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Interaction of *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) with Owl Monkey Kidney Cells in Enhancing the Yields of *Herpesvirus saimiri* (HVS) and Its Antigens (41890)

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Abstract. Pre- and posttreatment with *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG) of owl monkey kidney (OMK) cells infected with *Herpesvirus saimiri* (HVS) resulted in one to three logs higher yields of virus, depending upon the dose of MNNG. A higher percentage of cells also showed HVS early antigen (EA) and late antigen (LA) by immunofluorescence when OMK cells infected with HVS were fed with medium containing MNNG. The high yields of HVS were also observed by electron microscopy. MNNG did not induce HVS-EA in HVS nonproducer lymphoblastoid T cells, nor did it enhance TPA-induced EA to LA. The data suggest that MNNG could be useful in obtaining high yields of virus and/or antigen-producing cells for immunofluorescence or other biochemical experiments, especially from those strains of HVS which grow poorly *in vitro*. The interaction of MNNG and HVS could also be useful for *in vitro* transformation or *in vivo* enhancement of the malignant process.

N-Methyl-*N'*-nitro-nitrosoguanidine (MNNG) is a carcinogenic and mutagenic alkylating agent which has been used to induce experimental gastric and duodenal adenocarcinomas in rats (5, 18). MNNG was also able to transform human skin fibroblasts of patients with hereditary adenomatosis of the colon and rectum (16) and clones of osteosarcoma cells (15). Henderson *et al.* (10) showed that MNNG treatment increased the frequency of establishing permanent human lymphoblastoid cell lines, and also facilitated transformation of human cord blood leukocytes with Epstein–Barr virus (EBV) (11). More recently, we have been able to show that human cord blood lymphocytes treated with MNNG required lower amounts of EBV for transformation. In addition, MNNG pretreatment of Raji cells led to enhanced early antigen levels following superinfection with nontransforming EBV (8).

We report here on the interaction of MNNG with owl monkey kidney cells (OMK) in increasing the yields of two strains of HVS which cause leukemia and/or lymphoma in marmosets, owl monkeys, and rabbits (2, 4). Similarities between EBV and HVS have led to the use of HVS in a nonhuman primate model for investigating the role of herpesvirus-induced malignancies similar to the nasopharyngeal carcinomas (NPC) and Burkitt's lymphomas (BL) involving EBV (7, 9).

Materials and Methods. *Virus strains.* Plaque-purified HVS of the prototype strain S295C ("HVS-P") and the highly pathogenic "OMI" strain of HVS were grown in mycoplasma-free owl monkey kidney (OMK) cell cultures using Dulbecco's minimum essential medium (D-MEM) and 10% heat-inactivated fetal calf serum (FCS). HVS titers, expressed in $TCID_{50}$ units, were determined on OMK cells as described below.

Virus titration. Both HVS-P and HVS-OMI were titered in OMK cells grown in 24-well plastic plates. The cells were grown in D-MEM with 4.5 gm/liter glucose and 10% heat-inactivated fetal calf serum (Advanced Biotechnology, Inc., Silver Spring, Md.). When the cells were nearly confluent, 0.1-ml aliquots of serial 10-fold dilutions of the virus to be assayed was inoculated into 4 wells/dilution. The virus was absorbed for 1 hr at 37°C, the supernatant was removed, and medium containing 2% serum was added to each well and incubated at 37°C. Titers were based on the

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development of characteristic cytopathic effects (CPE) and the $TCID_{50}$ was calculated after 2 weeks incubation. The OMK cells, either pretreated with MNNG or infected first with HVS and then treated with MNNG (Tables I and II) were observed for CPE and when >90% of the cells showed rounding giant cell formations (4+ CPE), the virus was prepared as follows: Most of the supernatant from infected cultures was separated from nonadherent cells by low-speed centrifugation at 4°C. After pipetting of all but 2 ml of the supernatant virus, the cell pellet was resuspended, quick-frozen, and thawed in a dry-ice and alcohol bath and centrifuged at 2000 rpm for 15 min. The latter supernatant was mixed with the original supernatant, filtered through a 0.45-μm pore size filter and titered in OMK cells as described above.

The effect of MNNG on the growth of HVS was assessed by observing cytopathic effect (CPE), indirect immunofluorescence (IF), and electron microscopy. The detection of early antigen (EA) was carried out with HVS-infected cells treated with 100 μg-ml phosphonoacetic acid (PAA) according to published procedures (1).

Indirect immunofluorescence. The HVS-infected and uninfected OMK cells, with or without MNNG treatment, were removed by shaking the flask, washed three times with PBS without calcium and magnesium and then approximately 5×10^5 cells were deposited on the wells of Teflon-coated glass slides. The cells were air-dried and fixed in acetone at 4°C for 15 min. The fixed slides were then used for examining HVS-LA (late antigen)- and EA (early antigen)-producing cells using HVS antibodies.

An owl monkey serum (7874) from an animal which developed an HVS-P-induced lymphoma was used, since it had an HVS antibody titer of 1:160 and an HVS-EA titer of 1:40. A second serum was obtained from a squirrel monkey (S-175) naturally infected with HVS. This serum had no detectable EA antibody (at 1:10) and an HVS-LA antibody titer of 1:80. The treatment and dilutions of sera used are given in Table III. Appropriately diluted sera were deposited on the fixed cells (4), incubated at 37°C in a humid chamber for 45 min, and washed three times in PBS for 10 min using a shaker. The slides were air-dried and then the cells were incubated for 45 min with a 1:20 dilution of fluorescein-labeled anti-human IgG antibody (H + L chains, Cappel Laboratories, Inc.). After washing the slides three times in PBS, they again were air-dried, mounted, and then examined under the fluorescent microscope. The total number of cells in 10 different fields per slide were counted and divided by the number of IF-positive cells to obtain the percentage of IF-positive cells exhibiting HVS-LA and -EA (Table III).

Electron microscopy. Cultures of HVS-infected cells with or without MNNG treatment were washed briefly with PBS, fixed *in situ* for 2–5 min with 2.5% glutaraldehyde in PBS, and then scraped loose and pelleted in 0.4-ml microfuge tubes. The pellets were fixed an additional 30–60 min in glutaraldehyde, post-fixed 1 hr in 1% osmium tetroxide in PBS, dehydrated with ethanol and propylene oxide and embedded in Epon–Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate.

MNNG stock. MNNG was prepared as a stock solution in PBS, as described previously (8), and was frozen at −20°C. The stock solution was further diluted in tissue culture medium containing 5% FCS.

Results and Discussion. Table I summarizes our findings on the effect of pretreating OMK cells with 0.5, 1.0, and 2.0 μg/ml of MNNG for 48 hr prior to infection with HVS-P and HVS-OMI. In every case, a 1–3 log increase in titer was observed compared to controls, even for HVS-OMI, which while highly tumorigenic in monkeys, grows poorly in OMK cells. Extracts of uninfected cells treated with MNNG did not contain virus, showing that the enhanced titers were not due to the induction of virus from the original cells. This increase in titer due to MNNG pretreatment was reflected in an earlier onset of cytopathic effect in MNNG-treated cultures, which generally appeared within 6–8 hr in monolayers pretreated with MNNG and then used for titrations.

Table II summarizes experiments carried out with MNNG in the culture medium during the period of virus replication. Although no pretreatment was included in these cultures, it is evident that a significant enhancement of virus yield could be obtained. It was

TABLE I. EFFECT OF PRETREATMENT OF OMK CELLS WITH MNNG PRIOR TO HVS INFECTION ON VIRUS YIELDS

Pretreatment	Dose of MNNG (μg/ml)	Virus strain	Titer[a] ($\log_{10}$)	Average log enhancement
None	—	HVS-P	6.47 ± 0.25	—
48 hr MNNG	0.5	HVS-P	7.40 ± 0.17	0.9
	1.0	HVS-P	7.97 ± 0.25	1.5
	2.0	HVS-P	9.17 ± 0.29	2.7
	2.0	—	0	—
None	—	HVS-OMI	3.30 ± 0.17	—
48 hr MNNG	1.0	HVS-OMI	4.40 ± 0.36	1.1
	2.0	HVS-OMI	4.97 ± 0.25	1.7

[a] At 4+ CPE cultures (when more than 85% of cells are showing cell degeneration, clumps of rounded and enlarged cells, looking like bunches of grapes) were frozen and thawed, filtered through a 0.45-μm pore size filter, and titered. Control cultures were treated in the same way. Values are the average of three determinations.

noteworthy, however, that continuous treatment with MNNG after infection led to significantly greater cell damage (not related to viral-induced CPE) than did the pretreatment protocol. Virus obtained from wells used to titer virus derived from MNNG treated cultures was further titered in OMK cells. No differences in the titers were obtained, when compared to controls (<0.5 log), suggesting that MNNG treatment had no detectable genetic effect on the HVS grown in treated cultures.

When HVS-infected OMK cells were treated with MNNG and examined by indirect immunofluorescence, significantly higher levels of both EA- and LA-producing cells were seen as compared to untreated controls. Table III shows that OMK cells infected with 1000 $TCID_{50}$/ml of HVS-P, and treated with 1 μg/ml of MNNG led to 71% LA-positive cells in comparison to 48% positive cells in controls. Secondly, treatment of HVS-P-infected OMK cells with 20 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) produced a 17% in-

TABLE II. EFFECT OF MNNG ON HVS YIELDS IN OMK CELLS WHEN CELLS WERE CONTINUOUSLY TREATED AFTER VIRUS ADSORPTION

Treatment	Dose of MNNG (μg/ml)	Virus strain	Virus titer[a] ($\log_{10}$)	Average log enhancement
1. None	0	HVS-P	6.40 ± 0.14	—
2. MNNG after infection	2.0[b]	HVS-P	8.40 ± 0.17	2.0
	1.0	HVS-P	8.40 ± 0.36	2.0
	0.5	HVS-P	8.07 ± 0.12	1.7
3. None	0	HVS-OMI	4.6[c]	—
4. MNNG after infection	1.0	HVS-OMI	6.2	1.6
	0.5	HVS-OMI	5.5	0.9
5. None	0	—	0	—
6. MNNG time-matched to treatment 2 above	2.0	—	0	—
	1.0	—	0	—
	0.5	—	0	—

[a] Average of three measurements for HVS-P. When cultures were 4+ CPE, they were quick-frozen, thawed, and filtered through a 0.45-μm pore-size filter prior to titration.

[b] MNNG (2 μg) was somewhat toxic and led to morphological alterations and reduced viability. However, it did not affect virus titer, but significantly reduced the number of cells available for immunofluorescence.

[c] Average of two experiments.

TABLE III. EFFECT OF MNNG AND MNNG PLUS TPA ON HSV-EA AND -LA PRODUCTION[a]

Cell line	EA (%)[b]				LA (%)[b]			
	TPA (20 ng/ml)	MNNG (1 μg/ml)	MNNG + TPA	No treatment	TPA (20 ng/ml)	MNNG (1 μg/ml)	MNNG + TPA	No treatment
MLC[c]	5–7	—	5–7	—	—	—	—	—
OMK infected with HVS	≤35	≤40	≤45	30	≤65	≤71	≥80	≤48
OMK	—	—	—	—	—	—	—	—

[a] OMK cells were infected with 1000 $TCID_{50}$ of HVS. After 2 hr adsorption at 37°C, the virus was removed. The cells were washed once with medium containing no serum. The OMK cells for EA were treated with 100 μg/ml of PAA (1) and were fed with medium containing TPA or MNNG or with both together. All cultures were simultaneously examined when untreated infected cells exhibited moderate CPE.

[b] Serum from a naturally infected squirrel monkey, containing no EA antibody, was used as a negative control for the detection of EA. An owl monkey serum from an animal that developed an HVS-induced tumor contained antibodies to HVS EA and LA was used for both EA and LA detection (used at dilutions of 1:10 to detect EA and 1:40 to detect LA). Both sera were heat-inactivated at 56°C for 30 min prior to use in the IF test. Fluorescein-conjugated anti-human IgG (heavy and light chains) was obtained from Cappel Laboratories, Inc., West Chester, Pa.

[c] The MLC cell line is derived from a lymphoma induced in a cotton-topped marmoset by HVS-P.

crease over the controls in late antigen expression. Moreover, an additive effect was observed when both TPA and MNNG were applied to HVS-infected OMK cells, as was previously oberved with EBV-nonproducer Raji cells (8). This observation suggests that a carcinogen and a promoter can interact to enhance virus expression.

The tumor promotor TPA has also been found to increase the level of transformation of mononuclear cells of adult marmosets by EBV (17) and to enhance antigen levels and virus production in EBV- and HVS-infected cells (3, 13, 19). Since we have shown that MNNG could enhance the level of HVS production in acutely infected cells, we were interested in determining if MNNG could mimic or enhance the effect of TPA on nonproducer marmoset lymphoid cells (MLC) which contain 47 HVS genomes/cell. The rationale for using TPA on the HVS-nonproducer MLC line was to induce EA and to assess its expression in these cells. All HVS nonproducer cell lines are not superinfectable with virus. Thus, the only way to induce EA is by the use of a tumor promotor. Treatment of $\geq 10^6$/ml MLC cells (viability ≥90%) with 1–2 μg/ml MNNG did not induce EA expression nor did it enhance the TPA-mediated induction of EA-positive cells (≥5%) to LA-positive status (Table III). These observations suggest that MNNG is mediated through a different mechanism than TPA.

The enhanced yield of HVS when MNNG was used on OMK cells was also observed by electron microscopy. Figures 1 and 2 are representative of the levels of virus observed in cultures infected for 48 hr either without (Fig. 1) or with (Fig. 2) pretreatment with MNNG. In this case, 48 hr was chosen because maximal CPE was observed in the MNNG-pretreated sample. Untreated infected cultures had not reacted to this extent of CPE at 48 hr and it is possible that the lower level of virus being observed in these cells could be partially due to a less advanced state of infection. In MNNG-pretreated cells, it is apparent that there is a much larger number of capsids in the nucleus, and higher levels of incomplete and complete virions in the cytoplasm and extracellular environment. We have also observed, however, that control cultures held for longer periods prior to fixation for EM eventually developed CPE as extensive as in MNNG-pretreated cultures, but that the level of all types of virion structures, as seen by electron microscopy, always remains much lower than in MNNG-pretreated cultures. Thus, the virions shown in Figs. 2B–D are shown because these stages of budding from nuclear membranes and larger extracellular aggregates are relatively infrequently seen in untreated cultures.

These findings are consistent with observations made by Lin *et al.* (14) showing that MNNG increased the number of EBV ge-

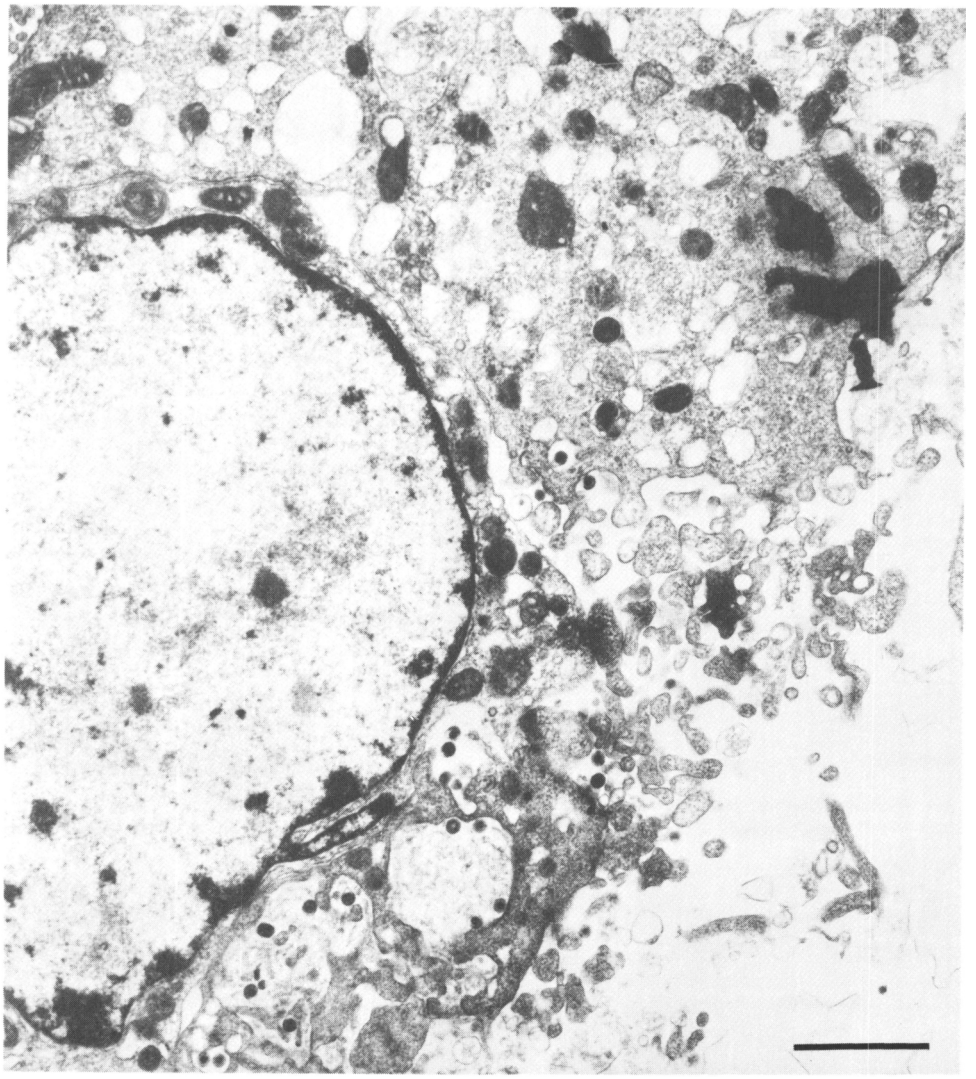

FIG. 1. Representative micrograph of OMK cells infected with HVS-P for 48 hr. At this stage of infection the cells contain moderate levels of capsids in the nucleus, and virions in the cytoplasm and in vacuoles. Budding through the nuclear and cytoplasmic membranes is only occasionally observed. At later times these cultures would contain higher levels of immature and mature virions, but at no time are the levels equivalent to those seen in MNNG-pretreated cultures. The magnification bar represents 1 μm.

nomes in EBV producer cells (P3HR-1) but had no such effect on EBV nonproducer Raji cells. Moreover, concomitant treatment of EBV-producer cells with MNNG and TPA further enhanced the number of EBV genome copies per cell, while pretreatment of Raji cells with MNNG, followed by super infection with P3HR-1 EBV, resulted in a 35% enhancement of EBV–DNA replication. Our data, however, also show that the final titers are greatly enhanced by MNNG. The EM observations showing greatly enhanced levels of virions suggest that the elevated PFU titers described above following MNNG treatment are due to an increase in virus production rather than an improvement in the PFU to physical particle ratio.

The capacity of viruses and chemical carcinogens or promotors to interact to produce enhanced levels of transformation has been

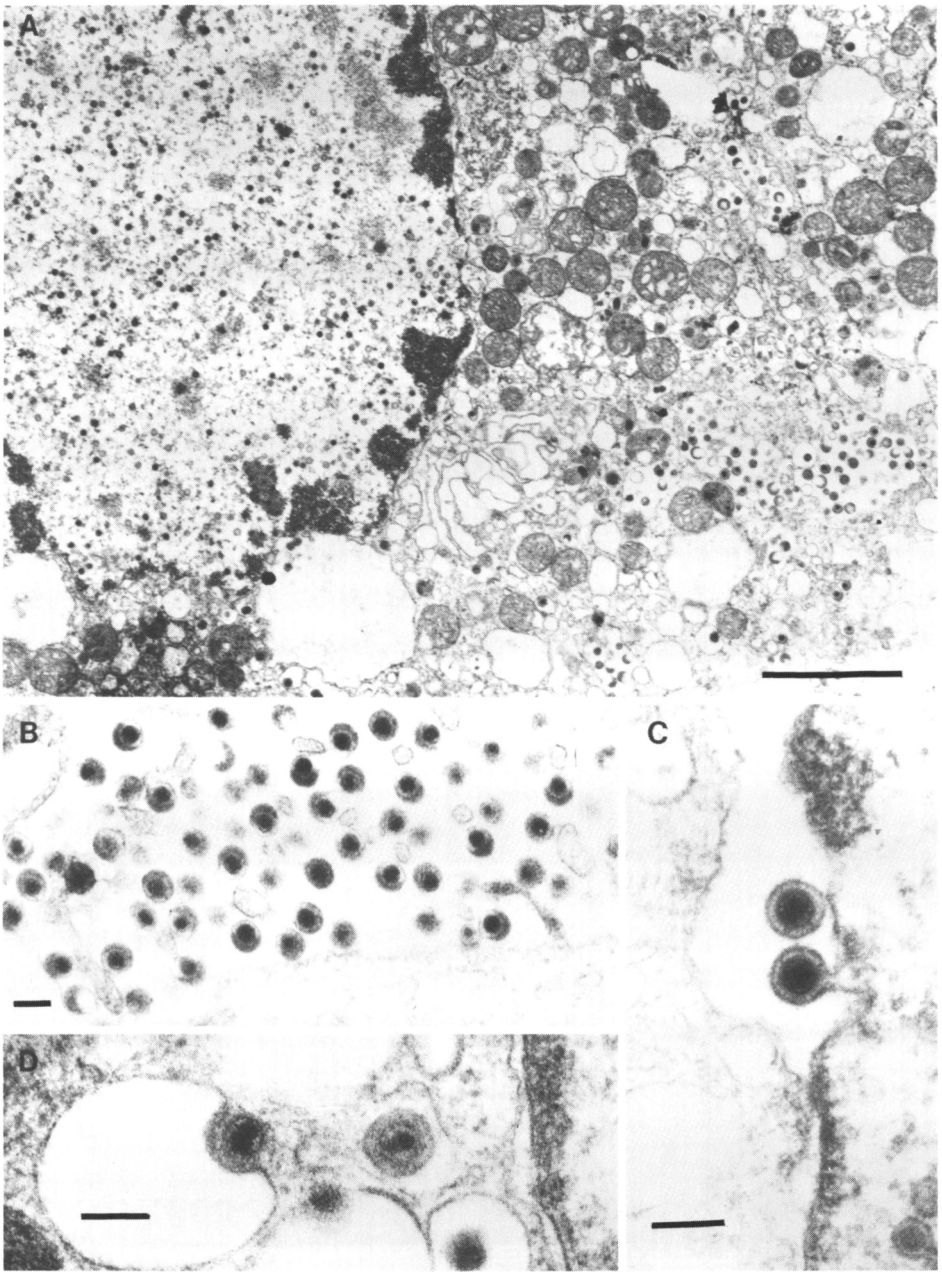

FIG. 2. Panel A is a micrograph of OMK cells pretreated for 48 hr with 1 μg/ml MNNG and infected with HVS-P for an additional 48 hr. The cells of this culture contained much higher levels of both nuclear capsids and cytoplasmic virions than seen in the control culture. The magnification bar represents 1 μm. Panels such as B–D, although typical of replicative forms of herpesviruses, are less frequently seen in micrographs of cultures not pretreated with MNNG. Aggregates as seen in (B) are rarely seen in HVS-infected OMK cells. Magnification bars represent 0.1 μm.

observed by others (6, 12). In this report, we describe how MNNG can significantly enhance the yield of infectious *Herpesvirus saimiri* grown in owl monkey kidney cells, but does not significantly affect the expression of the HVS genome in nonproducer cells. HVS

is an oncogenic herpesvirus capable of inducing lymphomas in marmosets, owl monkeys, and rabbits (2, 4), but not in its species of origin, the squirrel monkey. It would be of interest to determine if MNNG will enhance the oncogenicity of HVS in any of these animal systems as well as aid in transforming lymphoblastoid or monolayer cells from owl, marmoset, squirrel monkeys, and inbred rabbits.

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