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Cell Killing by Simian Virus 40: Variation in the Pattern of Lysosomal Enzyme Release, Cellular Enzyme Release, and Cell Death During Productive Infection of Normal and Simian Virus 40-Transformed Simian Cell Lines

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Simian virus 40 (SV40) growth on rhesus kidney cells and on the T-22 line of SV40-transformed green monkey kidney (GMK) cells is largely limited by the low plating efficiency of SV40 on these cells. In addition, a fraction of the rhesus kidney and T-22 cells are resistant to infection by SV40. Nevertheless, 72-h viral yields per infected rhesus kidney and T-22 cell are nearly equivalent to that obtained on normal GMK cells and are independent of the multiplicity of infection. Despite the production of high viral yields, infected rhesus kidney and T-22 cells are killed slowly by SV40. Monolayers of these cells are also refractory to plaque formation by SV40. SV40 induces the release of lysosomal *N*-acetyl-β-glucosaminidase into the cytoplasmic fractions of rhesus kidney and T-22 cells to an extent equal to that observed during infection of rapidly killed normal GMK cells. In contrast, damage to the plasma membrane, as indicated by the release of the cellular enzymes lactic dehydrogenase and glutamic oxaloacetic transaminase into the overlay media, occurred to a much greater extent in the normal GMK cells than in the rhesus kidney or T-22 cells. Neither a lysosomal hydrolase mechanism nor viral release appear to be responsible for this phenomenon. The different rates and extents of the SV40 cytocidal process on these cells do not result from the differences in the viral plating efficiency on them.

The mechanisms by which cytocidal viruses kill cells are largely unknown. This is so, in part, because it is difficult to distinguish cytocidal events from the many other phenomena that accompany productive infection. The studies on cell killing by simian virus 40 (SV40) reported here follow from the observation that host cell factors determine the rate of cell killing during productive SV40 infection. Since SV40 yields are nearly equivalent during the rapidly cytocidal and slowly cytocidal infections described below, phenomena associated with the cytocidal process can be distinguished from the epiphenomena that accompany productive infection.

In the original descriptions of the relationship between SV40 and rhesus kidney cells in culture (9, 19), it was noted that growth of the virus and resultant cellular destruction took much longer than in cultures of green monkey kidney (GMK) cells. Results reported below indicate that SV40 growth on rhesus kidney cells is largely limited by the low plating efficiency of this virus on these cells. We find that SV40 yields on rhesus kidney cultures approach that obtained on GMK cultures under conditions of high virus-to-cell input multiplicity. Nevertheless, even under these conditions, cytopathic effect is still minimal on rhesus kidney cells. A similar but not so striking dissociation of viral growth from cell death was also observed with the T-22 line of SV40-transformed GMK cells. As described below, we have taken advantage of the partial dissociation of SV40 growth and cell death on rhesus kidney and T-22 cells to determine whether the virus-induced release of lysosomal enzymes into the cytoplasm and/or damage to the plasma membrane correlate with cell killing.

Lysosomal hydrolytic enzymes are released into the cytoplasm during infection by a number of cytocidal viruses including poliovirus (13), mouse hepatitis virus (2), vaccinia virus (2), Newcastle disease virus (15), herpes simplex virus (1), and SV40 (1). It has been suggested that this phenomenon, referred to as "lysosomal activation," might lead to cellular degeneration and death (2). We measured the levels of lysosomal enzyme release during SV40 infection of normal GMK, rhesus kidney, and

T-22 cells. Our results indicate that lysosomal activation follows the same time course and occurs to the same extent on all three cell types. Therefore, we suggest that lysosomal activation per se is not primarily responsible for the death of SV40-infected cells.

We also examined the effect of SV40 on the integrity of the plasma membrane during infection of normal GMK, rhesus kidney, and T-22 cells. Damage to the plasma membrane, as indicated by the release of cellular enzymes into the overlay media, occurred to a much greater extent on the normal GMK cells and was correlated with cell killing.

MATERIALS AND METHODS

Cell cultures. Normal GMK cell lines CV-1 and BSC-1 were obtained from the American Type Culture Collection and T. L. Benjamin, respectively. The SV40-transformed GMK cell lines T-22 and GMK/PARA-7-1 (particle aiding replication of adenovirus) were kindly supplied by Janet S. Butel. The T-22 cell line was transformed by Shiroki and Shimojo (17) using a heavily UV-irradiated defective virion fraction of SV40 (T-fraction). GMK/PARA-7-1 was transformed by Butel using PARA-adenovirus 7. The LLC-MK_2 line of rhesus kidney cells was obtained from the American Type Culture Collection. A-31 BALB 3T3 cells were obtained from H. L. Ozer. All cells were cultivated in Dulbecco modified Eagle medium (DME, GIBCO), containing 10% fetal calf serum (Flow Laboratories), in a humidified 5% CO_2 atmosphere. Cultures did not contain mycoplasma as indicated by autoradiography (18).

Virus. Small plaque SV40 (strain 777) was obtained from T. L. Benjamin. This virus was used in all experiments unless otherwise indicated. Virus preparations were grown on CV-1 cells from plaque isolates. SV40 was precipitated from crude lysates with methanol, extracted with trichlorotrifluoroethane (Allied Chemical), pelleted at 109,000 × *g* for 1 h, and resuspended in Hanks balanced salt solution (HBSS) containing 2% fetal calf serum.

Large plaque SV40 (Strain SV-L) was obtained from H. L. Ozer. Simian adenovirus 7 (SA-7) was obtained from T. L. Benjamin. Human adenovirus 5 (Ad 5) was obtained from the American Type Culture Collection.

Plaque assays. Subconfluent CV-1 monolayers were infected with 0.05 ml of appropriate viral dilutions in HBSS containing 2% fetal calf serum. Adsorption was for 2 h at 37 C in a humidified 5% CO_2 atmosphere. The overlay contained DME, 0.09% agar (Difco), 10% fetal calf serum, and 0.01 M HEPES [(*N*-2-hydroxyethyl)piperazine] (Sigma). Assays were incubated at 37 C in a humidified 5% CO_2 atmosphere and, unless otherwise indicated, stained 8 to 10 days postinfection with the above overlay (minus the serum) containing 0.009% neutral red.

Antisera and immunofluorescence techniques. Anti-SV40 horse serum with a homologous titer of 1:640 versus 500 mean tissue culture dose of SV40 strain 911 was obtained from Flow Laboratories. Incubation of 10^7 PFU/ml of SV40 strain 777 with an equal volume of a 50-fold dilution of this antisera for 1 h at 37 C results in a 10^4-fold decrease in titer.

To detect production of the SV40 V or T antigen by indirect immunofluorescence cells were grown on 18-mm-square glass cover slips. Cover-slip cultures were washed with HBSS, air dried, and fixed in cold acetone for 10 min. The horse anti-SV40 sera diluted 1:5 in phosphate-buffered saline (PBS) followed by fluorescein-conjugated rabbit anti-horse globulins (Miles Research Laboratories) were used to detect V antigen. Hamster antiserum to SV40 T antigen (Flow Laboratories) diluted 1:5 in PBS followed by fluorescein-conjugated rabbit anti-hamster globulins (Miles Research Laboratories) were used to detect the SV40 T antigen. Incubation with all antibodies was for 1 h at 37 C. Cover slips were washed several times with PBS after exposure to each antibody and then wet mounted with buffered glycerol and examined under dark field UV illumination.

Viable cell count. Attached cells were resuspended with 0.025% trypsin and added to the original growth media containing detached cells. Trypan blue (GIBCO) dissolved in HBSS was added to a final concentration of 0.08%. After 5 min a total cell count and a stained cell count were made using a hemocytometer.

Cell cloning. Cell suspensions were examined microscopically to confirm the absence of cell clumps. Cells were then plated in Falcon micro test plates at 0.1 cells per well. Any wells containing more than one cell, as revealed by microscope examination 24 h later, were eliminated from further consideration.

Assays for infectious centers. Monolayers containing approximately 2×10^5 cells were infected with 0.05 ml of SV40 at the indicated MOIs. After adsorption for 2.0 h at 37 C the monolayers were washed five times with HBSS and remaining unadsorbed virus was neutralized for 1 h at 37 C with anti-SV40 horse serum diluted 1:50 in HBSS. Monolayers were then washed five more times with HBSS. The infected monolayers were then resuspended with 0.025% trypsin, diluted in DME containing 10% fetal calf serum, and plated as infectious centers onto CV-1 monolayers. Results obtained with the A-31 cells indicate that essentially all unadsorbed virus is removed by these procedures (Table 1). At 22 h postinfection the liquid growth medium was removed and replaced with an agar overlay as for a plaque assay. One-step growth experiments, using these procedures, indicate that insignificant amounts of extracellular virus are released by the time the agar overlay is added (Fig. 1). Assays were stained with neutral red on day 7 as for a plaque assay.

Preparation of homogenates. Microscope examination and measurement of latent enzyme activity were used to assess the quality of homogenates prepared in different ways. The procedure that was found to give most rapid cell breakage with maximal retention of latency was the following. Monolayers were washed with HBSS and resuspended into a solution containing 40 mM KCl and 10 mM EDTA at pH 7. After 4 min on ice greater than 90% cell

TABLE 1. *Plating efficiency and viral yields of SV40 on LLC-MK$_2$, T-22, CV-1, and GMK/PARA-7-1 cells*

MOI	Infectious centers[a]					Viral yield (PFU/infectious center[a])			
	LLC-MK$_2$	T-22	CV-1	GMK/ PARA-7-1	A-31	LLC-MK$_2$	T-22	CV-1	GMK/ PARA-7-1
0.01	40		10^3			0.6×10^3		6×10^3	
0.1	4×10^2	2×10^2	6×10^3			0.8×10^3	2×10^3	3×10^3	
1.0	2×10^3	4×10^3	6×10^4	10^5	<40	0.8×10^3	10^3	3×10^3	10^3
10.0	2×10^4	3×10^4	7×10^4	10^5		2×10^3	10^3	3×10^3	10^3
100.0	10^5		10^5		60	10^3	2×10^3	4×10^3	

[a] Infectious centers were titered as described in Materials and Methods. Parallel infected cultures were harvested at 72 h, frozen and thawed, and further disrupted by sonication. Viral yields were titered by plaque assay on CV-1 monolayers.

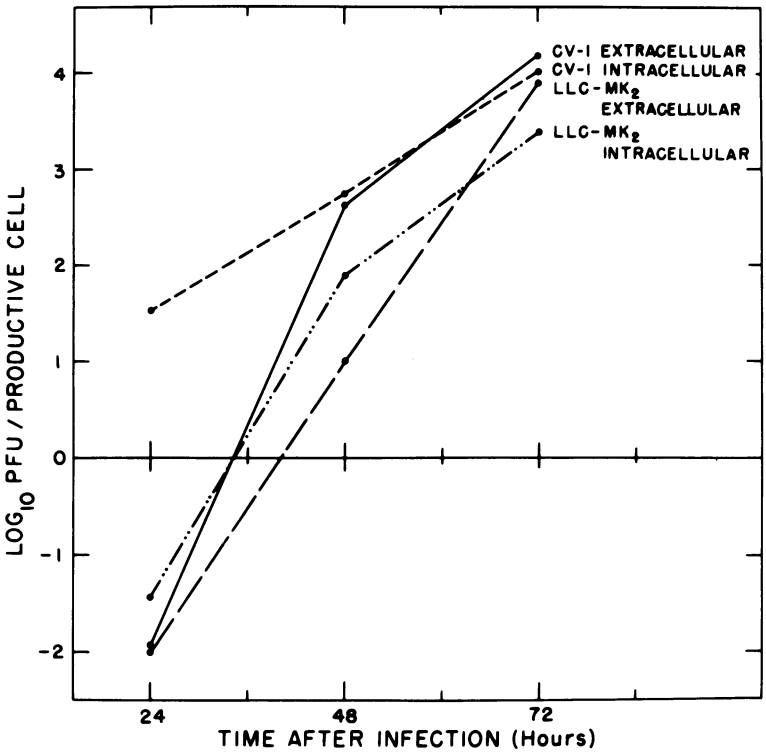

FIG. 1. *One-step growth of SV40 on CV-1 and LLC-MK$_2$ cells. Cells were infected at an MOI of 100 PFU/cell. Unadsorbed virus was removed and infectious centers were titered as described in Materials and Methods. Extracellular fluid and washed monolayers from parallel infected cultures, treated in the same way, were harvested separately at the indicated times. Extracellular fluid was clarified of any cell debris by low-speed centrifugation. Monolayers were frozen and thawed three times and further disrupted by sonication. Virus yields were determined by plaque assay on CV-1 monolayers.*

breakage was then achieved with 30 strokes of the Dounce homogenizer. An equal volume of a solution containing 0.24 M KCl and 10 mM EDTA at pH 7 was then added. The homogenate was sedimented at 20,000 × *g* for 20 min at 4 C. The supernatant containing released lysosomal enzymes was removed with a Pasteur pipette and the pellet containing intact lysosomes was resuspended into an equal volume of a solution containing 0.14 M KCl and 10 mM EDTA at pH 7. All samples were then frozen and thawed three times.

Enzyme assays. Lysosomal *N*-acetyl-β-glucosaminidase (EC 3.2.1.30) activities in supernatant and pellet fractions were assayed by the procedure of Findlay et al. (10). Activity in each fraction is assayed by measuring the release of *p*-nitrophenol

from the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide (Sigma) as indicated by the absorbance at 400 nm. Triton X-100 in the incubation mixture assures the activation of all particle-bound enzyme. Protein was measured by the method of Lowry et al. (16).

Lactic dehydrogenase (LDH) (1.1.1.27) was assayed by monitoring the rate at which pyruvate is reduced to lactate. This reduction is coupled with the oxidation of $NADH_2$ which is followed spectrophotometrically in terms of reduced absorbance at 340 nm. For this purpose Sigma kit number 340-LD was used.

Glutamic oxaloacetic transaminase (GOT) (2.6.1.1) was assayed using Worthington Biochemical Corp. determatube SGO.

RESULTS

SV40 plating efficiency and yields on T-22 and LLC-MK_2 cells. Results in Table 1 indicate that the plating efficiency of SV40 is approximately 25-fold lower on both LLC-MK_2 and T-22 cells than on CV-1 cells. This is indicated by the relative numbers of infectious centers produced on these cell lines at low input multiplicities. If the MOI is increased to 10 PFU/cell then comparable numbers of infectious centers are produced on each cell type within an order of magnitude. It is important to note that the viral yields per infected cell are nearly equivalent on LLC-MK_2, T-22, and CV-1 cells, and are independent of the MOI (Table 1). At an MOI of 10 PFU/cell SV40 growth on LLC-MK_2 and T-22 cultures is comparable to that obtained on CV-1 cultures within an order of magnitude. These results are taken to mean that viral production on LLC-MK_2 and T-22 cells is largely limited by the plating efficiency of SV40 on these cells.

Results in Fig. 1 indicate that release of SV40 from infected LLC-MK_2 cells is comparable to that on CV-1 cells. Similar results were obtained with the T-22 cell line.

A noninfectable cell fraction of T-22 and LLC-MK_2 cultures. Assays for infectious centers indicate that the number of infected LLC-MK_2 and T-22 cells approaches that of CV-1 cells at an MOI of 10 PFU/cell. Nevertheless, not all of the LLC-MK_2 and T-22 cells are susceptible to infection. As seen in Table 2, assays infectivity by the indirect immunofluorescent antibody technique for the production of the SV40 T and V antigens indicate that not more than about 50% of the LLC-MK_2 and T-22 cells become infected at an input multiplicity of 50 PFU/cell. In contrast, 100% of the CV-1 cells are infected at this MOI.

It is not yet clear why so large a fraction of the LLC-MK_2 and T-22 cells are refractory to SV40. The plating efficiency of SV40 is not limiting since not more than about 60% of the T-22 and LLC-MK_2 cells become infected at input multiplicities of 1,000 PFU/cell (Table 2). Heterogenicity of the LLC-MK_2 and T-22 cultures is not a factor because similar levels of infectivity are obtained with clonal isolates of these cell lines (Table 2). The fraction of LLC-MK_2 cells producing T antigen correlates with the fraction of productively infected cells, as shown by the production of viral antigen. Thus, the block to infection is early, before the production of T antigen.

TABLE 2. *Production of SV40 T and V antigens on CV-1, LLC-MK_2, and T-22 cells*[a]

Cell	MOI	Antigen-containing nuclei (%)	
		T antigen	V antigen
CV-1	50	100	100
CV-1	1,000	100	100
LLC-MK_2	50	47	52
LLC-MK_2	1,000	56	61
T-22	50		47
T-22	1,000		63
LLC-MK_2 clones			
1	50	58	52
2	50	61	55
3	50	44	45
T-22 clones			
1	50		42
2	50		44
3	50		46
4	50		51

[a] Cover-slip cultures were fixed for immunofluorescence at 48 h postinfection. Clonal isolates were obtained as described in Materials and Methods. The range of errors in the above values due to sampling is ± 5% to ± 8%.

Dissociation of viral growth and cell death on T-22 and LLC-MK_2 cells. When high input multiplicity infections are studied (MOI = 100 PFU/cell), the viral yields on CV-1, T-22, and LLC-MK_2 cultures are nearly equivalent. Nevertheless, the cytocidal process occurs much more slowly on LLC-MK_2 and T-22 cultures than on CV-1 cultures under these conditions (Table 3).

It is important to note the total cell number per culture every time the nonviable cell fraction is measured since those cells that were refractory at the time of infection may be increasing in number during the experiment. This would result in an underestimate of the rate at which infected cells are killed.

In the experiment presented in Table 3 the total cell number per culture was determined

TABLE 3. *Susceptibility of CV-1, LLC-MK$_2$, and T-22 cells to the SV40 cytocidal effect*

Days	Infected	CV-1		LLC-MK$_2$		T-22	
		Total cells	Nonviable fraction	Total cells	Nonviable fraction	Total cells	Non-viable fraction
0				4.6×10^4		4.1×10^4	
1	+	3.3×10^4	0	5.4×10^4	0.03	8.5×10^4	0.03
1	−	3.0×10^4	0	5.4×10^4	0.03	7.3×10^4	0.01
2	+	3.0×10^4	0.82	9.4×10^4	0.02	11×10^4	0.08
2	−	5.5×10^4	0	9.0×10^4	0.02	23×10^4	0.01
3	+	4.4×10^4	0.94	11×10^4	0.06	12×10^4	0.31
3	−	11×10^4	0	12×10^4	0.06	27×10^4	0.01
4	+			12×10^4	0.15	13×10^4	0.54
4	−			13×10^4	0.04	24×10^4	0.15
5	+			11×10^4	0.19	16×10^4	0.79
5	−			13×10^4	0.08	26×10^4	0.37

Days	CV-1[a]		LLC-MK$_2$[a]		T-22[a]
	T	V	T	V	V
1	96	88	26	1	14
2	100	100	66	46	60

[a] Percentage of antigen-containing nucleic. Cells were infected at an MOI of 200 PFU/cell.

every time the nonviable cell fraction was measured. In this experiment an attempt was also made to limit cell growth by using media partially depleted of serum growth factors. The fraction of infected cells was determined by immunofluorescent staining. Sixty-six percent of the cells of the infected LLC-MK$_2$ cultures expressed the SV40 T antigen at 2 days postinfection. It is difficult to determine the fraction of infected LLC-MK$_2$ cells before 48 h by immunofluorescence because the latent period on these cells is somewhat longer than on CV-1 cells (Fig. 1, Table 3). At any rate, little if any further cell growth occurred in the infected LLC-MK$_2$ cultures between day 2 and day 5. By 5 days only about 11% of the cells of the infected LLC-MK$_2$ culture were killed by SV40. Therefore, only about 17% of the infected LLC-MK$_2$ cells (0.11/0.66) are killed by SV40 in 5 days.

The rate of SV40-induced killing of the T-22 cells is intermediate between that of the CV-1 and LLC-MK$_2$ cells (Table 3). Sixty percent of the cells of the infected T-22 culture expressed the SV40 V-antigen at 2 days postinfection. There was little, if any, further cell growth in this culture during the next 2 days. By 4 days only 39% of the cells of the infected T-22 culture were killed by SV40. Therefore, about 65% of the infected T-22 cells (0.39/0.60) are killed by SV40 in 4 days. In contrast, 82% of the infected CV-1 cells are killed by 2 days.

Attempts to use T-22 and LLC-MK$_2$ cells in SV40 plaque assays. Numerous unsuccessful attempts were made to produce SV40 plaques on T-22 and LLC-MK$_2$ monolayers using both small plaque and large plaque strains of SV40. In these experiments monolayers were infected at either 50 or 90% confluency. Various overlays were used containing either DME, MEM, or F-12 media supplemented with either 5 or 10% calf or fetal calf serum. Infected monolayers were kept alive for as long as 35 days. Nevertheless, plaques were never observed on these cell lines, even on plates receiving 10^4 PFUs. In contrast, plaques were readily observed on CV-1 and BSC-1 monolayers by 8 days postinfection under these conditions. It may be noted that pretreatment of the T-22 and LLC-MK$_2$ monolayers with DEAE-dextran (25 μg/ml for 10 min) and/or the presence of DEAE-dextran in the overlay (10 μg/ml) did not enhance plaque formation in monolayers kept alive for 35 days. This treatment is known to enhance uptake of SV40 (5).

Passage of both small and large plaque strains of SV40 on T-22 and LLC-MK$_2$ cells did not result in virus capable of producing plaques on these cells.

There is no inherent inability of T-22 and LLC-MK$_2$ cells to produce virus plaques since these cells gave rise to SA-7 plaques as efficiently as BSC-1 and CV-1 cells. In addition, T-22 cells gave rise to human adenovirus 5 plaques as efficiently as the human cell line, L-132. These results with the T-22 cell line are in agreement with earlier observations that T-22 cells express the human adenovirus helper function as shown by their ability to support

growth of human adenovirus 7 (8).

Lysosomal enzyme redistribution. In the following experiment, we take advantage of the partial dissociation of viral growth from cell death on LLC-MK_2 and T-22 cells to determine whether lysosomal activation is correlated with the SV40-induced cytocidal process. The distribution of lysosomal *N*-acetyl-β-glucosaminidase was followed since this hydrolase displays a greater degree of latency than either acid phosphatase or β-glucuronidase (7).

Lysosomal enzyme redistribution resulting from infection is expressed as the difference between infected and control cultures with respect to the percentages of cellular *N*-acetyl-β-glucosaminidase in cytoplasmic fractions and as the specific activity of the enzyme that is achieved in the cytoplasmic fractions (Table 4). Viewed in either way the results indicate that the release of lysosomal enzyme into the cytoplasm occurs to the same extent on CV-1, LLC-MK_2, and T-22 cells. The levels of activity in the cytoplasmic fractions of the control cells are not unusual (7) and probably reflect disruption of some lysosomes during homogenization.

The fractions of nonviable cells at 48 and 72 h in parallel infected cultures are also presented in Table 4. The fractions of nonviable cells at 48 h do not correlate with the levels of *N*-acetyl-β-glucosaminidase in the cytoplasmic fractions at this time. Furthermore, the accumulation of the lysosomal hydrolase in the cytoplasmic fractions of the infected LLC-MK_2 and T-22 cultures at 48 h does not correlate with cell death over the next 24 h. These results indicate that the SV40-induced activation of lysosomal hydrolases per se is not responsible for cell death.

We also studied the time course of lysosomal enzyme release during SV40 infection of CV-1, LLC-MK_2, and T-22 cells. In three out of five experiments lysosomal activation was observed on all infected cultures by 24 h. In one experiment lysosomal activation did not occur on any cell line by 24 h. However, in all experiments similar levels of lysosomal activation were observed on each of the infected cultures by 48 h.

In some experiments a somewhat higher shift of enzyme activity to the cytoplasm resulted from infection at an MOI of 100 PFU/cell than from infection at 1,000 PFU/cell. This was apparently not due to interference at the higher MOI since equivalent viral yields were obtained at the two input multiplicities (data not shown).

Release of *N*-acetyl-β-glucosaminidase into the overlay media has not been considered in the experiments reported thus far. As described below in a different context, infected and control cells release notable amounts of activity into the overlay fluid by 48 h (Table 5). The results reported in Table 5 were obtained with cells incubated under serum-free conditions after infection. Serum-free conditions were necessary in these experiments because the levels of these enzymes in serum are high enough to obscure virus-induced changes. We tried many

TABLE 4. *N-acetyl-β-glucosaminidase activity in lysosomal (L), cytoplasmic (C), and whole cell (WC) fractions of infected and control cells at 48 h*[a]

Cell	MOI	Activity (U × 10^3/ml)			Recovery (%)	Cell-associated activity in cytoplasm (infected − control, %)[b]	Sp act in cytoplasm (U/g of protein)	Nonviable cells (%)	
		L	C	WC				48 h	72 h
CV-1	1,000	0.31	0.68	0.99	100	15	50	54	99
CV-1	100	0.22	0.87	0.96	113	26 (25)[c]	36	56	78
CV-1	0	0.56	0.66	2.1	58		16	0	0
LLC-MK_2	1,000	2.6	2.6	6.0	95	11	32	7	20
LLC-MK_2	100	1.8	4.0	5.1	113	30 (29)	50	1	8
LLC-MK_2	0	3.6	2.3	7.2	82		16	0	5
T-22	100	0.74	1.1	ND[d]	ND	20 (15)	ND	8	18
T-22	0	1.5	0.99	ND	ND		ND	1	6

[a] Results are the average of duplicate samples. One unit of activity is defined as the amount of enzyme necessary to release 1 μmol of *p*-nitrophenol per min.

[b] Difference between infected and control cultures with respect to the percentages of cell-associated activity in the cytoplasmic fraction.

[c] The data in the parentheses represent the differences between the infected and corresponding control cultures with respect to the percentages of total activated enzyme (cytoplasmic activity plus extracellular activity). Percentages of total activated enzyme were obtained by adding the fractions of extracellular activities (Table 5, 48 h) to the corresponding products of the fractions of cytoplasmic cell-associated activities (above) by the fractions of cell-associated activities (Table 5, 48 h).

[d] ND, Not done.

times to measure the activities of *N*-acetyl-β-glucosaminidase in lysosomal, cytoplasmic, and extracellular fractions under serum-free conditions to determine the total fraction of "activated" enzyme. However, we have not yet been able to obtain an acceptable level of latency and/or recovery when fractionating cells that have been incubated under serum-free conditions. To "correct" for the SV40-induced leakage of *N*-acetyl-β-glucosaminidase we combined data obtained in different experiments carried out in the presence and absence of serum. This "corrected" data appears in the parenthesis of Table 4. It was obtained as described in the legend to that table. We do not know whether leakage into the overlay fluid occurs to a similar extent in the presence of serum. At any rate, when viewing the corrected data the overall impression is that the levels of virus induced activation of lysosomal *N*-acetyl-β-glucosaminidase on CV-1, T-22, and LLC-MK_2 cultures are similar and not correlated with the rates or extent of cell killing.

TABLE 5. *Percentage of total cellular enzyme activities released into the overlay media of infected and control cultures*

Cell	MOI	Hours	Activity in overlay media (%)		
			LDH	GOT	*N*-acetyl-β-glucosaminidase
CV-1	100	24	0	0	15 (0)[a]
	0	24	0	0	15
	100	48	82 (60)	54 (50)	49 (25)
	0	48	22	4	24
LLC-MK_2	100	24	0	0	7 (1)
	0	24	0	0	6
	100	48	6 (6)	1 (1)	13 (5)
	0	48	0	0	8
	100	72	32 (32)	19 (13)	22 (9)
	0	72	0	6	13
T-22	100	24	0 (−5)	3 (−3)	16 (−3)
	0	24	5	6	19
	100	48	39 (10)	21 (4)	33 (3)
	0	48	29	17	30
	100	72	66 (20)	29 (7)	45 (11)
	0	72	46	22	34

[a] Data in parentheses are the differences between infected and control cultures. All results are the average of duplicate samples.

Leakage of cellular proteins. The release of cellular proteins from virus-infected cells has been noted previously (6, 11, 15). The damage to the plasma membrane that this phenomenon reflects is followed below by monitoring the release of the cytoplasmic enzymes, LDH and GOT, as well as lysosomal *N*-acetyl-β-glucosaminidase into the overlay media.

Since serum contains high levels of these enzymes, cultures were washed and overlayed with serum-free media at 4 h postinfection.

As seen in Table 5, infected CV-1 cells, in comparison to control cells, release notable amounts of the cytoplasmic enzymes, LDH and GOT, as well as lysosomal *N*-acetyl-β-glucosaminidase into the overlay media by 48 h. Much lower levels of enzyme are released into the overlay fluid by the infected LLC-MK_2 and T-22 cultures relative to the control cultures, at this

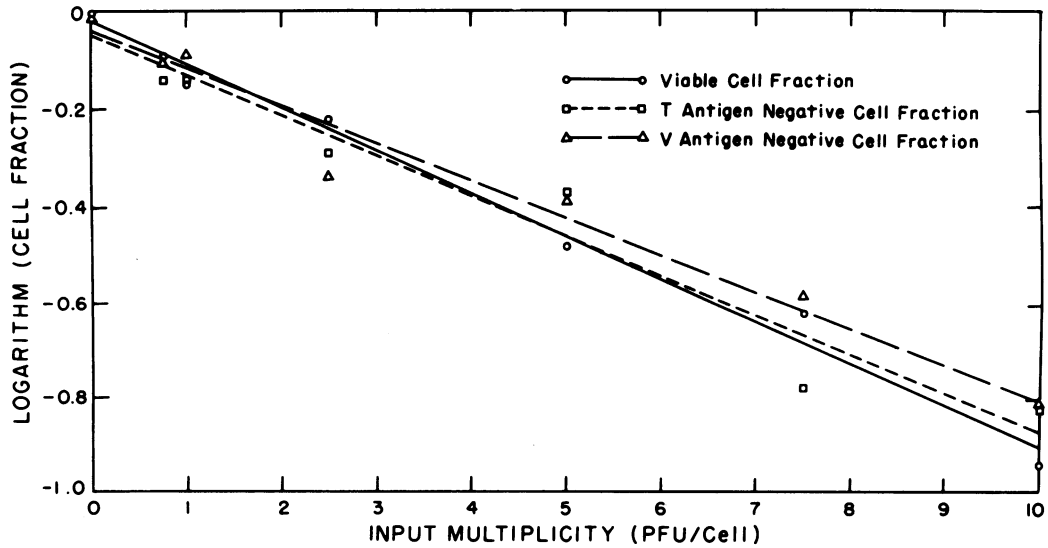

FIG. 2. *Relationship between cytopathogenicity and antigen-forming ability of SV40 on CV-1 cultures as a function of the MOI. Antigen-producing cell fractions were determined at 48 h and viable cell fractions were determined at 72 h.*

time. Even at 72 h the virus-induced enzyme leakage from LLC-MK_2 and T-22 cells is much less than that found with CV-1 cells at 48 h. This is most clear in the case of GOT. These results are taken to mean that damage to the plasma membrane, as measured by enzyme leakage from the cell, is correlated with the rate of cell killing.

MOI and cytopathic effect. Since the plating efficiency of SV40 is more than 20-fold higher on CV-1 cells than on either LLC-MK_2 or T-22 cells (Table 1), the effective MOI with a given viral inoculum may be considerably higher on the CV-1 cells than on the other two cell lines. For this reason it is necessary to determine if the rate of cell killing and/or plasma membrane damage are multiplicity dependent.

The experiment illustrated in Fig. 2 shows the relationship between the fraction of infected CV-1 cells and the fraction of cells subsequently killed at input multiplicities ranging from 0.75 to 10 PFU/cell. The fractions of CV-1 cells producing T and V antigen were determined at 48 h. The nonviable cell fractions were determined at 72 h. The results were analyzed as follows. According to the Poisson distribution the relationship between the surviving cell fraction, f_s, and the virus dosage, x, is given by $f_s = e^{-x}$, or the equivalent $1nf_s = -x$, assuming one-particle-to-kill kinetics. The graph of the values of $1nf_s$ versus the virus dosage should be a straight line. A similar relationship should exist between the logarithm of the nonantigen producing cell fraction and the virus dosage. Thus, best fit straight lines were drawn through the data in Fig. 2 using the method of least squares. The results are consistent with one-hit kinetics. The coincidence of the lines indicates the following. The fraction of T antigen-producing cells correlates with the fraction of V antigen-producing cells. This shows that once infection is initiated, as indicated by the expression of the viral T-antigen, productive infection ensues. The correspondence between the antigen-producing cell fractions at 48 h and the nonviable cell fractions at 72 h indicates statistically that infected cells are killed by 72 h regardless of the MOI. In this context it may be noted that viral yields per infected cell are independent of the MOI (Table 1).

We also measured the extent to which enzyme leakage is dependent on the MOI. Leakage of LDH and GOT from CV-1 cells was somewhat multiplicity dependent over a range of 1 to 100 PFU/cell. Nevertheless, more enzyme is released from CV-1 cells infected at an MOI of 1 PFU/cell than from LLC-MK_2 or T-22 cells infected at an MOI of 100 PFU/cell (data not shown).

DISCUSSION

The results reported above indicate that SV40 growth on both rhesus kidney and T-22 cells is largely limited by the relatively low plating efficiency of SV40 on these cells. In addition, a fraction of the rhesus kidney and T-22 cells are refractory to infection by SV40. Despite the presence of resistant cell fractions, we were able to show that infected rhesus kidney and T-22 cells are killed more slowly than infected CV-1 cells, even though the viral yields per infected cell are nearly equivalent on each cell type. In the original descriptions of the interaction of SV40 with rhesus kidney cells in culture (9, 19), these infections were characterized by slow viral growth as well as by minimal cell killing. It appears that low input multiplicity infections were being followed in these studies.

The dissociation of viral growth from cell death that we have observed during SV40 infection of rhesus kidney cell cultures is quite striking. This is surprising in view of the fact that DNA virus production is generally correlated with cell killing. Although it is known that host cell factors can influence the killing of cells by RNA viruses, our results indicate that host cell factors can also determine the time or rate at which the cytocidal process is set in motion during infection by DNA viruses as well.

To date, no single hypothesis can explain the mechanism by which viruses kill cells. Ginsberg (12) suggested that virus-induced cell damage might result from a passive role of the virus such as depletion of cellular components essential for life or mechanical harm due to excessive production of virus and its components. Our studies suggest that this is not the basis for cell killing by SV40 since infection of rhesus kidney and T-22 cells results in normal virus yields accompanied by slow cell death.

Very little is known about the virus-induced biochemical derangements that might lead to cell death. In cases where virus-induced metabolic depressions are recognized, such as the inhibition of host RNA and protein synthesis which accompanies infection by many viruses including vaccinia virus, mengovirus, and poliovirus, there is little evidence to connect these events to the virus-induced cell death (i.e., 3, 4, 14). In the case of SV40-infected simian cells there is no known inhibition of cellular metabolic functions.

One promising suggestion that has received much attention is that virus-induced redistribution of lysosomal hydrolases might lead to cellular degeneration and death (2). As noted above, "activation" of lysosomal hydrolases

occurs during infection by a number of cytopathic viruses (i.e., 1, 2, 13, 15). In particular, Allison and Black (1) have noted lysosomal activation by histochemical methods in SV40-infected GMK cells. Nevertheless, our studies comparing lysosomal activation during SV40 infection of rhesus kidney, T-22, and normal GMK cells indicate that lysosomal activation is not correlated with cell killing.

It may be noted that Allison and Black (1) also observed a less extensive, transient lysosomal activation during SV40 infection of 3T3 mouse cells. These infections are neither productive nor cytocidal. We were unable to detect lysosomal activation on SV40-infected 3T3 cells using the procedures described above (data not shown). Therefore, we suggest that lysosomes participate in SV40 growth or release, in some as yet unspecified manner, without directly causing cell death.

Damage to the plasma membrane, as indicated by the release of cellular enzymes into the overlay medium, was found to be correlated with the cytocidal process. It occurred to a much greater extent on the CV-1 cells than on either the rhesus kidney or T-22 cells.

Although we do not yet know the mechanism by which SV40 induces the observed permeability changes, a lysosomal hydrolase mechanism is not consistent with our results. Also, enzyme leakage is not simply associated with virus release (see Table 5 and Fig. 1). Much more enzyme is released into the overlay media by CV-1 cells at 48 h than by rhesus kidney cells at 72 h. Nevertheless, much more virus is released by rhesus kidney cells at 72 h than by CV-1 cells at 48 h. From this we suggest that the leakage of enzymes and virus from cells reflect different types of injury. As suggested by Gilbert (11), cellular damage may be reversible at the stage of virus release, but not after the release of enzymes.

The relatively slow SV40 cytocidal process on LLC-MK_2 and T-22 cells is not sufficient to account for the inability of SV40 to produce plaques on monolayers of these cells. No SV40 plaques are produced on these cell lines in as many as 35 days. Our results in no way suggest that virus-producing cells remain viable for this length of time. The relatively low plating efficiency of SV40 on these cells is also not a sufficient explanation since no SV40 plaques are produced on monolayers infected with as many as 10^4 PFUs. The inability of SV40 to produce plaques on these cells might involve the refractory cell fraction as well as some combination of the above factors.

The presence of SV40-resistant fractions among clonal isolates of both LLC-MK_2 and T-22 cells indicates that there can be heterogeneity of susceptibility to infection among presumably homogeneous cells. Our results suggest that resistant cells occur at random in the population and that resistance is transiently expressed.

A comparison of the interaction of SV40 with the two SV40-transformed permissive cell lines, GMK/PARA-7-1 and T-22, indicates the following dissimilarities. There is no evidence for an SV40-resistant fraction of GMK/PARA-7-1 cells. Also, SV40 produces plaques on GMK/PARA-7-1 monolayers with an efficiency equal to that on normal GMK cells (data not shown). Insofar as GMK/PARA-7-1 cells can be considered to be transformed by SV40, the comparison with T-22 cells is of interest with respect to the wide variation that has been observed in other aspects of the interaction of SV40 with various SV40-transformed GMK cells (i.e, 8). In this context, we did not detect a significant difference between T-22 and GMK-PARA-7-1 cells with respect to the rate of cell killing by SV40 (data not shown).

It may be noted that rhesus kidney cultures infected at an MOI of 100 PFU/cell, and subsequently passaged, remain viable. In the course of 3 months these cultures progress to a stable carrier state. Concurrently, all the cells acquire several properties of the transformed phenotype and express the SV40 T antigen (manuscript in preparation).

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