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Inactivating and Mutagenic Effects of Nitrosoguanidine on Simian Virus 40*

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Abstract. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) in high concentrations inactivates extracellular simian virus 40. No mutants were found in stocks of virions partially inactivated by NTG under the experimental conditions employed. In contrast, NTG in low concentrations inhibits the intracellular replication of simian virus 40 and is an effective mutagen of the intracellular virus. Six temperature-sensitive mutants of simian virus 40 have been isolated for genetic studies of the virus and its interaction with mammalian cells.

Simian virus 40 (SV40) is capable of transforming cells *in vitro*[1,2] and of inducing neoplasms in animals.[3,4] Several lines of evidence suggest that the DNA of the virus is integrated into the DNA of transformed cells[5,6] and can be recovered from them in the infectious form.[7] In most transformed cells only a portion of the genome is expressed.[8–12] The important question remains whether the portion of the genome thus expressed is responsible for the maintenance of the transformed state. Because of the small size of the SV40 genome,[13,14] genetic analysis of all essential viral functions should be possible through the isolation of conditional lethal mutants. Such analysis could provide specific information on the replication of SV40, the integration of genetic material into mammalian cells, the control of genetic expression in cells, and the mechanism of virus-induced neoplastic transformation.

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) has been used extensively as an effective mutagen for DNA-containing organisms and viruses.[15–17] This communication describes the effect of NTG on the infectivity of extracellular virions and extracted DNA and on the replication of intracellular virus. These effects are then correlated with the induction of temperature-sensitive and plaque mutants of SV40 by NTG. A preliminary report concerning the isolation of two of the temperature-sensitive mutants described here has been previously presented.[18]

Materials and Methods. Cell culture: Grivet monkey kidney cells, line AH-1,[19] were used for the replication, assay, and cloning of SV40. The cells were always cultivated in Eagle's basal medium with 10% fetal calf serum.

SV40 virus: SV40 strain VA 45-54[20] was genetically purified by four consecutive plaque passages at 39°C. The resulting parental clone forms plaques of intermediate size and has been designated SV40-W. All stocks of virus were prepared by inoculat-

ing AH-1 cells with dilute suspensions of virus. When cell destruction was marked, the cultures were frozen and thawed twice, spun at 1200 × *g*, and stored at −60°C.

Virus assay: AH-1 monolayers were grown in 35-mm Falcon plastic Petri dishes. The virus inoculum of 0.2 ml per plate was allowed to adsorb for 2 hr at 39°C. The plates were then overlaid with 3 ml of Eagle's basal medium with 1% agar, 0.5% lactalbumen hydrolysate, and 5% fetal calf serum and incubated at the appropriate temperature in an atmosphere of 5% CO_2. A second agar overlay of 2 ml containing neutral red (1:40,000) was added after a 10-day incubation.

Extraction and assay of infectious DNA: Viral DNA was extracted from cells late in the course of infection and assayed on AH-1 cells by the method of Pagano.[21] Infected cells were suspended in phosphate-buffered saline free of Ca^{2+} and Mg^{2+} and containing 0.5% sodium dodecyl sulfate and 0.008% ethylenediaminetetraacetate, pH 7.2. An equal quantity of cold water-saturated phenol was added, and the mixture was shaken for 10 min at 4°C. After centrifugation at 750 × *g* for 15 min, the aqueous phase was re-extracted twice. The final extraction was dialyzed in phosphate-buffered saline overnight at 4°C to remove the phenol. The DNA was plaque-assayed for infectivity in the buffer containing 1 mg/ml DEAE-dextran, mol wt 2 × 10^6 (Pharmacia, Uppsala, Sweden). The infectious DNA was treated with neutralizing antibody and with DNase to confirm that the extracted DNA and not residual virions accounted for its infectivity. Prior to use for this purpose, 5 ml of rabbit anti-SV40 serum (Microbiological Associates) was precipitated with 5 ml of saturated $(NH_4)_2SO_4$ and the precipitate was dissolved in 100 ml of the buffer without Ca^{2+} or Mg^{2+}. The DNA suspension was exposed to an equal volume of anti-SV40 antibody for 30 min at 31°C. Bovine pancreatic DNase purified by electrophoresis was obtained from Worthington Biochemical Corp. Extracted DNA was exposed to 20–200 Kunitz units of DNase in the buffer with Ca^{2+} and Mg^{2+} at pH 5.5, 31°C for 30 min.

Preparation of nitrosoguanidine: NTG (Aldrich Chemical Co.) was dissolved in phosphate-buffered saline and sterilized by filtration. Solutions were kept frozen in the dark until used. The final pH of the solutions was 7.0.

Exposure of extracellular virions and DNA to NTG: Pools of virions were filtered through 50-nm membranes (Millipore Corp.) to obtain nonaggregated virus.[22] Virions and extracted DNA suspended in phosphate-buffered saline were each diluted tenfold in solutions of NTG at 31°C. Samples of 1 ml were removed at intervals, diluted in 9 ml of cold PBS, and dialyzed at 4°C overnight against large quantities of buffer. The samples were stored at −60°C. Prior to examination for the presence of mutants, a portion of the virus stock exposed to NTG for 3 hr was regrown in AH-1 cells (input multiplicity 0.2 plaque-forming units/cell) at 31°C for 5 days so that any potential mutation would be represented in both strands of the viral DNA.

Exposure of replicating virus to NTG: Tube cultures of AH-1 cells were infected with a multiplicity of 5 plaque-forming units/cell. After adsorption for 2 hr at room temperature, the cultures were washed three times, and medium with varying concentrations of freshly thawed NTG was added. The tubes were incubated in the same medium for 5 days at 30°C and then harvested as described above. Control cultures were inoculated with either virus or NTG alone and were observed for cytopathic effect.

Examination of treated virus for the presence of temperature-sensitive mutants: Virus exposed to NTG was appropriately diluted in Hanks' salt solution and 0.2 ml were adsorbed to AH-1 cell monolayers in Petri dishes at 31°C for 2 hr. The inoculum was removed and the cells were overlaid with nutrient agar. After 24-days incubation at 31°C, well-isolated plaques were picked by gentle aspiration with a capillary pipette. The agar plug was suspended in 2 ml Hanks' solution with 2% fetal calf serum and stored at −60°C. To detect temperature-sensitive mutants, 0.2 ml of each undiluted suspension was added to AH-1 monolayers at 39°C. After a 2-hr adsorption period, the cells were overlaid with agar and incubated at 31 and 39°C. The clones which yielded no plaques at 39°C but which produced many plaques or lysed the plates at 31°C were scored as temperature-sensitive mutants. For further studies each mutant was cloned a

second time by plaque purification and virus stocks were prepared in AH-1 cells in 32-oz bottles at 31°C.

Evaluation of treated virus for the presence of plaque mutants: Undiluted suspensions of most of the clones which were tested for temperature sensitivity contained enough virus to completely lyse the AH-1 monolayers. Those plates in which the monolayers were not completely lysed at 31°C were also evaluated for plaque size.

Results. The effect of temperature of incubation on the replication of SV40: Prior to the search for temperature-sensitive mutants, preliminary experiments were performed to determine the temperature characteristics of SV40-W growth in AH-1 cells. Tube cultures of AH-1 cells were inoculated at a multiplicity of approximately 50 plaque-forming units/cell, washed after a 2-hr adsorption at 25°C, and incubated at a wide range of temperatures. Individual cultures were harvested at intervals and the yield of infectious virus was titered after freezing and thawing three times. The effect of incubation temperature on the maximal yields of SV40 is presented in Figure 1. The virus replicated well at temperatures ranging from 31 to 42°C. The attainment of maximal levels of production of new virus was followed shortly by extensive cytopathic effect. At 25°C the production of virus was markedly reduced and lysis of cells was not observed. At 44°C the number of plaque-forming units released per cell culture was less than the number that remained immediately after adsorption and washing. Extensive degenerative changes were observed in cells of both infected and control cultures maintained at this temperature.

The effect of the temperature of incubation on the time required for the production of initial and maximal yields of SV40 is shown in Figure 2. The time required to reach maximal production decreased from 120 hr at 31°C to 60 hr at 39°C and then increased to 72 hr at 42°C.

The efficiency of plaque formation by SV40 in AH-1 cells did not vary appreciably over the temperature range from 31 to 39°C. However, the time required for the development of well-defined plaques was 20 days at 31°C and 10 days at 39°C. Based on this information, 31°C was selected as the permissive temperature and 39°C was used as the restrictive temperature in the subsequent attempts to identify temperature-sensitive mutants.

Effects of exposure of extracellular SV40 to NTG: A 1-ml suspension of 10^8 plaque-forming units of virions in phosphate-buffered saline and a 1-ml suspension containing 10^5 units extracted viral DNA in the buffer were added to solutions of 9 ml NTG. Samples were removed, diluted, dialyzed, and titrated for infectivity at hourly intervals. Virus exposed to 100 μg/ml NTG for 3 hr was not measurably inactivated. After exposure to 5000 μg/ml NTG for 3 hr both virions and extracted DNA lost more than 90% of their infectivity (Fig. 3). Preparations of extracted DNA were shown to be free of residual virions by their susceptibility to DNase, their resistance to neutralizing antibody, and their requirement for DEAE-dextran in plaque titration (Table 1).

In one experiment the NTG solution was preincubated at 31°C for 24 hr before mixture with the virus suspension to determine the effect of these conditions on the stability of NTG. It was found that the capacity of NTG to inactivate the virus was reduced by more than half in this preincubation.

Exposure of SV40-infected cells to NTG: Monolayers of AH-1 cells which had

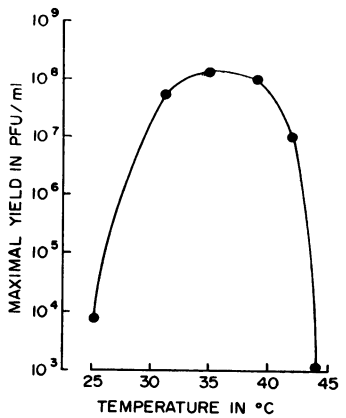

FIG. 1.—Maximal yields of SV40 at different temperatures following a single cycle of replication in AH-1 cells inoculated at a multiplicity of 50 plaque-forming units/cell.

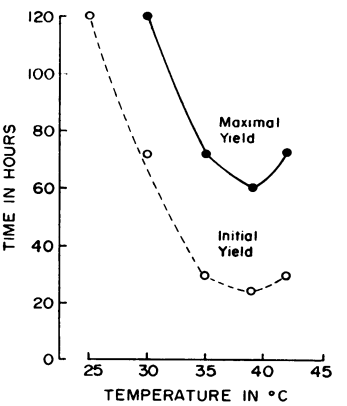

FIG. 2.—Time required for the production of initial and maximal yields of SV40 at different temperatures in AH-1 cells inoculated at a multiplicity of 50 plaque-forming units/cell.

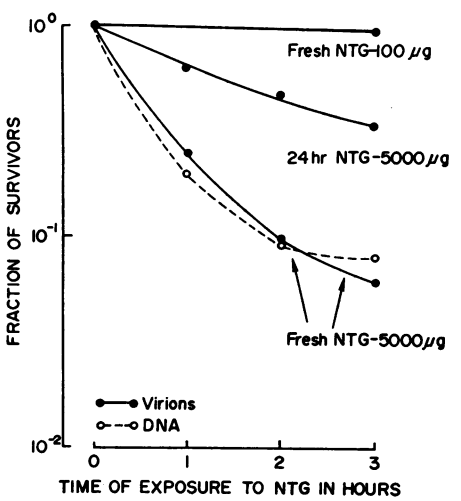

FIG. 3.—The inactivating effect of NTG on extracellular SV40 virions and infectious DNA at 31°C, pH 7.0. Fresh NTG was used immediately after a frozen stock was thawed; 24-hr NTG was part of the same frozen stock but was preincubated at 31°C for 24 hr prior to inactivation of SV40. Each value is based on duplicate determinations.

adsorbed SV40 for 2 hr were continuously exposed to NTG in concentrations ranging from 0.625 to 40 μg/ml, and the yield of virus from a single growth cycle at 30°C was subsequently determined (Fig. 4). At the high concentrations of NTG, virus yield did not differ significantly from the amount of the residual virus inoculum, and the toxic effect of the drug on uninfected cells was marked. At low concentrations of NTG the yield of virus approached that of control cultures without NTG, and no toxic changes were observed in cultures without SV40. At intermediate concentrations of NTG ranging from 2.5 to 10 μg/ml, virus replication was partially inhibibited. Cultures exposed to NTG alone at these concentrations showed the loss of approximately 25–75% of cells from

TABLE 1. *Survival and titration of virions and DNA under different conditions.*

Condition	Residual Infectivity Virions (%)	DNA (%)
DNase (20 Kunitz units)	~100	<1
DNase (200 Kunitz units)	~100	<1
Titration without DEAE-dextran	~100	<1
Anti-SV40 antibody	<1	~100

the monolayer. Cultures recovered fully if medium without NTG was added after 5 days.

Isolation of mutants: Virus exposed to NTG was plaqued on AH-1 monolayers at 31°C and 1342 well-separated plaques were picked.

The plaque progeny of SV40 treated extracellularly for 3 hr with 5000 μg NTG/ml were found to contain no temperature-sensitive mutants among 250 clones isolated directly and among 250 clones isolated after regrowth at 31°C for 5 days as described in *Materials and Methods*. The suspensions of 141 of these clones were sufficiently dilute to allow assessment of plaque size on plates inoculated with undiluted suspension. No plaque mutants were found.

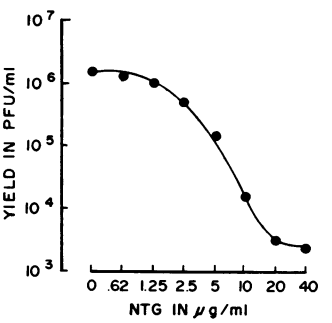

FIG. 4.—The inhibition of SV40 replication in AH-1 cells at 30°C by different concentrations of NTG after inoculation at a multiplicity of 5 plaque-forming units/cell.

TABLE 2. *Mutagenesis of SV40 by NTG.*

State of virus	NTG μg/ml	Decrease in infectivity ($\log_{10}$)	Mutants Temperature-sensitive	Plaque
Extracellular	5000	1.4	0/500	0/141
Intracellular	5	1.3	6/842	6/174

TABLE 3. *Plaque characteristics of parental SV40-W and temperature-sensitive mutants propagated at 31°C.*

Virus	Plaque-forming units 31°C (20 days) / Plaque-forming units 39°C (10 days)	Time of Plaque Appearance in Days 31°C	39°C
SV40-W	0.9	20(I)*	10(I)
NTG-1	>10^5	20(I)	0†
NTG-2	>10^4	20(I)	17(M)
NTG-3	>10^4	20(I)	24(MF)
NTG-4	>10^4	20(I)	0
NTG-5	>10^4	20(I)	17(MF)
NTG-6	>10^4	20(LG)	24(MF)

* Plaque morphology. MF = minute, fuzzy; M = minute; I = intermediate; LG = large
† 0 = no plaques after 24 days.

Among 842 plaque progeny isolated from SV40 replicating in AH-1 cells in the presence of 5 μg/ml NTG, six clones were found to form confluent or nearly confluent plaques after 20 days at 31°C but no plaques after 10-days incubation at 39°C. It was possible to assess the size of the plaques induced by 174 of the original 842 clones in the same plating of undiluted suspensions at 31°C. Six clones formed uniformly minute plaques and were stable in this characteristic through two plaque passages. None of the minute plaque formers was temperature sensitive (Table 2).

Plaquing characteristics of temperature-sensitive mutants: All of the temperature-sensitive mutants exhibited a greater efficiency of plaquing at the permissive than at the restrictive temperature by a factor of 10^4 or more (Table 3). After periods of incubation longer than 10 days at 39°C, four of the clones formed minute, and often poorly defined, plaques (Table 3). Monolayer cultures which were inoculated at a multiplicity of more than one temperature-sensitive virus per cell and incubated at 39°C showed an irregular pattern of staining with neutral red and no clearly defined plaques were formed.

Discussion. SV40-W has been shown to replicate well and produce complete cytopathic effect in AH-1 cells over a range of 31–42°C. The time required for the initial and maximal yields of new virus decreases approximately twofold with an increase of the temperature from 31 to 39°C. The finding of viral replication at 42°C is consistent with reports by Jerkofsky and Rapp[23] that SV40 replicates to normal yields at 41°C and Kitahara and Melnick[24] that SV40 produces T and V antigens in slightly diminished titers at temperatures up to 43°C. At 25°C we have found that the rate of SV40 replication is significantly slowed and the yield is markedly reduced. These findings are in accord with ability of SV40-infected cells to produce T antigen but very little V antigen at 23°C.[24]

NTG has been shown to inactivate extracellular infectious virions. Aqueous solutions of NTG in concentration approaching saturation decreased the infectivity of monodisperse SV40. The inactivation decreased with time at a variable rate. This finding can be attributed to the instability of NTG in solution since NTG which had been preincubated under experimental conditions for 24 hr caused less inactivation when subsequently added to virus.

The mechanism of inactivation of SV40 by NTG was further investigated by parallel exposure of virions and extracted infectious DNA to NTG. The rates of inactivation of virions and viral DNA by 5000 μg/ml NTG were similar. These findings suggest that the viral DNA is the target of NTG inactivation and that the protein coat of SV40 provides no significant impediment to the action of NTG.

None of the pools of virions which had been partially inactivated extracellularly by NTG were found to contain temperature-sensitive or plaque mutants. The possibility of mutagenic action of NTG under extracellular conditions other than those employed in these studies has not been eliminated. It is clear, however, that loss of infectivity alone cannot be used as a reliable indication of successful mutagenesis of SV40 by NTG.

Prior studies of the mutagenic effects of NTG on animal viruses have been

concerned solely with effects on extracellular virus.[25-28] The present study has extended these findings to demonstrate that NTG can mutate virus during its intracellular phase. NTG in concentrations one-thousandth of those required to inactivate extracellular virions effectively inhibited replication of intracellular infectious virus when added to infected cultures immediately after adsorption of the virus. Uninfected cultures treated with the same concentrations of NTG showed a partial and temporary toxic effect manifest by a loss of approximately 50% of the cells from the surface of the culture vessel. Virus stocks derived from the NTG-treated cultures contained approximately 3.4% plaque mutants and 0.7% temperature-sensitive mutants. NTG has been reported to have a similar effect on DNA-bacteriophage.[16] In concentrations of 15–50 μg/ml NTG inactivated and mutated intracellular, but not free, coliphage T2. Although the kinetics of intracellular viral mutagenesis have not yet been defined, it is clear that NTG can mutate intracellular SV40 and that cytotoxicity does not prevent mutagenesis. The mechanism(s) by which NTG induces mutations and inhibits viral replication remains unclear.

Although plaque mutants have been identified as an additional indication of mutagenesis, their usefulness for studies of functional viral genetics is extremely limited. The temperature-sensitive mutants, however, will be further used for the study of specific genetic functions of SV40. Similar studies with the closely related polyoma virus have already demonstrated the value of temperature-sensitive mutants in the investigation of tumor viruses.[29-31]

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