Superinfection of Simian Virus 40-Transformed Permissive Cells with Simian Virus 40

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Evidence that the resistance of simian virus (SV40)-transformed permissive cells to superinfection with SV40 is due to lack of virus uptake is presented. When virus uptake is enhanced, the events of infection proceed as in normal permissive cells, resulting in production of infectious virus.

In a previous study (14), we showed that simian virus 40 (SV40)-transformed permissive cells were resistant to superinfection with SV40 but were susceptible to SV40 deoxyribonucleic acid (DNA). The fact that minimal amounts of virus were adsorbed to such cells suggested the possibility that the resistance to superinfection with SV40 might be related to the changes in the cell surface of SV40-transformed cells (9, 16). In preliminary experiments, it was observed that pretreatment of cells with homogenates of mouse embryonic fibroblasts infected with polyoma virus enhanced the adsorption of SV40 to SV40-transformed cells (14). Further observations have shown that, employing a freshly prepared homogenate of mouse embryo fibroblasts (MEF) infected with polyoma virus, the adsorption of SV40 to the SV40-transformed cells was increased more than 20-fold. In addition, several chemical compounds displayed the same enhancing effect. It was, therefore, of interest to investigate whether enhanced adsorption of the virus to SV40-transformed cells would result in synthesis of infectious virus.

MATERIALS AND METHODS

Cells. The following cells were used throughout this study: (i) African green monkey kidney (AGMK) cells after one cell transfer in tissue culture; (ii) CV-1, a continuous line derived from AGMK cells (5); and (iii) EVa, clone 2A-1, AGMK cells transformed by SV40 (8). Cells were grown in Eagle's basal medium supplemented with 10% fetal bovine serum (FBS). They were maintained, after infection with SV40, in the same medium supplemented with 2% FBS (maintenance medium).

Viruses. The Rh 911 strain of SV40 was grown in CV-1 cells as described previously (14). The prepara-

tion and purification of the virus, isotopically labeled with ³H-thymidine or with ³H-leucine, have been described (1, 14). The purified preparations of labeled virus had an infectious titer of 5×10^{10} plaque-forming units (PFU) per ml and contained 2×10^6 counts per min per ml. The extraction of SV40 DNA from purified virus preparations was also described previously (14).

Preparation of an homogenate of mouse embryo fibroblasts infected with polyoma virus. Polyoma virus wild strain (kindly supplied by V. Defendi) was used to infect MEF at an input multiplicity of 0.1 to 0.01 PFU/cell. Twelve to 15 days after infection, when 75 to 100% of the cells showed cytopathic effect, the cultures were frozen and thawed once and subjected to sonic oscillation (Raytheon, model 10KC, twice for 1 min each time at 1 amp and 100 v). The cell lysate was then centrifuged at $1,600 \times g$ for 10 min at 4°C, and the supernatant was decanted and stored at 4°C. The pellet was resuspended in 5 ml of maintenance medium containing 250 units per 0.5 ml of receptor-destroying enzyme (RDE). After incubation at 37°C overnight in an atmosphere of CO₂, the mixture was centrifuged at $1,600 \times g$ for 10 min. The sediment containing cell debris was discarded, and the supernatant fluid was pooled with the supernatant fluids of the first centrifugation. Homogenates of mouse embryo fibroblasts infected with polyoma virus (MEF-Py) had an hemagglutinating titer of 128 to 256 hemagglutinating units per ml for guinea pig red blood cells and an infectious titer of 1.3×10^8 to 2.6×10^8 PFU per ml on MEF. Purification of polyoma virus from the homogenate of infected MEF was performed by centrifugation at equilibrium in CsCl as already described for SV40 (1). Homogenates of noninfected MEF were prepared in the same way with (MEF-1) or without (MEF-2) RDE treatment.

Chemicals. The following chemicals were used: lipase (Worthington Biochemical Corp., Freehold, N.J.); phospholipase C (Calbiochem, Los Angeles, Calif.); phospholipase D (Calbiochem); pancreatic deoxyribonuclease (deoxyribonuclease I, Sigma Chemical Co., St. Louis, Mo.); phosphodiesterase (Sigma Chemical Co.); sodium desoxycholic acid

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(Nutritional Biochemicals Corp., Cleveland, Ohio); RDE (Behring Diagnostics, Woodbury, N.Y.), diluted in maintenance medium to a final concentration identical to that of RDE contained in MEF-Py; neuraminidase (Sigma Chemical Co.), diluted in Brinster's medium (3), pH 5.5, and tested at concentrations ranging from 1,000 to 0.1 $\mu\text{g}/\text{ml}$; trypsin (Flow Laboratories, Rockville, Md.) diluted in phosphate-buffered saline (PBS) and tested at concentrations ranging between 0.1 and 0.0025%; histones (Worthington Biochemical Corp.), diluted in a small volume of 0.1 N HCl and brought to a concentration of 500 $\mu\text{g}/\text{ml}$ with Brinster's medium, final pH, 7.2; diethylaminoethyl (DEAE)-dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) dissolved in PBS without calcium and magnesium at a concentration of 500 $\mu\text{g}/\text{ml}$; heparin, sodium salt, 160 USP/mg (City Chemical Co., New York, N.Y.), and dextran sulfate, sodium salt (Pharmacia), dissolved in maintenance medium at a concentration of 500 $\mu\text{g}/\text{ml}$.

Uptake of SV40 by pretreated cells. Confluent monolayer cultures containing 3×10^6 cells were infected with either ^3H -thymidine- or ^3H -leucine-labeled SV40 at an input multiplicity of 100 PFU/cell (input radioactivity, 1.5×10^4 counts per min per culture). After incubation at 37 C for different time intervals, the inoculum was removed. The cells were washed three times with PBS, dispersed with trypsin, and disrupted by sonic oscillation. The acid-precipitable radioactivity contained in the cell lysate was then determined as described previously (1).

Infection of cells with purified SV40 and with SV40 DNA. When the susceptibility of cells to SV40 was tested, purified unlabeled virus at an input multiplicity of 30 PFU/cell was employed. Adsorption was carried out for 2 hr at 37 C. After addition of maintenance medium, cells were incubated at 37 C and harvested 24 and 72 hr after infection.

Cells were infected with SV40 DNA at an input multiplicity of 0.4 PFU/cell by a method already described (14). Virus titrations were made by plaque assay in CV-1 cells (14). In all experiments, the moment of exposure of the cells to the virus or to its DNA was considered as the starting time of infection.

Disruption of cells and separation of the nuclear and cytoplasmic fractions. At various intervals after infection, cell monolayers were washed three times with PBS. The cells were dispersed with trypsin and sedimented by centrifugation at $1,000 \times g$ for 4 min at 4 C. The pelleted cells were suspended in NTM buffer [0.14 M NaCl, 0.01 M tris-(hydroxymethyl)-aminomethane (Tris)-hydrochloride, 0.0015 M MgCl_2 , pH 7.4] and sedimented again. The cells were then disrupted by resuspending them in 2 ml of 0.5% Nonidet P-40 (NP-40) nonionic detergent (Shell Chemical Co., New York, N.Y.) in NTM buffer (2, 10). After incubation at 0 C for 10 min with occasional shaking, the suspension was centrifuged at $1,600 \times g$ for 2 min at 4 C. The cytoplasmic supernatant fluid was decanted, and the sedimented nuclei were washed once in NTM buffer. The treatment of nuclear and cytoplasmic fractions with deoxyribo-

nuclease and phosphodiesterase was described previously (1).

Isopycnic centrifugation in CsCl solution of SV40 recovered from nuclei of infected cells. Nuclei isolated from infected cells were suspended in 0.6 ml of 0.5% NP-40 in NTM buffer. Sodium deoxycholate (DOC) at a final concentration of 1%, lipase at a final concentration of 2 mg/ml, phospholipase C at a final concentration of 0.5 mg/ml, and phospholipase D at a final concentration of 1 mg/ml were added. (Tenfold concentrated stock solutions of these substances were previously prepared in distilled water.) The mixture (final volume 1 ml) was incubated for 15 min at 37 C with frequent agitation and sedimented at $1,600 \times g$ for 10 min at 4 C. The supernatant was then centrifuged at equilibrium in a CsCl solution by a technique previously described (1). Fractionation of gradients and determination of the total radioactivity were also described (1).

Immunofluorescence. The presence of the viral coat protein (VP) antigen in cells at different times after infection with SV40 or with SV40 DNA was detected by an indirect immunofluorescence test, employing a rabbit anti-SV40 serum and a fluorescein-conjugated goat anti-rabbit gamma globulin (Hyland Laboratories, Los Angeles, Calif.).

Autoradiography. At various intervals after infec-

TABLE 1. Uptake of simian virus 40 by pretreated cells^a

Pretreatment with	Per cent of input ^3H -thymidine-SV40 adsorbed to cells	
	2A-1	AGMK
Maintenance medium..	0.2 (9) ^b	13.1 $\pm$ 1.7 (3)
MEF-1	1.3 $\pm$ 0.1 (2)	14.2 $\pm$ 2.1 (2)
MEF-2	1.8 $\pm$ 0.3 (3)	15.6 $\pm$ 2.0 (3)
MEF-Py	4.2 $\pm$ 0.7 (9)	21.3 $\pm$ 2.8 (3)
Purified polyoma virus..	0.2 (2)	
RDE	0.6 (2)	
Neuraminidase ^c	0.6 (2)	
Trypsin ^b	0.4 (2)	
Histones ^c	3.4 $\pm$ 0.5 (3)	
DEAE-dextran ^c	1.8 $\pm$ 0.2 (2)	
Heparin	0.1 (1)	
Dextran sulfate	0.2 (1)	

^a African green monkey kidney cells were incubated for 90 min at 37 C with 1 ml of maintenance medium, MEF-1, MEF-2, and MEF-polyoma virus (Py). 2A-1 cells were incubated for 90 min at 37 C with 1 ml of the first four preparations as well as with purified polyoma virus, and for 30 min at 37 C with 1 ml of receptor-destroying enzyme (RDE), neuraminidase, histones, DEAE-dextran, heparin, and dextran sulfate. Cells treated with trypsin were incubated for 20 min at room temperature and then washed with medium containing 10% serum. All of the cultures were then washed three times with phosphate-buffered saline and infected with SV40. After adsorption for 2 hr at 37 C, the cells were processed as described in the text.

^b Numbers in parentheses indicate number of experiments.

^c Cells show slight toxic effect.

^d Used at concentration showing highest adsorption of SV40.

tion with ^3H -thymidine- or ^3H -leucine-labeled SV40, cells seeded on cover slips were fixed in methanol and covered with strips of photographic emulsion (Kodak AR.10). After 20 days of exposure, autoradiograms were developed and stained with Giemsa.

RESULTS

Uptake of SV40 by cells. The results of pre-treating cells prior to adsorption of SV40 (Table 1) indicate that 2A-1 cells pretreated with maintenance medium adsorb about 60 times less virus than do secondary AGMK cells. 2A-1 cells pretreated with a homogenate of mouse embryo fibroblasts infected with Py adsorbed 20 times more virus than did untreated 2A-1 cells. Pretreatment of 2A-1 cells with histones resulted in 17-fold higher adsorption of SV40. DEAE-dextran and a homogenate of noninfected mouse embryo fibroblasts also enhanced the adsorption of SV40 to 2A-1 cells. No effect was observed after the 2A-1 cells were pretreated with purified polyoma virus, RDE, neuraminidase, trypsin, and polyanions (heparin and dextran sulfate). Pretreatment with MEF-1, MEF-2, and MEF-Py increased the adsorption of SV40 to AGMK cells, although to a lesser degree than to 2A-1 cells.

Because the MEF-Py homogenate was most effective on 2A-1 cells, this preparation was used in all subsequent experiments.

Kinetics of adsorption of SV40 onto 2A-1 cells pretreated with MEF-Py. Within 15 min after exposure, 2.2% of the input radioactivity of the ^3H -thymidine-labeled virus became cell-associated. The virus uptake then increased until 90

FIG. 1. Kinetics of uptake of SV40 by 2A-1 cells. 2A-1 cells were pretreated with MEF-Py as described in footnote a, Table 1. Cells were then infected with ^3H -thymidine-labeled SV40 and incubated at 37°C. At intervals after infection, they were harvested and processed as described in the text.

FIG. 2. Distribution of SV40 proteins and SV40 DNA between the cytoplasm and the nuclei of infected cells and sensitivity of the viral DNA to nucleases. Monolayer cultures containing 6×10^6 2A-1 cells were pretreated with MEF-Py and infected with ^3H -thymidine- or ^3H -leucine-labeled SV40 at an input multiplicity of 100 PFU/cell (input radioactivity, 3×10^4 counts per min per culture). Cells were harvested at intervals after infection, and the nuclei were separated from the cytoplasm as described in Materials and Methods. The acid-precipitable radioactivity contained in the cytoplasmic (a) and in the nuclear (b) fractions was determined. In cells infected with ^3H -thymidine-labeled virus, the acid-precipitable radioactivity of the fractions was determined also after treatment with deoxyribonuclease and phosphodiesterase. Each point represents mean values from four independent experiments. Symbols: ●, ^3H -leucine radioactivity; ■, ^3H -thymidine radioactivity; ○, ^3H -thymidine radioactivity refractory to the action of deoxyribonuclease and phosphodiesterase.

min after infection, when 4.5% of the total input radioactivity was adsorbed to the cells. Between 90 and 120 min after infection, a slight decrease in the cell-associated viral radioactivity was observed (Fig. 1).

Distribution of viral components in the cytoplasm and nuclei of infected cells: kinetics of uncoating of SV40. The distribution of the ^3H -leucine-, ^3H -thymidine-labeled, or both, viral components be-

FIG. 3 Autoradiography of 2A-1 cells infected with ^3H -thymidine-labeled or ^3H -leucine-labeled SV40. (A) 2A-1 cells infected with ^3H -thymidine-labeled SV40 and (B) with ^3H -leucine-labeled SV40 at 1.5 hr after infection. (C) 2A-1 cells infected with ^3H -thymidine-labeled SV40 and (D) with ^3H -leucine-labeled SV40 at 2.5 hr after infection.

tween the cytoplasm and the nuclei of SV40-infected 2A-1 cells was determined from 0.5 hr until 24 hr after infection. At the same time, the uncoating of SV40 in the cytoplasm and in the nuclei

was investigated by assaying the sensitivity of the viral DNA to the action of nucleases (Fig. 2).

The time dependence of the distribution of the ^3H -viral protein and ^3H -viral DNA followed a

FIG. 3 (cont'd). Autoradiography of AGMK cells infected with ^3H -thymidine-labeled or ^3H -leucine-labeled SV40. (E) AGMK cells infected with ^3H -thymidine-labeled SV40 and (F) with ^3H -leucine-labeled SV40 at 2.5 hr after infection.

similar pattern in the nuclei and in the cytoplasm. At 2.5 hr after infection, approximately 50% of both the ^3H -thymidine and ^3H -leucine cell-associated radioactivity was recovered in the nuclei. The highest concentration of isotope with each radioactive precursor in the cytoplasm was observed at 4 hr after infection when the radioactivity of the nuclear fraction decreased. Later, a progressive decline of the acid-precipitable radioactivity was observed in both the nuclear and cytoplasmic fractions. About 25% of the total cell-associated ^3H -thymidine or ^3H -leucine acid-precipitable radioactivity was recovered in the medium between 2 and 24 hr after infection.

SV40 DNA contained in the cytoplasmic fraction (Fig. 2a) was sensitive to the action of nucleases by 0.5 hr after infection. At 4 hr after infection, 47% of the acid-precipitable ^3H -thymidine radioactivity contained in the cytoplasm was degraded by nucleases. The sensitivity to nucleases of the intranuclear viral DNA started between 1 and 2.5 hr after infection (Fig. 2b). At this time, 34% of the acid-precipitable ^3H -thymidine radioactivity contained in the nuclei became sensitive to the action of the enzymes.

Autoradiography of 2A-1 cells infected with either ^3H -leucine- or ^3H -thymidine-labeled SV40 confirmed the presence of both viral proteins and

viral DNA in the nuclei of infected cells soon after infection. Indeed, from 1 to 1.5 hr after infection, both ^3H -leucine- and ^3H -thymidine-labeled materials appeared to be in the process of penetrating the nuclei. Between 2 and 3 hr after infection, about 40% of the cells exhibited selective concentration of the label in the nuclei. At the same time, about 95% of AGMK cells showed presence of the label, and these cells, in contrast to 2A-1 cells, contained many more grains per nucleus (Fig. 3).

Isolation of SV40 from nuclei of infected cells. Intact SV40 virions banding in CsCl at a density of 1.34 g/cm³ were recovered 2.5 and 6 hr after infection in the nuclei of 2A-1 cells infected with ^3H -thymidine-labeled SV40 (Fig. 4).

Production of VP and infectious virus by 2A-1 cells superinfected with SV40. The experiments described above have shown that, if the viral uptake is enhanced, SV40 reaches the nucleus of 2A-1 cells within a short time after penetration.

Since the 2A-1 cells are susceptible to SV40 DNA (14), it was interesting to investigate whether, under conditions of increased viral adsorption, they became susceptible also to superinfection with the whole virus. To investigate this problem, 2A-1 cells were pretreated with MEF-Py and then infected with SV40; the percentage

FIG. 4. Isolation of parental SV40 from nuclei of infected cells. Confluent monolayer cultures containing 6×10^6 2A-1 cells were pretreated with MEF-Py and infected with ^3H -thymidine-labeled SV40 at an input multiplicity of 100 PFU/cell (input radioactivity, 3×10^4 counts per min per culture). At 2.5 and 6 hr after infection, cells were harvested and the nuclei were isolated as described in Materials and Methods. Nuclei were disrupted and analyzed by equilibrium centrifugation in CsCl density gradients. In parallel, purified and labeled SV40 used for the infection of cells was analyzed. Symbols: $\square$, density at 25°C ; $\bullet$, total radioactivity.

of cells showing positive staining for VP antigen was observed. In addition, the amount of infectious virus produced in these cells was determined in comparison to that produced in AGMK cells and in 2A-1 cells infected with SV40 without MEF-Py pretreatment. In the same experiments, AGMK and 2A-1 cells were also infected with SV40 DNA and examined for production of VP and infectious virus (Table 2). The results showed

that when 2A-1 cells were treated with MEF-Py before superinfection with SV40, the percentage of cells producing VP was 1,500 times higher than in the culture of untreated 2A-1 cells, and that the virus yield was 6.3 log/ml 3 days after infection. In contrast, only 4.6 log/ml of SV40 was recovered from 2A-1 cells without MEF-Py pretreatment, and no increase in virus yield was observed between the 1st and 3rd day after infection. The percentage of 2A-1 cells producing VP was ten times less than the percentage of VP-positive AGMK cells 3 days after infection. The virus yield of SV40-infected 2A-1 cells, pretreated with MEF-Py, was seven times less than the virus yield of AGMK cells 3 days after infection.

Conversely, 2A-1 and AGMK cells produced comparable amounts of VP and infectious virus at 3 days after infection with SV40 DNA. The percentage of VP-positive cells then increased until 7 days after infection in the culture of AGMK cells, whereas it stayed constant in 2A-1 cells. Control 2A-1 cells not superinfected with SV40 did not show presence of VP and did not produce infectious virus.

DISCUSSION

The results presented in this report indicate that it is possible to enhance adsorption of SV40 on SV40-transformed permissive cells, which without pretreatment adsorb only negligible amounts of the superinfecting virus. Under the conditions of increased virus adsorption, viral particles penetrate the cells and can be recovered in the nuclei. After uncoating, the VP is synthesized in the cells which, in contrast to untreated cells, produce infectious virus.

Even after treatment with MEF-Py, uptake of SV40 by 2A-1 cells was still lower than by the AGMK cells. This difference alone may account for the lesser number of grains seen on autoradiography in the nuclei of 2A-1 cells in comparison to nuclei of AGMK cells, for the lower percentage of 2A-1 cells synthesizing VP antigen, and for the production of lesser amounts of infectious virus.

The fact that the amount of virus recovered from the nuclei of 2A-1 cells was only one-twelfth of the adsorbed virus as compared to one-sixth in the case of AGMK cells (1) indicates that penetration of the virus into the nucleus of 2A-1 cells may be hampered. Finally, one should not overlook the possibility that nuclear uncoating of the virus in 2A-1 cells is inhibited, as indicated by the fact that a lesser amount of deoxyribonuclease-sensitive viral DNA was recovered from the nuclei 2.5 hr after infection as compared to the nuclei of AGMK cells (1) and that no peak corresponding to the uncoated virus (1) could be detected by

TABLE 2. Production of infectious virus by cells exposed to SV40 or SV40 DNA^a

Cell type	MEF-Py ^a pretreat- ment	Infection with SV40	Infection with SV40 DNA	Percentage of VP-positive cells after infection				Virus yield		
				Day 2	Day 3	Day 5	Day 7	Log PFU/ml after infection		Avg PFU/cell
								Day 1	Day 3	
AGMK	—	+	—	38.0	84.5	96.3	NT ^b	5.8	7.1	12.5
2A-1	+	+	—	1.0	8.3	9.6	7.8	5.5	6.3	2.0
2A-1	—	+	—	0	0.005	0.007	NT	4.7	4.6	0.04
2A-1	—	—	—	0	0	0	0	0	0	0
AGMK	—	—	+	0.2	1.9	4.2	6.8	NT	5.4	0.2
2A-1	—	—	+	0.3	1.7	1.9	1.8	NT	5.0	0.1

^a Abbreviations: VP, viral protein; SV40, simian virus 40; MEF-Py, mouse embryonic fibroblasts infected with polyoma virus; PFU, plaque-forming units; AGMK, African green monkey kidney.

^b Not tested.

CsCl centrifugation of the intranuclear virions at 6 hr after infection. The inhibition of the uncoating mechanism has been postulated by Sauer and Hahn (13) to be the main factor of resistance of SV40-transformed cells to superinfection.

Regardless of the nature of the actual mechanism or combination of mechanisms which are operative in 2A-1 cells, the failure of viral uptake seems to be the primary mechanism of their resistance to SV40. Challenge of these cells with SV40 DNA results in the same percentage of VP-positive cells and in production of comparable amounts of infectious virus as does challenge of AGMK cells.

These findings also corroborate previous results (6, 14) which indicate the absence of a repressor (4) of SV40 replication in SV40-transformed cells. This is further supported by the results of Rapp and Trulock (12) who found SV40-transformed BSC-1 cells resistant to superinfection with SV40 but capable of supporting the replication of a defective SV40 genome, encased in an adenovirus capsid, and linked with adenovirus DNA which penetrates the cells.

It is doubtful that the enhancing effect of MEF-Py and other compounds on virus uptake is mediated by a specific virus-receptor interaction. Indeed, in contrast to AGMK cells (1), 2A-1 cells exhibit a decrease in the amount of virus adsorbed between 90 and 120 min after exposure. This is followed by elution of considerable amounts of virus into the medium between 2 and 24 hr after exposure.

The mechanism of stimulation of SV40 uptake by SV40-transformed cells upon the different treatments employed in this study is only a matter of speculation. That the enhancing effect of MEF-Py on virus uptake is caused by polyoma virus can be ruled out because purified polyoma virus showed no such activity. The increase of viral

uptake obtained by pretreating 2A-1 cells with polycations, such as histones and DEAE-dextran, suggests the possibility that the effect of these compounds is mediated by the modification of the electric charges on the surface of SV40-transformed cells. Similar observations on enhancement of viral uptake by polycations were reported for rabies virus (7), poliovirus (11), and rubella virus (15). The presence of a positively charged compound in MEF homogenates, which is released in higher concentrations by the lytic effect of polyoma infection, could be postulated within the context of this hypothesis.

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