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A Rapid *in Vivo* Assay for SV40 Tumor Immunity in Hamsters* (33531)

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The appearance of several viral or viral stimulated neoantigens in SV40 transformed hamster cells is well documented. These include the virion or V antigen(s), a class of intracellular tumor or T antigens reactive in both the complement fixation and fluorescent antibody test, a cell surface reacting S antigen detected by fluorescent microscopy and transplantation antigen detected by transplant rejection tests *in vivo*. The detection of the presence of transplantation antigen in a tumor cell preparation capable of stimulating an immune reaction and of suppressing the development of SV40 induced tumors in hamsters is a prolonged procedure requiring several hundred days for completion (1).

In previous studies, radiation inactivated, 5-iododeoxyuridine-treated or disrupted SV40 tumor cell preparations were evaluated for immunizing efficacy against SV40-induced tumors in hamsters or against challenge with homologous tumor cells (2, 3). The observation was made that both systems of assay provided similar results with equal specificity. The tumor cell challenge assay required some 40–60 days after completion of immunization to evaluate the efficacy of a preparation.

The present report describes a 5-day *in vivo* method for detecting transplantation immunity in a homologous hamster system employing immunodiffusion chambers. The role of a diffusable immunity factor in tumor transplant rejection is discussed.

Materials and Methods. Hamsters. Four to 7-week-old random bred Syrian golden hamsters were used in this study (Lakeview Hamster Colony, Newfield, New Jersey). Males and females in equal proportion were used, however, males were used in recent work to eliminate problems associated with abdominal surgery on female hamsters.

SV40 hamster tumor cells. The virus-free F5-1 line (4–6) of SV40 hamster tumor was used. The cells were cultivated in medium 199 containing 5% heat-inactivated calf serum plus antibiotics. Cell cultures from passage 80 to 125 *in vitro* were removed from bottle culture by brief trypsinization, washed once with Hank's balanced salt solution (HBSS), resuspended to the desired concentration and employed.

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Muscle tissue. Hamster muscle tissue was prepared from retired breeders by carefully removing a portion of the biceps femoris and sartorius muscle from the front of the thigh. The muscle was rinsed with warmed medium 199 and minced thoroughly with sterile scissors under aseptic conditions. The mince was suspended in medium 199 and disaggregated cells were collected after repeated passage of the fluid through several thicknesses of sterile gauze mesh. For immunization, the cells were standardized to the desired concentration (5×10^6 viable cells/ml) in HBSS and irradiated.

Irradiation. X-irradiation was performed in plastic petri plates at a distance of 22 cm from the source of the General Electric Maxitron "300" X-ray machine equipped with 0.5 mm aluminum and 0.42 mm copper filters. The unit was set at 250 kVp, 20 mA delivering 540 R/min as determined by a Victoreen condenser R meter.

Virus. SV40 virus, strain 45-54 was used to immunize hamsters. Intraperitoneal injections of 0.2 ml of virus prepared in primary grivet monkey renal cell culture with a titer of $10^{7.0}$ $TCID_{50}$/0.2 ml were given at 1-week intervals.

Preparation of diffusion chambers. Lucite rings of the following dimensions were used as chambers: outside 20 mm, inside 10 mm, thickness 3 mm with radial hole of 1 mm diameter, (Piedmont Plastics, Inc., Charlotte, North Carolina). The chambers were manufactured with soap lubricant *in lieu* of cutting oil in milling. The rings were soaked in ether for 6 hr to remove toxic products, then soaked and washed in hot tap water containing tissue culture soap (7X, Linbro Chemical Co., New Haven, Connecticut) and rinsed repeatedly with distilled water. Only flat, non-chipped chambers were used. Millipore filters (no. GSWP 018-00, 18 mm, Millipore Filter Corp., Bedford, Massachusetts) were glued to the Lucite ring with Dekadhese Cement (A. H. Thomas and Co., Philadelphia, Pennsylvania). Sterilization of the chambers was accomplished in sealed glass petri dishes (bottom of dish covered in filter paper) by placing in an 80° oven for 12 hr, followed by a 12-hr incubation period at 37°, followed by an additional 12–18-hr exposure to 80°. Sample chambers were routinely tested for sterility in beef heart infusion, thioglycollate and Sabouraud broth culture.

Loading the chambers. The chambers were soaked in medium 199 without calf serum for 2 hr before use. Defective, leaky chambers can be spotted at this time. Chambers were removed from the soak bath to Lucite holders which maintained the chambers in a verticle position. Inoculum (0.24-ml capacity) was introduced into the chamber carefully through the radial hole with an 22-gauge, 1.5 in. needle. Paraffin was used to seal the chamber. The loaded, sealed chambers were immediately returned to a chilled 199 bath to await implantation.

Implantation of chambers. Anesthetized hamsters were shaved over the entire abdominal region and wiped thoroughly with 70% ethanol. A lateral incision about 2 cm in length was made 1 cm to the left of the midline of the abdomen and the chamber carefully introduced into the peritoneal cavity with curve-tipped forceps. The peritoneum lining, muscle, and epidermis were gathered into position and the incision was closed with several stainless steel staples. A few drops of fresh, 3% hydrogen peroxide were placed on each closed incision. No antibiotic therapy was necessary and 100% survival of hamsters following surgical implantation was routinely observed.

Removal of chambers. At the selected interval, chambers were located by making a new incision into the abdomen to the right of the original incision. Chambers were recovered and carefully removed with forceps, wiped gently with a sterile gauze soaked with sterile HBSS and placed in a Lucite holder for unloading.

Unloading the chambers. Paraffin was removed from the radial hole with a 22-gauge needle, and the chamber fluid was removed, carefully observing the exact volume with the aid of a 1-ml calibrated syringe fitted with a 22-gauge needle. The fluid was saved and 0.15 ml of 0.5% pronase solution (Cal-Biochem, Los Angeles, California) was intro-

duced into the chamber and the chamber was allowed to stand for 10 min at 22–25°.The pronase solution was removed after gentle mixing by pumping the syringe and pooled with the original chamber contents. The total volume of the collected fluid is adjusted to 0.3 ml if required with medium 199 (almost never necessary since chambers uniformly contain 0.15 ml of initial fluid). For counting, 0.15 ml of trypan blue solution was added and the enumeration and viability of cells was performed in the hemocytometer. Contamination of the chamber resulting from leakage could easily be detected by observing the fluid for the presence of debris, erythrocytes, or lymphoid cells. Contaminated chambers appeared rarely and were discarded. Cell contamination controls containing only medium 199 were routinely implanted for each group of chambers.

Immunization of hamsters. Hamsters were routinely immunized at 1-week intervals by the administration of two to three intraperitoneal injections of X-irradiated F5-1 cells (5 to 7.5 $\times$ 10^6 cells/dose). Chambers were not prepared and implanted until days 10–14, after the last immunization. Following removal of chambers, hamsters were saved and, upon recovery from surgery, were inoculated with 5 $\times$ 10^4 F5-1 cells subcutaneously in the right subscapular region to determine the status of immunity to live cell challenge in each animal.

Results. When care was exercised to prepare, handle, and unload the diffusion chambers in the manner just described no leakage of the chambers was observed. Chamber fluids were clear except for added cells. Pronase treatment to release the proportion of cells attached about the inside of the chamber was essential to total recovery of the cell population. The enzyme was not found to be specifically deleterious to F5-1 tumor cells or muscle cells over many hours of exposure. The impermeability of these diffusion chambers to peritoneal cells has been established (7) in mice and we obtained similar results in hamsters.

Chamber cultuure of SV40 tumor cells. Trypsinized, washed F5-1 tumor cells adapted readily to the chamber environment in the peritoneal cavity of the hamster and replicated as demonstrated in Fig. 1. Proliferation ensured rapidly after the third day. Similar results were obtained for smaller inoculum levels.

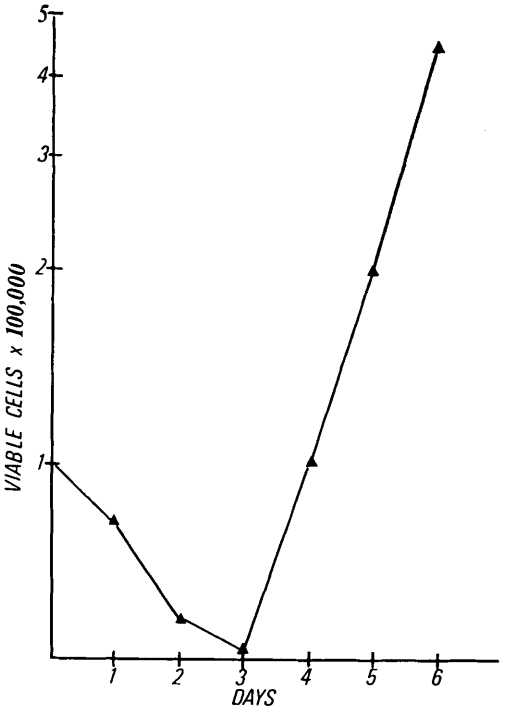

FIG. 1. Growth of SV40 transformed hamster cells in immunodiffusion chambers in the peritoneal cavity of normal hamsters.

Destruction of SV40 tumor cells in immune hamsters. Groups of hamsters were immunized with either irradiated F5-1 tumor cells, SV40 virus or irradiated salt solution control fluid on three successive weekly occassions. Ten days after the immunization schedule was completed, diffusion chambers inoculated with 15,000 viable tumor cells (trypan blue-dye exclusion test) were implanted. Chambers were collected on the fifth day after implantation and cells were counted. The results show that F5-1 tumor cells were inhibited from proliferation and, in fact, destroyed in chambers implanted in hamster immunized with homologous tumor cell material or with SV40 virus.

The specificity of the immunity for the

TABLE I. Destruction of SV40 Tumor Cells in Diffusion Chambers in the Hamster Peritoneal Cavity.

Hamster immunized against:	Av no. of viable tumor cells/chamber at 5 days postimplantation[a]	Range for 10 chambers[b]	% Viability (av)
Unvaccinated control	37,000	21–72,000	99
SV40 tumor cells (5000 R X-ray)	4300	0–9000	23
SV40 virus	3750	0–6000	27
HBSS (5000 R X-ray)	41,600	12–51,000	93

[a] Chambers inoculated with 0.15 ml of a carefully standardized suspension of tumor cells containing 15,000 viable cells in medium 199 without calf serum.

[b] The differences observed for results obtained for animals immunized with tumor material or virus were reliable at the 1% confidence level as determined by the Wilcoxon test.

F5-1 target cell was suggested by the results obtained when chambers containing homologous muscle cells were implanted into animals immunized according to the regimen described for Table I. The results (Table II) show that no diminution of muscle cell survival or proliferation was observed in hamsters immunized with either irradiated SV40 tumor cells or with SV40 virus indicating the specificity of the inhibitory effect for SV40 tumor cells.

The number of SV40 tumor cells present in the diffusion chamber effected the degree of cell killing observed for target cells in immunized hamsters as shown in Table III. The presence of 50,000 cells in chambers implanted in hamsters immunized as described for Table I reduced the effectiveness of cell killing and the majority of chambers contained the same number of cells after the fifth day that had initially been present. Since growth experiments have demonstrated that a predictable drop in the number of viable tumor cells occurs between days 0 and 3 in chambers in normal hamsters, our results suggest that some cell division must have occurred in the majority of chambers since slight increases in the number of viable cells over those present in the original inoculum were apparent on the fifth day of culture.

Hamsters were coded and, upon removal of the chambers and recovery from surgery, were challenged with a concentration of tumor cells which produces 90–100% tumors at the site of inoculation in 40–50 days in nonimmune hamsters. Hamsters immunized with either F5-1 cells or SV40 were uniformly immune to this concentration of tumor cells following chamber removal and no correlation could therefore be drawn between animals yielding chambers with high survival values and weak tumor immunity.

The importance of three episodes of immu-

TABLE II. Specificity of SV40 Transplantation Immunity for Tumor Cells Determined by Diffusion Chamber Technique.

Hamster immunized against:	Av no. of viable muscle cells/chamber at 5 days postimplantation[a]	Range for 10 chambers[b]	% Viability (av)
Unvaccinated control	33,600	21–60,000	81
SV40 tumor cells	28,200	21–36,000	86
SV40 virus	28,000	12–39,000	83
Irradiated HBSS control fluid	23,400	15–30,000	84

[a] Chambers inoculated with 0.15 ml of medium 199 without calf serum containing 15,000 viable muscle cells.

[b] No statistically significant difference was detected between any of the treatments studied as determined by the Wilcoxon test.

TABLE III. Effect of Increased Inoculum Size on the Detection of SV40 Tumor Immunity by Diffusion Chambers.

Hamster immunized against:	Av no. of SV40 tumor cells in fifth day postimplantation[a]	Range for 20 chambers	% Viability (av)
Homologous muscle cells (irradiated 5000 R X-ray)	228,000	144–447,000	97
SV40 tumor cells	52,000[b]	9–107,000[b]	77
Unvaccinated control	262,000	201–329,000	99
HBSS (irradiated 5000 R X-ray)	216,000	144–291,000	97

[a] Chambers inoculated with 50,000 viable SV40 tumor cells in 0.15 ml of medium 199 without calf serum.

[b] Cell counts from hamsters immunized with SV40 tumor cells (irrad. 5000 R X-ray) were significantly different from counts obtained from animals receiving other treatments at the 1% confidence level as determined by the Wilcoxon test.

nization with 5×10^6 viable, irradiated SV40 tumor cells is shown in Table IV. In this experiment hamsters received either two or three immunizations. In cell challenge studies two such immunizations produce a protective effect in 40–50% of animals challenged with 400–500,000 tumor cells whereas three immunizations produce 90–100% protection against this challenge. The results indicate that chambers from animals receiving two immunizations contained more tumor cells than chambers from thrice immunized hamsters at the same inoculum level. Further, complete destruction of all cells in any given chamber was not detected in animals receiving only two immunizations whereas several

TABLE IV. Effect of Immunization and Number of Target Cells on Detection of SV40 Tumor Immunity in Diffusion Chambers.

Hamster immunized against irradiated:	No. of vaccine doses	Initial inoculum viable cells/chamber	Av no. of tumor cells sixth day post-implantation[c]
HBSS		15,000	145,000[a]
SV40 tumor cells	2	15,000	72,000
Homologous muscle		15,000	146,000
HBSS		50,000	208,000
SV40 tumor cells	2	50,000	78,000
Homologous muscle		50,000	209,000
HBSS		100,000	207,000
SV40 tumor cells	2	100,000	55,000
Homologous muscle		100,000	130,000
			—[b]
HBSS	3	15,000	37,000
SV40 tumor cells		15,000	4300
HBSS		50,000	216,000
SV40 tumor cells	3	50,000	52,000
Homologous muscle		50,000	228,000

[a] Counts averaged from 8–10 chambers.

[b] Chamber removed and counts performed on fifth day.

[c] Differences between counts obtained for HBSS or muscle cell controls and SV40 tumor cell counts were significantly different at the 1% confidence level as determined by the Wilcoxon test. All preparations were pretreated with 5000 R of X-irradiation.

chambers containing no survivors were observed in hamsters given three immunizations. At all inoculum levels, however, it is important to note that two immunizations always resulted in a marked diminution in the number of tumor cells present in hamsters immunized with SV40 tumor cell antigen when compared with controls.

A tumor cell dose of 50,000 cells/chamber provided an excellent level of target cells for assay since few cells died initially upon implantation of the chamber and "crowding" phenomena observed for higher cell levels did not occur. In numerous confirming experiments, we have never failed to detect the cytostatic inhibition of tumor cell growth in chambers implanted in hamsters immunized with irradiated SV40 tumor cell antigen.

Discussion. Transplantation immunity in both the adenovirus and SV40 hamster model systems is often depicted as resulting from a set of immunologic conditions in delicate balance between tumor rejection and immunologic enhancement. If true, preparations from homologous, virus-induced tumor cells containing low concentrations of transplantation antigen would actually stimulate tumor development when used as immunogen to interrupt virus tumorigenesis in neonatally infected hamsters. Similar preparations with higher antigenic content might stimulate an immune response leading to tumor rejection. Such a situation, indeed, seems to exist (2).

Berman (8) reported that adaptive and passive transfer experiments in the adenovirus 12 intracerebral challenge system suggested that the bulk of transplantation immunity was mediated by immune lymphoid cells. Serum antibody played no major role, either in causing cytotoxic destruction of tumor cells or in immunologic enchancement *in vivo* according to Berman. Hellström and Sjögren (9) demonstrated *in vitro* tumor-specific antigenicity to adenovirus 12 tumor employing the colony inhibition test. Immunization of mice against syngeneic, adenovirus 12 mouse tumor cells yielded serum with cytostatic activity against adenovirus 12 tumor cells of mice and hamsters *in vitro*.

In previous work, Coggin *et al.* (3) demonstrated that peritoneal lymphoid cells from hamsters immunized against irradiated SV40 tumor cells were incapable of destroying homologous target cells *in vitro* under a variety of conditions but these lymphoid cells did stimulate transplant rejection when admixed with live tumor cells *in vitro* and inoculated into hamsters. In numerous attempts, we have repeatedly failed to detect cytotoxic or cytostatic inhibition of SV40 tumor cells *in vitro* using serum from hyperimmunized hamsters which were highly immune to SV40 tumor cell challenge.

Results reported here demonstrate the presence of a diffusable, noncellular factor which caused limited destruction of homologous target cells in immunodiffusion chambers *in vivo*. Cell destruction was only detected when low concentration of target cells were present. Cytostasis of tumor cell replication was evident at higher target cell concentrations. It is unclear why serum from hamsters similarly immunized fails to effect the normal growth of SV40 tumor cells *in vitro* with or without fresh guinea pig complement.

Several important questions regarding the nature of the response measured by the diffusion technique remain unanswered and are the substance of present investigation in this laboratory. We assume that the inhibitory factor present in hamsters immunized with either SV40 tumor cells or virus is humoral antibody. Confirmatory efforts are presently underway to detect the presence of S antibody on the surface of tumor cells in diffusion chambers.

The inhibitory effect on SV40 tumor cells detected in diffusion chambers does not seem to result from a subtle histoincompatible response within the random bred hamster used as demonstrated by the failure to detect the inhibition of homologous muscle tissue proliferation. The specificity of the test for SV40-induced tumor cells alone when compared with activity against adenovirus-induced tumor cells is being determined.

Although the diffusion test gives a clear indication of the presence of transplantation immunity in immunized hamsters, it is important to determine whether or not the im-

munity measured is identical to the mode of immunologic response responsible for tumor cell rejection in the cell challenge test. Experiments are now in progress to answer this question employing, among other things, disrupted tumor cell preparations as vaccine in the diffusion chamber test. These preparations failed to stimulate transplantation immunity in the virus newborn-hamster or cell challenge system (*2*, 3). If the immune response measured by the diffusion chamber technique is identical to that occurring in transplant rejection, one might anticipate a corresponding absence of an inhibitory effect on target cells in chambers in hamster immunized with these preparations.

The development of this rapid test for monitoring transplantation immunity will greatly expedite the evaluation of numerous tumor cell components and subfractions for transplantation antigen. Further, the possibility that humoral antibody may play an important role in the inhibition of tumor development or even in the destruction of tumor cells *in vivo* is indicated. These results suggest that transplant rejection may result from a combination immune response. Antibody may function to limit the proliferation of tumor cells early in rejection in tumor immune hamsters, allowing time for the migration and destructive action of the lymphoid cell population which eventually eliminates the tumor cell from the animal completely. The diffusion chamber technique affords a new approach for the study of transplantation immunity in this homologous hamster system.

Summary. Hamsters immunized with either SV40 tumor cell material or with SV40 virus possessed a diffusable factor in the peritoneal cavity which destroys or inhibits the proliferation of homologous SV40 tumor cells in diffusion chambers. The diffusable factor had no activity against hamster muscle cells from homologous hamsters and its activity was dependent, to some degree, on the target cell concentration and on the number of immunizations. The diffusion chamber environment was free from lymphoid cell penetration under the conditions employed and the immunity detected is believed to result from humoral antibody.

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