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Research Article

DNA fragmentation in leukocytes following repeated low dose sarin exposure in guinea pigs†

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Abstract. The objective of this study was to determine levels of DNA fragmentation in blood leukocytes and parietal cortex from guinea pigs following repeated low-level exposure to the chemical warfare nerve agent (CWNA) sarin. Guinea pigs were injected (s.c.) once a day for 10 days with saline, or 0.1, 0.2, or 0.4 LD_{50} (50% mean lethal dose) sarin dissolved in sterile physiological saline. Blood and parietal cortex was collected after injection at 0, 3, and 17 days recovery and evaluated for DNA fragmentation using single-cell gel electrophoresis (Comet assay). Cells were imaged using comet analysis software and three parameters of DNA fragmentation measured: tail length, percent DNA in the tail, and tail moment arm. Repeated low-dose exposure to sarin produced a dose-dependent response in leukocytes at 0 and 3 days post-exposure. There was a significant increase in all measures of DNA fragmentation at 0.2 and 0.4 LD_{50}, but not at 0.1 LD_{50}. There was no significant increase in DNA fragmentation in any of the groups at 17 days post-exposure. Sarin did not produce a systematic dose-dependent response in parietal cortex at any of the time points. However, significant increases in DNA fragmentation at 0.1 and 0.4 LD_{50} were observed at 0 and 3 days post-exposure. All measures of DNA fragmentation in both leukocytes and neurons returned to control levels by 17 days post-exposure, indicating a small and non-persistent increase in DNA fragmentation following repeated low-level exposure to sarin.

Keywords. Chemical warfare agents, sarin, comet assay, DNA fragmentation, guinea pigs, lymphocytes, brain, parietal cortex.

Introduction

Chemical warfare nerve agents (CWNAs) remain an ongoing threat to military and civilian populations. For example, sarin was used previously when terrorists released sarin gas in Matsumoto and Tokyo, Japan, in 1994 and 1995, respectively [1, 2]. Furthermore, in March of 1991, a munitions storage complex at Khamisiyah, Iraq, was destroyed, resulting in the presumed exposure of troops to low levels of sarin and

† Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulation relating to animals and experiments involving animals and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, NIH publication 85-23. The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense (para 4-3), AR 360–365.

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cyclosarin [3]. A recent study of 1991 Gulf War veterans has linked low-level CWNA exposure in troops with reduced white matter and increased right and left ventricle volumes [4]. As a result, research into the effects of low-level exposure has become particularly important. Personnel responding to an attack as well as people located away from the epicenter of an attack are likely to receive low-level exposure to CWNAs. Also, occupational exposure as chemical weapons stockpiles are destroyed is another potential risk for repeated low-dose exposure. Such chemical terrorist attacks as well as accidental exposure pose a definite threat to both civilians and military personnel in the United States and overseas, and as such, research into methods of evaluating, and protecting against, CWNA-induced tissue and brain injury is of great importance.

CWNAs such as soman (GD), sarin (GB), and VX are extremely toxic, irreversible cholinesterase inhibitors that can be used in military operations, or by terrorists. Sarin (GB) is an extremely toxic, man-made CWNA, similar to organophosphate insecticides. Sarin irreversibly inhibits acetylcholinesterase (AChE), the enzyme that breaks down acetylcholine, resulting in the buildup of acetylcholine at neuronal synapses and neuromuscular junctions. This results in overstimulation of cholinergic systems and, when exposure is sufficient, leads to tetany of skeletal muscles, respiratory depression, seizures, coma, and possibly death. In addition, it has been shown that organophosphates have non-cholinergic effects on various organ systems, including mutagenic and hematotoxic effects as well as immunosuppression. Traditionally, anti-cholinergic compounds have been demonstrated to provide variable degrees of neuroprotection against organophosphates [5]. Recent studies have also attributed involvement of NMDA (N-methyl-D-aspartate) receptor modulation, in conjunction with the anticholinergic properties of neuroprotective drugs like caramiphen, against organophosphate toxicity [6].

Neuronal DNA fragmentation following traumatic brain injury and ischemic injury is well documented. However, neuronal DNA fragmentation following nerve agent-induced injury is less well studied. Several recent studies have shown alterations in immune function following low-dose CWNA exposure [7–12], making further study into leukocyte integrity of interest. Previously, we reported that low-dose soman exposure showed DNA fragmentation in leukocytes, providing a potential biomarker for assessment of cellular damage following low-dose (non-symptomatic) CWNA exposure [13]. In the present study, we extended our earlier findings to determine whether low-dose sarin exposure would also produce DNA fragmentation similar to soman exposure. DNA fragmentation in circulating leukocytes and parietal cortex following repeated low-dose exposure to sarin were quantified using single-cell gel electrophoresis (comet assay). Comet assay is a sensitive method for evaluating DNA damage in cells where denatured, cleaved DNA fragments will migrate out of the nuclei on electrophoresis, allowing assessment of DNA damage [13].

Materials and methods

Male guinea pigs (400–500 g, n = 5 per group) were injected (s.c.) with saline, or 0.1 LD_{50} (50% mean lethal dose), 0.2 LD_{50}, or 0.4LD_{50} sarin [14] dissolved in sterile physiological saline, Monday through Friday, once a day at 0800 h for 10 days (animals were not injected on the weekend). At 1200 h on day 12 (0 day post-injection), day 15 (3 days post-injection), or day 29 (17 days post-injection), animals were anesthetized with pentobarbital (325 mg/kg) and sacrificed by decapitation. (Agent administration was conducted at USAMRICD using all applicable safety regulations and standard operating procedures). Blood was collected in tubes containing EDTA, and kept on ice until processed for comet analysis. Brain tissues were removed, and parietal cortex was dissected out and kept on ice until further processing.

Whole blood (100 µl) from each animal was added to 1 ml of ice-cold Ca^{2+}, Mg^{2+}-free PBS (phosphate-buffered saline) with 20 mM EDTA. The mixtures were centrifuged at 1000 *g* for 10 min to remove plasma, and the cell pellet was resuspended in 1 ml of the same solution. Cells were washed by this method two more times to remove cell debris. The final pellet was suspended in 1 ml of ice-cold PBS with 20 mM EDTA, and 50 µl was removed and combined with 500 µl of low-melting-point agarose (LMAgarose: Trevigen, Gaithersburg MD) warmed to 42 °C. For the rest of the procedure the manufacturer's (Trevigen, Gaithersburg, MD) protocol was used for the comet assay. Briefly, a 50-µl aliquot of this mixture was transferred to specially treated slides and allowed to cool. Slides were then immersed in ice-cold lysis solution (pH 9.6; Trevigen) and incubated for 30 min at 4 °C. Slides were then moved to an alkali buffer solution (0.6 g of NaOH and 100 µl of 0.5 M EDTA in 50 ml of purified water, pH 12.8) for 20–30 min at room temperature. Slides were then washed twice in Tris-borate EDTA solution (TBE buffer, pH 8.4), 5 min each, and then placed in the electrophoresis chamber. Electrophoresis was run for 10 min at 25 mV in TBE buffer. Slides were removed from the apparatus and were fixed for 5 min with ice-cold 100% MeOH, followed by 5 min in

ethanol, and then were dried in the dark at room temperature.

Dissected parietal cortex tissue samples were individually forced through cell strainer (100 μm Nylon; BD Falcon, Bedford, MA) in 10 ml ice-cold Ca^{2+}, Mg^{2+}-free PBS with 20mM EDTA. After three washes in PBS the cell pellets were resuspended in 1 ml of PBS, and 50 μl was combined with 500 μl of low-melting-point agarose at 42 °C. Fifty microliters of this mixture was transferred to comet slides. The above-mentioned protocol was followed for the rest of the procedure. For quantification of DNA fragmentation, 30 leukocytes or neurons were imaged per animal, with 5 animals in each group (150 individual leukocytes or neurons per group). Specially designed comet analysis software (Loats Associates, Westminster, MD) was used to analyze the degree of DNA fragmentation in individual leukocytes or neurons. Slides were covered with 50 μl of 0.01 % SYBR Green (Trevigen) in Tris-EDTA buffer and imaged with a monochrome digital camera connected to an Olympus BX60 fluorescent microscope using a 20× objective. Three parameters of DNA fragmentation were measured: comet tail length, percent DNA in the tail, and tail moment arm.

Statistical analysis

Inferential statistics were calculated using the SAS (Cary, NC) statistical software package. Between-group effects for dependent measures of 'tail length', 'percent DNA in the tail', and 'moment arm' were assessed with ANOVA (analysis of variance) using the Proc GLM procedure. Multiple contrasts comparing sarin-exposed groups with vehicle control groups at each time period were evaluated with two-tailed Dunnett's t-tests. For the latter tests, significance was assumed when significant t-values were observed and a significant dose by time interaction at the same time level.

Results

No clinical signs were observed following exposures to repeated doses of sarin up to 0.4 LD_{50}. Leukocytes with DNA fragmentation were rare in blood samples from the saline-injected animals, but were significantly more numerous in blood from sarin-injected animals. The representative images in Fig. 1a show a normal leukocyte from a saline-injected animal, as compared with a leukocyte taken from a sarin-injected animal after 10 days of exposure (0 day post-exposure). Typically, normal leukocytes had smooth edges, and little or no DNA was observed in the comet tail. When DNA fragmentation was present, the fluorescent signal in the nucleus appeared granular, the edges of the nucleus were uneven, and some of the DNA had migrated out of the nucleus into the comet tail (a hallmark of apoptosis).

Repeated low-dose exposure to sarin produced a dose-dependent response in leukocytes at 0 and 3 days post-exposure (Fig. 2). There was a significant increase in all measures of DNA fragmentation after repeated injection with 0.2 and 0.4 LD_{50}, but not at 0.1 LD_{50}. There was no significant increase in DNA fragmentation in any of the groups at 17 days post-exposure. Sarin injection did not produce a systematic dose-dependent response in parietal cortex at any of the time points (Fig. 3). However, significant increases in DNA fragmentation were observed after repeated injection of 0.1 LD_{50} on day 0 and at 0.4 LD_{50} on day 3. All measures of DNA fragmentation in both leukocytes and neurons returned to control levels by 17 days post-exposure.

Discussion

The results of the present study shows that repeated exposure to low-dose sarin produces DNA fragmentation of the neurons and leukocytes in guinea pigs as measured by single-cell gel electrophoresis or comet assay. Repeated low-dose exposure to sarin produced a dose-dependent response in leukocytes at 0 and 3 days post-exposure. There was a significant increase in all measures of DNA fragmentation at doses higher than 0.1 LD_{50}. There was no significant increase in DNA fragmentation in any of the groups at 17 days post-exposure, indicating that the effects of the repeated exposures were not persistent. Moreover, sarin did not produce a systematic dose-dependent response in parietal cortex at any of the time points, although a significant increase in DNA fragmentation was observed following repeated injection of 0.1 LD_{50} on day 0 and at 0.4 LD_{50} on day 3. Furthermore, the reason for the lack of any DNA fragmentation in neurons following 0.2 and 0.4 LD_{50} on day 0 is not clear. All measures of DNA fragmentation in both leukocytes and neurons returned to control levels by 17 days post-exposure. In contrast to the results of the present study, in an earlier study we reported that low-level repeated soman exposure produced leukocyte DNA fragmentation that persisted up to 17 days after the repeated exposures at all the doses studied (0.1–0.4 LD_{50}) [13]. Although we do not know why we observed these differences in DNA fragmentation between soman and sarin, it may be related to differences in the agents with respect to the rate of spontaneous

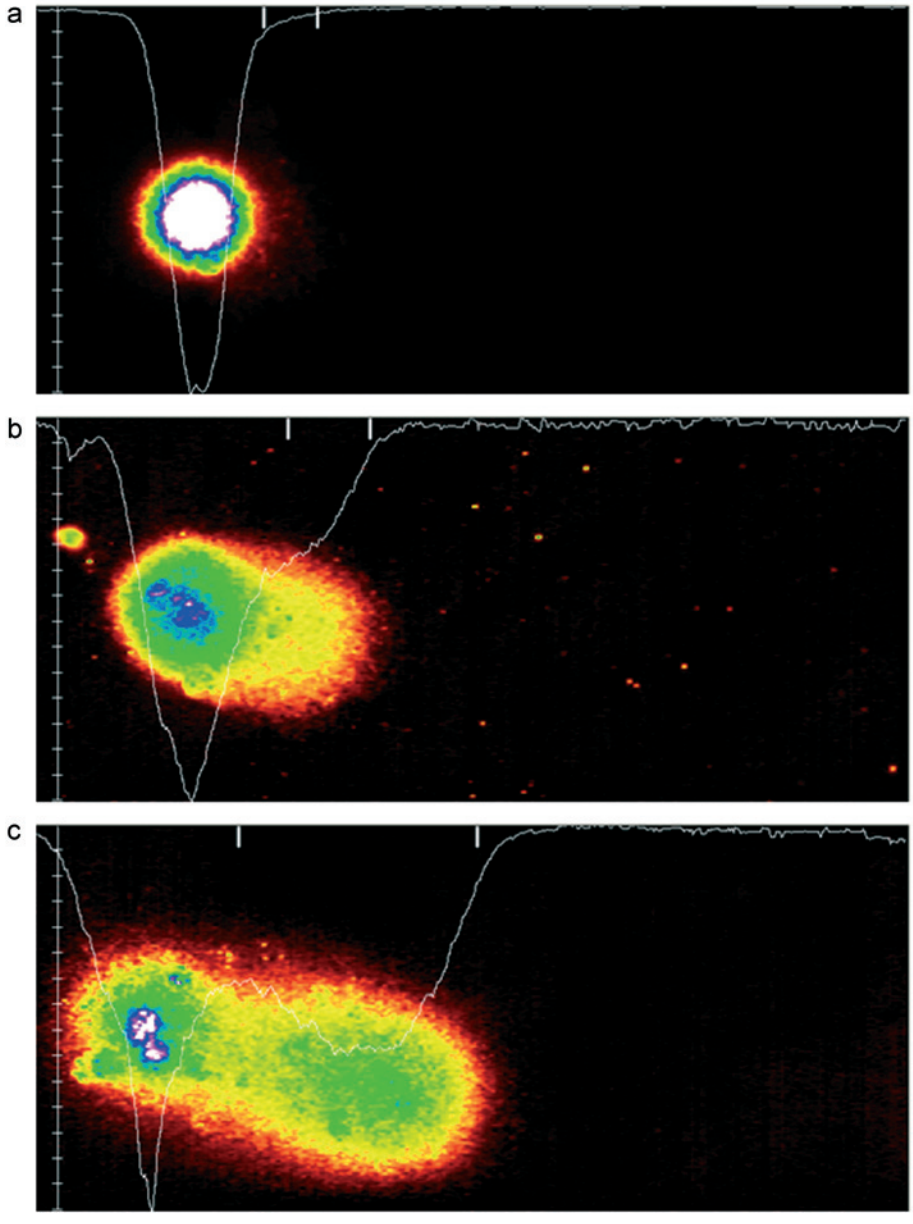

Figure 1. Representative images of leukocytes from control (*a*), 0.2 LD_{50} (*b*), and 0.4 LD_{50} (*c*) sarin-injected guinea pigs 3 days after the last injection. The fluorescence intensity for each vertical strip of pixels in the image is summed and shown as an overlaid graph. Tail length is a measure of the linear length of the comet tails, while percentage DNA is a measure of the total cellular DNA found in the tail (the fluorescent intensity in the tail $\times 100$ divided by the total fluorescent intensity in the cell). The moment arm is a measure of the average DNA migration from the nucleus.

reactiviation of phosphyrylated enzyme and the rate of enzyme dealkylation (i.e., aging). Further, in case of soman exposure, a high degree of correlation was previously observed between striatal AChE inhibition and toxic signs, which was not seen in the case of sarin exposure [15]. Although in the present study increased DNA fragmentation could be detected in blood leukocytes over 3 days after exposure to 0.2 LD_{50} or higher doses of sarin, no obvious symptoms of toxicity were observed.

Organophosphates such as soman and sarin exert their toxic effects primarily through a potent inhibitory action on AChE, the enzyme that degrades the neurotransmitter acetylcholine. This leads to increased concentrations of acetylcholine in the brain and at neuromuscular junctions, resulting in epileptic seizures and muscle tetany. The neuropathological sequelae of severe sarin toxicity include neural lesions of the hippocampus, piriform cortex, and thalamus [16]. The effects of low levels of sarin poisoning on the immune system have also been characterized [7–9]. An earlier study reported that both symptomatic and asymptomatic doses of sarin are able to produce long-term reductions in leukocyte responsiveness at 6 and 12 months after exposure [17], but the mechanism by which such long-term leukocytic damage occurs is not known. Though speculative, these long- and short-term changes in immune function may be related to leukocyte DNA fragmentation, as we observed in the present study. It is also possible that organophos-

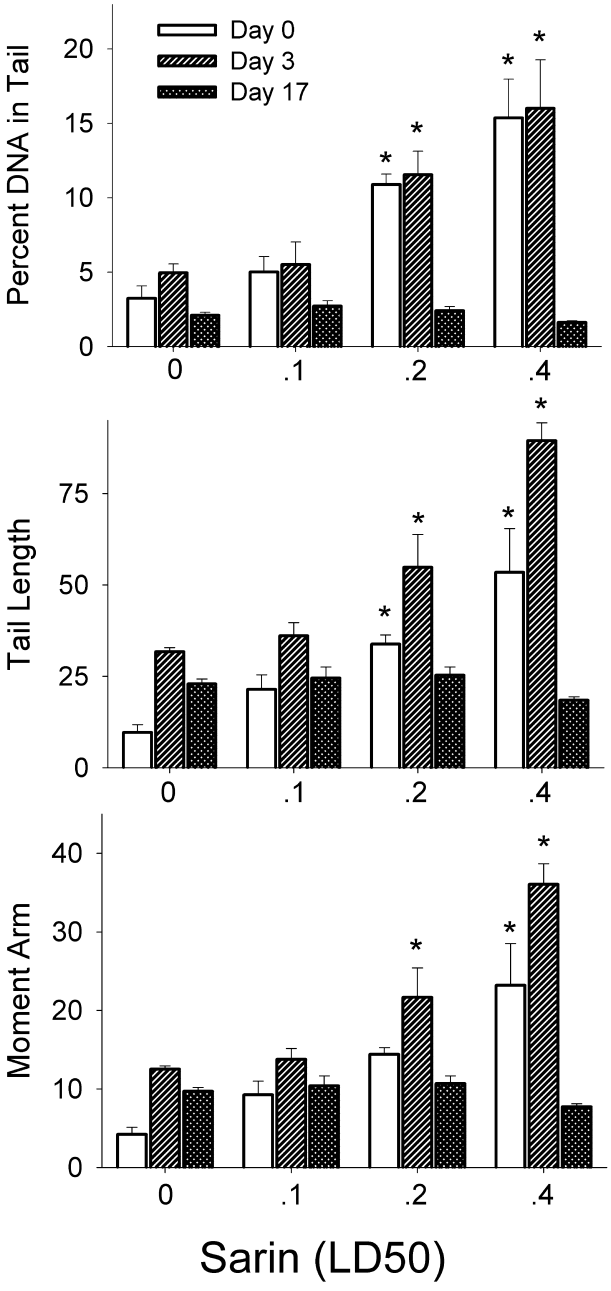

Figure 2. Measures of DNA damage in leukocytes after 0, 3 or 17 days following last sarin exposure. Values marked with an asterisk are significantly different from controls ($p < 0.05$). Error bars represent the standard error of the mean.

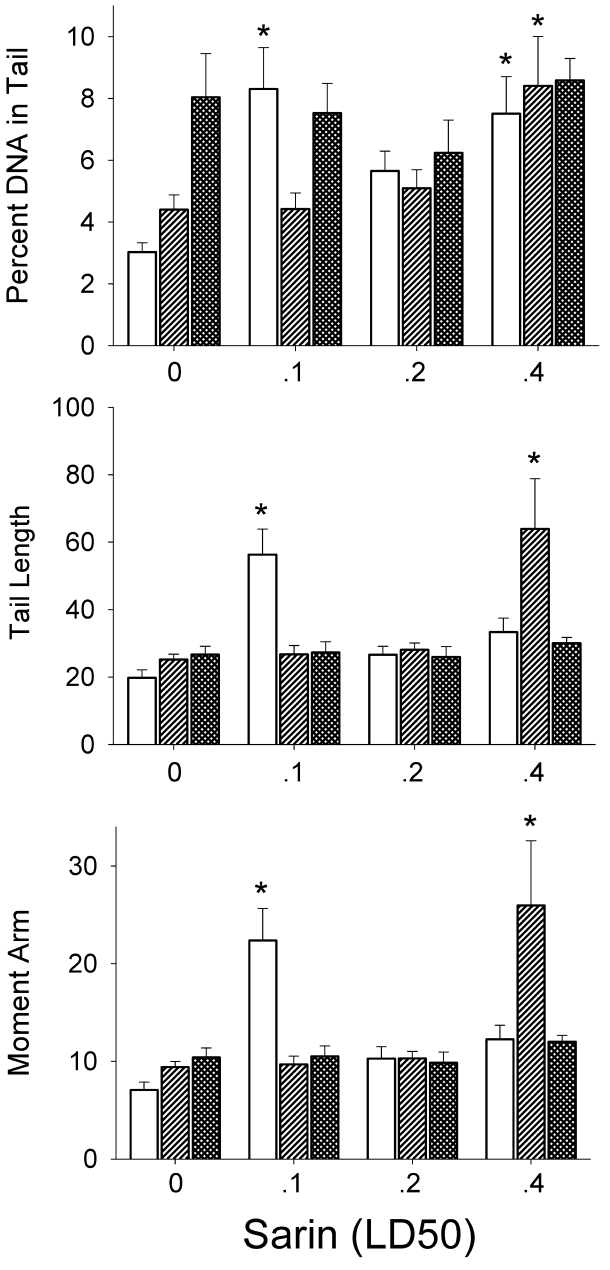

Figure 3. Measures of DNA damage in neurons from parietal cortex after 0, 3 or 17 days following the last sarin exposure. Values marked with an asterisk are significantly different from controls ($p < 0.05$). Error bars represent the standard error of the mean.

phates cause damage at the level of stem cells in the bone marrow, which could then lead to increased apoptosis in the derived leukocytes.

While the link between organophosphate poisoning and leukocyte or neuronal apoptosis remains uncertain, it is interesting to note that an earlier study observed necrotic neuronal cell death following toxic doses of organophosphate vs. apoptotic neuronal cell death involving oxidative stress following sublethal doses of organophosphate [18]. Other studies also suggest that reactive oxygen molecules may be involved in neuronal apoptosis after exposure to the cholinesterase inhibitor pyridostigmine bromide [19]. It was found that the release of reactive oxygen species after pyridostigmine bromide treatment was mediated by muscarinic acetylcholine receptors, and NMDA glutamate receptors, and that pretreatment with atropine or MK-801 blocked formation of reactive oxygen species. It is possible that similar mechanisms

could be involved in the leukocyte or neuronal damage observed in the present study after sarin exposure. If confirmed in the case of CWNA exposure, current organophosphate poisoning treatments may benefit by cotreatment with antioxidant compounds, and inhibitors of reactive oxygen species generation. In conclusion, these results suggest that DNA fragmentation in leukocytes observed in this study may play a role in the altered immune function shown in earlier studies.

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