Characterization of Tumor-Specific Transplantation Immunity Reactions in Immunodiffusion Chambers *in Vivo*[1] (34357)

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(Introduced by N. G. Anderson)

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Tumor rejection in both allogeneic and syngeneic animal model systems is generally considered to result from cell-mediated responses similar or identical to those described for homograft rejection (1, 2). The observations of several investigators (3–5) clearly suggest that the serum of tumor-bearing or tumor immune animals contains antibody which effectively suppresses the proliferation of tumor cells *in vitro* employing the colony inhibition (CI) procedure. Hellström *et al.* (5) observed that patients with neuroblastomas possessed serum antibody and lymphoid cells which suppressed the growth of autochthonous tumor *in vitro* again using the CI test procedure.

We recently reported that the presence of SV40 tumor-specific transplantation immunity (TSTA) could be detected in mature hamsters employing syngeneic target cells trapped in a membrane-bound diffusion chamber (6). The porosities of the membranes allowed for the exchange of fluids but prevented direct lymphocyte contact with the target cells (6, 7). At low target cell concentrations tumor cells were destroyed when placed in the peritoneal cavity of hamsters specifically immunized against SV40 TSTA and at higher target cell concentrations the tumor cells were rendered nonproliferative. The present report describes the specificity of the diffusion chamber test, characterizes the growth response of target cells, and extends the usefulness of this assay method to adenovirus-induced TSTA immunity.

Materials and Methods. Hamsters. Four to 6-week-old male Syrian golden hamsters were used in this study (Lakeview Hamster Colony, Newfield, New Jersey).

Virus-induced tumor cell lines. The F5-1 line of SV40 hamster tumor cells which has been shown to be virus free and to possess T and S antigen (8, 9) was maintained *in vitro* in medium 199 containing 10% heat-inactivated calf serum with antibiotics. Cell cultures from passage 90 to 116 were used. Adenovirus 7 hamster tumor cells (Pinckney strain) (10) were cultivated *in vitro* in double strength basal medium of Eagle (BME) in Hanks' balanced salt solution (HBSS) with 10% heat-inactivated calf serum plus antibiotics. Passage levels 170 to 190 were used. Adenovirus 31 tumor cells derived *in vivo* following neonatal infection of Syrian hamsters with virus was established *in vitro* and maintained with medium 199 containing 10% heat-inactivated calf serum and antibiotics. This cell line possessed adenovirus 31 T antigen, TSTA, and was neoplastic upon transplant into hamsters (11). Cells from passage 15 to 25 were used and were never found to yield infectious adenovirus 31 by cocultivation with human embryonic kidney cells.

Kidney cells. Hamster kidney cells were removed from retired breeders, trimmed, and washed in prewarmed HBSS. After thorough mincing under aseptic conditions, the cells

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were suspended in HBSS and strained through sterile stainless steel grids to obtain disaggregated cells.

Muscle cells. Fibroblasts obtained from muscle homogenates were prepared as previously described (6).

Chemical immunosuppressives. 6-Mercaptopurine (Mann Research, N. Y., N. Y.) was prepared as a sodium salt at a final pH of 8.5. The cyclophosphamide (Mead-Johnson, Evansville, Ind.) was reconstituted in distilled water. All solutions of immunosuppressives were prepared daily and filter-sterilized before use. Hamsters were given daily intraperitoneal doses of cyclophosphamide (87 mg/kg of body wt) for 5 consecutive days. After a 2-day rest period, the hamsters were immunized with a single intraperitoneal injection of 10^7 irradiated SV40-transformed F5-1 cells. Two hr following the immunization and for 10 consecutive days, the hamsters were given daily intraperitoneal injections of 6-mercaptopurine, (9 mg/kg).

Preparation of irradiated tumor cells. Tumor cells were harvested from bottle culture by brief trypsinization, washed, and exposed to 5000 R of X-irradiation as previously described (6). Hamsters were immunized with three weekly intraperitoneal injections of 5×10^6 viable irradiated tumor cells.

Preparation of sonified cells. The SV40 tumor cells were removed from bottle culture with a rubber policeman, washed with HBSS, and resuspended to give a final, viable cell concentration of 5×10^6 cells/ml. The cells, chilled to 4° in an ice bath, were subjected to sonic oscillation employing a Branson sonifier (Branson Instruments, Inc., Stamford, Conn.) operating at a setting of 7 and delivering 9–10 A for four 30-sec intervals. The sonified cell preparation contained only cell fragments and no detectable viable cells as determined by the trypan blue dye-exclusion test. Hamsters were immunized with three weekly intraperitoneal injections of 1 ml of the sonified cell preparation.

Immunodiffusion chamber assay. The preparation and use of diffusion chambers for monitoring SV40 transplantation immunity has been extensively described in a previous paper (6). Briefly, cleaned Lucite rings were fitted with Millipore filters of 0.22-μ porosity and sterilized. After soaking in medium 199 or BME, the immunodiffusion chambers were loaded through the radial hole with 0.15 ml of a tumor cell suspension standardized so that each chamber received 20,000 or 50,000 viable tumor cells as required. The holes were sealed with paraffin and the chambers were returned to a chilled solution of medium 199 or BME prior to implantation. The chambers were implanted into the peritoneal cavity of anesthetized hamsters through a small lateral incision in the shaved abdomen. The incisions were closed with sterile autoclips. At the selected day, the chambers were removed and the hamsters were saved for a later subcutaneous challenge of 5×10^4 tumor cells in the subscapular region to determine the status of immunity to a live tumor cell challenge. Chambers were cleaned externally with gauze moistened with medium 199, the paraffin plug was removed, and the chamber fluid was collected and saved. A solution of 0.5% pronase (Cal-Bio-Chem, Los Angeles, California) was introduced into the chamber and following a 20-min incubation at 25°, the pronase solution was removed and added to the original chamber fluid. The final volume was brought to 0.3 ml with fresh medium 199 or BME. Viable cells were enumerated in a hemocytometer by the trypan blue dye exclusion procedure. Cell counts reflected the total number of viable cells per individual chamber.

Results. Transplantation immunity and the chamber assay. Hamsters were rendered immune to SV40 tumor cell challenge by three weekly intraperitoneal injections of irradiated SV40 tumor cells or SV40 virus. Diffusion chambers were implanted and removed on day 5 thereafter. Surviving cells in each chamber were enumerated and the hamsters were coded to indicate the specific chamber that had been implanted in a particular hamster. Following recovery from surgery the hamsters were challenged with 5×10^4 SV40 tumor cells subcutaneously in the right subscapular region to determine their status of immunity. The effects of immunization on tumor cell survival in chambers are correlated with the percentage of hamsters de-

TABLE I. Inhibition of SV40 Tumor Cells in Diffusion Chambers in Immune Hamsters.

Hamster immunized against:	Av no. viable tumor cells/chamber after 5 days	Tumor bearers/survivors following chamber removal and challenge (No.)	(%)
Expt. 1			
HBSS (5000 R X-ray)[a]	164,100	14/15	96
SV40 tumor cell (5000 R X-ray)	49,700[b]	4/15	26
Homologous muscle tissue (5000 R X-ray)	174,000	15/15	100
Expt. 2			
HBSS	230,000	8/10	80
SV40 tumor cells (5000 R X-ray)	18,000[b]	0/10	0
Homologous muscle tissue (5000 R X-ray)	200,000	9/10	90

[a] HBSS = Hanks' balanced salt solution placebo exposed to 5000 R of X-ray prior to injection.

[b] The average number of surviving cells in SV40 tumor immunized animals was significantly different from HBSS or muscle immunized animals at the 1% level as determined by the Wilcoxon test.

veloping tumors in each group in Table I. The results clearly indicated that hamsters immune to SV40 tumor cell challenge possessed a diffusible, inhibitory factor detectable by the chamber procedure and this factor was not present in nonimmune, control animals.

Figure 1 demonstrates the influence of placebo immunization (HBSS) or immunization with SV40 irradiated tumor cells on proliferation of SV40 tumor cells in chambers as a function of time after implantation of the chamber. All immunized animals received

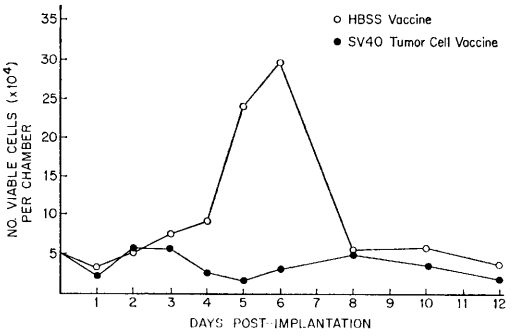

FIG. 1. Proliferation of SV40 hamster tumor cells in diffusion chambers in the peritoneal cavity of hamsters immunized with three injections of either salt solution (HBSS) or irradiated, SV40 tumor cells.

three weekly injections of vaccine and were rested 10 days prior to chamber implant. No discernible difference could be detected between the immunizations prior to day 4. After this time, however, a rapid increase in the number of tumor cells in chambers from hamsters receiving placebo immunization occurred whereas cells in chambers from hamsters receiving the irradiated tumor cell preparation containing tumor specific transplantation antigen (TSTA) did not increase in number. Inhibitory effects of specific immunization were maximally detectable on days 5 and 6 postimplantation of the chamber. Growth responses of SV40 tumor cells identical to those given for HBSS immunized hamsters (in Fig. 1) were obtained in similar studies employing hamsters immunized with irradiated kidney cells from another hamster or in hamsters rendered immune to adenovirus 31 tumor. A number of factors, discussed below, contributed to the decline in viable tumor cells observed in control chambers (HBSS vaccine) after day 6. Five hamsters were employed for each point indicated and similar results were obtained in several different experiments.

Immunologic specificity. To determine the

TABLE II. Immunological Specificity of SV40 Transplantation Immunity as Determined by Cytostasis in Diffusion Chambers.

Hamster immunized against:	Av no. of SV40 tumor cells 5 days postimplant[a]
Unvaccinated control	160,000
SV40 tumor cells (5000 R X-ray)	68,000[b]
(sonified)	207,000
Adenovirus 31 tumor cells (5000 R X-ray)	168,000
Homologous kidney cells (5000 R X-ray)	185,000

[a] Average values derived from 10 to 20 chamber /treatment.

[b] The differences observed for this result and the result for other vaccines were reliable at the 1% confidence level as determined by the Wilcoxson test.

immunologic specificity of the diffusion chamber test for detecting SV40 TSTA immunity, hamsters were immunized with heterologous, irradiated adenovirus 31 tumor cells, irradiated SV40 tumor cells, sonified SV40 tumor cells or irradiated hamster kidney cells. In previous work sonic disruption of SV40 hamster tumor cells had destroyed the capacity of the tumor cells to induce SV40 specific TSTA immunity (12). Kidney cells were used to establish the histocompatibility of the hamster tissue system. The results obtained from placing SV40 target cells in diffusion chambers in the peritoneal cavity of these immunized hamsters are given in Table II. Inhibition of SV40 hamster tumor cell proliferation was only detected in animals receiving SV40 irradiated tumor cells as immunogen. Use of sonically disrupted SV40 tumor cells as immunogen did not produce SV40–TSTA immunity detectable by the diffusion chamber test in agreement with results obtained using the virus–newborn model system (12) and with the cell challenge system (13). In those animals which received the sonified SV40 tumor cells an enhancement in tumor cell growth in the chambers was suggested. Hamsters receiving adenovirus 31 irradiated cells as vaccine are resistant to adenovirus 31 tumor cell challenge (11).

An experiment was conducted to be certain that the adenovirus 31 tumor cells used as heterologous TSTA antigen would stimulate adenovirus 31 transplantation immunity detectable by the chamber procedure. Hamsters were immunized with irradiated adenovirus 31 tumor cells, SV40 tumor cells, or kidney cells from syngeneic hamsters and chambers containing 20,000 adenovirus 31 tumor cells were implanted 10 days after the third immunization. Five days later the surviving cells were enumerated and the results indicated that animals immunized with adenovirus 31 tumor cells possessed an inhibitory factor against homologous target cells (Table III).

The specificity of the adenovirus 31 tumor cell TSTA-induced immunity was evaluated employing adenovirus 7 target cells in the diffusion chambers. In this experiment hamsters were immunized as before (Table III) with the additional vaccination of a group of hamsters with adenovirus 7 tumor cells inactivated with X-irradiation. The results in Table IV show that immunization with either adenovirus 31 or adenovirus 7 tumor cell preparations produced some inhibition of growth of adenovirus 7 tumor cells by the third day after implantation. On the fifth day after implantation marked inhibition of adenovirus 7 tumor cell proliferation was ob-

TABLE III. Destruction of Adenovirus 31 Tumor Cells in Diffusion Chambers of Immunized Hamsters.

Hamster immunized against:	Av no. of viable cells/chamber after 5 days[a]
Unvaccinated control	43,000
Adenovirus 31 tumor cells (5000 R X-ray)	8500[b]
Homologous kidney cells (5000 R X-ray)	45,000
SV40 tumor cells (5000 R X-ray)	47,000

[a] Chambers originally inoculated with approximately 20,000 viable adenovirus 31 tumor cells.

[b] The differences observed for results obtained for animals immunized with adenovirus 31 tumor material and other vaccines were reliable at the 1% confidence level as determined by the Wilcoxson test.

TABLE IV. The Detection of Adenovirus 7 Tumor Immunity by Diffusion Chambers.

Hamsters immunized against:	Av no. of viable adenovirus 7 cells[a]; postimplant day: 3	5
Unvaccinated control	23,800	206,900
Adenovirus 7 tumor cells (5000 R X-ray)	10,600	21,300[b]
Adenovirus 31 tumor cells (5000 R X-ray)	9600	120,000
Homologous kidney cells (5000 R X-ray)	26,500	377,600

[a] Chambers originally inoculated with 50,000 viable adenovirus 7 tumor cells.

[b] The differences observed for this result and other results from chambers from animals receiving other vaccinations were reliable at the 1% confidence level as determined by the Wilcoxson test.

vious in homologously immunized animals but some inhibition of normal growth (50% of control) of adenovirus 7 target cells was evident in hamsters immunized with adenovirus 31 tumor cells. This suggestion of cross-reactivity between adenovirus 7 and adenovirus 31 transplantation antigens was observed on two other occasions using the chamber assay procedure but has not been confirmed using the cell challenge assay.

Antibody as inhibitory factor. Careful examination of the chambers upon removal from TSTA-immunized hamsters failed to reveal any usual or abnormal collection of clot material or lymphocyte aggregation at the external membrane surface which could account for the inhibition effect exerted against the target cells.

We used several procedures to establish that the inhibitory effect observed on target cell growth or survival in the chambers resulted from an interaction between the cell and antibody generated by the specific immunization. The most direct method employed was to see if the tumor target cells were coated with antibody. The procedure selected was that described by Tevethia *et al.* (14) wherein an attempt was made to demonstrate an S antibody on the surface of the target cells in SV40–TSTA immunized hamsters. The SV40 tumor cells were collected from chambers in the usual manner after 5 days implantation in hamsters immunized with either irradiated SV40 tumor cells, irradiated HBSS, or irradiated kidney cells from a syngeneic animal. The cells were washed thoroughly and were placed on cover slips, thus beginning Tevethia's procedure at the stage following adsorption of tumor cells with hamster antiserum directed against SV40 S antigen. We assumed that the cells from chamber immunized against irradiated SV40 tumor cell antigen would already be coated with the antibody directed against the S antigen if the antibody were present. Following the washing procedure the cells were absorbed with fluorescein conjugated baboon antihamster IgG globulin and examined for surface fluorescence. The results clearly showed the presence of S antibody at the surface of SV40 tumor cells from hamster receiving homologous vaccination and the absence of S antibody on tumor cells from animals receiving kidney or HBSS.

Immunosuppression. An indirect approach was employed to determine if the observed inhibition of tumor cells in transplant immune hamsters detected by diffusion chambers resulted from antibody action. From studies to be reported elsewhere, (15) we have established the regimens of 6-mercaptopurine (6MP) and cyclophosphamide (CY) which effectively suppress gamma globulin (IgG) and macroglobulin (IgM) synthesis in hamster after the procedures described by Schwartz *et al.* (16). The levels of 6MP and CY used and the timing of drug administration employed markedly inhibited the hamster's ability to produce agglutinin antibody to sheep erythrocytes. Hamsters received one injection of irradiated tumor cells or control preparations. Immunization with this level of tumor antigen was expected to produce a significant level of immunity (50–75% protection against tumor development) and the vaccination period was scheduled in one group of animals with dosages of 6MP and CY which inhibited IgG and IgM synthesis. Chambers containing SV40 tumor cells were

TABLE V. Effect of Chemical Immunosuppression on the Inhibition of SV40 Tumor Cell Growth in Diffusion Chambers.

Immunized with:[a]	Control: no 6MP or CY treatment		Immunosuppressed: 6MP + CY treatment	
	Av no. of viable tumor cells/chamber	% with challenge tumor[b]	Av no. of viable tumor cells/chamber	% with challenge tumor
SV40 tumor cells (5000 R X-ray)	29,000	15	45,000	11
Muscle brei (5000 R X-ray)	97,000	87	84,000	100
HBSS (5000 R X-ray)	71,000	100	NT[d]	NT
Unvaccinated controls	66,000	71	71,000[c]	100

[a] Each hamster received one immunization with the indicated preparation. Tumor cells for vaccination were standardized to contain 10^7 dye-excluding cells. Chambers were removed 5 days postimplantation.

[b] Hamsters were saved after chamber removal, allowed to recover from surgery, and then challenged in right subscapular space with SV40 tumor cells. Results show percentage developing challenge tumors out of 13–15 animals used in each group.

[c] SV40 tumor cells were exposed to 6MP (4.5 mg) and CY (28.5 mg) for 24 hr *in vitro* in 50 ml of medium 199 harvested, standardized, inoculated into chambers and implanted.

[d] NT = not tested.

implanted at the termination of immunosuppressive therapy. The results (Table V) showed that a 3-fold increase in SV40 tumor cell survival was detected in immunosuppressed hamsters receiving one immunization with the irradiated SV40 tumor cell preparation. It was not technically possible with available procedures to determine quantitatively whether the cells from chambers from immunosuppressed hamsters receiving SV40 tumor cells as immunogen had less S antibody on their surface than cells from control hamsters.

A slight cytotoxic effect of the immunosuppressive chemicals on the tumor cells was detected by treatment *in vitro* of SV40 tumor cells with 28.5 mg of CY and 4.5 mg of 6MP in 50 ml of medium 199 with 10% calf serum. The tumor cells were allowed to grow in this medium at 37° for 24 hr prior to their use as target cells in chambers for animals receiving neither immunosuppressives nor vaccine. Hamsters receiving immunosuppressives did not possess the cytostatic antibody to the same extent as nonimmunosuppressed controls, however, these animals were observed to demonstrate immunity to tumor cell challenge comparable to immunized controls (Table V).

Discussion. Data presented here confirm the reliability of the diffusion chamber assay as an index of TSTA immunity under the usual conditions of test. Immunized hamsters, demonstrating the cytostatic effect on tumor cells in chambers, were correspondingly immune to live cell challenge. The assay was observed to be specific for SV40-stimulated TSTA since animals rendered immune to adenovirus 31 tumor TSTA did not develop antibody inhibitory to the growth of SV40 tumor cells. Interestingly, hamsters administered sonified SV40 tumor cells, a preparation that does not possess functional SV40 TSTA (12), failed to develop cytostatic antibody (Table II) and these animals uniformly yielded chambers with increased numbers of tumor cells when compared with other control groups. Such findings correlate well with the virus-newborn hamster system and the cell challenge system where immunization with cell sonicates leads to enhanced tumor appearance.

The diffusion chamber procedure showed marked cytostasis of tumor cell proliferation in SV40 tumor immune hamsters. Tumor cells in chambers in normal hamsters or nonimmune hamsters lagged in proliferation for several days and then rapidly increased in cell numbers for the next 2–3 days. After day 6, a rapid decline in cell viability was usual-

ly observed in control animals. Holub (17) reported that "clot" formation and occlusive cell accumulation at the outer membrane surface of diffusion chambers lowers the diffusion quality of the chamber environment by a factor of eight over a 10-day period. Fox (18) reported that L-fibroblast cells and sarcoma 180 cells grew exponentially *in vivo* in chambers but with a longer generation time than *in vitro* because of low diffusion rates and cell hinderance on the outer membrane surface. We recently conducted a thorough study of the cell population accumulating about the outer membranes of chambers in both immune and nonimmune hamsters after 6-days implantation. We were unable to detect, either qualitatively or quantitatively, any difference in the number or type of cells attaching to the outer membranes in immune or normal hamster using electron microscopy. All chambers became increasingly occluded with intermembrane deposits of amorphous structure after 7-days postimplantation. Earlier (6), we had shown that the population density in the chamber dramatically affected the longevity of cells in the chamber. High inoculum levels caused a quicker cessation of growth because a shorter time was required to reach the critical cell density (500–800,000 cells/chamber). Thus, we conclude with others (19), that permeation of the membrane is severely impaired after 7–10 days *in vivo* and, coupled with a population limit, afford an explanation for the death of cell populations after day 7.

Immunization of hamsters with adenovirus 31 tumor cells induced an inhibitory effect on homologous tumor cells (Table III) and heterologous vaccination of hamsters with SV40 tumor cells had no inhibitory effect on adenovirus 31 tumor cells in chambers.

Hamsters rendered immune to adenovirus 31 tumor cells demonstrated considerable cytostasis against adenovirus 7 target cells in chambers whereas heterologous immunization with SV40 tumor cells showed no inhibition. The adenoviruses that induced these tumors fall into two distinct categories with respect to their guanosine–cytosine ratios (20) and tumor (T) antigens (21). Sjögren *et al.* (22) and Sjögren and Ankerst (23) recently showed a cross-reactivity between surface antigens on tumor cells induced by adenovirus 5, 7, and 12 using both mouse and rabbit antiserum directed against the heterologous virus-induced tumor. These antigens may reflect common TSTA's present in tumor cells induced by these viruses. Our results suggest a cross-reactivity between adenovirus 7 and 31 tumor immune hamsters with respect to cytostasis of adenovirus 7 tumor cells in chambers. These hamsters were immune to their respective homologous tumor cell challenges. We are presently seeking to determine the extent of cross-reactivity of TSTA-induced immunity to cell challenge.

That the cytostasis noted in specifically immunized SV40 tumor immune hamsters results from antibody-target cell interaction seems highly probable. The presence of a specific IgG globulin at the surface of inhibited cells (S antibody) and the absence of inhibition of cell growth when hamsters are chemically immunosuppressed supports our original contention that the inhibition resulted from antibody attachment to the surface of the tumor cells. Whether the IgG immunoglobulin(s) present is directed specifically against SV40 or adenovirus TSTA is not clear. In particular, hamsters which did not produce cytostatic antibody because of immunosuppressive therapy were, nevertheless, immune to cell challenge following chamber removal. These findings coupled with the repeated observation that the effect on target cell population is one of cytostasis rather than true cytotoxicity introduce the possibility that the antibody monitored under the condition of assay employed may not be totally or, in fact, partially responsible for ultimate tumor rejection. Nonetheless the presence of cytostatic antibody, as detected in the 5-day chamber assay, provides a true index of the existence of transplantation immunity under ordinary circumstances. The antibody described may be necessary for optimum interaction between target cell and lymphoid cell in a role similar to that described by Chambers and Weiser (24) for cytophilic antibody in macrophage–target cell adherence.

In preliminary trials we have been seeking

to detect the first measurable antibody response induced against target cells by implantation of chamber simultaneous with, and shortly after, one immunization with irradiated tumor cells. Results show that cytotoxic antibody appears as early as 4 days after immunization and subsides by days 12-17, after which time only slight cytotoxicity can be noted. It is possible that the early cytotoxic antibody is a macroglobulin of the IgM type whereas the cytostatic antibody is of the 7S or IgG variety (S antibody) which appears in significant quantity only after several rounds of immunization and late in time. Exploitation of these possibilities is the subject of future experiments.

Summary. A diffusible, cytostatic factor present in hamsters rendered immune to SV40-stimulated tumor-specific transplantation antigen (TSTA) was studied. The factor appears to be an antibody of the IgG class and is present only in hamsters rendered specifically immune to SV40 tumor transplant. Sonified SV40 cell preparations did not stimulate the appearance of the inhibitory antibody nor did immunization with heterologous tumor. These findings agree well with results obtained in the SV40–newborn hamster system and in the cell challenge system. Hamsters immunized against SV40–TSTA did not inhibit adenovirus 31 tumor cell growth in chambers although some cross-reactivity was noted between adenovirus 7 and adenovirus 31 TSTA immunization. The existence of a specific immunoglobulin in hamsters rendered immune to SV40 and adenovirus TSTA's provides a convenient assay to rapidly monitoring TSTA immunity. The role of cytostatic antibody in tumor transplant rejection is discussed.

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