110,000–120,000 shown in Fig. 1. No species of molecular weight 280,000 to 350,000 was detected by the complement fixation procedures of Sever (22), but this antigen species was reproducibly detected by the more sensitive complementfixation technique of Wasserman and Levine (27).

Tumor antigens from SV40 tumor cells grown in tissue culture. Tumor antigen, extracted from SV40 tumor cells grown in tissue culture, was analyzed similarly by rate-zonal centrifugation in 5 to 20% linear sucrose gradients. Three different antigen preparations were tested (complement fixation titer 1:16–1:32), and a typical result is given in Fig. 4. Only one species of antigen, showing symmetrical distribution, was identified. From the position of this antigen with reference to the position of horse alcohol dehydrogenase, the molecular weight was estimated to be 65,000 to 75,000. No other complement-fixing antigen species was detected in tests using the complement fixation technique of Wasserman and Levine (27).

"T" Antigens from SV40-infected BSC-1 cells. Figure 5 gives the results of rate-zonal centrifugation tests of 5 to 20% linear sucrose gradients of "T" antigen extracts from SV40 virus-infected BSC-1 cells (complement-fixing titer 1:64) centrifuged at 100,000 × *g* for 12 hr. Three

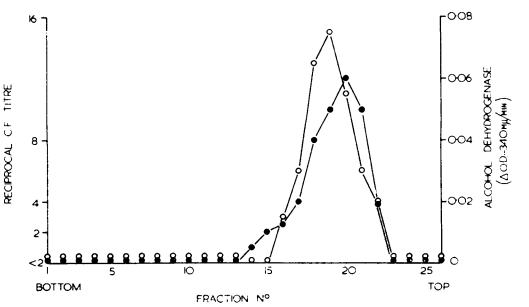

FIG. 3. *Rate-zonal centrifugation of SV40 tumor antigen extracted from hamster tumors. Of SV40 tumor antigen extract, 0.25 ml, containing 15 μliters of horse alcohol dehydrogenase, was layered on a 5 to 20% linear sucrose gradient and centrifuged at 100,000 × g for 12 hr. Fractions were collected by bottom puncture and assayed for alcohol dehydrogenase (○) and CF activity [(●) by the method of Sever (22)].*

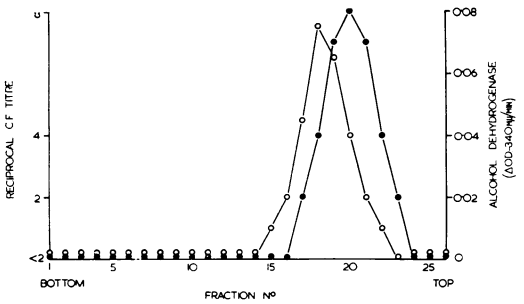

FIG. 4. *Rate-zonal centrifugation of SV40 tumor antigen extracted from hamster cells cultured in vitro. Of SV40 tumor antigen extracted from hamster tumor cells cultured in vitro containing 15 μliters of horse alcohol dehydrogenase, 0.25 ml was layered on a 5 to 20% linear sucrose gradient and centrifuged at 100,000 × g for 12 hr. Fractions were collected by bottom puncture and assayed for alcohol dehydrogenase* (○), *and CF activity* [(●) *by the method of Sever* (22)].

species or molecular forms of antigen were detected by the complement fixation technique of Sever (22). The estimated molecular weight of these antigens with reference to aldolase was 300,000 to 330,000, 110,000 to 120,000, and 65,000 to 75,000.

Antigen preparations from SV40 virus-infected BSC-1 cells were treated with ribonuclease at a final concentration of 50 to 150 μg/ml to test the possibility that the largest antigen species was a complex of antigen with ribonucleic acid (RNA). However, rate-zonal centrifugation of ribonuclease-treated "T" antigen preparations revealed the same spectrum of antigen forms as found in untreated preparations (Fig. 5).

DISCUSSION

The present study was undertaken to characterize the SV40 tumor antigen by rate-zonal centrifugation prior to attempts to purify the antigen to investigate its chemical form and function. In addition, a comparison was made of SV40-induced "T" and tumor antigens as Gilden (6) reported differences between adenovirus 12 T and tumor antigens. Our results indicate that extracts from transplanted SV40 tumors of hamsters contain three species or molecular forms of tumor antigen with estimated molecular weights of 65,000 to 75,000, 110,000 to 120,000, and approximately 300,000. Previous studies of extracts from SV40-transformed human fibroblasts reported a single species (8) and two species (4) of tumor antigen. The significance of multiple forms of tumor antigen is not clear at present, but multiple molecular forms of tumor antigen have been described in extracts from hamster cells transformed in vitro by adenovirus 12 (26).

The major component of the SV40 tumor antigen extracted from transplanted hamster tumors was identified at a position in centrifuged linear sucrose gradients corresponding to a molecular weight of 65,000 to 75,000. This was considerably less than the molecular weight estimation of 600,000 reported by Gilden et al. (8) for SV40 tumor antigen from extracts of SV40-transformed human fibroblasts. However, Delvillano et al. (4), using similar extracts, found that this large antigen was a complex of RNA and antigen with a sedimentation constant of 4*S*. This latter value is comparable to the molecular weight estimation of 65,000 to 75,000 for the major tumor antigen species found in the present study.

Molecular weight estimations of SV40 tumor antigen in extracts of tumors would give high values if the antigen were bound to cellular RNA, and would give relatively low values if the antigen-RNA complex were degraded by cellular ribonuclease to release unbound antigen. However, the addition of bentonite to buffers used in the extraction of tumor antigen to adsorb ribonuclease did not give preparations containing an antigen species of molecular weight corresponding to that described in extracts from SV40-transformed human cells (8). In addition, the antigen extraction procedures of Gilden et al. (8) and Delvillano et al. (4) did not include measures to prevent the activity of native ribonuclease.

The two larger molecular species of SV40 tumor antigen were identified in relatively small amounts during the present study. The smaller of these two species, molecular weight 110,000 to 120,000, corresponds to the second of two molecular forms identified by Delvillano et al. (4) and to the molecular weight of the single antigen species identified by Kit et al. (16). The position of the largest molecular forms of SV40

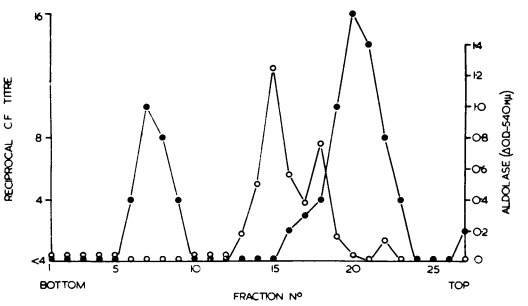

FIG. 5. *Rate-zonal centrifugation of SV40 "T" antigen extracted from SV40-infected BSC-1 cells. Of SV40 "T" antigen, 0.25 ml, containing 20 μliters of aldolase, was layered on a 5 to 20% linear sucrose gradient and centrifuged at 100,000 × g for 12 hr. Fractions were collected by bottom puncture and assayed for aldolase* (○) *and CF activity* [(●) *by the method of Sever* (22)].

tumor antigen indicated a molecular weight of approximately 300,000. Black (1) calculated that the entire length of the SV40 virus DNA could not code for a protein of molecular weight as large as 300,000; therefore, this antigen species probably represents an aggregate form.

The failure to detect more than one molecular form of SV40 tumor antigen in extracts from SV40 tumor cells grown in tissue culture was attributed to the lower complement fixation titers of antigen in these preparations. However, it is possible that all antigen forms are not present in all tumor cells. Thus, selection of the more tumorgenic cells, and the antigen forms they contain, may have occurred during tumor formation in vivo and resulted in an alteration in the antigen content of tumor extracts as compared with that of cells cultured in vitro. Further studies with cloned lines of SV40-induced hamster tumor cells are required to test this possibility.

The three species of antigen identified in hamster tumor extracts were also identified in "T" antigen preparations from SV40 virus-infected BSC-1 cells. The antigen species corresponding to a molecular weight of approximately 300,000 constituted a major component (20 to 30%) of the complement-fixing activity of "T" antigen preparations, whereas it was only identified in trace amounts in tumor extracts. Analysis by rate-zonal centrifugation of SV40 "T" antigens treated with ribonuclease indicated that this antigen species was stable to ribonuclease activity. A similar large SV40 "T" antigen species, molecular weight >250,000, was described by Kit et al. (17) in extracts previously treated with ribonuclease. The present findings were reproducible in all tumor and "T" antigen preparations, but the significance of the quantitative differences in antigen formation is not known. It remains possible that the largest species or aggregate form of SV40 "T" antigen is associated with viral replication in BSC-1 cells and is not found in significant quantities in SV40-induced tumor cells. Further studies are required to determine whether these quantitative differences in the expression of "T" and tumor antigens may relate to the different cell species from which they were extracted.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of G. Ellis, and the advice and criticism of M. G. Ellis, and the advice and criticism of M. G. McEntegart and C. H. Stuart-Harris.

This investigation was supported by a research grant from the British Empire Cancer Campaign.

LITERATURE CITED

1. Black, P. H. 1968. The oncogenic DNA viruses: a review of *in vitro* transformation studies. Ann. Rev. Microbiol. 22:391–426.
2. Black, P. H., W. P. Rowe, H. C. Turner, and R. J. Huebner. A specific complement-fixing antigen present in SV40 tumor and transformed cells. Proc. Nat. Acad. Sci. U.S.A. 50:1148–1156.
3. Defendi, V., R. I. Carp, and R. V. Gilden. 1966. Cellular antigen induced by viruses. p. 269–287. *In* W. J. Burdette (ed.), Viruses inducing cancer. University of Utah Press, Salt Lake City, Utah.
4. Delvillano, B. C., R. I. Carp, L. A. Manson, and V. Defendi. 1968. Studies on the cellular distribution and purification of SV40 T antigen. Transplantation 6:632–634.
5. Ehrenberg, A., and K. Dalziel. 1958. The molecular weight of horse liver alcohol dehydrogenase. Acta Chem. Scand. 12:465–469.
6. Gilden, R. V. 1968. Isolation and properties of the adenovirus type 12 nuclear T and tumor antigens. Transplantation 6:637–638.
7. Gilden, R. V., T. G. Beddow, and R. J. Huebner. 1967. Production of high-titer antibody in serum and ascitic fluid of hamsters for a variety of virus-induced tumor antigens. Appl. Microbiol. 15:657–660.
8. Gilden, R. V., R. I. Carp, F. Taguchi, and V. Defendi. 1965. The nature and localisation of the SV40-induced complement-fixing antigen. Proc. Nat. Acad. Sci. U.S.A. 53:684–692.
9. Habel, K. 1965. Specific complement-fixing antigens in polyoma tumors and transformed cells. Virology 25:55–60.
10. Hoggan, M. D., W. P. Rowe, P. H. Black, and R. J. Huebner. 1965. Production of "tumor-specific" antigens by oncogenic viruses during acute cytolytic infections. Proc. Nat. Acad. Sci. U.S.A. 53:12–191
11. Hopps, H. E., B. C. Bernheim, A. Nisalak, J. H. Tjio, and J. E. Smadel. 1963. Biological characteristics of a continuous kidney cell line derived from African green monkey. J. Immunol. 91:416–424.
12. Huebner, R. J. 1966. Nonvirion T or neoantigen and hybrids, *In* W. J. Burdette (ed.), Viruses inducing cancer. University of Utah Press, Salt Lake City, Utah. p. 219–228.
13. Huebner, R. J. 1967. Adenovirus-directed tumor and T antigens. *In* M. Pollard (ed.), Perspectives in virology, vol. 5. Academic Press, Inc., New York.
14. Huebner, R. J., W. P. Rowe, H. C. Turner, and W. T. Lane. 1963. Specific adenovirus complement-fixing antigens in virus-free hamsters and rat tumours. Proc. Nat. Acad. Sci. U.S.A. 50:379–389.
15. Klein, G. 1968. Tumour-specific transplantation antigens: G. H. A. Clowes Memorial Lecture. Cancer Res. 28:625–635.
16. Kit, S., D. R. Dubbs, P. H. Frearson, and J. L. Melnick. 1966. Enzyme induction in SV40-infected green monkey kidney cultures. Virology 29:69–83.
17. Kit, S., J. L. Melnick, M. Anken, D. R. Dubbs, R. A. de Torres, and T. Kitahara. 1967. Nonidentity of some simian virus 40-induced enzymes with tumor antigen. J. Virol. 1:684–692.
18. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: applications to protein mixtures. J. Biol. Chem. 236:1372–1379.
19. Potter, C. W., and J. S. Oxford. 1969. Specific tumour antigen, induced by chick embryo lethal orphan (CELO) virus. J. Gen. Virol. 4:287–289.
20. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent end points. Amer. J. Hyg. 27:493–497.
21. Sabin, A. B., and M. A. Koch. 1964. Source of genetic information for specific complement-fixing antigens in SV40 virus-induced tumours. Proc. Nat. Acad. Sci. U.S.A. 52:1131–1138.

22. Sever, J. L. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. 88:320–329.
23. Sibley, J. A., and A. L. Lehninger. 1949. Determination of aldolase in animal tissues. J. Biol. Chem. 177:859–871.
24. Stoker, M. G. P. 1969. Effects of tumour viruses on the growth regulation in cultured cells. Lancet 1:362–365.
25. Theorell, H., and R. Bonnichsen. 1951. Studies on liver alcohol dehydrogenase. Acta Chem. Scand. 5:1105–1126.
26. Tockstein, G., H. Polasa, M. Pina, and M. Green. 1968. A simple purification procedure for adenovirus type 12 T and tumor antigens and some of their properties. Virology 36:377–386.
27. Wasserman, L., and E. Levine. 1961. Quantitative microcomplement fixation and its uses in the study of antigenic structure by specific antigen-antibody inhibition. J. Immunol. 87:290–295.