

Lack of Genotoxicity in VX and Soman-Treated Cultured Human Cells by Comet Assay Analysis

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ABSTRACT

Nerve agents are not considered to be genotoxic or carcinogenic. However, repeated low-dose exposure of the nerve agent soman (GD) in guinea pigs has shown DNA fragmentation in isolated blood leukocytes using the Comet assay. We questioned whether the Comet assay would uncover similar DNA fragmentation in cells of human origin grown under in vitro conditions and exposed to both GD and VX. Human small airway epithelial cells (SAEC) and normal human epidermal keratinocytes (NHEK) in culture were exposed to either VX (0.1 μ M to 50 μ M) or GD (0.1 μ M to 100 μ M) concentrations for 2 hrs. The agents were removed, cells re-fed with fresh media, and then incubated for an additional 24 hrs. The cells were harvested and processed for genotoxicity analysis by the Comet assay and for cytotoxicity via flow cytometry. A minimum of 50 cells for each VX and GD concentration was analyzed by fluorescence microscopy using the Comet Assay Analysis System by Loats. No statistical change in Comet moment (a measure of DNA damage) or cytotoxicity in GD- or VX-exposed SAEC and NHEK was seen. We concluded that GD and VX have no direct effects on DNA fragmentation or cytotoxicity in SAEC or NHEK under these acute exposure conditions.

INTRODUCTION

Genotoxic damage, such as chromosomal alterations, occurs in people that are repeatedly exposed to pesticides during agricultural or manufacturing processes [Lieberman et al., 1998]. The alkaline single cell electrophoresis assay (Comet assay) has been used to evaluate leukocytes of individuals involved in the manufacture of pesticides. These repeatedly exposed workers showed much greater DNA damage (measured by Comet moment) than did control workers [Grover et al., 2003].

Although nerve agents are chemically similar to organophosphorus pesticides used in agriculture, they are 3 to 4 orders of magnitude more potent as anticholinesterases than their agricultural cousins. These compounds are a significant threat to personnel either through accidental exposure during demilitarization or by potential exposure during chemical warfare or terrorist attacks. Nerve agents are well known for their lethal effects via cholinesterase and the cholinergic pathways, but are not considered to be either genotoxic or carcinogenic [Munro et al., 1994].

A recent study using repeated low-dose exposure of guinea pigs to a potent nerve agent, soman (GD) has shown DNA fragmentation in isolated blood leukocytes at various times following exposure [Moffett et al., 2003]. This DNA fragmentation was analyzed by the Comet assay under alkaline conditions and could be found at doses of GD that did not exhibit any pathology normally seen with nerve agent exposure. In another study of guinea pigs to repeated low-dose sarin (GB) blood leukocytes were analyzed [Dave et al., 2007] and also showed DNA fragmentation. The mechanism that is responsible for this fragmentation in mammalian cells remains unidentified.

Since our laboratory studies DNA fragmentation resulting from alkylating agents in cultured human cells, the observation that nerve agents produced DNA fragmentation in mammalian cells prompted us to study whether nerve agent could also cause DNA fragmentation in human cells. Concentrations of VX (0.1 nM - 0.1 μ M) representing subacute dermal exposures to human were studied to determine whether DNA fragmentation occurs at 24 hours in NHEK following exposure, but no DNA fragmentation or cytotoxicity was found [Gross et al., 2008]. In this report, we used the Comet assay to investigate whether higher doses of nerve agents could cause DNA fragmentation in human cells by direct exposure. We have investigated concentrations of VX (0.01 μ M - 50 μ M) and GD (0.1 μ M - 100 μ M) to determine whether direct damage to DNA could be seen. These concentration ranges of VX and GD were studied in normal human epidermal keratinocytes and human small airway epithelial cells, two in vitro systems used in our laboratory that represent dermal and inhalation exposures models. The direct effects of VX and GD exposures on DNA fragmentation were determined by the Comet assay and cytotoxicity was analyzed by flow cytometry in both cell types.

MATERIALS AND METHODS

Materials

NHEK and SAEC cultures were obtained from Lonza Corp (Walkersville, MD). Tissue culture vessels were purchased from Corning Corporation (Corning, NY) or Falcon Corporation (Newark, NJ). Comet assay was performed using Trevigen CometAssay™ kits (Gaithersburg, MD). Propidium iodide was purchased (Sigma, St Louis, MO) and flow cytometry measurements were

performed on a BDFACSAria™ II flow cytometer (Becton Dickinson, San Jose, CA) utilizing FlowJo software (San Carlos, CA). DNA damage was measured by fluorescence microscopy on an Olympus BH-2 fluorescence microscope. VX (O-Ethyl-S-(2-diisopropylaminoethyl) methyl phosphonothiolate), CAS Registry Number 50782-69-9, and soman (Pinacolyl methyl phosphonofluoridate; GD), CAS Registry Number 96-64-0, were obtained from the Edgewood Chemical Biological Center, APG, MD at > 99% purity assessed by GS-MS or GC-FID. Agents were frozen in saline at -80 C and used within 90 days of preparation. Experimental concentration ranges were selected to comply with USAMRICD surety regulations.

Cell culture

NHEK and SAEC cultures were purchased as second passage cells and cultured in their appropriate supplemented media in T-75 flasks at 37°C in a 5% CO₂ incubator. Cells were grown until estimated confluencies of 60 - 80% were reached before experimentation began.

GD or VX exposure to NHEK or SAEC

Tertiary cultures of cells grown in T-75 tissue culture flasks were exposed to GD or VX by thawing the frozen agent and diluting it into the appropriate tissue culture media before adding the suitably diluted agent to the tissue culture flasks in a surety approved hood. The vessels were incubated in the surety hood for 2 hrs (no loss of viability without CO₂ was determined in previous studies) and the media, which contained either VX or GD, was vacuum-aspirated into a side-arm flask and decontaminated. The tissue culture vessels were then washed five times with phosphate-buffered saline and all five washes were removed by vacuum aspiration and decontaminated as above. The cells were replenished with the appropriate fresh media and incubated at 37°C in a 5% CO₂ incubator for 24 hrs.

Methods

Isolation of cells

After agent exposure and incubation for 24 hrs, cells were isolated from flasks by conventional trypsin-EDTA treatment. The trypsin was neutralized and the cells were pelleted by centrifugation, washed in phosphate buffered saline, and resuspended in 1.75 mL of Ca⁺⁺ and Mg⁺⁺ free phosphate buffered saline. The cells were divided into 0.5 mL aliquots for cell concentration measurements by Coulter counting, viability determinations by flow cytometry, and alkaline Comet analysis.

Assessment of viability

Cell viability was established by using propidium iodide uptake. Cells were stained with propidium iodide and analyzed by flow cytometry. A 0.5-mL aliquot of cells ($\sim 10^6$ /mL) was incubated with 30 μ L of propidium iodide (50 μ g/mL) for 2 min in the dark and analyzed by flow cytometry. A minimum of 10,000 cells were analyzed using the BDFACSAria™ II flow cytometer, and the fluorescence of the vital dye uptake was measured. The data were analyzed by FlowJo flow cytometry analysis software and viability was expressed as the percentage of unexposed (control) cells.

Preparation of DNA fragmentation (positive control)

A positive control for DNA fragmentation was prepared using cells not exposed to nerve agent, but treated with hydrogen peroxide (H_2O_2), a treatment known to produce DNA damage [Szmigiero and Studszian, 1988]. Briefly, a 0.25 mL aliquot of unexposed control cells was treated with 50 μ L of 0.03% H_2O_2 for 5 min on ice and diluted to 1×10^5 /mL with Ca^{++} and Mg^{++} free phosphate buffered saline. A 50 μ L aliquot of this cell suspension was then prepared for Comet analysis as described below.

Comet analysis

Comet analyses were performed using Trevigen CometAssay™ kits and following the manufacturer's instructions. Briefly, 50 μ L of cells (1×10^5 /mL) was added to 500 μ L of 37°C low melting agarose. An aliquot (50 μ L) of this suspension was pipetted into each well of the Trevigen double-well slide and spread with the tip of the pipette.

The slides were placed in the refrigerator for 30 min to allow the agarose to solidify and then immersed in pre-chilled (4°C) lysis solution (Trevigen) for 60 min. Slides were drained and placed into freshly prepared alkaline solution (0.3 M NaOH—0.001M EDTA) for 45 min, drained, and placed in the electrophoresis slide tray. The slide tray was then placed in the Comet Assay™ electrophoresis tank containing ~ 950 mL of freshly made pH 13 electrophoresis solution (0.2 M NaOH—0.001 M EDTA) and covered with the slide tray overlay. The power supply was set to 21 volts and electrophoresis continued for 30 min. The slides were rinsed twice in distilled H_2O , immersed in 70% ethanol for 5 min, dried for 30—45 min at 45°C, and then stored in the dark overnight.

Slides were stained with freshly prepared SYBR® Green (1 μ L/10 mLs of 10 mM Tris-HCL, pH 7.5 – 1mM EDTA) for 10 min at 4°C, drained and then dried in the dark at room temperature. The slides were analyzed by fluorescence microscopy and comet moment (i.e. % DNA in tail X tail length) was determined by the use of Comet Assay Analysis System by Loats Associates, Westminster,

MD. Data in figures were analyzed by t-test and the significance level was $p < 0.05$ (*) when compared to untreated controls.

RESULTS

An image of the Comet Assay results, i.e., the single cell gel electrophoresis profile of a cell, provided by the software and that accounts for background and noise is shown in figure 1. In figure 1A on the left, the cell has a Comet moment of ~ 0.2 . Figure 1B shows a cell treated with a positive control for DNA fragmentation (hydrogen peroxide), and has a Comet moment of ~ 35 .

The direct effect of a 2 hr exposure of SAEC and NHEK to VX was investigated and the results are shown below in table 1. The DNA fragmentation patterns measured by Comet moment in all VX concentrations studied did not appear to be different from patterns in the unexposed controls.

Application of the t-test ($p < 0.05$) to the above data showed no statistical differences in DNA fragmentation between control cells and the VX-exposed cells. Only the H_2O_2 –treated positive control was statistically different from the unexposed control or agent-exposed cells. Therefore, VX exposure did not induce fragmentation in these cells.

The question of whether these concentrations of VX were directly cytotoxic to SAEC or NHEK was also investigated. Cells that were incubated for 24 hrs, after a 2 hr VX exposure, and isolated by conventional tissue culture methods were then analyzed for viability by the uptake of the fluorescent dye, propidium iodide. Figure 2 (below) shows identical viability profiles seen for both types of cells compared to their respective control cells. There appears to be no effect of VX on viability of either cell type over a 5000-fold range of concentration.

The direct effect of 2 hr exposure of SAEC and NHEK to GD was also studied and the results are shown in table 2. The DNA fragmentation patterns measured by Comet moment for all five concentrations of GD studied did not appear to be different from unexposed controls. The Comet moments of these cells, as well as the H_2O_2 positive controls in table 2, are much lower than in table 1 because of a change in the microscope-camera setup. This adjustment allowed us to scan more comets at a lower magnification and intensity than in table 1.

Application of the t-test ($p < 0.05$) to the preceding data showed no statistical differences between the control and the five GD concentrations. Only the H_2O_2 –treated positive control was statistically different from the unexposed control or agent-exposed cells.

The question of whether these concentrations of GD were directly cytotoxic to NHEK or SAEC was also investigated. Cells that were incubated for 24 hr after a 2 hr GD exposure and isolated by conventional tissue culture methods were analyzed for viability by the uptake of the fluorescent dye, propidium iodide. Figure 3 shows the viability profiles seen for both types of cells compared to their respective untreated control cells. There also appears to be no effect of GD on the viability of either cell type over a 1000-fold range of agent concentration.

DISCUSSION

Nerve agents are organophosphorus compounds that are potent anticholinesterases. Their powerful effects on both cholinesterase and cholinergic pathways are well known and responsible for their lethality. They are generally considered to be neither genotoxic nor carcinogenic. In earlier studies, nerve agents have not been shown to be mutagenic using microorganisms and mammalian cell cultures. When the Ames Salmonella assay was employed with and without metabolic activation, no significant increases in mutation were seen [Bakshi et al., 2000] However, it appeared that sister-chromatid exchange occurred in Chinese hamster ovary cells exposed to G-agents [Nasr et al., 1988]. Recent studies [Moffett et al., 2003] using the Comet assay have shown that DNA fragmentation of leukocytes occurs after repeated low-dose exposure to GD in guinea pigs. This increased fragmentation could be found at doses of GD that did not exhibit any pathology normally seen with nerve agent exposure. The mechanism responsible for this fragmentation is unidentified. With these results, sister chromatid exchange and DNA fragmentation, it appeared that nerve agent exposure might produce a measurable genotoxic change and the sensitive Comet assay could be used as a rapid tool to determine latent genotoxicity of other organophosphorus compounds.

The effects of GD, or another more potent anticholinesterase, VX, on DNA fragmentation in human tissues is unknown. Our *in vitro* models allowed us to study whether acute exposure of cells of human origin could directly cause DNA fragmentation. Two *in vitro* models of skin and lung cells were exposed to 0.1 to 100 μ M GD or 0.01 to 50 μ M VX for 2 hrs. The agents were removed, and cells were allowed to recover for 24 hrs in fresh media and then isolated by using conventional tissue culture methods. When the cells were analyzed for DNA fragmentation using the single cell gel electrophoresis (Comet) assay, neither cell type exhibited any DNA fragmentation. Comet moment measurements at each nerve agent concentration were indistinguishable from untreated controls. Comet moment increases were seen only with the positive control, H₂O₂, which is known to cause both single and double strand breaks in DNA [Szmigiero and Studszian, 1988]. No direct breakage of DNA was seen after exposure with either agent.

Flow cytometry analysis of propidium iodide uptake in these cultured human cells also showed the absence of cytotoxicity by these lethal agents. These studies have confirmed the lack of genotoxicity and cytotoxicity seen with nerve agents in other systems. Our results indicate that organophosphorus nerve agents do not directly induce DNA fragmentation in NHEK or SAEC.

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TABLES

Table 1: Exposures to VX

VX Concentration	SAEC (Comet Moment)	NHEK (Comet Moment)
None	9.4 ± 1.16	0.9 ± 0.22
0.01 µM	11.9 ± 1.97	0.5 ± 0.17
0.10 µM	14.6 ± 2.06	0.9 ± 2.23
1.00 µM	13.3 ± 2.26	0.9 ± 0.22
10.0 µM	13.8 ± 2.07	0.9 ± 0.21
50.0 µM	13.1 ± 1.79	1.1 ± 0.24
H ₂ O ₂	118.7 ± 56.92*	81.7 ± 22.63*

SAEC or NHEK were exposed to the indicated VX concentrations for 2 hr. Agent was removed and cells were incubated for 24 hr. Cells were isolated and processed for Comet assay analysis. DNA fragmentation was measured by Comet moment using Loats's Comet Assay Analysis software. Each value represents the mean ± SD for 50 comets / experiment; n= 3 experiments. Data were analyzed by t-test and levels of significance were p < 0.05 and designated as (*).

Table 2: Exposures to GD

GD Concentration	SAEC (Comet Moment)	NHEK (Comet Moment)
None	0.2 ± 0.18	0.6 ± 0.39
0.1 µM	0.1 ± 0.03	0.9 ± 0.70
1.0 µM	0.2 ± 0.07	0.8 ± 0.77
10.0 µM	0.1 ± 0.01	0.8 ± 0.5
50.0 µM	0.2 ± 0.04	1.0 ± 0.66
100.0 µM	0.1 ± 0.03	1.0 ± 0.97
H ₂ O ₂	37.4 ± 5.61*	39.5 ± 5.07*

SAEC or NHEK were exposed to the indicated GD concentrations for 2 hr. Agent was removed and cells were incubated for 24 hr. Cells were isolated and processed for Comet assay analysis. DNA fragmentation was measured by Comet moment using Loats's Comet Assay Analysis software. Each value represents the mean ± SD for 50 comets / experiment; n= 3 experiments. Data were analyzed by t-test and levels of significance were p < 0.05 and designated as (*).

FIGURES

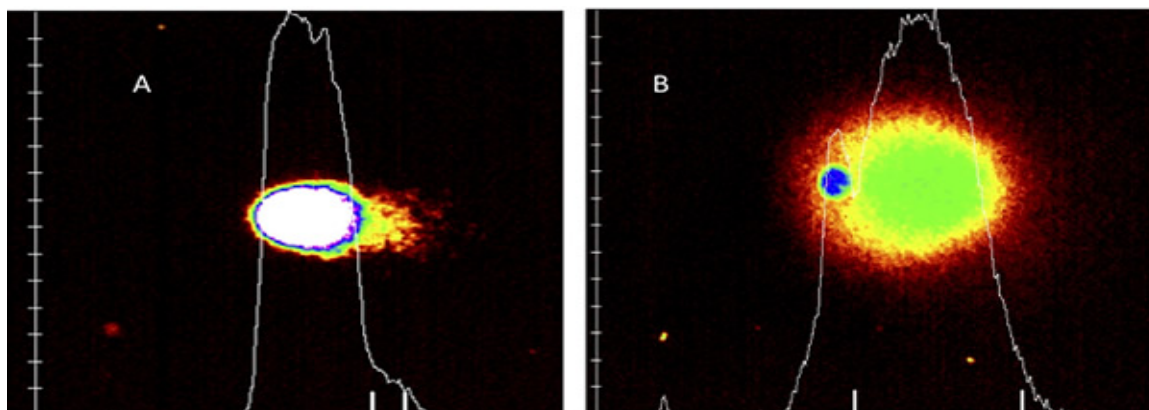


Figure 1: Pictorial view of representative Comets. Panel A represents a Comet from control or agent exposed cells with a Comet moment of 0.2. Panel B represents a Comet from H₂O₂ exposed cells that has a Comet moment of 35. Comet moment is defined as the (% DNA in tail) multiplied by (tail length) and is a measure of DNA fragmentation. It is the area enclosed by the 2 small white bars in figure 1 and is calculated by the Loats's Comet Assay Analysis software.

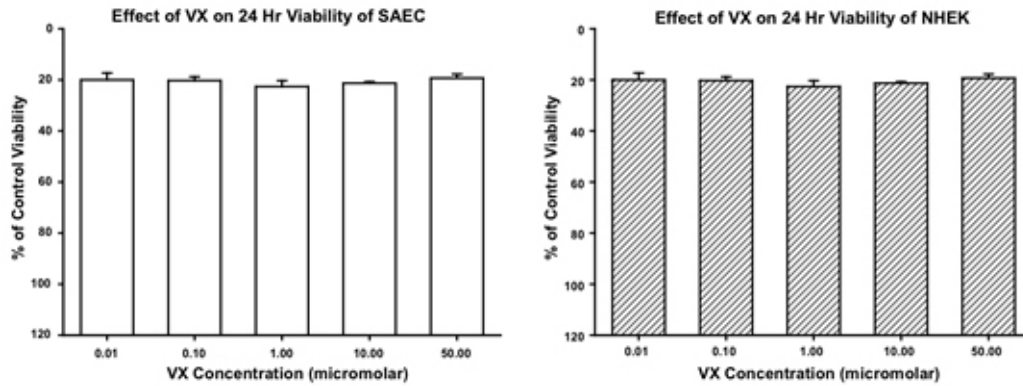


Figure 2: VX exposure. SAEC or NHEK were exposed to the indicated VX concentrations for 2 hr. Agent was removed and cells were incubated for 24 hr. Cells were isolated and viability was determined by propidium iodide uptake via flow cytometry. A least 5000 cells were counted for each experiment. Bars represent mean values \pm SD; (n=3).

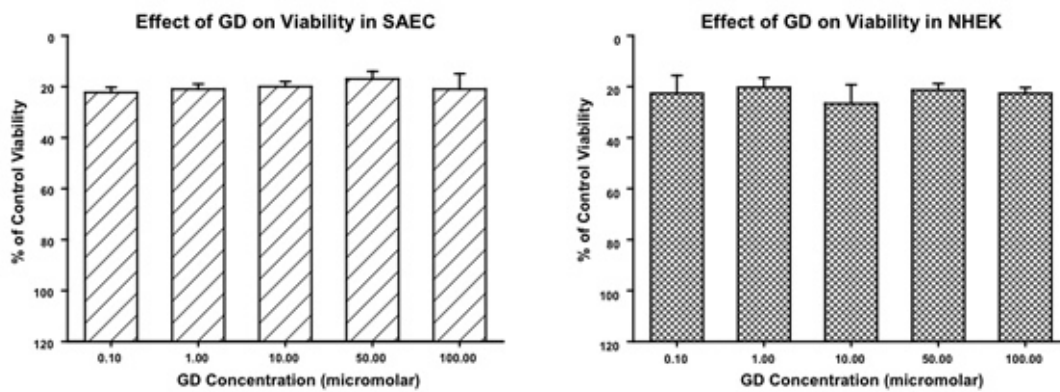


Figure 3: GD exposure. SAEC or NHEK were exposed to the indicated GD concentrations for 2 hr. Agent was removed and cells were incubated for 24 hr. Cells were isolated and viability was determined by propidium iodide uptake via flow cytometry. At least 5000 cells were counted per experiment. Bars represent mean values \pm SD; (n=3).

REFERENCES

Bakshi KS, Pang, SNJ, and Snyder R (eds) Review of the U.S. Army's health risk assessments for oral exposure to six chemical warfare agents. *J.Toxicol. Environ. Health A*. 2000, 59; (5-6):281-526.

Dave JR, Connors RA, Genovese RF, Whipple RA, Chen RW, DeFord SM, Moran AV, and Tortella EC. DNA fragmentation in leukocytes following repeated low-dose sarin exposure in guinea pigs. *Cell Mol. Life Sci*. 2007; 64: 2823- 2828.

Gross, CL, Nealley, EW, Miller, AL, Nipwoda, MT, and Smith WJ. Effect of VX on genotoxicity in cultured human epidermal keratinocytes. 16th Biennial Medical Defense Bioscience Review, 2008; 157, Hunt Valley, MD

Grover, P, Danadevi, K, Maahboob, M, Rozati, R, Salehu Banu, B, and Rahman, M. Evaluation of genetic damage in workers employed in pesticide production utilizing the Comet assay. *Mutagenesis* 2003. 18: 201-205.

Lieberman, AD, Craven, MR, Lewis, H, and Nemenzo, JH. Genotoxicity from domestic use of organophosphate pesticides. *J. Occup. Environ. Med* 1998., 40: 954-957.

Moffett, JR, Price, RA, Anderson, SM, Sipos, ML, Moran, AV, Tortella, FC, and Dave, JR. DNA fragmentation in leukocytes following subacute low-dose nerve agent exposure. *Cell Mol. Life Sci.*, 2003; 60: 2266-2271.

Munro, NB, Ambrose, KR and Watson, AP. Toxicity of the organophosphate chemical warfare agents GA, GB, and VX: Implications for public protection. *Environ. Health Perspec*. 1994; 102: 18-38.

Nasr, ML, Goldman, M, Klein, AK, and Dacre, JC. SCE induction in Chinese hamster ovary cells (CHO) exposed to G-agents. *Mutat. Res*. 1988; 204: 649-654.

Szmigiero, L and Studszian, K. H₂O₂ as a DNA fragmenting agent in the alkaline elution interstrand crosslinking and DNA-protein crosslinking assays. *Anal. Biochem*. 1988; 168: 88-93