Inactivation of Human Cytomegalovirus by the Chemical Carcinogen 4-Nitroquinoline 1-Oxide

(Accepted 30 May 1979)

SUMMARY

The infectivity of cytomegalovirus (CMV), strain Davis, was inactivated by 4-nitroquinoline 1-oxide (NQO). A series of survival curves indicates that the rate of inactivation was directly dependent on the concentration of NQO over a range of 5 to 200 μg/ml. At concentrations of 1 μg/ml or less, inactivation of virus stock was not observed and at concentrations in excess of 200 μg/ml, the cellular toxicity of residual NQO prevented quantification of the relatively low surviving infectivity. At a concentration of 200 μg/ml NQO or less, the loss of virus infectivity could be clearly shown to result from the interaction of NQO with virus and not with cells, since the addition of similar doses of NQO to assay cells simultaneously with virus did not adversely affect the sensitivity of the assay cells to measure virus infectivity. Similarly, the dimethylsulphoxide carrier at concentrations of 5% or less was shown to have a negligible effect on both virus infectivity and on the sensitivity of human skin muscle cells to assay virus infectivity. NQO inactivation of virus infectivity appeared to depend very little on white light, since the kinetics of inactivation in the presence and in the absence of white light were similar.

Several studies have suggested that human cytomegalovirus (CMV) has oncogenic potential. Limited studies have implicated CMV in associations with human cervical cancer (Pasca et al. 1975; Muñoz, 1976), prostate cancer (Geder et al. 1977) and colon carcinoma (Huang & Roche, 1978). Furthermore, the oncogenic potential of CMV has been demonstrated in vitro by the transformation of hamster embryo fibroblasts (Albrecht & Rapp, 1973) and human embryo lung cells (Geder et al. 1976). These transformed cells were shown to be malignant when transplanted into syngeneic hamsters or athymic nude mice. Although CMV transformation studies have been successful, the transforming frequency of this virus appears to be relatively low. Thus, it would seem prudent to examine the possibility that other factors, such as mutagens and carcinogens, might be involved in co-carcinogenic processes which might increase the frequency of transformation or further affect the properties of the transformed cells. One possible mechanism by which transformation might occur is through the direct interaction of chemicals with virus, resulting in decreased infectivity and enhanced oncogenic potential of the virus similar to that observed following limited u.v. irradiation of virus stock.

This report presents an investigation of the in vitro effects of NQO on the infectivity of CMV. NQO was selected for this study because the molecular mechanisms of the interaction of NQO and its metabolites with nucleic acids of microbial and mammalian cells are to a certain extent understood (Nagao & Sugimura, 1976). The inactivation kinetics of CMV, strain Davis, following treatment of virus stock with NQO are described. CMV, strain Davis (Weller et al. 1957), was obtained from Dr Thomas Weller and has been subcultured a total of 49 to 54 times in human cells.

Human embryo skin-muscle cells (SM), prepared locally, were dispersed in new glass...
prescription bottles containing growth medium: Eagle's minimum essential medium (MEM) with Earle's balanced salt solution plus 10% γ-irradiated foetal calf serum (γFCS), 0.075% sodium bicarbonate (NaHCO₃), penicillin (100 units/ml) and streptomycin (100 µg/ml). Cells were incubated at 37 °C. Confluent cell cultures were maintained with MEM containing 5% γFCS and 0.15% NaHCO₃ and antibiotics as before (maintenance medium).

For the virus infectivity assay, SM cells were cultivated in 35 x 10 mm Falcon plastic tissue culture dishes. Dishes were seeded with SM cells in growth medium containing 0.225% NaHCO₃ and incubated at 37 °C in a 5% CO₂ atmosphere. The infectivity of the CMV used in these studies was 1 x 10⁶ to 3 x 10⁸ p.f.u./ml. Virus stocks of CMV were prepared as described previously (Albrecht & Rapp, 1973) by inoculating confluent SM cell cultures with virus stock diluted 1:5 to 6 to provide a m.o.i. of 0.02 to 0.08 per cell.

The NQO employed in this study was supplied through the NCI Carcinogenesis Research Program by IIT Research Institute, Chicago, Ill. and was stored at -20 °C. Stock solutions of NQO were prepared in dimethylsulphoxide (DMSO) at a concentration of 10 mg/ml. Final dilutions of NQO were made with maintenance medium. The final concentration of DMSO never exceeded 5% in the test systems. NQO was always prepared immediately prior to use with care to protect it from light until it was mixed with virus stock. Just prior to treatment with NQO, CMV stocks were sonicated briefly and then centrifuged at 3200 g for 15 min to minimize the aggregates of CMV commonly observed in cell debris and to decrease subcellular components in the virus stock which might interfere with determining the actual kinetics of CMV inactivation. Supernatant fluids containing about 1 x 10⁵ to 9 x 10⁵ p.f.u./ml were used for determining the survival of virus treated with NQO. These clarified fluids were mixed with equal volumes of NQO solution prepared as previously described and appropriately diluted to obtain final concentrations of 1 to 500 µg/ml NQO. For controls, maintenance medium containing the maximum concentration of solvent (DMSO) used in the experiment was added to an equal volume of virus suspension. Samples of virus and chemical or control were incubated in a water bath at 37 °C. At various times the surviving CMV infectivity was determined by plaque assay as previously described (Albrecht & Rapp, 1973).

Our first concern was to determine whether DMSO could inactivate virus infectivity and whether it was a more appropriate solvent than acetone. CMV was treated with various concentrations of DMSO and acetone (0.05, 0.5 or 5%) by mixing virus stock with an equal volume of MEM containing twice the desired final concentration of DMSO or acetone. CMV stock treated with MEM was used as a control. The results suggested that the infectivity of CMV was sufficiently stable at 37 °C in the presence of up to 5% DMSO to determine the effect of NQO on the survival of virus infectivity. The infectivity of virus stock incubated with 0.5 or 0.05% DMSO was not appreciably different from that of the control virus stock through 24 h. No loss in virus infectivity was observed in virus stock treated with 5% DMSO during the first 8 h incubation. Over 24 h, a gradual decrease in virus infectivity occurred which was only slightly greater than that observed with control virus stock. In contrast, virus stock incubated in 5% acetone lost more than 99.9% of its infectivity during the period from 8 to 24 h after mixing.

The rate of inactivation of CMV during exposure to a wide range of NQO doses (1, 10 and 100 µg/ml) for periods from 0.25 to 8 h (Fig. 1) was first investigated. In this series of experiments no precautions were taken to maintain the virus-NQO mixed samples in the dark. Samples were maintained under laboratory fluorescent 40 W lamps in an open 37 °C water bath. When CMV was incubated with 100 µg/ml NQO, inactivation of virus infectivity was first noted at 2 h and after 8 h incubation about 99.9% of the virus infectivity had been lost. At a concentration of 10 µg/ml NQO, inactivation of virus infectivity was not
Fig. 1. Survival of human cytomegalovirus at 37 °C in 4-nitroquinoline 1-oxide (NQO) at concentrations of 1 μg/ml (○—○), 10 μg/ml (△—△) and 100 μg/ml (□—□) and 1 % DMSO (●—●). Samples were prepared by mixing equal volumes of clarified (3200 g) virus stock with NQO which had been dissolved in DMSO and diluted in Eagle's MEM (supplemented with 5 % foetal calf serum, 0·15 % NaHCO₃, 100 μg/ml streptomycin and 100 units/ml penicillin). The sample containing a final concentration of 100 μg/ml NQO also contained 1 % DMSO. Other NQO virus samples contained proportionately less DMSO. Surviving virus infectivity was determined by plaque assay on human embryo skin-muscle cells with an agarose overlay as described in Methods.

observed until after 4 h incubation and after 8 h incubation about 84 % of the virus infectivity was destroyed. The maximum loss of infectivity with 1 μg/ml NQO was approx. 35 % of the control (Fig. 1).

It was possible that the observed loss of infectivity with increased NQO doses (Fig. 1) might be in part the result of the carcinogen producing a decreased sensitivity to virus in the assay cells. The observation that at 0 h incubation, virus incubated with the three concentrations of NQO had about the same infectivity as the control suggested that the sensitivity of cells exposed to these concentrations of NQO was not adversely affected. However, by assaying surviving infectivity in 2 × virus samples that were separately incubated from 2 × NQO solutions and combined just before assay, it would be possible to determine the effect of the various concentrations of NQO on the sensitivity of the assay cells to estimate the infectivity of CMV. The data from one such experiment are shown in Fig. 2. In Fig. 2a the consistency of the virus infectivity measured immediately after mixing separately
Fig. 2. The effect of 4-nitroquinoline 1-oxide (NQO) on (a) the efficiency of plating human cytomegalovirus and on (b) the kinetics of inactivation of virus infectivity. Solutions of NQO were prepared by dissolving NQO in DMSO and making appropriate dilutions in Eagle's MEM with 5% foetal calf serum, 0.15% NaHCO₃, 100 µg/ml streptomycin and 100 units/ml penicillin. Virus stock was clarified by centrifugation (3200 g) and combined with an equal volume of NQO appropriately diluted in MEM (b) or sealed in separate tubes from the diluted NQO (a). All samples, NQO mixed with virus, NQO without virus and virus stock alone, were incubated at 37 °C. At the times specified, surviving virus infectivity was determined in the mixed sample (b) and in the samples that were separately incubated and combined immediately before infectivity assay. Infectivities were determined by plaque assay under an agarose overlay. Each member of the family of curves is derived from assays of virus stock treated with various doses of NQO for set periods of time (□—□, 0 h; △—△, 2 h; ◇—◇, 4 h; □—□, 6 h; Δ—Δ, 8 h; ○—○, 10 h; ●—●, 12 h). Symbols in parentheses indicate that within the limits of the assay, surviving virus infectivity was not detected at these doses nor at larger doses of NQO at these exposure intervals (not plotted in the figure). Data were normalized to the control data points for virus stock incubated with 5% DMSO for each period of incubation.

incubated virus stock with various concentrations of NQO revealed no substantial decrease in cell sensitivity to plaquing CMV over a 12 h period. At dilutions of 1:10 or more of these NQO mixed virus stocks, cellular toxicity was not observed and plaque counts were proportional to dilution of these virus stocks. In contrast, samples of virus stock incubated together with the various concentrations of NQO showed a steady loss of infectivity at a rate dependent on the duration of exposure and concentration of NQO (Fig. 2b). Several observations can be made from this plot. The survival curves are multicomponent with both the first and the second components having an increasingly negative slope as the concentration of NQO is increased. There was a logarithmic loss of infectivity which was directly
dependent on the concentration of NQO through a minimum dose of 25 µg/ml. No adverse effect on the biological activity of CMV stock treated with 5 µg/ml was observed until 10 h after treatment.

Since we have recently demonstrated that human embryo lung cells persistently infected with CMV developed a morphologically transformed phenotype following exposure to NQO (Li & Albrecht, 1979), we were interested in determining the effect of NQO on the biological activity of stocks of CMV. This paper establishes that there can be a direct interaction between virus and NQO. The data presented in Fig. 1 and 2 indicate that the loss of biological activity of CMV was directly dependent on the concentration of the chemical carcinogen, NQO and the duration of exposure to NQO. The curves obtained by plotting the surviving virus infectivity as a function of NQO dose (Fig. 2b) are multicomponent and are strikingly similar to those observed for the u.v. light inactivation of CMV (Albrecht et al. 1974). The extent to which host cell reactivation or multiplicity reactivation is responsible for the second component is currently under study. Loss of virus infectivity was a result of NQO interacting with virus and not with cells since residual NQO apparently had little, if any effect on the efficiency of SM cells in plating CMV (Fig. 2a).

NQO has been shown to form at least two types of free radicals (Kawazoe, 1971; Nagata, 1971) and to have photodynamic activity (Nagata et al. 1967). Although photodynamic activity with NQO has been observed by a number of investigators (Epstein et al. 1964; Malkin & Zahalsky, 1966; Nagata et al. 1966; Kodama & Nagata, 1967), in several experiments we have not been able to detect a substantial photodynamic component in survival curves of CMV treated with NQO.

Although the molecular mechanism of NQO inactivation of CMV is unclear at this time, several alternative explanations are possible. It could be anticipated that a substance capable of forming highly reactive free radicals, such as NQO, might bind to or change the characteristics of the virus envelope and/or capsid and prevent adsorption, penetration or uncoating. It has been demonstrated that NQO can react with -SH compounds in vitro (Okabayashi, 1953) and in the skin (Hayashi, 1959; Takayama & Oota, 1961) and with aromatic amino acids (Okano et al. 1968). A second mechanism which could readily account for our results involves the reduction of NQO in virus infected cell lysates to 4-hydroxyaminoquinoline 1-oxide (HAQO). The inactivation of CMV infectivity might then occur by the interaction of HAQO with virus nucleic acid. The reduction of NQO to HAQO has been observed in mammalian cells (Sugimura et al. 1966), so it would not be unusual to find a similar reduction in lysates of human fibroblasts (SM). HAQO has been shown to inactivate isolated transforming DNA (Ono, 1964) and bacteriophage in vitro (Ishizawa & Endo, 1967) whereas similar inactivations have not been observed with NQO. In contrast to NQO, HAQO can only form one type of free radical (Nagata, 1971) and thus, as we observed, photodynamic activity would not be anticipated. Further study is necessary to distinguish between these two hypotheses.

The authors express their gratitude to Drs R. Fleischmann, H. Johnson, L. Olson, and S. Baron for their critical review of this manuscript. This investigation was supported by Contract no. NOI-CP-6-1042 from the Division of Cancer Cause and Prevention, National Cancer Institute, United States Public Health Service.

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(Received 7 February 1979)