Induction of Tumor-Specific Transplantation Immunity in Hamsters with Cell Fractions from Adenovirus and SV40 Tumor Cells[1] (34208)

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The presence of several neoantigens in tumor cells transformed by adenoviruses and SV40 virus is well documented. Among these new antigens is the class termed tumor-specific transplantation antigen (TSTA) that is immunogenic against tumors induced by the transforming virus or against cell challenge with the homologous tumor type. Repeated efforts to obtain subcellular components from hamster tumor cells containing immunogenic levels of SV40-TSTA have failed (1–3). Recently adenovirus 7 hamster tumor cells, rendered nonproliferative by formalin treatment or disrupted by freezing and thawing, were observed to be immunogenic against adenovirus 7 tumor cell challenge (3). Tevethia *et al.* (4) reported that SV40-induced tumor cells could be lysed hypotonically; this procedure yielded cell ghosts which, when administered to hamsters within 24 hr after birth, stimulated a tolerant state to TSTA immunization with SV40 in later life. No indication of the effectiveness of these ghosts to stimulate transplantation immunity against cell challenge was presented.

Our results describe efforts to effect TSTA immunity against SV40 and adenovirus 31 tumor cell challenge with tumor homogenate, subcellular components, or cell ghosts from homologous tumor.

[1] Research conducted under the joint NIH-AEC Molecular Anatomy (MAN) Program supported by the National Cancer Institute, the National Institute of General Medical Sciences, the National Institute of Allergy and Infectious Diseases, and the U. S. Atomic Energy Commission, under contract with the Union Carbide Corporation Nuclear Division.

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Materials and Methods. Hamsters. Golden Syrian hamsters (Lakeview Hamstery, Newfield, New Jersey), uniformly histocompatible for skin grafts (5) and considered to be immunologically syngeneic, were used in this study.

Virus. SV40 strain VA45-54 (6), propagated in primary grivet monkey kidney (GMK) cell culture with a titer of 10^7 $TCID_{50}$/0.2 ml for GMK cells, was employed. Adenovirus 31 was propagated in primary human embryonic kidney (HEK) cell culture and possessed a titer of $10^{7.3}$ $TCID_{50}$/0.2 ml in HEK cells.

Tumor cell lines. The F5-1 line (7–9) of SV40 hamster tumor cells grown in roller culture in medium 199 containing 10% heat-inactivated calf serum and antibiotics was used throughout the study to prepare cell ghosts. A derivative of an adenovirus 31-induced hamster tumor transplant (Flow Laboratories, Rockville, Maryland), passaged at 7–10-day intervals in mature hamsters, was used in initial efforts to prepare plasma membranes. This transplant line of tumor cells did not shed infectious adenovirus 31, as determined by co-cultivation with HEK cells *in vitro*, but possessed specific adenovirus 31 tumor antigen, as determined by complement fixation testing (10) or by the fluorescent antibody procedure (11).

Adenovirus tumor cell challenge assay. Weanling hamsters were given three intraperitoneal injections of tumor cell suspension or cellular components in 1.0-ml aliquots at 1-week intervals. Ten to 14 days after the third immunization, hamsters were challenged in the right subscapular area with standardized concentrations of viable tumor cells

suspended in Ca^{2+}–Mg^{2+}-free Hanks' balanced salt solution (HBSS). Hamsters were then palpated weekly until the first tumor appeared and several times a week thereafter. All animals were examined weekly for the appearance of tumors at the site of immunization and were autopsied and examined for internal tumors at death or at the termination of the experiments.

SV40 cell challenge assay. The procedure previously described (2) for evaluating vaccines in the SV40 tumor cell challenge assay was used in this study. Immunized hamsters and controls were challenged with 5×10^4 viable tumor cells 10–14 days after immunization was completed. Tumors develop rapidly by 40 days, and we find that animals failing to develop tumors by day 60 remain tumor free.

Irradiated cell vaccines. Adenovirus 31 tumors, even when small and relatively necrosis free, contain a large proportion of dead tumor cells, as determined by the trypan blue exclusion test. To obtain a sufficient number of living cells to prepare irradiated tumor cell control antigen, a method was devised to avoid rapid destruction of cells by prolonged mincing and trypsinizatiation of the whole tumor. Two small tumors (2 cm diam) were removed aseptically, rinsed and freed of connective tissue. The tumors were minced to fragments between 1–2 mm in size and placed in a 30-ml trypsinizing flask with 5 ml of medium 199 without calf serum or trypsin. Minute tumor fragments were disaggregated with a magnetic, Teflon-coated stir bar that was too large to rotate in the flask but produced a violent bumping action when placed on a magnetic stirrer. Ten to 15 min of this bumping was adequate to produce a disaggregated cell suspension, and cells were harvested by passing the mixture through fine wire mesh. This procedure preserved the viability of the cell population at 50–60%.

Irradiation was carried out on standardized cell suspensions as previously described (1). The preparations contained 5×10^6 dye-excluding cells per ml after exposure to 5000 R of X-irradiation. Cells prepared in this manner and not irradiated were used for cell challenge later in the experiment.

Preparation of adenovirus 31 tumor cell fractions. Small tumors (1–2 g) were removed from the right dorsal surface of hamsters where they had been transplanted 10–14 days earlier. Aseptic technique was employed. The tumors were freed of connective tissue and necrotic regions and pooled. Forty to 60 g of pooled, fresh tumor were placed in 8.5% sterile sucrose solution to a final volume of 800 ml. The tumor was mixed 2 min in an Omnimixer and frozen quickly to —20°. The homogenate was thawed slowly 1 hr to 4°, homogenized 2 min in the Omnimixer, refrozen to —20°, and thawed as before. After a third freezing and homogenizing cycle, the disrupted tumor cells (homogenate) were centrifuged at 4° (20-ml aliquots) in a No. 30 Spinco rotor to an $\omega^2 t$ (12) $= 10^7$. The supernatant (15 ml) was removed and the pellet (5-ml volume/tube) was saved. The supernatant was centrifuged in a No. 50 rotor to an $\omega^2 t = 5 \times 10^9$, collected from the pellet (2-ml volume), and centrifuged a third time to an ω^2 value $= 10^{11}$. The 10^7, 5×10^9, and 10^{11} pellets (2-ml volume) collected at each centrifugation were pooled within groups, dialyzed 18 hr at 4° against three changes of Miller–Golder buffer (13), pH 7.0, and frozen until use at —60°. Homogenate maintaining constant volume was likewise dialyzed. Protein estimates were determined on each fraction by the method described by Lowry (14) in a Technicon automated system (Technicon Corp., Tarrytown, New York), modified by Elrod (15). The amount of adenovirus 31 tumor antigen was determined on each fraction by a microcomplement fixation procedure (10).

Preparation of cell ghosts. The method of Haughton (16) was employed to prepare cell ghosts from SV40-induced hamster tumor cells of the F5-1 cell line. Cells (3.3×10^8) were removed from roller bottle culture by scraping resuspended in 50-ml Tris buffered saline (pH 7.4), and centrifuged at 4° for 10 min at 1500 rpm in an International PR6 centrifuge (No. 253 rotor). The supernatant was removed and cells were resuspended in 50 ml of 3% sodium chloride in distilled water. Sterile reagents were used throughout. The cells were immediately centrifuged at 4°

TABLE I. Protein and Tumor Antigen Complement Fixation Titer of Adenovirus 31 Tumor Cell Homogenate and Cell Fractions.

Preparation	Final vol (ml)	Protein (mg/ml)	Tumor antigen CF titer (reciprocal)
Homogenate	800	6.35	80
Nuclear fraction ($\omega^2 t = 10^7$)	168	9.70	80
Crude membranes ($\omega^2 t = 5 \times 10^9$)	129	17.60	160
Pellet ($\omega^2 t = 10^{11}$)	108	7.70	160[a]
Supernatant ($\omega^2 t = 10^{11}$) cell sap	390	1.85	20

[a] Average of two evaluations.

for 10 min at 1500 rpm. The pellet was collected and resuspended in 6% saline and, following centrifugation, in 15% saline. Cells in 15% saline were collected as a flocculant pellet by centrifugation at 3000 rpm for 15 min, and the pellet was washed two additional times in 15% saline. After the third wash, cells were resuspended in distilled water to effect lysis and centrifuged at 4° for 15 min at 3000 rpm. The pellet was observed to contain structurally intact ghost cells, but no viable cells were evident as judged by the dye exclusion procedure. Ghosts were resuspended in distilled water to give a final concentration that represented 8×10^7 original viable cells per ml and held at 4° for inoculation. One-ml aliquots of the cells were administered intraperitoneally to weanling hamsters weekly for 3 weeks. Fresh ghost preparations were used each week. Controls included tumor cells inactivated by exposure to X-ray or disrupted by repeated cycles of freezing and thawing as previously described (1).

Washed, F5-1 tumor cell ghosts were pelleted and fixed for electron microscopy. Ghosts from each preparation were also inoculated subcutaneously (0.1 ml) into the right subscapular space of neonatal hamsters to provide an oncogenicity control on each preparation.

Preparation of F5-tumor cell ghosts for electron microscopy. The F5-1 cell ghosts were fixed with Palade's Veronal-buffered osmium (17) in the cold for 1 hr, dehydrated, infiltrated, and embedded in Epon 812 according to the procedure of Luft (18).

Results. Adenovirus 31 tumor cell fractions as immunogen. Weanling hamsters were immunized with either X-irradiated adenovirus 31 tumor cells, tumor cell homogenate, or membrane fraction, nuclear fraction, cell sap, or smaller organelles from the homogenate separated by differential centrifugation. The sedimentation character, protein content and complement fixation (CF) titer for tumor antigen of each component used as vaccine is given in Table I. Serum from adenovirus 31 tumor-bearing hamsters was employed in the CF test.

Three weekly injections of each preparation listed in Table I, as well as injections of adenovirus 31 tumor cells rendered nonproliferative by X-irradiation, were employed. Hamsters were subsequently challenged with 4×10^6 or 8×10^6 viable, homogenously suspended, homologous tumor cells, and the results of immunization or tumor appearance are given in Table II.

Dialyzed, unseparated tumor cell homogenate (starting material used to prepare fractions) was not observed to stimulate immunity to adenovirus 31 cell challenge. This observation has been made repeatedly for adenovirus 7 tumor cells or SV40 tumor cells whether the homogenate was preserved at ultralow temperatures, injected immediately or treated and held under a great variety of conditions. Irradiated tumor cells were completely effective as vaccine against the homologous tumor cell challenge.

The material pelleted in the 8.5% sucrose solution at $\omega^2 t = 5 \times 10^9$ was observed to stimulate a degree of transplantation immunity comparable to that induced by immunization with irradiated whole cells. Other components of the homogenate (10^7 pellet, 10^{11}

TABLE II. The Efficacy of Adenovirus 31 Hamster Tumor Cells and Cell Fractions as Immunogens against Homologous Tumor Cell Challenge.

Immunization with	Challenge level:	Tumors/survivors[a]	
		4×10^6 cells	8×10^6 cells
		(%)	(%)
Nothing		7/8 (87.5)	7/8 (87.5)
Buffer solution		6/8 (75)	7/7 (100)
Unseparated cell homogenate		8/12 (66)	10/12 (83)
Whole irradiated tumor cells (5000 R X-ray)		0/12 (0)	0/10 (0)
Adenovirus 31		2/12 (16)	3/12 (25)[b]
Nuclear fraction ($\omega^2 t = 10^7$)		12/16 (75)	9/14 (65)
Crude membrane-containing fractions ($\omega^2 t = 5 \times 10^9$)		1/16 (6)	2/16 (13)
Pellet ($\omega^2 t = 10^{11}$)		8/12 (66)	13/16 (81)
Supernatant cell sap ($\omega^2 t = 10^{11}$)		7/16 (43)	15/16 (93)

[a] Results after 156 days' postchallenge; tumors appeared first in nonimmunized controls on day 47.

[b] Results 116 days' postchallenge.

pellet, and 10^{11} cell sap) were not effective in stimulating adenovirus 31 tumor transplant immunity with the highest challenge level. A second experiment gave similar results; however, the protein concentrations present in subsequent experiments varied slightly with those given in Table I. The supernatant components which did not pellet at $\omega^2 t = 10^{11}$ appeared to stimulate some degree of immunity against a challenge of 4 million cells, but the immunity was not evident against a challenge of 8 million cells. Sixty-three percent tumors occurred in the second experiment group.

Hamsters immunized with the crude membrane fraction ($\omega^2 t = 5 \times 10^9$) received approximately 52 mg of protein, whereas animals immunized with homogenate received only 19 mg of protein. To eliminate the possibility that the homogenate failed to stimulate immunity because of the simple lack of sufficient antigenic mass (using protein content as a convenient index), the homogenate was administered at 3 times the previous level (60 mg) in the same manner as described, and the hamsters were challenged as usual. No protection was observed.[3]

Electron micrographs of the crude membrane fractions ($\omega^2 t = 5 \times 10^9$) disclosed the material to consist of vesicle-like structures (Fig. 1), membranous fragments, and occasional organelles. The vesicles were typical of the double membrane structures described by Boone (18). Few intact nuclei were observed to contaminate the crude membrane fraction. The objective of this work was first to be certain, using drastic methods, that no intact cells were present, and second that the surviving major fractions were at least partially separated.

SV40 hamster tumor ghosts. Mature hamsters were administered three intraperitoneal injections of SV40 virus, whole X-irradiated, dye-excluding SV40-tumor cells, a frozen and thawed extract of tumor cells, or tumor cell ghosts. A single injection of each vaccine preparation was injected subcutaneously into neonatal hamsters to supply oncogenicity control. We have found that neonatal challenge can reliably detect the presence of small numbers of viable tumor cells; thus, this system provides an excellent method for evaluating cell extracts for proliferative, viable cell contaminants.

The results of these immunizations and of the oncogenic potential of each cell preparation or extract are given in Table III. Irradiated SV40 tumor cells or SV40 virus conferred absolute immunity to the hamsters

[3] In other studies administration of gram quantities of tumor homogenate failed to stimulate transplantation immunity.

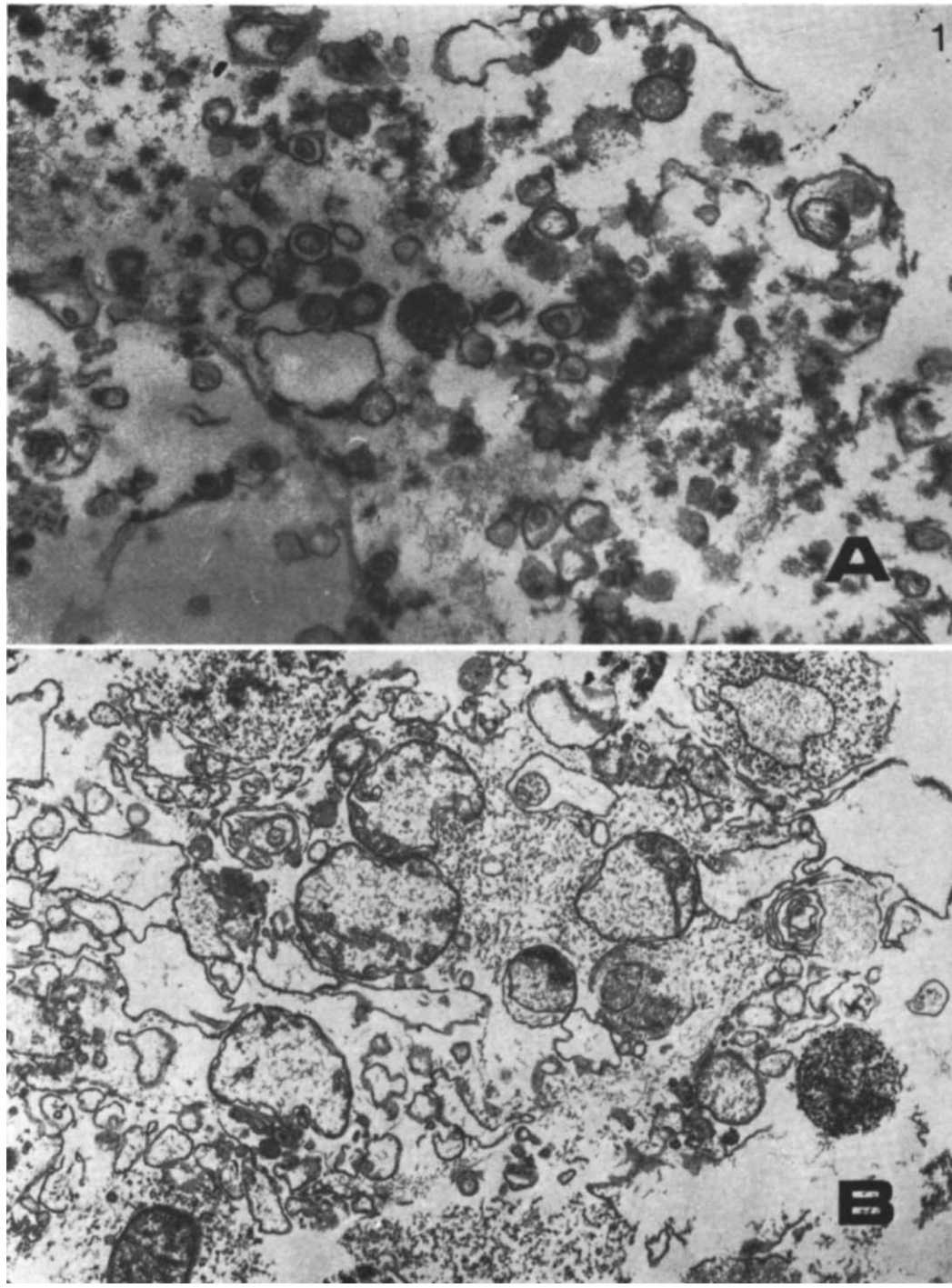

FIG. 1. Crude membrane containing fraction from adenovirus 31 hamster tumor cells ($\omega^2 t = 5 \times 10^9$) consists of vesicles, membranous fragments, and occasional mitochondria; (A) ×42,000; (B) ×20,000.

TABLE III. The Immunogenic Efficacy of SV40-Hamster Tumor Cell Ghosts against Homologous Cell Challenge.

Immunization with[a]	Animal age	Tumors/survivors 70 days postchallenge with 5×10^4 tumor cells (%)
Control; nothing	4 weeks	7/8 (87)
Irradiated SV40 tumor cells (5000 R)	4 weeks	0/10 (0)
SV40 virus (3 weekly injections, 0.2 ml 10^7 $TCID_{50}$/0.2 ml)	4 weeks	0/10 (0)
SV40 tumor cell ghost	4 weeks	0/11 (0)
SV40 tumor cells disrupted by freezing and thawing	4 weeks	9/10[c] (90)
Oncogenicity control[b]		Vaccine tumors/survivors (day of tumor appearance)
Irradiated SV40 tumor cells (5000 R)	Neonate	4/11 (day 75)
SV40 freeze–thaw disrupted cells	Neonate	9/9
SV40 tumor cell ghosts	Neonate	0/15

[a] One ml given ip on three weekly occasions. Fresh vaccines were prepared each week.

[b] One-tenth ml of the preparation used in the first round of the immunization above was given subcutaneously in right subscapular region to neonates within 24 hr after birth to provide an oncogenicity control.

[c] In addition, four vaccine (ip)-induced tumors were detected.

against tumor cell challenge. Similarly, SV40 cell ghosts stimulated complete transplantation immunity against cell challenge with a concentration of tumor cells that produced 87 and 90% tumors in control animals or animals immunized with tumor cells disrupted by freezing and thawing, respectively. It should be noted that SV40 tumor cell ghosts were observed to have no oncogenic potential in the neonatal hamster, whereas cell extracts or, surprisingly, even irradiated tumor cells produced tumors when inoculated into newborns. No "vaccine-induced" tumors appeared in mature animals immunized with irradiated tumor cells and later challenged. We conclude it is unlikely that any intact viable cells survived the distilled water lysis step used to prepare ghosts. Many cells survived the freeze–thaw extraction procedure, and some cells obviously recovered sufficiently from X-irradiation damage to again become oncogenic in neonates.

The ghost preparations were fixed and examined by electron microscopy, and the structures observed are shown in Fig. 2. The basic predominating components in the preparation were numerous, large, empty saccules, as well as smaller vacuoles, and vesicles. Occasional saccules contained intact nuclei, and many cells appeared intact with unbroken membranes at the plane of cut used to prepare the specimen. The predominant characteristic of ghosts prepared by the Haughton method appears to be loss of the cell sap and the term "ghost" here is used in the same context as defined by Haughton (16) and Tevethia and Rapp (4).

Discussion. Adenovirus 31 tumor cell homogenate. The negative results obtained in efforts to effect transplantation immunity with frozen and thawed adenovirus tumor homogenates confirm previous work with similar homogenates using SV40 tumor cells (1, 2). Recently frozen and thawed cell fragments from adenovirus 7 tumor cells were reported to produce 20–25% protective effect against homologous cell challenge (3). Our observation, that a crude membrane fraction could be prepared from homogenates of adenovirus 31 tumor cells which retained apparent tumor-specific transplantation antigen (TSTA), was surprising. Other components of the homogenate lacked sufficient TSTA to stimulate detectable immunity in the assay system described. The obvious question derived from this finding is, why was the TSTA not "active" or functional in stimulating transplantation immunity when

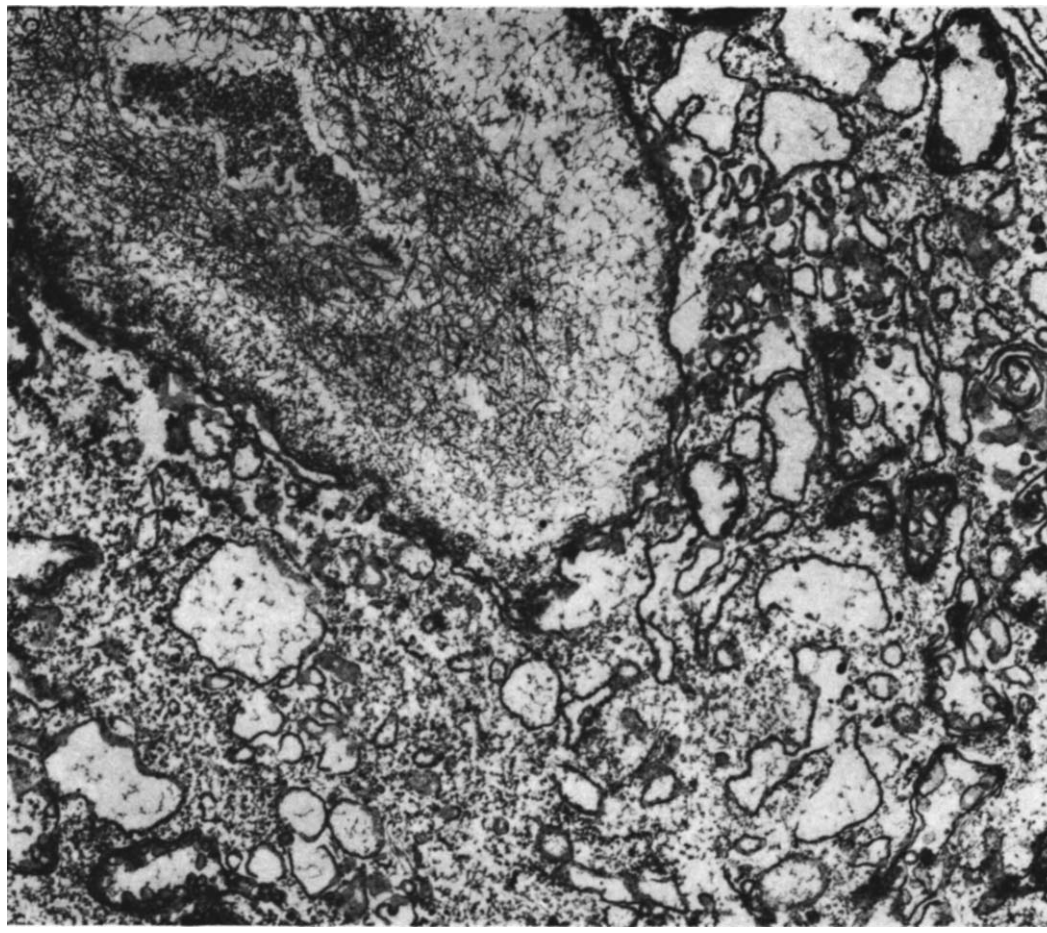

FIG. 2. Cell lysate preparations from SV40 hamster tumor cells show vesicles of many different sizes and an intact cell which appears to have lost the cell sap. The preparations are not analogous to erythrocyte ghosts (×16,692).

present in the unseparated homogenate? No clear answer is available at the present time; however, several explanations are possible. It may be that other components of the homogenate contain sufficient antigen of a type that stimulated the synthesis of blocking antibodies to effectively prevent transplant rejection by humoral and/or cellular mechanisms. This possibility is being evaluated by reconstitution experiments performed with the fractions. That is, the membrane fraction is mixed with nuclei, or the 10^{11} pellet, or supernatant components and used as immunogen to determine if the presence of these latter fractions interferes with the expected immunity induced by the membranes alone.

It may also be possible that the various components "mask" each other antigenically by spatial interference or by stimulating the synthesis of antibodies directed against two or more components, only one of which is the TSTA. Immunity against the combination antigen may not be functional in transplant rejection. Tumor antigen was found in all components; however, the greatest concentration prevailed in the plasma membrane component ($\omega^2 t = 5 \times 10^9$) and in the pellet collected at $\omega^2 t = 10^{11}$. The presence of the tumor antigen(s) in the fractions seems to be unrelated to the interference effect. The possibility cannot be eliminated that the antigenic "integrity" of the adenovirus 31 transplan-

tation antigen(s) may not properly be retained in fractionated cells but that the "integrity" can be reinstated by removal or separation of the antagonistic inhibitory or masking components. Specifically, several levels of chemical and physical interaction between the components of the homogenate may serve to distort the steric arrangement of TSTA in the plasma membrane component. Immunity directed against the distorted TSTA antigen might then be nonfunctional against the nondistorted TSTA at the surface of the intact cells used to challenge immunity. Other possibilities exist and are the subject of future work.

Induction of transplantation immunity by SV40 tumor cell lysates. Tevethia and Rapp (4) reported that a tolerant-like state could be induced in hamsters to stimulate transplantation immunity by immunization of mature animals with SV40 virus. Neonates exposed to a single injection of SV40 tumor cell ghosts prepared by the method of Haughton (16) could not be successfully immunized in later life by SV40 virus inoculation and were susceptible to SV40 tumor cell challenge. It seems reasonable to assume that these cell ghosts must possess SV40 TSTA and, if so, should be evaluated for their potential to stimulate transplantation immunity in adult hamsters. Three immunizations totaling three times the level previously used to stimulate TSTA tolerance in neonates was employed, and this regimen was observed to produce complete transplantation immunity. The water-lysed cell preparations contained large and small saccules of cell membrane internal components and occasional lysed, but morphologically intact, cells. These findings suggest that previous attempts to disrupt SV40 tumor cells (1, 2) by freezing and thawing, sonic oscillation, formalin treatment, or pressure cell explosion may have been too severe for the either weak or unstable SV40 transplantation antigen. The availability of these TSTA-containing SV40 cell lysates as starting material for the purification of SV40-stimulated transplantation antigen should greatly expedite the identification of this important antigen in tumor cells. These results provide a starting point for studies on the isolation of membrane fractions of higher purity and for the isolation of the antigen itself.

Summary. The specific induction of transplantation immunity against adenovirus 31- and SV40-induced hamster tumor cell challenge employing either a crude membrane fraction of homologous adenovirus 31 tumor cells or SV40 tumor cell fragments is described. In each case immunity was equivalent or superior to that induced by whole, irradiated tumor cells previously described to contain TSTA, or to that induced by vaccination of mature hamsters with the live virus. Whole tumor homogenate of adenovirus 31 hamster cells, from which the membrane fraction was obtained failed to induce TSTA immunity. The possibility exists that a masking or enhancing component is present in the homogenate to interfere with TSTA immunity induction or detection.

Addendum. Hamsters immunized with SV40 tumor cell ghost lysates were uniformly resistant to an initial cell challenge as shown in Table III. Several months later the survivors were challenged a second time and 42% of the hamsters developed tumors by day 50. One hundred percent of normal control hamsters similarly challenged developed tumors at this time.

The authors are grateful to Mrs. Betty W. Harrell of Oak Ridge National Laboratory for the skillfully prepared electron micrographs of the cell ghosts, and to E. L. Candler, Oak Ridge National Laboratory, for technical assistance.

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Received May 8, 1969. P.S.E.B.M., 1969, Vol. 132.