Systematic Variables Affecting Simian Virus 40-Induced T-Antigen Expression and Transformation in Human Cells

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Simian virus 40 (SV40) infection of human skin fibroblast and human tumor cells resulted in the expression of T-antigen and transformed foci. By examining various conditions of input virus multiplicity and initial cell density, the systematic variation of T-antigen determination was minimized. The most uniform results were obtained at multiplicities of about 275 plaque-forming units/cell. Within limits (5×10^4 to 2×10^5 cells/dish), initial cell density had little effect on T-antigen expression. Volume of virus inoculum was critical for some cell lines, but not for others. Cell passage level had no general effect on T-antigen expression, although specific cell lines demonstrated increased or decreased levels of T-antigen expression with serial passage for no apparent reason. T-antigen expression correlated with virus-induced cell transformation (focus formation) at two different multiplicities. In addition, T-antigen assays at 3 days gave consistently more reproducible results than transformation assays at 21 days in seven cell lines tested at two multiplicities of infection. These results defined input multiplicity as the major source of systematic variability and will permit development of a more reproducible tool in the evaluation of individuals at high risk of cancer.

The incidence of transformation and/or T-antigen expression in cultured fibroblasts infected with simian virus 40 (SV40) is reported to be increased among individuals with certain genetic disorders (7, 8, 11, 12). Since some of these genetic conditions are associated with susceptibility to malignant neoplasms (3), the test may provide an epidemiological tool in the evaluation of cancer risk. However, the techniques commonly used for measuring T-antigen expression in human fibroblasts tended to yield highly variable results in our hands. To develop a reproducible assay for epidemiological application, a series of experiments was performed to examine the potential sources of variability in T-antigen expression which limit the application of this technique. We here wish to communicate the results of these methodological studies, which have been used subsequently to study the relationship between T-antigen expression and cancer risk in various normal and high-risk populations (A. S. Lubiniecki, J. F. Fraumeni, Jr., and W. A. Blattner, *The Genetics of Human Cancer*, Raven Press, New York, in press).

MATERIALS AND METHODS

Cell cultures. Biopsies were obtained from clinically normal individuals and persons with genetic predisposition or strong familial susceptibility to cancer (3). Sixteen human fibroblast strains were grown from explanted biopsy specimens in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (GIBCO, Grand Island, N.Y.) as previously described (5). These strains were derived from clinically normal individuals (1346, 1395, 1419, and 1569), cancer patients (1543 and 1617), relatives of cancer patients (525, 1597, 1600, 1611, and 1676), inbred populations (1558 and 1781), aplastic anemia patients (833 and 2241), and a myotonic dystrophy patient (36). Two human tumor cell lines (A172 and A498) were derived as described (4). All cell cultures were incubated at 37°C in a humidified atmosphere of 10% CO₂-90% air.

Virus. The SV40 virus employed was a small-plaque variant having a titer of 1.0×10^6 plaque-forming units (PFU)/ml, propagated and assayed on African green monkey cells (9).

Standard method of infection. Confluent monolayers were trypsinized, and cell suspensions were seeded in plastic cell culture dishes (60 by 15 mm) at a density of approximately 2×10^5 cells/plate. Before infection, 24 h after seeding, actual cell densities were determined on duplicate plates. The input multiplicity of infection (MOI) was then adjusted to the actual cell count at the time of infection (between 100 and 500 PFU/cell, according to experi-

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ment). Before infection, monolayers were washed once with sterile DMEM salts, and inoculated with 0.5 ml of SV40 virus (unless otherwise indicated). Infected cultures were then rotated every 15 min for 3 h at 37°C. After this, the inoculum was removed, and the cultures were washed once with serum-free DMEM medium. The cultures were then incubated in 5.0 ml of complete DMEM medium plus hyperimmune SV40 rabbit antiserum (GIBCO) for 3 days unless otherwise specified. The rabbit antiserum to SV40 was included in sufficient concentration to totally inhibit the development of plaques by 10^6 PFU of SV40 in African green monkey kidney cells. Thus, those cells that exhibited T-antigen were assumed to result from initial infection with SV40 rather than from progeny virus released from other infected cells.

T-antigen assay. Preliminary experiments showed maximal expression of T-antigen at 3 days after infection in 8 of 10 cell strains, which is similar to the results of Potter and Potter (7). This time period was employed in all studies except those assessing SV40 transformation, described below. Cell cultures in petri dishes were fixed in methanol, treated with hamster antibody to T-antigen followed by fluorescein-conjugated antihamster globulin, and counterstained with rhodamine contrast stain as previously described (5). Plates were examined in a systematic fashion using 100 microscope fields to determine the proportion of T-antigen-positive cells. A weighted mean proportion of T-antigen-positive cells was calculated by adjustment for the number of cells examined, which ranged from 6×10^3 to 2×10^4 per 100 fields per replicate plate. Uninfected cultures employed in all experiments exhibited only nonspecific rhodamine fluorescence when stained by this method.

Counts made by two trained operators did not vary significantly between operators or with time, using several sets of significantly different cell strains examined several times. All plates were coded at the time of infection; codes were broken only after examination for T-antigen was completed.

Transformation assay. Cultures were initially examined for T-antigen formation at 21 days postinfection. The incidence of T-antigen-positive cells and of antigen-containing colonies was determined simultaneously. Entire T-antigen-containing colonies were scored the same as one positive cell. The cultures were then washed with phosphate-buffered saline and treated with 10% (vol/vol) formalin in phosphate-buffered saline for 15 min. The formalin was removed, and the plates were stained by adding 1% (vol/vol) Harris hematoxylin (Fisher Scientific Co.). After 1 h, the stain was removed and the plates were washed with water and diluted ammonium hydroxide (12 mM) was added briefly (5 to 10 s). The transformation frequency was then determined for each cell strain by a microscopy examination method similar to that of Todaro (10).

RESULTS

Effect of MOI. Preliminary experiments indicated that failure to control cell numbers or

multiplicities (i.e., to infect an unknown but presumed equal number of cells from several different strains with a constant amount of virus) resulted in input multiplicities ranging from 50 to 350 by actual cell count. To document this, the following experiment was performed to test the relationship between MOI and the magnitude and variability of T-antigen formation, holding cell density constant. Replicate cultures of nine different cell strains and lines were seeded at 2.0×10^5 cells/ml and were infected 24 h later with SV40 virus at input multiplicities ranging from 100 to 500 PFU/cell, in 50 PFU/cell increments. Cultures were then fixed and stained as described in Materials and Methods.

Figure 1 shows computer-fit least-squares regression lines, each line representing 50 to 200 separate determinations for each cell line and 8 to 20 separate experiments. The cell lines and strains tested tend to form three groups on the basis of the slope of their regression lines. A172 and 1611 show a strong effect of MOI (relatively steep slope) on the proportion of cells expressing T-antigen (%T⁺). The majority of the lines showed considerably less MOI effect (1558, 1597, 1617, 1346, 1419). Two lines (A498 and 525) showed no MOI effect.

Despite the strong linear relationships observed between MOI and %T⁺, some cell lines showed persistent between-experiment variability with the most marked variability at extreme MOI values and the least variability at an MOI of approximately 275 PFU/cell. This

FIG. 1. Effect of input virus multiplicity on T-antigen expression by SV40-infected human skin fibroblasts and tumor cells. Code numbers used to identify regression lines correspond to cell line codes.

MOI value was employed for all further work unless otherwise specified.

Effect of cell density. To determine the optimal cell density for T-antigen formation, the following experiments were performed employing diploid cell strains. Cell suspensions were transferred into petri dishes (60 by 15 mm) at a cell density of 1.0×10^4 , 5.0×10^4 , 7.0×10^4 , 1.0×10^5 , or 2.0×10^5 cells/plate. Cells were counted 24 h later and infected with 275 PFU/cell. T-antigen formation was assessed by the standard method.

Table 1 indicates that the initial cell density at the time of seeding had no reproducible effect upon the expression of T-antigen. Further, the lack of effect was observed in cell strains with both high and low values of %T⁺. Cultures with very low cell densities (1×10^4) did not survive the 4-day test period. All further experiments employed an initial cell density of 2.0×10^5 cells/plate.

Effect of inoculum volume. Since the standard protocol required infecting cells at constant MOI, each cell line received a given volume of virus stock based on the average cell count for that line. It was of interest to determine whether inoculum volume had any significant effect on the expression of T-antigen. To provide maximum sensitivity, five skin fibroblast lines were selected on the basis of their very high levels of %T⁺. Cultures were infected with a constant MOI of 275 PFU/cell in various volumes from 0.1 to 1.5 ml. Cultures were incubated, harvested, and assayed in the usual manner.

Table 2 shows that the volume of the virus inoculum had a significant effect on %T⁺ in some cell strains, but not in others. Therefore, future experiments were performed at a constant inoculum volume of 0.5 ml; MOI was ad-

TABLE 1. Weighted mean percentage of T⁺ cells in cultures of cell lines seeded at various cell densities

Expt	Cell line	%T ⁺ at initial density (cells/plate) of:					P value ^a
		1.0×10^4	5.0×10^4	7.0×10^4	1.0×10^5	2.0×10^5	
1	1419	— ^b	0.29	0.39	0.20	0.58	>0.05
	1611	—	0.627	1.79	0.78	2.03	>0.025
	1617	—	6.69	4.09	3.89	7.35	>0.01
	1676	—	2.74	2.13	2.55	3.73	>0.01
2	1419	ND ^c	1.22	0.50	0.72	0.85	>0.10
	1617	ND	2.98	3.26	3.67	4.88	>0.10

^a Each strain was tested by analysis of variance, one-way classification. Throughout this work, statistical significance is assumed at $P \leq 0.01$.

^b No cell growth.

^c ND, Not done.

justed to 275 PFU/cell by dilution of a small amount of high-titered stock to 0.5 ml.

Effect of cell passage level. To test the effect of cell passage history, 13 different cell lines and strains were infected at an MOI of 100 and 500 at each of various serial levels from 4 to 28. T-antigen assays were performed as usual. Table 3 indicates that for 5 of the 13 lines the cell passage level had a significant effect. These effects were more frequently observed at the lower MOI; however, the relationship between cell passage level and %T⁺ did not appear to depend on readily identifiable factors, such as karyotype or clinical history.

The relationship between cell passage level and %T⁺, if significant, was quite linear. Figure 2 indicates that the value of %T⁺ generally decreased with passage (but not always). In addition, the slopes of computer-fit regression lines for the 500 PFU/cell data were uniformly

TABLE 2. Effect of inoculum volume on weighted mean percentage of T-antigen expression

Cell line	%T ⁺ at 275 PFU/cell in:					P value ^a
	0.10 ml	0.25 ml	0.50 ml	1.00 ml	1.50 ml	
36	10.2	10.0	11.8	10.9	10.3	≥ 0.100
833	18.9	25.9	28.0	33.0	34.2	>0.025
1395	19.4	20.5	16.1	16.2	11.3	≤ 0.005
1561	8.7	9.0	11.5	9.0	8.6	≤ 0.005
2241	10.8	13.8	11.1	15.9	— ^b	>0.010

^a Each strain was tested by analysis of variance one-way classification employing replicate data at each volume tested.

^b Samples lost.

TABLE 3. Effect of cell passage level on T-antigen expression

Cell code	Pas-sage level (range)	%T ⁺ (range)	Significance (P value) ^a of relationship at MOI of:	
			100 PFU/cell	500 PFU/cell
1419	5-16	0.8-2.0	<0.01	>0.05
1346	5-13	1.0-3.0	>0.05	<0.01
1611	5-19	2.0-4.0	>0.05	>0.05
525	5-18	2.0-4.0	>0.05	>0.05
1676	6-10	2.0-4.0	>0.05	>0.05
1543	5-10	3.0-5.0	>0.05	>0.05
1597	6-10	4.0-5.0	>0.05	>0.05
1617	4-11	4.0-6.0	>0.05	>0.05
1558	5-11	4.0-7.0	<0.01	>0.05
1781	5-13	5.0-9.0	<0.01	>0.05
1600	5-20	10.0-14.0	<0.01	<0.01
A498	10-25	0.2-0.7	>0.05	>0.05
A172	15-28	6.0-11.0	>0.05	>0.05

^a Each line or strain was tested at each MOI by analysis of variance, one-way classification. At each passage level, 4 to 10 single determinations were employed for each line and MOI.

FIG. 2. Effect of cell passage level on T-antigen expression in some SV40-infected human skin fibroblast strains at two different input virus multiplicities. Solid lines represent MOI values of 500 PFU/cell, and dashed lines indicate MOI values of 100 PFU/cell.

steeper than for 100 PFU/cell data, yet fewer lines showed statistically significant effects of passage level at the high MOI. This apparent discrepancy probably reflects the inherently greater variability in %T⁺ values at extremely high MOI than at low MOI. To minimize these effects, since they apparently cannot be predicted, we routinely employ cell lines and strains at the lowest available passage level (usually <10).

Relationship between %T⁺ and transformation. To confirm the relationship between T-antigen formation and transformation in our system, the following experiment was performed. Standard petri dish cultures were counted 24 h after seeding and infected with SV40 virus at an MOI of 100 or 500 PFU/cell. Cultures were incubated for 3 h at 37°C with frequent shaking. Cells were then washed with fresh medium, dispersed with 0.1% trypsin, and reseeded at a cell density of 1.0×10^4 cells/plate. The transformation frequency (transformed colonies/cells plated) and T-antigen expression were then determined at 21 days. Parallel plates that were not subcultured after infection were also examined at 3 days for T-antigen formation.

The results (Fig. 3) are for %T⁺ at 3 days versus transformation at 21 days. Both functions have been mathematically transformed by the arc sin $\sqrt{(\%)}$ function to permit direct comparison of the various cell lines (unpub-

lished observations). The relationship between the expression of percentage antigen and virus-induced transformation appeared strongly linear after mathematical transformation. Transformation was highly correlated with %T⁺ at both MOI values tested and with both day 3 and day 21 values of %T⁺. T-antigen expression on day 21 tended to be lower than on day 3 for some cell lines, as found by other investigators (1, 7); however, the results of the day 3 and day 21 assays for the various cell lines were highly correlated with colony formation.

Table 4 shows that the number of T-antigen-positive cells required for expression of one transformed clone varied with MOI. The higher MOI produced more transformants than are expected from the modest increases in %T⁺ produced by adding more virus particles. The actual values of the number of T-antigen-positive cells per transformant are similar to reported value for SV40-infected human cells at similar multiplicities (1, 2, 7, 8).

Table 4 and Fig. 3 strongly suggest the existence of a threshold phenomenon in the expression of virus-induced transformation, in which a certain minimal fraction of cells must express T-antigen (or a function that covaries with it) before one of these cells becomes visibly transformed.

FIG. 3. Relationship of virus transformation to T-antigen expression in SV40-infected human skin fibroblasts and tumor cells at two different input virus multiplicities. Symbols: (●) weighted mean data (4 to 10 separate determinations) at MOI values of 500 PFU/cell; (○) MOI values of 100 PFU/cell. The solid and dashed regression lines represent MOI data at 500 and 100 PFU/cell, respectively.

TABLE 4. *Effect of multiplicity on the expression of T-antigen and transformation*

Cell code	MOI (PFU/cell)					
	100			500		
	%T ⁺ ^a	% Transformation ^a	Ratio ^b	%T ⁺	% Transformation	Ratio
1346	0.92	0.002	460	1.91	0.041	46.1
1419	0.67	0	— ^c	1.59	0.039	40.7
1611	2.94	0.025	118	3.92	0.109	36.0
1676	2.71	0.050	54.2	3.77	0.206	18.3
1600	4.37	0.097	45.1	6.69	0.384	17.6
A498	0.05	0 ^d	—	0.20	0 ^d	—
A172	5.82	0 ^d	—	11.61	0.260	44.6

^a T-antigen (3 days) and virus-specific transformation (21 days) expressed as weighted mean percentages, averages of 6 to 12 determinations.

^b Number of T-antigen-positive cells per transforming event.

^c Indeterminate form.

^d Unlike zero value obtained with 1419, these zero values represent failure to observe virus-specific transformed colonies. Both tumor lines exhibited significant numbers of spontaneous "transformed" colonies in uninfected cultures. Unless significantly more transformed colonies were found in infected cultures, zero values are shown for virus-specific transformation. None of the skin fibroblast cultures showed any spontaneously transformed colonies.

The greater reproducibility of the T-antigen assay is demonstrated in Table 5. Of the seven lines tested, four can be compared directly at both multiplicities. The data indicate that T-antigen assays were considerably more reproducible than transformation assays (on the average, by fourfold). Of the seven lines, 1419 and A498 exhibited relatively low values of %T⁺ and virtually no transformed foci. Since enumerative functions, such as cell counts, used to generate %T⁺ are approximately distributed as Poisson functions, the very low values for 1419 and A498 are expected to possess greater variability. In contrast, A172 had high values of %T⁺, and therefore had relatively low coefficients of variation for %T⁺.

DISCUSSION

The present report describes the effect of input virus multiplicity, cell density at seeding, and cell passage level on SV40 T-antigen expression by infected human cells, and its relationship to virus-induced transformation. The most important factor appeared to be multiplicity. Aaronson and Todaro (2) previously described the effect of multiplicity on T-antigen expression in three human cell strains, but at

TABLE 5. *Comparison of variability between T-antigen and transformation assays*

Cell code	σ/μ at MOI (PFU/cell) ^a			
	100		500	
	%T ⁺	% Transformation	%T ⁺	% Transformation
1346	0.23	2.00	0.17	0.95
1419	0.36	— ^b	0.39	0.46
1611	0.17	0.93	0.11	0.45
1676	0.22	0.76	0.19	0.48
1600	0.24	0.39	0.31	0.45
A498	0.75	—	0.61	—
A172	0.20	—	0.19	1.85

^a Coefficient of variation (standard deviation [σ]/weighted mean [μ]) at two input multiplicities for both T-antigen expression (3 days) and virus-specific transformation (21 days).

^b Indeterminate form ($\mu = 0$).

levels of virus generally lower than those employed here. Our studies of nine human cell strains and lines were done at slightly higher MOI values to maximize accuracy and minimize the coefficient of variation for T-antigen expression, since the raw data approximately followed Poisson distribution.

Cell density had no significant effect, and the influence of inoculum volume and cell passage level was unpredictable from one cell line to another. We therefore employed a constant MOI of 275 PFU/cell in a volume of 0.50 ml, an initial cell density of 2.0×10^5 cells/plate, and the lowest available cell passage level to minimize systematic variability of T-antigen expression at 3 days after infection. These studies defined sensitive and reproducible methods for examining T-antigen expression in human cells and collectively indicated that those methods for determining %T⁺ which do not closely control virus multiplicity (1, 2, 7, 8) may yield results that are considerably less reproducible than those obtained by our method.

Several additional factors may influence the T-antigen expression assay as described in the present communication. The stage of the cell cycle at the time of SV40 infection did not seriously affect either virus adsorption or T-antigen expression in permissive CV₁ monkey cells (6). The importance of this factor in the assay system reported here for relatively nonpermissive human fibroblasts is currently under study. The influence of various assay components, such as virus strain, type of medium, and type and concentration of serum, may be predicted with a high degree of certainty, but these factors are easily controlled or standardized. In addition, we are currently examining

the possible effect of different virus stock preparations on T-antigen expression. The experiments described herein are not expected to identify all possible variables in this assay, but rather the most obvious, most easily detected, or most easily controlled factors. Others surely exist, and additional studies to further control the T-antigen expression assay in human fibroblasts are in progress.

As reported previously, T-antigen expression and transformation were highly correlated in SV40-infected human cells (1, 2, 7, 8). Although the exact relationship between these two virus-induced events remains obscure, it is apparent that T-antigen assays are more sensitive and rapid than transformation assays and may represent a useful tool in the epidemiological evaluation of cancer risk in diverse groups. Further studies are being carried out on a large number of cell strains to assess the applicability of the assay in predicting cancer risk. For this reason, we hesitate to discuss the ramifications of the actual %T⁺ values reported here for the very small sample of normal and abnormal skin cell strains examined. However, the modified T-antigen assay described herein has been used in carefully controlled studies to demonstrate significant elevation of T-antigen expression in a number of clinical groups at high risk of cancer (Lubiniecki et al. and Blattner, *The Genetics of Human Cancer*, Raven Press, New York, in press).

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LITERATURE CITED

1. Aaronson, S. A. 1970. Susceptibility of human cell strains to transformation by simian virus 40 and simian virus 40 deoxyribonucleic acid. *J. Virol.* 6:470-475.
2. Aaronson, S. A., and G. Todaro. 1968. SV40 T-antigen induction and transformation in human skin fibroblast cell strains. *Virology* 36:254-261.
3. Fraumeni, J. F., Jr. 1973. Genetic factors. In J. F. Holland and E. Frei (ed), *Cancer medicine*. Lea and Febiger, Philadelphia.
4. Giard, D. J., S. A. Aaronson, G. J. Todaro, P. Arnstein, J. H. Kersey, H. Dosik, and W. P. Parks. 1973. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J. Natl. Cancer Inst.* 51:1417-1423.
5. Kaplan, M. M., D. J. Giard, W. A. Blattner, A. S. Lubiniecki, and J. F. Fraumeni, Jr. 1975. An improved method for immuno-fluorescent detection of SV40 T-antigen in infected human fibroblasts. *Proc. Soc. Exp. Biol. Med.* 148:660-664.
6. Pages, J., S. Mantevil, D. Stehelin, M. Fiszman, M. Marx, and M. Girard. 1973. Relationship between replication of simian virus 40 DNA and specific events of the host cell cycle. *J. Virol.* 12:99-107.
7. Potter, A. M., and C. W. Potter. 1975. Transformation of human cells by SV40 virus. *Br. J. Cancer* 31:348-354.
8. Potter, C. W., A. M. Potter, and J. S. Oxford. 1970. Comparison of transformation and T-antigen induction in human cell lines. *J. Virol.* 5:293-298.
9. Takemoto, K. K., G. J. Todaro, and K. Habel. 1968. Recovery of SV40 virus with genetic markers of original inducing virus from SV40-transformed mouse cells. *Virology* 35:1-8.
10. Todaro, G. J. 1969. In K. Habel and N. P. Salzman (ed.), *Fundamental techniques in virology*. Academic Press, New York.
11. Todaro, G. J., H. Green, and M. R. Swift. 1966. Susceptibility of human diploid fibroblasts to transformation by SV40 virus. *Science* 153:1252-1254.
12. Todaro, G. J., and G. M. Martin. 1967. Increased susceptibility of Down's syndrome fibroblasts to transformation by SV40. *Proc. Soc. Exp. Biol. Med.* 124:1232-1236.