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Expression of Tumor-Specific Transplantation Antigen in Cell Lines Transformed by Wild-Type or *tsA* Mutant Simian Virus 40

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The simian virus 40-induced tumor-specific surface antigen(s) (TSSA) and tumor-specific transplantation antigen(s) (TSTA) were detected in cells transformed by wild-type or temperature-sensitive mutant simian virus 40 by an antibody-mediated cytolytic assay for TSSA and an immunization test for TSTA. Cells transformed by *tsA* mutants, which lose their transformed phenotype when grown at nonpermissive temperatures, nonetheless do express TSSA and TSTA as well as T-antigen at both temperatures.

Mammalian cells exhibit striking changes in their pattern of growth in tissue culture after transformation by simian virus 40 (SV40). These changes include: increased "saturation density," lower serum requirements, ability to overgrow a monolayer of normal cells, and ability to form clones in soft agar or methylcellulose suspensions.

In addition, SV40 transformation causes antigenic modification of the cell surface. Early work, using transplantation rejection tests, demonstrated the tumor-specific transplantation antigen(s) (TSTA) to be a common antigen(s) in SV40-transformed cells originating from several different species (12, 15, 19, 30). This TSTA, moreover, was shown to be highly specific for the virus; for example, no significant cross-reactivity occurs between cells transformed by SV40 and polyoma, a similar but distinct papovavirus (8, 12). Several in vitro methods were subsequently introduced that sought to measure changes in surface antigens (13, 14, 32, 37, 38, 39). Among these assays, the cytolytic assay was observed to be quite specific for antigen(s) induced by SV40 transformation (28, 29, 31, 32, 39). The antigens detected by these specific in vitro tests are designated tumor-specific surface antigens (TSSA).

Since only the cell surface is in direct contact with the surrounding environment, it is of interest to determine to what extent the antigenic changes on the surface after viral transformation correlate with the altered pattern of cell growth in culture or with the growth in vivo.

Recently, several investigators have made use of the temperature-sensitive group *A* (*tsA*) mutants of SV40 to study the role of this virus in transformation. Only group *A* mutants are defective in their ability to initiate (34) and maintain the transformed phenotype at the nonpermissive (restrictive) temperature, suggesting a unique and critical role of gene *A* in transformation (4, 7, 16, 25, 27, 35, 36). The group *A* mutants induce transformation at permissive, but not at nonpermissive, temperatures; cells transformed by group *A* mutants and shifted to nonpermissive temperatures showed, with few exceptions, a markedly restricted ability to grow to high density, to grow in low serum, and to form colonies on plastic substrata, on normal cells, and in soft agar. Furthermore, these changes were reversed after return to the permissive temperature. In most cases, including the cell lines employed in this study, both the group *A* mutant transformants and the nontransformed cells grew to confluence at the nonpermissive temperature.

The group *A* mutants cluster in one portion of the early region of the genetic map of SV40 (21). Expression of TSTA requires information from a similar region of the viral genome (22). Although expression of nuclear T-antigen has been studied in some of the group *A* transformants (4, 7, 16, 27, 35), no data exist on the expression of TSTA and TSSA. Therefore, we have investigated TSSA and TSTA in cell lines transformed by *tsA* mutants to see whether expression of TSSA and TSTA is modulated in parallel with changes in cell growth in culture.

MATERIALS AND METHODS

Cell types: AL/N mouse cell lines. An established normal AL/N cell line (AL/N ME) was used; it was originally derived from an AL/N mouse embryo and

has been shown to be non-tumorigenic at 10^7 cells (29). SV40-transformed (SV AL/N) and spontaneously transformed (T AL/N) lines have been described previously (31).

BALB-3T3 mouse cell lines. BALB-3T3 cells (1), BALB-3T3 clone A-31 (2), an SV40-transformed subclone 11-A-8 (33), and a polyoma-transformed line PY-3T3-4a were kindly supplied by G. J. Todaro and S. Aaronson. A BALB-3T3 cell line transformed by the wild-type, small-plaque SV40 virus (strain 776) isolated by one of us (J.L.A.) is designated *3T3WTJ*. A second wild-type-transformed BALB-3T3 line (*3T3WTB1a*) and a *tsA255*-transformed BALB-3T3 line (*3T3A255B1b*) were kindly provided by William Brockman. These cell lines all grow at 39.4°C, but the *3T3A2551b* cells fail to overgrow a normal monolayer or to clone in soft agar at 39.4°C, although they do at 33°C (William Brockman, personal communication). The wild-type transformants express the transformed phenotype at both temperatures.

Mouse brain cells. Normal NIH Swiss mouse brain (astroglial) cells, *MB* (formerly referred to as NMB), and cells transformed by the wild-type SV40 virus, *MBWT* (formerly referred to as SVWT-MB), by an early group *A* mutant, *MBA239* (formerly A239-MB), and by a group *C* mutant, *MBC219* (formerly C-219-MB), were used (4). The properties of these cells in culture have been reported (4).

Chinese hamster lung cells. Nontransformed (*CHL*) and wild-type (*CHLWT15, CHLWT16*) as well as group *A* mutant (*CHLA30L2, CHLA207L1, CHLA209SL1, CHLA239L1*) SV40 virus-transformed lines of Chinese hamster lung cells have been described (25, 36).

mKSA-ASC cell line. The mKSA-ASC BALB/c mouse cell line, an ascites tumor line (kindly supplied by L. Law) derived from the mKSA TU-5 cell line (17), was passaged semimonthly intraperitoneally (i.p.) in the syngeneic mice; it served as a tumor challenge line for in vivo tests. This cell line expresses strong SV40 TSTA activity and has a TD_{50} of 10^3 cells after i.p. injection (10).

Meth-1-A cell line. The Meth-1-A line, a methylcholanthrene-induced BALB/c tumor, which contains its own strong TSTA, carried in tissue culture, was used as a control, non-SV40 tumor line for in vivo challenge tests (24).

Cultivation of cells. Cells were cultivated in Eagle or in Dulbecco Vogt medium (NIH media unit) containing 10% fetal bovine serum (unless otherwise stated) in a humidified CO_2 incubator at 37°C. Chinese hamster and NIH Swiss mouse brain lines, both normal and transformed, were incubated at 33°C (permissive) or at 40.5°C (restrictive), whereas the restrictive temperature for the BALB-3T3 lines was 39.5°C.

Production of antiserum. Inbred female BALB/c mice, 5 to 8 weeks old, were obtained from the NIH breeding colony. Subconfluent 11-A-8 cells were washed three times with Tris-buffered saline (TBS), pH 7.4 (5). Cells were harvested by scraping with a rubber policeman and dispersed by gentle pipetting. Mice were injected i.p. six times with 5×10^6 cells/injection at intervals of 7 to 10 days. Two weeks after the final injection, the mice were bled from the heart; this serum is designated 11-A-8 antiserum. Sera were incubated at 56°C for 30 min to inactivate complement.

Direct ^{51}Cr cytolytic assay. Direct cytolytic assays, in the presence of complement, were carried out in a MicroTest tissue culture plate, as previously described (29, 31).

Indirect ^{51}Cr microcytolytic assay. A modified cell competition assay (29) was employed to estimate the number of cells required to inhibit the cytolytic activity of the antisera by 50%. Subconfluent monolayers were harvested by scraping with a rubber policeman. The cells were then washed three times with cold TBS. Varying numbers of these cells were added to antiserum (0.2 ml) diluted 100-fold in Eagle medium. The mixture was incubated at 4°C for 1 h unless otherwise specified. Cells were removed by centrifugation, and the supernatant was tested for residual cytolytic activity by approximately 500 SV AL/N target cells (29). All assays were performed in duplicate or triplicate. The percentage of lysis inhibition was plotted against the number of cells used to absorb the antibody, and the value for 50% inhibition of lysis (ID_{50}) was estimated by interpolation.

Solubilization of TSTA. Cells were harvested by scraping (generally 3×10^8 to 8×10^8 cells), washed three times with TBS, and mixed with detergent solution (0.5% Triton X-100 in TBS, with 100 μM phenylmethylsulfonyl fluoride) equal to five times the packed wet-cell volume. The cell-detergent suspensions were incubated for 30 min at 4°C, with occasional mixing, and centrifuged at $27,000 \times g$ for 20 min. The supernatant solutions were passed through 0.22-μm membrane filters (Millipore) (C. Chang et al., Int. J. Cancer, in press). The filtrates were used in TSTA assays. Portions were used for protein determination (23). The extracts contained no detectable virus by plaque assay.

TSTA assay in vivo. Inbred female BALB/c mice, 5 to 8 weeks old, were obtained from the NIH breeding colony. They were injected i.p. once, or twice 1 week apart, with the solubilized extract. Ten days after immunization, the mice were challenged with either mKSA-ASC tumor cells (i.p.) or Meth-1-A cells (subcutaneously). Tumor development was monitored for up to 7 weeks. Tumors usually appeared in 2 weeks, and death from malignant ascites occurred in 3 to 4 weeks.

RESULTS

Production of cytolytic antiserum and the direct cytolytic assay. Normal mouse serum was obtained from BALB/c mice prior to immunization with SV40-transformed BALB-3T3 cells (11-A-8). This normal serum was tested for cytolytic activity against SV AL/N and 11-A-8 target cells. No cytolytic activity was observed at serum dilutions of 1:2. Antiserum was obtained after six immunizations with 11-A-8 cells as described above. In tests with several cell lines, this antiserum was cytolytic only for the SV40-transformed SV AL/N and 11-A-8

cells; however, the cytolytic activity was much greater for the SV AL/N cells, with 50% cell lysis occurring at antiserum dilutions of 1:160, compared with the 11-A-8 cells, with 40% cell lysis at a dilution of 1:4. This antiserum did not have any detectable direct cytolytic activity against the normal AL/N line (AL/N ME), the spontaneously transformed cell line (T AL/N), or the clonal derivative BALB-3T3 line (A-31) (Fig. 1).

Specificity of the antiserum. Pancake and Mora (28) found that the SV40 TSSA may be detected by an inhibition assay at lower levels than are detected by the direct cytolysis assay, and that the cell line SV AL/N appeared to be superior as a target cell. The specificity of cell lysis by 11-A-8 antiserum was tested by assaying for cell lysis inhibition with several cell lines by using SV AL/N as the target cell line. All of the SV40-transformed cell lines absorbed 50% of the cytolytic activity at relatively low cell numbers (below 5×10^6), whereas the untransformed and non-SV40-transformed (negative for SV40 T-antigen) cells did not produce a 50% reduction of lysis with 10-fold or more cells than the corresponding SV40-transformed cells (Table 1). An example of such a titration is plotted in Fig. 2 for T AL/N, A-31, 11-A-8, and SV AL/N cells. With 1×10^8 or more cells negative for T-antigen, a maximum of only 20 to 30% reduction in cytotoxicity was obtained (for similar findings, see reference 29).

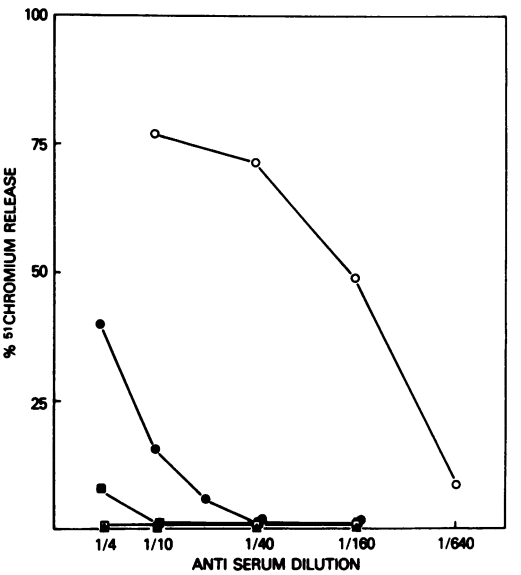

FIG. 1. *Direct cytolytic assay. Antiserum was prepared against the SV40-transformed BALB/c cell line (11-A-8) as described in text. Cells labeled with chromium were incubated with diluted antiserum in the presence of complement. The percentage of chromium release corresponds to the percentage of cells lysed as measured by a microcytolytic assay (29, 31). Data from duplicate experiments. Target cells and symbols: SV AL/N, ○; 11-A-8, ●; T AL/N, ■; AL/N ME, □; and A31, ▲.*

TABLE 1. *Specificity of inhibition with SV40-transformed cells of cell lysis by 11-A-8 antiserum*

Cell line	T-antigen[a]	No. of cells to reduce cell lysis by 50%[b]
AL/N ME	–	$>5 \times 10^7$
T AL/N	–	$>10^7$
SV AL/N	+	4×10^5
CHL	–	$>5 \times 10^7$
CHLWT16	+	3×10^6
A-31	–	$>10^7$
11-A-8	+	10^6
BALB-3T3	–	$>5 \times 10^7$
PY-3T3-4a	–	$>5 \times 10^7$
3T3WTJ	+	4×10^6

[a] From references 25, 28, and 33.

[b] See text for assay. Data from an average of three independent experiments.

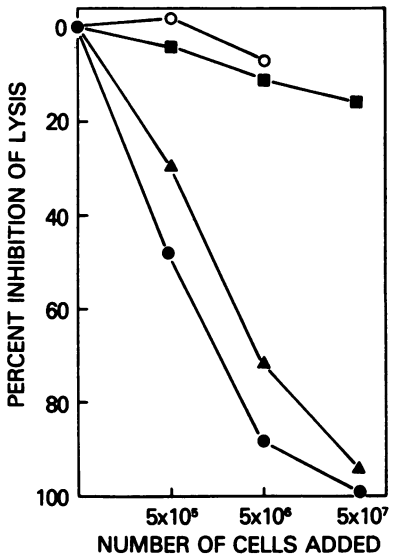

FIG. 2. *Inhibition of cell lysis by various cell lines. The 11-A-8 antiserum was diluted 100-fold with Eagle medium; the indicated number of cells were incubated, and the percentage of inhibition of lysis was assayed as described in text. Symbols: SV AL/N cells, ●; 11-A-8 cells, ▲; T AL/N cells, ■; A31 cells, ○.*

TSSA in temperature-sensitive SV40-transformed cell lines. Chinese hamster lung cells (*CHL*), two wild-type-transformed derivatives (*CHLWT15*, *CHLWT16*), and representative temperature-sensitive group *A* mutant transformants (*CHLA30L2*, *CHLA209SL1*, *CHLA239L1*) were tested for their ability to inhibit cytolytic activity of 11-A-8 antiserum. Table 2 shows that 1×10^6 to 7×10^6 *CHLA30L2*, *CHLA209SL1*, or *CHLA239L1*

TABLE 2. *Assays for TSSA with cells transformed by SV40 temperature-sensitive mutants*

Cell line	Temp (°C)	T-antigen[a]	Monolayer over growth[a]	No. of cells to reduce cytolytic activity by 50%[b] (ID_{50})
CHL	33	−	−	$>2 \times 10^7$, $>2 \times 10^7$
	40.5[c, d]	−	−	$>2 \times 10^7$[c], $>2 \times 10^7$[d]
CHLA30L2	33	+	+	1.2×10^6
	40.5[d]	+	−	1.3×10^6
CHLA209SL1	33	+	+	6.5×10^6
	40.5[d]	+	−	4×10^6
CHLA239L1	33	+	+	4×10^6
	40.5[c]	+	−	2×10^6
CHLWT15	33	+	+	1.8×10^6
	40.5[d]	+	+	3×10^6
CHLWT16	33	+	+	3×10^6
	40.5[c]	+	+	2.5×10^6[c]
MB	33[e]	−	−	$\gg 3 \times 10^6$[e]
MBA239	33	+	+	7×10^5
	40.5[f, g]	+	−	5×10^5[f], 5×10^5[g]
MBWT	33	+	+	6×10^5
	40.5[c]	+	+	4×10^5
MBC219	33	+	+	5×10^5
	40.5[c]	+	+	6×10^5
3T3	33	−	−	$>1 \times 10^7$
	39.5[d]	−	−	$>1 \times 10^7$
3T3A255B1b	33	+	+	8×10^5
	39.5[d]	+	−	9.5×10^5
3T3WTB1a	33	+	+	7.5×10^5
	39.5[d]	+	+	8.5×10^5

[a] See references 4, 25, and 36.
[b] See text; also cf. Table 1 and Fig. 2.
[c] Grown 6 days at 40.5°C. The cells were seeded at low cell densities and harvested after confluence had been achieved.
[d] Grown 4 days at the elevated temperature. The cells were seeded at 3×10^4 cells/cm² and harvested at confluence, approximately 3×10^5 cells/cm².
[e] Limited growth permitted only 3×10^6 cells to be collected for assay.
[f] Grown 4 days at 40.5°C. The cells were seeded at moderate cell densities and harvested after confluence had been achieved.
[g] Grown 14 days at 40.5°C. The cells were seeded at very low cell densities and harvested after confluence had been achieved.

cells grown either at the permissive or at the nonpermissive temperature inhibited the cytolytic activity by 50% (ID_{50}). A 50% reduction in cytolytic activity required a similar number (1.8×10^6 to 3×10^6) of *CHLWT15* or *CHLWT16* cells (wild-type-transformed cells). A substantially higher number of *CHL* control cells ($>2 \times 10^7$) did not inhibit the cytolytic activity by 50%.

The NIH Swiss mouse brain cell line grows poorly in vitro, with doubling times of 10 to 12 days (4), and hence only a moderate number of cells (3×10^6) could be obtained for testing. With this number of cells, no significant inhibition of cytotoxicity was observed. Wild-type-transformed cells, *MBWT*, a *tsA* transformant, *MBA239*, and a representative late mutant transformant, *MBC219* (4), were all strongly inhibitory ($ID_{50} \leq 7 \times 10^5$ cells) at the permissive temperature (Table 2). *MBA239* cells grown at the restrictive temperature for either 4 or 14 days were likewise strongly inhibitory. Similarly, *MBWT* and *MBC219* cells grown at 40.5°C actively bound antibody. As with the *CHL tsA* mutant transformants, the mouse brain line, *MBA239*, showed no significant difference in reducing the antibody titer at permissive versus nonpermissive temperatures (Table 2). In other control experiments, *MBA239* cells grown 4 days at the nonpermissive temperature also exhibited strong absorption ($ID_{50} = 5 \times 10^5$ cells) when incubated with 11-A-8 antibody at 40.5 instead of 4°C, eliminating the possibility that inactive TSSA was reactivated during the assay at 4°C. The mouse brain SV40-transformed cells had greater ca-

pacity to absorb antibody than the Chinese hamster-transformed cells.

Similar results were also found with mouse BALB-3T3 cells and their transformants. Less than 50% inhibition of cytolysis was observed with 10^7 nontransformed *3T3* cells, whereas with wild-type or a *tsA* transformant grown at 30 or 39.5°C (the nonpermissive temperature for expression of the transformed phenotype), 7×10^5 to 10×10^5 cells were sufficient to inhibit lysis by 50%.

Characterization of TSTA expression in wild-type- and *tsA*-transformed cells. To test for the presence of TSTA in representative transformed cells, soluble cell membrane extracts were prepared with 0.5% Triton X-100 in TBS (see above). Mice were injected once or twice with soluble extract containing 0.1 or 0.5 mg of protein from each cell line tested and subsequently challenged with mKSA-ASC tumor cells i.p. Extracts from *MBWT* (grown at 33°C) and *MBA239* (grown at 33 or at 40.5°C for 3 days) provided almost complete protection against challenges with 10^4 or 10^5 tumor cells, whereas all of the mice sham-injected with TBS or injected with an extract from NIH Swiss mouse brain died of ascites tumor. The differences between the control and immunized groups are significant at $P \leq 0.005$ level (Table 3).

Nontransformed BALB-3T3 or *CHL* cells failed to protect against a tumor challenge of 10^4 mKSA-ASC cells. On the other hand, significant protection was observed when mice were immunized once with soluble extracts containing 0.1 or 0.5 mg of protein from each of the wild-type- and *tsA*-transformed *3T3* and *CHL* cell lines grown either at the permissive or restrictive temperature (Table 3).

To test for delayed loss of TSTA, the *MBA239* cells were next incubated still longer (6 days) at 40.5°C prior to extraction; the cells were either encouraged to grow (in 25% serum supplement) or to rest (last 3 days in 2.5% serum). After immunization with extracts and challenge with tumor cells, the mice were observed for 7 weeks (Table 3). In the control group, 6 out of 10 mice died of ascites, whereas none of the 13 immunized mice developed tumors. Protection was complete whether cells were grown in high or low serum. Protection against ascites death was significant at $P < 0.03$ for the two immunized groups, or $P < 0.002$ for the combined immunized group.

In control experiments to determine the specificity of protection, mice were immunized with extracts of permissively grown cells or restrictively grown cells, or left unimmunized. Meth-1-A, a methylcholanthrene-induced non-SV40 tumor cell line that carries its own strong TSTA (24), was used for challenge. The incidence of tumors after 4 weeks is presented in Table 4. (The experiments described in the table were carried out at the same time as, and correspond to, the experiments in Table 3.) There were no significant differences between the control groups and the groups immunized with the SV40-transformed cell lines.

DISCUSSION

We observed that BALB/c mice hyperimmunized with the cloned SV40-transformed BALB-3T3 line 11-A-8 developed cytolytic antiserum to SV AL/N cells, but not to normal or spontaneously transformed AL/N cells or to the normal cloned BALB-3T3 line A-31. This antiserum was observed to be slightly cytolytic against the 11-A-8 cells at a dilution of 1:4. The SV AL/N cells proved considerably (40-fold) more sensitive to lysis by 11-A-8 antiserum than 11-A-8 cells. The difference in immunosensitivity of various SV40-transformed lines against cytolytic antiserum has been observed previously (10, 28); however, the factors responsible for this were not elucidated (cf. also reference 26). It has been shown that the surface density of antigens is important in determining the sensitivity of cells to lysis in the H-2 system (18). It is notable that SV AL/N cells, which are the most sensitive to lysis, also have the highest antibody-absorbing capacity despite their apparent small surface area.

We have modified the test system of Pancake and Mora (29) by using antiserum obtained from BALB/c mice hyperimmunized with the SV40-transformed BALB 11-A-8 cells. This antiserum was reacted against the SV40-transformed line SV AL/N (allogeneic in our assay) after absorption with still a third cell line, usually allogeneic or xenogeneic to the other two. This modified test system should provide a highly specific assay for SV40 tumor surface antigens; to inhibit target cell lysis, an antigen must be common to each of three different cell lines and also must be recognized as foreign by the mice immunized with the originally syngeneic SV40-transformed cells.

The specificity of this modified cytolytic test is supported by the finding that polyoma-transformed cells (PY-3T3-4a) behaved like normal *3T3* cells and did not inhibit cytolytic activity at high cell concentrations (28, 29). When 1×10^8 or more cells negative for T-antigen were used, up to 20 to 30% reduction of cytolytic activity could be obtained. We attribute this to nonspecific binding of antibody to the large number of cells used.

Our inhibition studies have demonstrated

TABLE 3. *Immunogenicity test of solubilized TSTA from cells transformed by SV40 wild type or tsA mutants*

Cell line	Temp of cell culture[a] (°C)	Immunizing dose (mg of extract/inoculum)	Challenge dose (no. of mKSA-ASC cells)	Death by ascites tumor/ total inoculated	
Expt 1					
Buffer[b]			10^5	8/8	15[c]
			10^4	7/7	15
NIH Swiss mouse brain cells[d, e]		0.5	10^5	9/9	17[c]
		0.5	10^4	8/8	17
MBWT[e]	33	0.5	10^5	1/9	2[c]
		0.5	10^4	1/7	16
MBA239[e]	33	0.5	10^5	0/8	0[c]
		0.5	10^4	0/5	13
	40.5	0.5	10^5	0/8	0[c]
		0.5	10^4	0/7	15
Expt 2					
Buffer[b]			10^4	6/10	
MBA239, 6 days, 25% serum	40.5	0.5	10^4	0/7 ($P < 0.03$)	
MBA239, 6 days, 2.5% serum	40.5	0.5	10^4	0/6 ($P < 0.03$)	
Expt 3					
Buffer[b]			10^4	10/10	
CHL	33	0.1	10^4	7/7	14[c]
		0.5	10^4	7/7	14
	40.5	0.1	10^4	7/7	12[c]
		0.5	10^4	5/5	12
CHLWT15	33	0.1	10^4	3/7	4[c]
		0.5	10^4	1/7	14
	40.5	0.1	10^4	2/7	3[c]
		0.5	10^4	1/6	13
CHLA30L2	33	0.1	10^4	0/7	0[c]
		0.5	10^4	0/7	14
	40.5	0.1	10^4	0/7	0[c]
		0.5	10^4	0/7	14
CHLA239L1	33	0.1	10^4	1/7	2[c]
		0.5	10^4	1/7	14
	40.5	0.1	10^4	2/7	2[c]
		0.5	10^4	0/4	11
Expt 4					
Buffer[b]			10^4	10/10	[c]
CHLA207L1	33	0.1	10^4	2/7	2[c]
		0.5	10^4	0/7	14
	40.5	0.1	10^4	4/7	4[c]
		0.5	10^4	0/5	12
CHLA209SL1	33	0.1	10^4	2/7	4[c]
		0.5	10^4	2/7	14
	40.5	0.1	10^4	2/6	3[c]
		0.5	10^4	1/6	12
3T3	33	0.1	10^4	7/7	14[c]
		0.5	10^4	7/7	14
	39.5	0.1	10^4	7/7	13[c]
		0.5	10^4	6/6	13

TABLE 3. *Continued*

Cell line	Temp of cell culture[a] (°C)	Immunizing dose (mg of extract/inoculum)	Challenge dose (no. of mKSA-ASC cells)	Death by ascites tumor/ total inoculated	
3T3WTB1a	33	0.1	10^4	3/7	3[c] / 14
		0.5	10^4	0/7	
	39.5	0.1	10^4	4/7	5[c] / 14
		0.5	10^4	1/7	
3T3A255B1b	33	0.1	10^4	2/6	3[c] / 13
		0.5	10^4	1/7	
	39.5	0.1	10^4	2/7	3[c] / 13
		0.5	10^4	1/6	

[a] Cells were incubated at the elevated temperature for 4 days unless otherwise specified. During this time the cells grew from subconfluence to confluence.

[b] TBS.

[c] The differences between the inoculated and uninoculated groups were significant at $P < 0.005$ values (11).

[d] Brain cells were extracted as described in text.

[e] Mice were immunized twice.

that SV40-transformed cells originating from a Chinese hamster or from three different strains of mice (AL/N, BALB/c, and NIH Swiss) can inhibit cytolytic activity at relatively small numbers of cells (ID_{50} = 4 × 10^5 to 4 × 10^6). Untransformed and non-SV40-transformed cells are unable to reduce the antiserum lytic activity by 50%, even with 10-fold or more cells than required by their SV40-transformed counterparts.

In the inhibition test of cytolytic activity, mouse or hamster cell lines transformed by temperature-sensitive group *A* mutants of SV40 used in this study expressed the SV40 TSSA at both permissive (33°C) and nonpermissive (40.5°C) temperatures. Furthermore, there was no sign of a reduction in the quantities of TSSA in cells grown at 40.5°C for 4 days or 2 weeks. Within 1 to 5 days after shift to the nonpermissive temperature, these *tsA* transformants display striking changes in growth in tissue culture consistent with loss of the transformed phenotype, but the T-antigen persists under these conditions (4). In contrast, the group *A* mutant-transformed cell lines studied by Brugge and co-workers lost surface antigen at the nonpermissive temperature (7). Although the modified test for inhibition of cytolytic activity appears to be quite specific to SV40-transformed cells, evidence to date appears insufficient to assume the identity of TSSA and TSTA.

Our observations concerning TSSA are consistent with the recent work of Kurth (20) in another tumor virus system. He demonstrated that TSSA were present in several cell lines transformed by avian leukosis sarcoma virus

TABLE 4. *Specificity of solubilized TSTA preparations from cells transformed by SV40 tsA mutants*

Cell line	Temp of cell culture[a] (°C)	Immunizing dose (mg of extract/ inoculum)	Tumors[b]/total inoculated
Expt 1			
Control (none)			9/10
NIH Swiss mouse brain cells[c, d]		0.5	7/9 (P = 0.37)
MBA239[d]	33	0.5	14/19 (P = 0.24)
	40.5	0.5	7/10 (P = 0.25)
Expt 2			
Control			7/9
CHLA30L2	33	0.1	4/5
	40.5	0.1	4/6
CHLA239L1	33	0.1	5/6
	40.5	0.1	5/6
CHLWT15	33	0.1	4/5
Expt 3			
Control			8/9
CHLA207L1	33	0.1	5/6
	40.5	0.1	4/6
CHLA209SL1	33	0.1	4/5
	40.5	0.1	5/5
3T3A255B1b	33	0.1	5/6
	39.5	0.1	4/6

[a] Cells grown as in Table 3 for 4 days at the nonpermissive temperature. All of these experiments were carried out at the same time as, and in parallel with, the experiments in Table 3.

[b] Large (>0.5 cm) nonregressing tumors at site of injection; all animals injected subcutaneously with 10^4 Meth-1-A cells.

[c] Extracted as described in text.

[d] Mice were immunized twice.

mutants, even though the growth phenotype of these cells had reverted toward normal at the nonpermissive temperature.

The *tsA* mutations map (21) in the region of the SV40 genome necessary for expression of TSTA (22). We assessed TSTA expression by in vivo testing of extracts from *tsA* mutant-transformed cell lines that were obtained in our laboratories. Three different parental cell types and five different *tsA* mutants were represented. To avoid possible changes in the expression of surface antigens on cells grown at 33 or 40.5°C during exposure of whole cells to mouse body temperature, we solubilized SV40 TSTA prior to injection. Injection of extracts of cells transformed by wild-type SV40 and cells transformed by *tsA* mutants grown at 33 or at 40.5°C provided strong transplantation immunity against subsequent challenge by the SV40-transformed mKSA-ASC cells (10^4 to 10^5 cells; 10 to 100 × LD_{50}). Extracts of the parental nontransformed cell lines containing equivalent amounts of protein were found to be completely inactive. Furthermore, these extracts did not provide significant immunity against methylcholanthrene-induced tumor cells. These results demonstrate that these *tsA* mutant transformants express TSTA whether they are grown at permissive or nonpermissive temperatures. The theoretical possibility that the TSTA is expressed in an inactive form at the nonpermissive temperature and is only reactivated upon extraction or after injection into the mouse cannot be eliminated. The extraction procedure also allowed us to demonstrate for the first time that TSTA activity can be extracted from SV40-transformed hamster cells and used to immunize mice.

Although *tsA* mutant-transformed cells lose their ability to grow in low serum and to form colonies on a normal cell monolayer or in soft agar under nonpermissive temperature conditions (4, 7, 25, 35, 36), the lines we have used strongly retain TSSA, TSTA, and T-antigen. However, a unique *tsA28* rat embryo-transformed cell line, which loses T-antigen at the nonpermissive temperature (27), also shows a concomitant quantitative decrease in TSSA and TSTA (3).

Our results do not exclude the possibility that TSTA is an early SV40 gene product. The *tsA* mutations, presumably point mutations of the missense type, may not influence the precise site(s) determining TSSA and TSTA. Indeed, on the basis of other experiments, we (3, 5, 6) and others (9) have proposed that T-antigen and TSTA represent different antigenic activities contained in the same virally encoded polypeptide.

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