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Induction of Transplantation Resistance with Soluble Simian Virus 40-induced Hamster Tumor-specific Transplantation Antigen[1]

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ABSTRACT

Multiple injections of 3 M KCl extracts of SV40-induced hamster sarcoma cells were found to have no inhibitory effect on tumor growth in syngeneic hamsters following homologous tumor challenge. When a single injection of the same extract was administered in the range of 0.5 mg of antigen protein, a significant delay in the appearance of tumors was repeatedly observed, and a significant number of challenged animals failed to develop tumors in two of three experiments. Subsequent studies established that a single injection of the tumor cell extract generally conferred 20 to 40% permanent protection, always with a marked delay in tumor appearance. The immunity was specific in that neither immunization with normal muscle extracts nor heterologous tumor challenge resulted in protection or delay in tumor appearance. Higher or lower amounts of homologous, soluble tumor transplantation antigen given in a single injection were either without effect or promoted tumor development. The resistance observed in animals receiving a single dose of soluble tumor antigen could only be detected if the hamsters were challenged with 5×10^5 tumor cells. Challenge with only 10^4 tumor cells did not lead to the detection of resistance. The immunity induced by a single optimal dose of tumor antigen could be transferred to normal, uninjected hamsters at 20 days postsensitization with lymph node cells but not with peritoneal exudate cells from injected donors, suggesting that the immunity detected in animals immunized with soluble tumor extracts was cell mediated. Taken together, these data indicate that antigen dose, regimen of administration to the host, and the challenge level used to detect transplantation resistance are all important parameters to consider when using cell-free, tumor-associated transplantation antigens. These data strongly support and extend an earlier related report derived in another model with a chemically induced tumor.

INTRODUCTION

One of the major thrusts in modern tumor immunology is the effort to purify and chemically identify TSTA.[2] It is extremely important to isolate and determine the precise chemical structure and specific cellular location of TSTA's because of their potential importance in tumor detection and for specifically identifying their role in tumor progression or rejection. The history of modern efforts to prepare cell-free, membrane-associated TSTA's goes back almost 2 decades now, and the chemical identity of purified TSTA in any system is still unknown.

The early attempts to isolate TSTA were fraught with disappointment in the oncodnavirus model system in hamsters (11). The typical finding was that any effort to release true TSTA from SV40-induced tumors of hamsters seemed to result in the apparent destruction of or altered the immunogenicity of the transplantation rejection antigen. Multiple injections of cell-free extracts of these sarcomas failed to induce protection against tumor transplantation or virus-induced tumor formation (10). Tevethia and Rapp (27), reported that SV40 tumor cell ghosts were immunogenic, and Coggin *et al.* (9) reported that concentrated membranes of SV40 or adenovirus-transformed hamster sarcoma cells were capable of conferring modest transplant resistance following multiple injections at weekly intervals. Soluble extracts were ineffective, and we often observed that soluble tumor products promoted tumor growth when injected at high concentrations on multiple occasions (8-10).

More recently, a number of papers have appeared suggesting that it may be possible to isolate soluble extracts which are capable of causing prophylactic protection against tumor transplantation in mice (1, 2, 5-7, 12, 14, 18, 23). SV40 does not produce tumors when injected into neonatal mice as it does when injected into neonatal hamsters. Mouse cells must be transformed under artificial circumstances *in vitro*, and the study of TSTA in SV40-transformed mouse sarcoma cells may not represent true, native TSTA since SV40 mouse sarcoma cells are unusually antigenic compared to SV40-induced hamster sarcoma cells.

Forbes *et al.* (14) reported that KCl-solubilized extracts of 3-methylcholanthrene-induced sarcomas produced a modest increase in survival (50% lethal dose) following homologous and heterologous challenge. These workers also noted an unexplained "low antigen dose" effect on the stimulation of lymphocytes from tumor bearer hosts using similar solubilized tumor antigen extracts from homologous tumors (14). Pellis and Kahan (24) reported that the capacity of similar soluble extracts administered prophylactically from chemically induced mouse sarcomas to retard the rate of tumor development following challenge was a function of the tumor antigen concentration administered and the immunization schedule, with high doses of antigen effecting enhanced tumor development.

In other more recent studies (16-18, 20, 22, 25, 26), Law *et al.* reported the successful isolation and cellular location of a transplantation resistance antigen from SV40-transformed mouse sarcoma cells. These results repeatedly suggested that the apparent TSTA from mouse sarcoma cells induced *in vitro*

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[2] The abbreviations used are: TSTA, tumor-specific transplantation antigen; T-antigen, tumor antigen; Adv 7, adenovirus 7; PEC, peritoneal exudate cells; LNC, lymph node cell preparations; PBS, phosphate-buffered saline (0.015 M phosphate: 0.85% NaCl solution, pH 7.2).

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by SV40 had been prepared in a cell-free form and retained the potential to induce specific transplantation resistance against tumor challenge following several injections of soluble preparation. In one of these repetitive reports, Rogers *et al.* (25) noted that the so-called TSTA obtained copurified and was inseparable from the T-antigen of these tumors. SV40-induced T-antigen in the SV40-transformed hamster sarcoma cell is specific for SV40-transformed cells but does not confer transplantation resistance to recipient test hamsters or copurify with TSTA, nor will T-antigen prevent SV40 oncogenesis in the hamster, the original model system in which SV40 T-antigen and TSTA were first identified (28). Girardi[3] repeatedly observed that SV40 T-antigen preparations from human cells transformed by SV40 (WI-26VA4) as well as F5-1 hamster cells transformed by SV40 would not protect hamsters against SV40-induced sarcoma.

We have been attempting to develop or utilize methods for isolating cell-free, tumor-associated antigens from viral and chemically induced sarcoma or fetal tissues of the hamster. The findings of Pellis and Kahan (24), using chemically induced sarcomas of mice, suggested the importance of careful control of antigen concentration in utilizing soluble antigen to stimulate transplantation resistance. In retrospect, such considerations as antigen dose, timing, challenge level, and number of immunizations logically address the problem of immunization with TSTA's, in our view. As we learn more about activation and regulation of the cellular arm of the immune system of animals, it is becoming clear that the type and quantity of antigen presented and the regimen of antigen used as vaccine may play a critical role in activating functional, cell-mediated resistance in the transplantation rejection assay.

The purpose of the present work is to present results of studies with 3 M KCl extracts using the SV40-induced sarcoma model in hamsters where we observed that antigen dose and cell challenge level markedly affected the result obtained and where we were able to show that the immunity detected using soluble extracts was truly cell mediated.

MATERIALS AND METHODS

Hamsters. The LVG/LAK strain of Syrian golden hamster from the Lakeview Hamster Colony, Newfield, N. J., was used throughout the study. The LVG strain has been used for over a decade as the classic model for measuring induced immunity to SV40 neoplasmas.

SV40 Tumor Cells. The F5-1 line of SV40-induced hamster fibrosarcoma (14), derived in the LVG strain, was used throughout the study and was maintained in tissue culture at passage levels between 40 and 70. The cells were cultured in Medium 199 containing 10% heat-inactivated, fetal calf serum with penicillin, streptomycin, and Fungizone. This tumor line is positive for SV40 T-antigen, is pleuropneumonia-like organism free, is negative for SV40 virion antigen production, and the transforming SV40 genome present is defective and can only be rescued by hybridization with a complementing SV40-transformed cell. Attempts to induce active virus production have failed repeatedly. This is an important test parameter in selecting tumor cell models for tumor antigen purification studies. If a given test line should shed infectious virus, even at very low concentrations, the virus released in antigen purification efforts might mimic TSTA when given to adult hamsters and produce false positive data, incorrectly suggesting the successful release of TSTA.

The tumor cells were grown at 37° in large roller bottles and were usually harvested 5 to 7 days after seeding in air-tight bottles.

An Adv 7-induced hamster fibrosarcoma produced in the LVG hamster served as a control, heterologous cell line (9).

Preparation of 3 M KCl Extracts. Five- to 7-day-old cultures of F5-1 cells (SV40-induced) grown in roller tubes were harvested by scraping with a sterile windshield wiper designed to fit into the long roller tube bottles. Trypsin harvest could also be used. The cells were washed in Medium 199, standardized to 5×10^5 living cells in Medium 199 plus 10% fetal calf serum, and cultured in siliconized spinner flasks with gentle stirring for at least 12 hr at 37° prior to use. The suspended cells, totaling 5 to 9.9×10^9 cells/ml in 3 trials reported, were then harvested by centrifugation and washed on 3 occasions by centrifugation at 1500 rpm in PBS at 25°. The viability of the cell suspension following the third wash was greater than 99% (trypan blue dye exclusion), and the cell pellet generally measured more than 5 ml of wet packed cells. The cell pack was resuspended in 10 ml of chilled PBS per ml of packed cells used. The suspended cells were exposed to the addition of anhydrous, reagent grade potassium chloride added slowly over a 30-min period to the cell suspension until a final concentration of 3 M KCl was obtained. The 3 M KCl extraction mixture was held at 4° for 16 hr with constant, slow stirring on a non-heat-generating Bellco magnetic mixer. Insoluble cell debris was sedimented by centrifugation at 164,000 × *g* (Ti-50 rotor; Beckman Instruments) for 1 hr at 4°. The supernatant containing the solubilized immunogenic component was dialyzed against a 50% sterile sucrose solution at 4° for a minimum of 5 hr and subsequently was dialyzed against 200 volumes of PBS for 16 hr at 4°. Precipitates forming in the dialysate were removed by centrifugation at 48,000 × *g* (Ti-50 rotor; Beckman Instruments) for 30 min at 4°, and the supernatant was passed through a 0.45-μm Unipore filter prior to standardization and use. The Lowry *et al.* (21) protein determination was performed on all supernatants as a means of standardizing concentration. The typical protein yield from 5 to 9.9×10^9 F5-1 cells was 60 to 131 mg of total protein in the final dialysate.

Immunizations and Challenge. Seven- to 8-week-old male hamsters were given injections of varying concentrations of the KCl extract of the SV40-induced sarcoma cell (F5-1) or the Adv 7-induced line by i.p. inoculation on one or more occasions as described. Animals so immunized were challenged s.c. in the right subscapular space at a site distant from the immunizing site 10 days following the final vaccine experience with standardized, freshly harvested SV40 tumor cell (F5-1) suspensions produced in tissue culture, and the animals were palpated weekly for tumor appearance. Control preparations included extraction fluid carried through all steps of the procedure using extracts of normal hamster muscle freshly collected on the day of the extraction or extraction fluid prepared in the absence of tissue.

Other hamsters were immunized with one injection of 10^7 F5-1-irradiated tumor cells exposed to 5000 R of X-irradiation.

[3] A. J. Girardi, personal communication

This procedure is known to yield absolute protection to the homologous SV40 tumor cell challenge.

Adoptive Immunity Assay. The adoptive transfer of lymphocytes was carried out by procedures already described elsewhere for this hamster model (11). Briefly, lymph nodes from vaccinated animals were harvested from the axillary, mesenteric, inguinal, and cervical lymph nodes, pooled in Medium 199 without calf serum, minced with sterile scissors, and pressed through a stainless steel wire mesh. The suspended cells were subsequently washed on 3 occasions with Medium 199, standardized to the desired level, and mixed at the desired ratio with freshly harvested living tumor cells.

Exudate cells were collected by another standardized procedure (11) from the peritoneal cavity of hamsters sensitized on 3 alternate days with sterile oyster glycogen to promote lymphocyte and monocyte influx. The exudate fluids were collected and washed thoroughly with warmed Medium 199 by centrifugation in plastic tubes. For some experiments, adherent cells were removed by 2 subsequent platings on sterile plastic Petri dishes for periods of 1 hr each, followed by gentle aspiration of suspended cells. Following 2 platings on plastic dishes, the cells were used.

Adoptive transfer studies using lymph node or exudate cells were carried out by admixing the standardized lymphoid and tumor cell suspensions together in a small siliconized tube in equal volume ratios (usually 1 ml each) for 30 min at 37°. After a 30-min incubation, the cells were resuspended by vigorous pipetting and injected s.c. into the right subscapular space of normal 6- to 8-week-old male recipients. The animals were palpated weekly at the site of injection for tumor appearance. Controls for adoptive transfer studies included animals sensitized to normal muscle extract or to extracts of Adv 7-transformed cells.

RESULTS

Hyperimmunization with 3 M KCl Extract. Initial studies to attempt to prepare cell-free TSTA from SV40-induced hamster sarcoma cells used 3 M KCl extracts of 10^9 SV40-induced sarcoma cells grown in tissue culture. A prolonged hyperimmunization schedule was used involving 6 i.p. injections of the extract standardized to specific protein concentrations at weekly intervals. On the tenth day, following the sixth injection, the hamsters were challenged s.c. with living SV40-induced sarcoma cells (10^4 or 5×10^5 cells/ml) from tissue culture. Controls included hamsters which had been given injections, according to the same protocol of 3 M KCl extracts of normal hamster muscle tissue or hamsters given injections of a placebo extraction fluid (no tissue) carried through the same procedure as that used for the tumor and muscle cell extracts. Another control group received 6 injections of intact, irradiated SV40-induced tumor cells, which were known to induce solid transplantation resistance to the syngeneic tumor challenge. All control groups contained at least 10 hamsters/group, and all groups receiving 3 M KCl extracts of the SV40 sarcoma cells numbered 15 animals. No nonspecific deaths occurred during the course of these experiments. The results obtained are summarized in Table 1.

The use of this hyperimmunization protocol with 3 M KCl extracts failed to confer any significant transplantation resistance at either challenge level. Hamsters immunized with the intact cells used for extraction and inactivated by irradiation exhibited complete protection to either level of tumor challenge. These results showed that hyperimmunization with preparations reported to induce protection against chemically induced sarcomas of mice failed to effect detectable transplantation resistance.

Table 1

Results of attempts to induce transplantation resistance against challenge with SV40 sarcoma cells following 6 injections of 3 M KCl extract of SV40 sarcoma cells grown in tissue culture

Vaccine[a]	No. of hamsters	% protection[b] after challenge with 10^4 cells	% protection[b] after challenge with 5×10^5 cells
Placebo vaccine solution	10	0	0
3 M KCl extract of SV40 sarcoma cells (0.5 mg protein/injection)	15	0	0
1.0 mg protein/injection	15	0	0
3 M KCl extract of normal hamster muscle (1.0 mg protein/injection)	10	0	0
X-irradiated SV40 sarcoma cells (5×10^6/ml)	10	100	100

[a] Each 4-week-old hamster received an i.p. injection of vaccine at 1-week intervals for 6 weeks.
[b] Determined at 150 days postchallenge.

Single Immunization with 3 M KCl. We prepared 3 M KCl extracts of SV40 tumor cells by the same method described for the hyperimmunization protocol, standardized SV40 tumor cell, or muscle extracts for specific protein concentrations and injected them i.p. Ten days following that single injection, the hamsters were challenged s.c. in the right subscapular space with a large SV40 tumor challenge (5×10^5 cells/ml) and a low tumor challenge level (10^4 cells/ml). Results are given in Charts 1 and 2. Challenge of identically immunized animals with 10^5 Adv 7 sarcoma cells constituted the heterologous tumor challenge control.

The results of these single immunization studies showed a consistent lag of 60 to 100 days ($p > 0.001$; Student's t test) in the appearance of sarcomas in animals immunized with 0.5 mg of KCl tumor extract when the animals were challenged with the high dose of tumor cells (Chart 1). Permanent protection of a significant level ($p > 0.005$) varying from 30 to 40% was noted in 2 of 3 experiments. Immunization attempts with 0.5 mg of protein from extracts of normal hamster muscle or a placebo extraction mixture containing no tumor cell extract produced tumors in the same time frame and number, and no protection was noted. Immunization with twice as much protein (1.0 mg/ml) from the 3 M KCl extract of the SV40-induced sarcoma cells resulted in a delay in tumor appearance and significant protective effect in only 1 of 3 trials. Specificity of the transplant resistance noted was established when it was observed that no protection was observed in hamsters in 2 of 2 of these trials using the same concentrations of Adv 7-induced hamster sarcoma cells as challenge in hamsters given a previous single sensitizing injection of 0.5 or 1.0 mg of F5-1 tumor cell extract (data not shown).

The protective effect against tumor development detected in the first and third trials did not change by 150 days in the surviving hamsters. When these animals were sacrificed and examined for microtumor foci, histopathological studies indicated no tumors at the site of challenge. All groups of hamsters

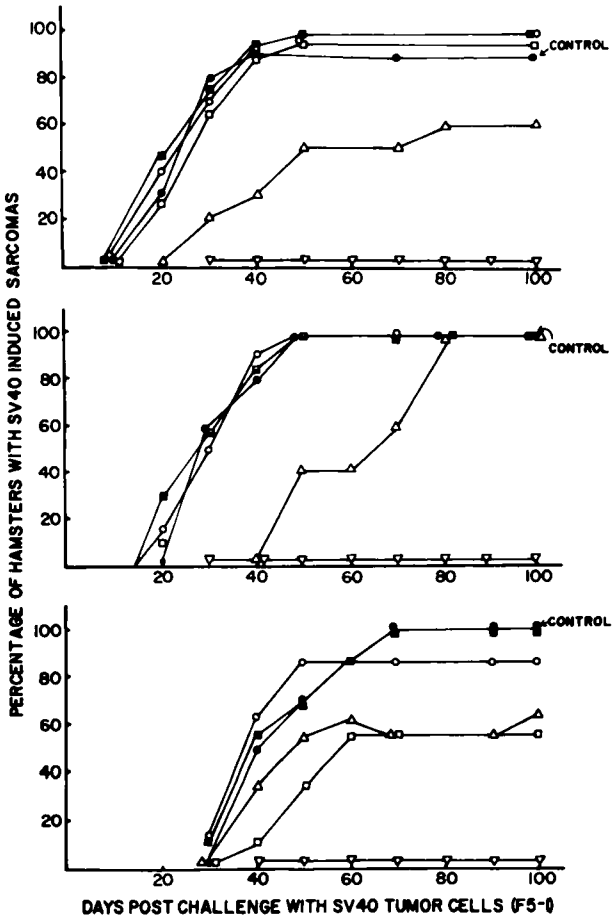

Chart 1. Results of 3 trials to induce transplantation resistance with 3 M KCl extracts of F5-1 cells against SV40 tumor cell challenge with 5×10^5 F5-1 sarcoma cells. Test vaccine: ●, 0.9% NaCl solution control; ■, 0.5 mg normal hamster muscle tissue extract in 3 M KCl; ▽, 5×10^6 X-irradiated F5-1 sarcoma cells; □, 0.1 mg of 3 M KCl extract of F5-1 sarcoma cells; △, 0.5 mg of 3 M KCl extract of F5-1 sarcoma cells; ○, 1.0 mg of 3 M KCl extract of F5-1 sarcoma cells. Adult hamsters received one i.p. injection of test vaccine and were challenged in the right subscapular space with 5×10^5 living F5-1 sarcoma cells 10 days later. Challenged animals were palpated at weekly intervals for tumor development.

sensitized to a single dose of 5×10^6 irradiated SV40-induced tumor cells showed total resistance to the highest level of tumor challenge. The results recorded in Chart 1 were at a high challenge level in each of the 3 experimental situations depicted.

In a parallel set of experiments, hamsters were vaccinated with a single injection of varying concentrations of the same extracts used in the previously discussed experiment. These animals were subsequently challenged with only 10^4 viable F5-1 cells (Chart 2). There was a slower appearance of tumors in the control groups compared to the control values of the higher challenge presented in Chart 1. Each experiment shown was an independent trial. Hamsters receiving very carefully standardized inocula of 10^4 F5-1 challenge cells on 4 separate trials showed tumor incidence variations from 10 to 80% tumors, illustrating the necessity to perform repeated, independent trials to obtain reliable data in the tumor challenge system when minimally oncogenic challenges are used. Significant delays in tumor appearance were not noted, nor was there any evidence of protection at any concentration of 3 M KCl of SV40 tumor cell extract presented in these single vaccination attempts using the smaller challenge of SV40-induced F5-1 tumor cells. As can be seen, the relationship of nonprotection was consistently obtained in 4 separate trials. To the contrary, note the apparent marked enhancement of tumor appearance in animals receiving any concentration of 3 M KCl extracts of

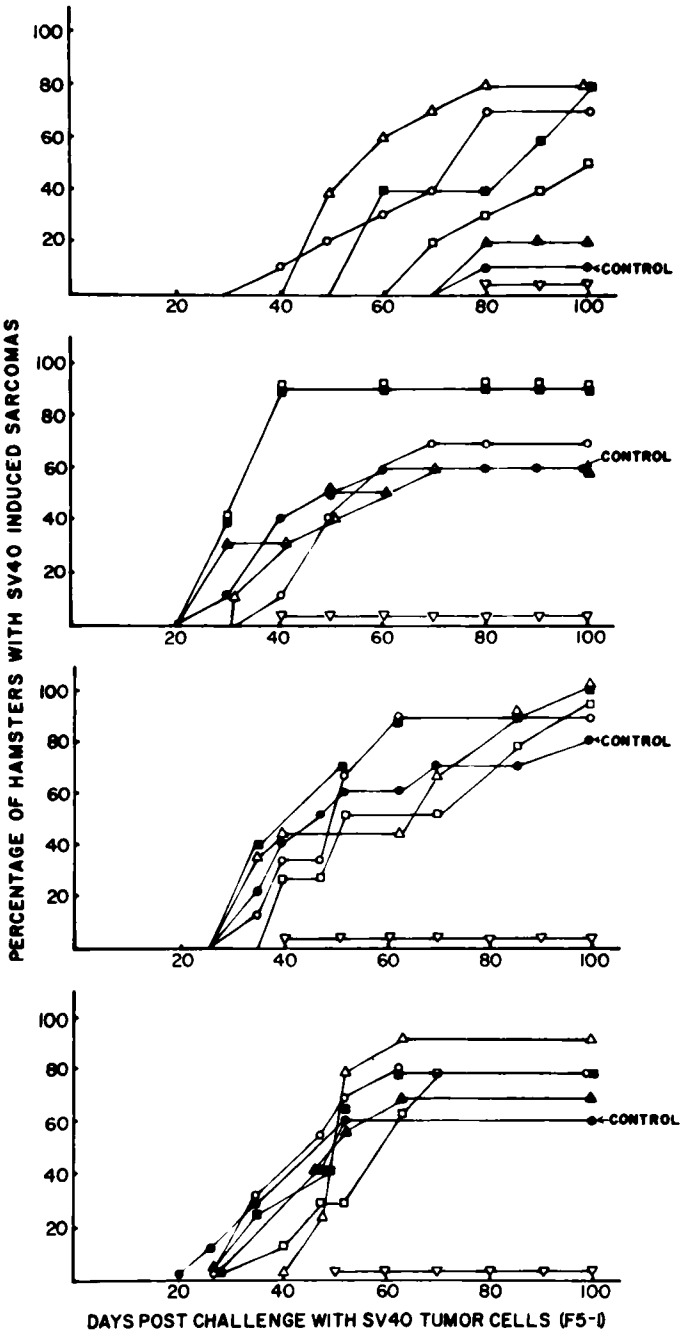

Chart 2. Results of 4 trials to induce transplantation resistance with 3 M KCl extracts of F5-1 cells against SV40 tumor cell challenge with 10^4 F5-1 sarcoma cells. Test vaccine: ▽, irradiated F5-1 cells; ●, 0.9% NaCl solution control; ▲, 0.5 mg of normal muscle extract in 3 M KCl; ○, 0.1 mg of 3 M KCl extract of F5-1 sarcoma cells; △, 0.5 mg of 3 M KCl extract of F5-1 sarcoma cells; □, 1.0 mg of 3 M KCl extract of F5-1 sarcoma cells; ■, 3.0 mg of 3 M KCl extract of F5-1 sarcoma cells. Same protocol for vaccine administration and challenge as in Fig. 1.

SV40-induced sarcoma cells that occurred in each trial with low challenge. In all cases, animals immunized with intact, irradiated SV40-induced tumor cells for each of these experiments showed complete resistance to tumor challenge at either dose of F5-1 cells used in Chart 1 or 2, and challenge of heterologous tumor control groups with Adv 7 sarcoma cells did not divulge any cross-reactive protection in hamsters given injections of SV40-induced sarcoma cell extracts.

Adoptive Transfer of Immunity. It was important to determine whether the transplantation resistance detected in single immunization trials against high challenge was truly cell mediated in animals receiving vaccinations with 0.5 mg of the 3 M KCl extract. Initial efforts were directed toward sensitizing the animals with the SV40-induced sarcoma extract preparations and harvesting either PEC or pooled LNC from animals given injections 10 days earlier with the tumor extract. Checks were performed using sample animals from each donor group to confirm that they showed the same lag in tumor appearance as that described in Chart 1. A lag in tumor development was noted in each case following challenge with 5×10^5 homologous tumor cells, and protection of 20 to 40% of animals receiving 0.5 mg of the F5-1 tumor cell extract given in a single injection was again noted. When PEC collected at 10 days postvaccination from matched donors were used to attempt to confer passive protection, we were unable to detect any protective effect with PEC at a ratio of 200 or 700 effector cells per tumor cell given as challenge (Chart 3). LNC collected 10 days after a single vaccination with 0.5 mg of 3 M KCl extract were similarly nonprotective (data not shown). PEC from donors given a single injection of intact, irradiated F5-1 cells were likewise not protective in the same assays. In subsequent trials, several levels of tumor challenge (10^6, 5×10^5, 10^4) were used with PEC, as well as LNC taken at 10 days postvaccination, and again, no protection was noted in any case.

The possibility existed that our sampling at 10 days postvaccination might not have allowed sufficient time for full cell-mediated immunity to be properly activated, even though matched donors challenged with 5×10^5 F5-1 cells 10 days postvaccination showed a significant lag in tumor development beginning 25 days postchallenge. The experiments above were repeated, and the harvest of both LNC and PEC was delayed until 20 days following the sensitizing injection. LNC harvested at 20 days postinjection of 3 M KCl tumor extract (0.5 to 1.0 mg) were able to confer reproducible and significant protection (Chart 4) in 3 of 3 trials, reflecting the resistance detected *in vivo* challenge experiments in Chart 1. Similar protection was noted with LNC or PEC collected from donors sensitized 20 days earlier with intact, X-irradiated F5-1 cells.

When PEC were tested 20 days postimmunization with 3 M KCl extracts of F5-1 cells and examined for the ability to confer protection in the same adoptive transfer experiments, no protection could be discerned at exudate cell:tumor cell ratios of 700:1 (Chart 5). It appears then that LNC and not PEC from hamsters receiving 0.5 to 1.0 mg of 3 M KCl extract from tissue culture represented appropriately activated effector cells capable of conferring protection. Controls included hamsters given injections of extracts from normal muscle or placebo mixture involving no tissue extract, and these failed to yield LNC or PEC capable of conferring protection. The resistance detected with 20-day LNC was specific since no protection

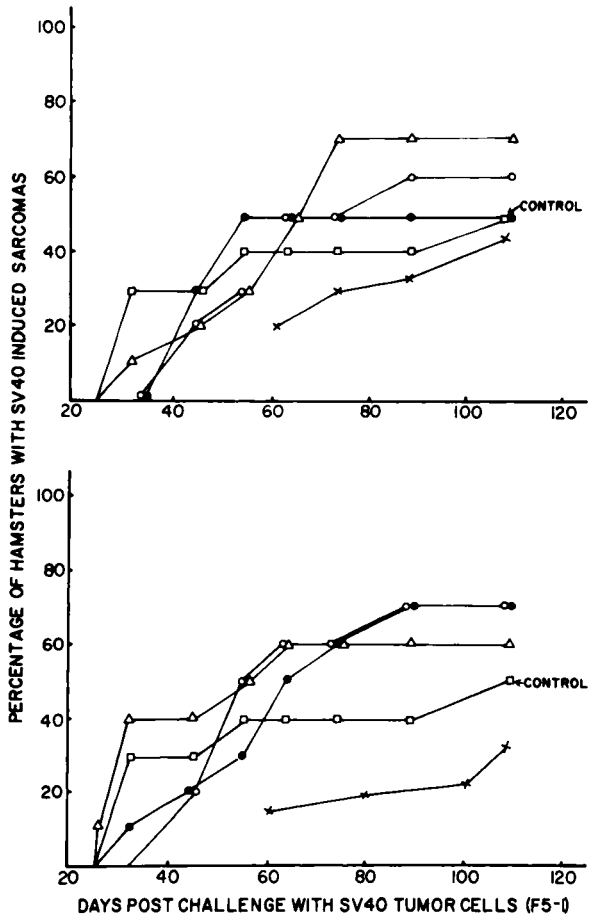

Chart 3. Attempts to passively transfer immunity to normal hamsters using PEC collected from donors vaccinated 10 days earlier on a single occasion i.p. with: □, 0.5 mg of 3 M KCl extract of normal hamster muscle; ×, intact, X-irradiated F5-1 tumor cells; ○, 0.1 mg of a 3 M KCl extract of F5-1 tumor cells; ●, 0.5 mg of a 3 M KCl extract of F5-1 tumor cells; △, 1.0 mg of a 3 M KCl extract of F5-1 tumor cells. Challenge was in the right subscapular space with 5×10^6 F5-1 cells admixed with a 1:200 ratio of PEC from donors (*top*) or 1:700 ratio of PEC (*bottom*).

was detected against challenge with 5×10^5 Adv 7 tumor cells (data not shown).

Four different preparations of SV40 hamster sarcoma cell (F5-1) extracts in 3 M KCl were tested for their potential to produce SV40 cytopathology on established, permissive CV-1 monkey kidney cells and carried for 3 blind passages without any detectable evidence of infectious virus.

DISCUSSION

KCl (3 M)-solubilized extracts of SV40-induced hamster tumor cells were capable of conferring transplantation resistance to syngeneic hamsters only when administered in a narrow range of protein concentration (0.5 to 1.0 mg). At greater or lesser levels of antigen or when multiple rounds of sensitization were attempted, the protection was variable or not detectable in direct challenge studies. The extracts used were crude but truly cell free (filtered) and were free of infectious SV40. The neoantigens were determined to be weak transplantation resistance-inducing antigens when used in soluble form. The immunity detected could be adoptively transferred to normal

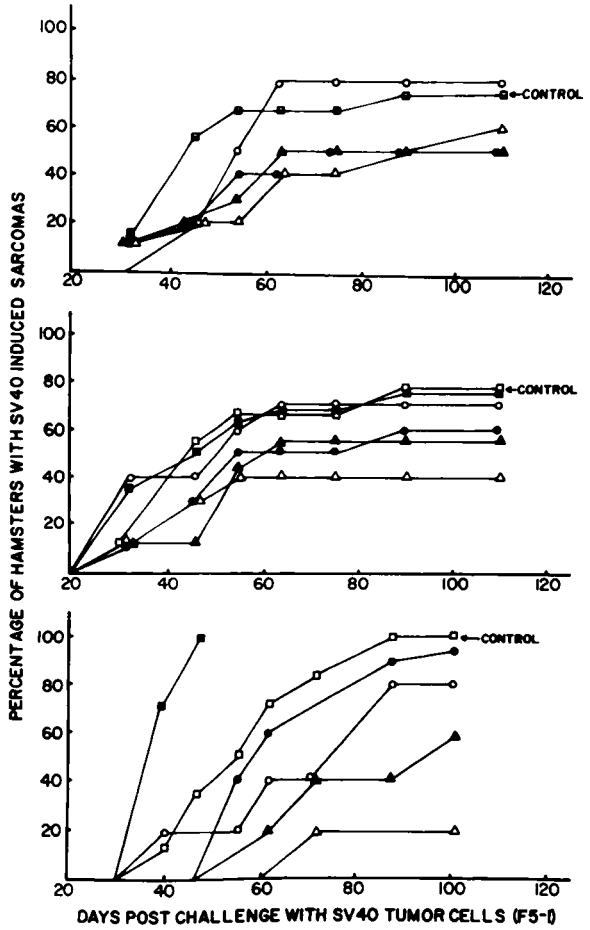

Chart 4. Detection of adoptive resistance in hamsters receiving LNC from syngeneic donors vaccinated i.p. 20 days earlier with: □, 0.9% NaCl solution placebo; ■, 3 M KCl extract of normal hamster muscle; ▲, intact, X-irradiated F5-1 tumor cells; ○, 0.1 mg of a 3 M KCl extract of F5-1 tumor cells; ●, 0.5 mg of a 3 M KCl extract of F5-1 tumor cells; or △, 1.0 mg of a 3 M KCl extract of F5-1 tumor cells. Challenge was in the right subscapular space with 5×10^5 F5-1 cells admixed with a 1:200 ratio of LNC from donors (*top* and *middle*) or 1:700 (*bottom*).

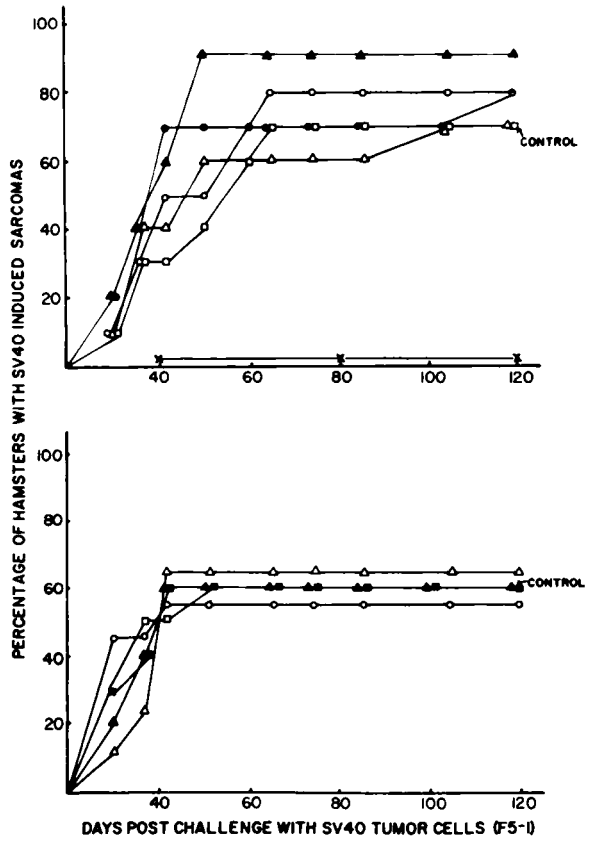

Chart 5. Attempts to passively transfer immunity to normal hamsters using PEC collected from donors immunized 20 days earlier on a single occasion i.p. with: □, 0.9% NaCl solution placebo; ▲, intact, X-irradiated F5-1 tumor cells; ○, 0.1 mg of a 3 M KCl extract of F5-1 tumor cells; ●, 0.5 mg of a 3 M KCl extract of F5-1 tumor cells; or △, 1.0 mg of 3 M KCl extract of F5-1 tumor cells. Ratios of target tumor cells to effector PEC was 1:700.

hamsters using lymphocytes from lymph node tissues taken 20 days after a single sensitization with appropriate tumor antigen concentrations. Peritoneal exudate cells were ineffective in passively conveying protection when derived from hamsters vaccinated with soluble tumor cell extracts. The transplantation immunity elicited by a single injection of the standardized SV40 tumor cell extract could only be detected with a high tumor cell challenge in direct challenge studies. If a low tumor cell challenge was given following an identical sensitization experience, reproducible enhancement of tumor development resulted.

These results clearly pose at least 3 experimental dilemmas. The first dilemma relates to the unusual observation that we could reproducibly detect transplantation resistance and a significant inhibition of tumor development in animals sensitized to an intermediate level of the tumor extract and given a relatively large tumor cell challenge. Hamsters sensitized to the same extract did not demonstrate any resistance against a more subtle homologous tumor cell challenge. This has not been previously reported. We cannot explain this reproducible phenomenon at the present time.

The only similar observation in tumor immunology which might be considered analogous is the phenomenon of "sneaking through" (3, 18). This classic observation, controlled by an as yet undefined immunological mechanism, occurs in animals that are sensitized to tumor by implantation of living tumor cells which are subsequently excised. Animals sensitized in this fashion will reject a subsequent heavy tumor cell challenge with the homologous tumor, but smaller levels of homologous challenge will frequently produce tumors in this classic phenomenon. It is conceivable that sneaking through is a parallel observation to our result reported here.

Our second dilemma is to explain why the higher concentrations of tumor extract have no effect or, conversely, produce enhanced tumor development. The effectiveness of the 0.5-mg dose of crude tumor extract did not vary significantly in the ability to confer protection from batch to batch, which we feel is most remarkable. A 1.0-mg dose induced only variable protection. The delay and/or protection observed using intermediate antigen levels given in a single dose followed by high challenge was completely reproducible in this system and relates directly to a similar observation by Pellis and Kahan (24) in their work in mice with chemically induced tumors. Our studies and theirs show clearly that the level of soluble antigen from sarcoma cells administered plays a critical role in whether transplantation resistance is successfully activated against an appropriate cell challenge in hamsters and in mice using either virally or chemically transformed tumor cells. Forbes *et al.* (14)

reported an increased 50% lethal dose in mice given injections of soluble extracts of methylcholanthrene-induced sarcomas and challenged with homologous cells.

The immunity detected with soluble extracts at a single intermediate dose was specific, causing no protection against Adv 7-induced hamster sarcoma cell challenge. Injections of higher concentrations (3.0 to 5.0 mg) of F5-1 protein extracts were never observed to be protective in any experiment against homologous F5-1 challenge.

In subsequent studies published elsewhere (8), we have reported that significant suppressor thymocyte (4) cell activity was noted in animals receiving higher levels of protein from homologous tumor cell extracts in the range of 3.0 to 5.0 mg using SV40-transformed hamster cells.

Our findings are indicative of a delicate balance in obtaining functional, protective effector cells. In current studies, we are attempting to determine whether suppressor T-lymphocytes, or macrophages, or soluble products from effector cells may be inhibitory to normal, functional transplantation resistance in animals receiving soluble SV40 tumor extracts at concentrations which promote enhancement.

The third interesting dilemma posed by our data was generated by the observation that peritoneal exudate lymphocytes harvested from hamsters that were known to be resistant to tumor challenge following an intermediate sensitization using a single vaccine dose of the soluble extracts could not passively transfer protection. Lymphocytes harvested from lymph nodes of the same donor at 20 days postsensitization were observed to be protective. In previous work (11), we had shown that a single presentation of intact, irradiated tumor cells as immunogen to hamsters would activate peritoneal exudate cells which were highly inhibitory in adoptive transfer experiments to the growth of simultaneously transplanted SV40 sarcoma tumor cells. These data taken together suggest that there may be inhibitory cells among the exudate cells which limit the ability of sensitized, cytotoxic lymphocytes to affect tumor cell killing, while lymphocytes taken from lymph nodes of the same animals may be devoid of these inhibitory cells and can confer protection at about the same level, as was detected *in vivo* by direct challenge.

The observation that absolute resistance to tumor challenge could not be obtained with any concentration or regimen of soluble tumor extract administration to adult hamsters, compared with the resistance induced with intact, cell-associated TSTA, is not surprising. First, intact, irradiated tumor cell preparations yield a small number of replicating oncogenic cells which may serve to superstimulate transplantation resistance. Cell-free preparations cannot duplicate the presentation of antigen that occurs when it is present in the native membrane. When a transplantation resistance determinant is released from the native membrane matrix, it would be expected to have reduced immunogenicity (8).

Recently, Law *et al.* (19) and Chang *et al.* (6) have reported that the numerous previous reports (12, 16–18, 20, 22, 25, 26) from their laboratories that they had isolated and partially purified SV40 TSTA from mouse sarcoma cells may have simply reflected the isolation of SV40 T-antigen. Since their preparations contained T-antigen and since they cannot distinguish T-antigen in their TSTA-like preparations from nuclear extracts of T-antigen from mouse or human cell, it would appear that they have not isolated true SV40 TSTA independent of T-antigen. We recently observed that several mouse sarcoma lines induced by SV40, including the lines used by Law *et al.* (19), would not interrupt SV40 oncogenesis in the newborn hamster, unlike SV40-transformed human, hamster, and rat cells. Law *et al.* (19) reported that, indeed, neither their mouse cell lines induced *in vitro* by SV40 nor their T-antigen-containing extracts were capable of conferring protection against the transplantation of SV40-induced hamster sarcoma cells.

Our findings extend an earlier report by Pellis and Kahan (24) in another system and open a number of new avenues of exploration of tumor enhancement or inhibition relating directly to antigen dose, method, and timing of administration. Furthermore, the results establish that the immunity generated with a single immunization with cell-free TSTA can be passively transferred. Our results obtained with varying challenge levels further suggest that the level of stimulation generated by the actual tumor challenge itself, with cancer cells carrying intact tumor-associated antigens, markedly affects the detection or lack of detection of tumor-specific transplantation resistance.

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