

Subclinical Doses of the Nerve Gas Sarin Impair T Cell Responses through the Autonomic Nervous System

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The nerve gas sarin is a potent cholinergic agent, and exposure to high doses may cause neurotoxicity and death. Subclinical exposures to sarin have been postulated to contribute to the Gulf War syndrome; however, the biological effects of subclinical exposure are largely unknown. In this communication, evidence shows that subclinical doses (0.2 and 0.4 mg/m³) of sarin administered by inhalation to F344 rats for 1 h/day for 5 or 10 days inhibited the anti-sheep red blood cell antibody-forming cell response of spleen cells without affecting the distribution of lymphocyte subpopulations in the spleen. Moreover, sarin suppressed T cell responses, including the concanavalin A (Con A) and the anti- $\alpha\beta$ -T cell receptor (TCR) antibody-induced T cell proliferation and the rise in the intracellular calcium following TCR ligation. These concentrations of sarin altered regional but not total brain acetylcholinesterase activity. Interestingly, serum corticosterone levels of the sarin-treated animals were dramatically lower than the control animals, indicating that sarin-induced immunosuppression did not result from the activation of the hypothalamus—pituitary—adrenal (HPA) axis. Pretreatment of animals with the ganglionic blocker chlorisondamine abrogated the inhibitory effects of sarin on spleen cell proliferation in response to Con A and anti-TCR antibodies. These results suggest that the effects of sarin on T cell responsiveness are mediated via the autonomic nervous system and are independent of the HPA axis. © 2002 Elsevier Science (USA)

Key Words: organophosphates; cholinergic agents; neuroimmune modulation; glucocorticoids; T cell mitogenesis.

Organophosphorus compounds including pesticides are potent cholinergic compounds that annually affect approximately 3 million people worldwide and result in 220,000 deaths (WHO, 1996). Sarin (isopropyl methyl phosphonofluoridate) is a powerful organophosphorus nerve agent that irreversibly inactivates cholinesterase (ChE) activity, elevating the synaptic levels of acetylcholine (Grob and Harvey, 1958). In high doses,

sarin causes seizures and even death (Marrs, 1993). Its lethality and low cost of production have made sarin the chemical of choice for terrorism. In the 1994 sarin attack of a Tokyo subway station, several people died and more than 6000 suffered sarin-related injuries (Nozaki *et al.*, 1995). Humans are exposed to sarin primarily through inhalation, and subclinical sarin exposures have been proposed as a factor in the development of the Gulf War illness (Abou-Donia *et al.*, 1996).

While people in the immediate vicinity of a sarin attack may be exposed to overt neurotoxic doses of sarin, others away from the epicenter of the attack are likely to receive subclinical amounts. High levels of organophosphates such as malathion, parathion, and dichlorvos impair cellular and humoral immune responses (Street and Sharma, 1975; Desi *et al.*, 1978). The immunological effects of subclinical doses of sarin have not been defined. Kassa *et al.* (2000) observed a decreased bacteriocidal activity of peritoneal macrophages in sarin-treated rats, and some survivors of the Tokyo subway attack died from opportunistic *Legionella* infection (Kamimura *et al.*, 1998), indicating that sarin may affect the immune system. However, the effects of sarin on the adaptive immune response are largely unknown. Sarin is a neuroactive agent and at subclinical doses affects the electrical activity of the brain (Burchfiel *et al.*, 1976). Because the brain communicates with the immune system (Blalock, 1994), it is possible that sarin would influence the immune system through the CNS. In this communication, we show that inhalation of subclinical doses of sarin suppresses T cell responses in the rat, and that the autonomic nervous system (ANS) may play a critical role in this immunosuppression.

MATERIALS AND METHODS

Animals. Pathogen-free, 8-week-old male Fischer 344 rats were purchased from Harlan Sprague–Dawley Farms (Indianapolis, IN). Food and water were provided *ad libitum* to the animals. Approximately 10-week-old animals were used for sarin inhalation.

Chlorisondamine (CHL) treatment. The ganglionic blocker CHL (Tocris, Ballwin, MO) was injected into rats (10 mg/kg body wt sc) 7 days prior to sarin exposure. This concentration of CHL blocks the behavioral responses to neuroactive substances for several months (Reuben *et al.*, 1998).

Sarin exposure. Briefly, sarin, dissolved in isopropyl alcohol, was obtained from the U.S. Army. The sarin nose-only exposure system, developed at Lovelace Respiratory Research Institute, was used to expose rats to 0 (vehicle-control; CON), 0.2 (low-dose sarin; LS), or 0.4 (high-dose sarin; HS) mg/m³ of sarin for 1 h/day for 1, 5, or 10 days according to the study protocol (Henderson *et al.*, 2002). None of the concentrations of sarin used in these experiments produced detectable histopathological changes in the brain or overt symptoms of behavioral neurotoxicity.

Isolation of tissues and cells. All animals were euthanized 1 day after the last sarin exposure between 7 and 9 a.m. Blood was collected by cardiac puncture. Clotted blood was centrifuged to obtain serum. Spleen cell suspensions were made as described (Singh *et al.*, 2000). Briefly, spleens were pressed through stainless-steel mesh, and red blood cells (RBC) were lysed by treatment with NH₄Cl solution. After washing, cells were counted and suspended in complete medium (RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, 50 mM 2-ME, and 10 µg/ml gentamicin).

Antibody-forming cell (AFC) assay. For determining the AFC response, CON and LS animals were injected iv with 5×10^8 sheep RBC (SRBC; Colorado Serum Company, Denver, CO) and euthanized 4 days later. The primary direct AFC response was determined by Cunningham and Szenberg's method as described (Singh *et al.*, 2000). Briefly, spleen cells were mixed with 2% SRBC and 25 µl of guinea pig complement (Accurate Chemicals, Westbury, NY) in 250 µl complete medium. Aliquots were distributed on Cunningham slides and incubated for 45 min at 37°C. Results are expressed as AFC/10⁶ spleen cells.

Enumeration of lymphocyte subpopulations. Number and purity of lymphocyte subpopulations were determined by FACS analysis on an EPICS C flow cytometer (Coulter) as reported (Geng *et al.*, 1995). Briefly, to quantitate the percentages of B cells and T cells, spleen cells were incubated with predetermined optimal amounts of FITC-conjugated anti-rat CD45RA or CD3 monoclonal antibodies (PharMingen, San Diego, CA), respectively. Cells were treated with the appropriate isotype-matched immunoglobulins to control for nonspecific binding. The percentage of positive cells was determined by subtracting the nonspecific (isotype control) from the specific fluorescent profiles.

Proliferative response to concanavalin A (Con A). The proliferative response of spleen cells from animals exposed to vehicle (CON), LS, HS, LS + CHL, and CHL to the T cell mitogen Con A (Sigma, St. Louis, MO) was determined as described (Singh *et al.*, 2000). Briefly, 5×10^5 cells were cultured in 0.2 ml complete medium in the presence of various concentrations of Con A in microtiter wells. The cultures were incubated for 3 days at 37°C in 5% CO₂. Proliferation was assayed by overnight pulsing of the culture wells with 0.5 µCi of [³H]thymidine (Tdr). Cells were harvested on a Skatron cell harvester (Skatron, Sterling, VA).

Proliferative response to anti-αβ-T cell receptor (TCR) mAb. Unlike the mouse, rat spleen cells proliferate in response to anti-CD3 or anti-αβ-TCR mAb in the absence of anti-CD28 (Kalra *et al.*, 2000). For proliferative response to anti-αβ-TCR (PharMingen), spleen cells were cultured with the indicated concentrations of the mAb under the conditions described for the Con A response. After 3 days cultures were labeled with [³H]Tdr and harvested.

Assay for intracellular ionized calcium [Ca²⁺]_i levels. Intracellular ionized Ca²⁺ levels were determined by spectrofluorometry as described (Kalra *et al.*, 2000). Briefly, spleen cells from CON, LS-, and HS-treated animals were loaded with acetoxymethyl ester of indo-1 (Sigma). Indo-1-labeled spleen cells were treated with mouse anti-rat αβ-TCR mAb followed by goat anti-mouse IgG (second Ab), and changes in the [Ca²⁺]_i concentration were determined in a PTI Deltascan fluorometer (Photon Technology International, South Brunswick, NJ).

Assay for serum corticosterone (CORT) levels. Serum CORT levels were determined in the CON and LS-treated animals by the CORT RIA-kit (ICN Biochemicals, Orangeburg, NY) according to the manufacturer's instructions.

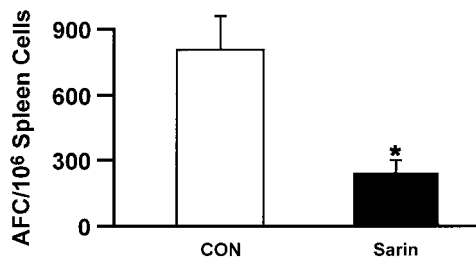


FIG. 1. Sarin inhibits the anti-SRBC AFC response. Animals (six per group) were exposed to 0.4 (LS) mg/m³ of sarin or vehicle for 1 h/day for 5 days, immunized with SRBC, and euthanized after 4 days. The anti-SRBC AFC response of spleen cells was determined as described under Materials and Methods. *AFC response of sarin-exposed animals was significantly different from CON ($p = 0.01$).

As stated by the manufacturer, under these assay conditions, normal rats have serum corticosterone levels of 50–400 ng/ml.

Statistical analysis. Statistical comparisons among the experimental groups were performed using a one-way analysis of variance. A Scheffé *post hoc* test was used to determine the significance among groups. A Student's *t* test was used to compare the means between the CON and sarin-treated animals. These statistical procedures were performed using ABSTAT software (Anderson-Bell Corp., Parker, CO).

RESULTS

Sarin Suppresses the AFC Response to SRBC

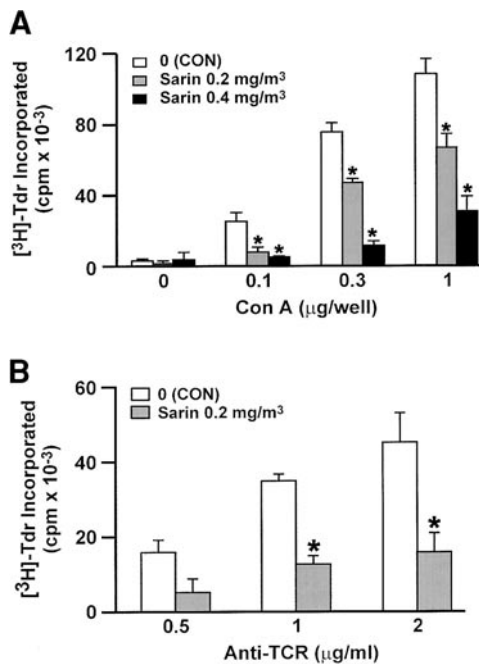
Exposure to LS for 1 h/day for 5 days caused a significant reduction in the AFC response to SRBC, indicating that sub-clinical levels of sarin suppress the Ab response (Fig. 1).

Sarin Inhibits Con A- and Anti-αβ-TCR-Induced T Cell Proliferation

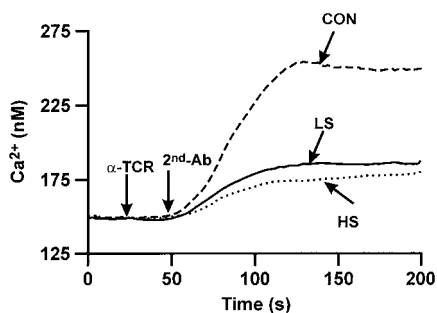
Con A-induced T cell proliferation was not significantly affected by the 1-day exposure (data not shown); however, animals exposed to both LS and HS (1 h/day for 5 days) exhibited a dose-dependent reduction in T cell proliferation (Fig. 2A). Proliferative response of spleen cells to anti-αβ-TCR mAb from animals exposed to LS (1 h/day for 10 days) also showed a significant reduction in proliferation (Fig. 2B). Similar results were obtained with the anti-CD3 antibody (data not shown). Moreover, the number and distribution of T cells (CD3⁺) and B cells (CD45RA⁺) within splenic cells were not significantly different between CON and sarin-treated animals (not shown). Thus, exposures to concentrations of sarin that do not cause detectable neurotoxicity or changes in splenic lymphocyte populations produce a significant reduction in the proliferation of T cells to mitogens and antigens.

Sarin Inhibits the Anti-TCR-Induced Rise in [Ca²⁺]_i

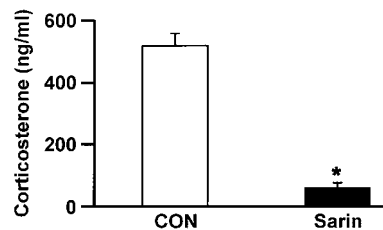
An early event in the TCR-mediated T cell activation is an increase in [Ca²⁺]_i (Clapham, 1995). A representative experiment (Fig. 3) shows that, compared to CON, the rise in [Ca²⁺]_i



was significantly reduced in spleen cells from animals exposed for 5 days to LS and HS. However, there was no significant difference in the inhibition of Ca^{2+} response caused by LS and HS treatments. These data suggest that sarin inhalation may affect the antigen-mediated T cell activation cascade at step(s) proximal to the rise in $[\text{Ca}^{2+}]_i$.



Results are representative of four separate CON/sarin-treated animal combinations.



Although, the corticosterone assay kit manufacturer (ICN Biochemicals) cites that normal rat serum corticosterone concentration is in the range of 50–400 ng/ml, the corticosterone value of approximately 500 ng/ml in control animals (Fig. 4) is very high and might reflect the effects of restraining the animals for the nose-only exposure. Surprisingly, despite immunosuppression (Figs. 1 and 2), the serum CORT levels of animals treated with LS for 5 days were dramatically lower than CON rats (Fig. 4). These data suggest that even subclinical doses of sarin inhibit serum glucocorticoid levels, and the immunosuppressive effects of sarin inhalation are not the result of increased serum CORT levels produced by the activation of the HPA axis.

Sarin Decreases Serum CORT Levels

Sarin is a neuroactive substance and could affect the immune system through the CNS. The hypothalamus–pituitary–adrenal (HPA) axis modulates the immune system through elaboration of glucocorticoids (Turnbull and Rivier, 1999). To examine whether sarin inhalation suppressed the immune system by elevating glucocorticoids, we compared the serum CORT levels of CON and sarin-treated animals. Although, the corticosterone assay kit manufacturer (ICN Biochemicals) cites that normal rat serum corticosterone concentration is in the range of 50–400 ng/ml, the corticosterone value of approximately 500 ng/ml in control animals (Fig. 4) is very high and might reflect the effects of restraining the animals for the nose-only exposure. Surprisingly, despite immunosuppression (Figs. 1 and 2), the serum CORT levels of animals treated with LS for 5 days were dramatically lower than CON rats (Fig. 4). These data suggest that even subclinical doses of sarin inhibit serum glucocorticoid levels, and the immunosuppressive effects of sarin inhalation are not the result of increased serum CORT levels produced by the activation of the HPA axis.

Effects of Sarin on T Cell Proliferation Are Abrogated by CHL

The ANS innervates and modulates the immune system (Felten and Felten, 1994). Because sarin-induced suppression of T cell responses was not caused by increased glucocorticoid production, we investigated whether the ANS played a role in this suppression. The inhibitory effects of LS (1 h/day for 10 days) on Con A- (Fig. 5A) and anti-TCR- (Fig. 5B) induced T cell proliferation were attenuated by treatment with the ganglionic blocker CHL. Pretreatment with CHL alone did not significantly affect T cell mitogenesis (Fig. 5). These results suggest that the ANS may play a critical role in mediating sarin-induced immunosuppression.

DISCUSSION

Our results show that inhalation of subclinical doses of sarin suppressed the T-dependent Ab and T cell proliferative re-

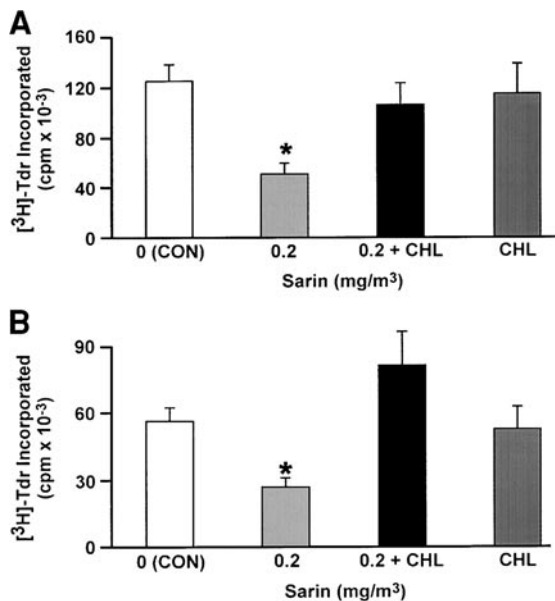


FIG. 5. Pretreatment with CHL abrogates the effects of sarin on T cell proliferation. Spleen cells from CON, 10 day LS, LS + CHL, and CHL were cultured with 5 $\mu\text{g}/\text{ml}$ of Con A (A) or 2 $\mu\text{g}/\text{ml}$ of anti- $\alpha\beta$ -TCR mAb (B) for 3 days. Proliferation was assayed as described under Materials and Methods. *Differences in T cell proliferation between CON and sarin-treated animals were significant ($p < 0.05$). However, CON, sarin + CHL-, and CHL-treated groups were not significantly different.

sponses. In these studies, we examined only the T cell function; whether sarin affects the function of other cell types is not known at this time. Because sarin exposure inhibited the ability of T cells to elevate $[\text{Ca}^{2+}]_i$ in response to TCR ligation, sarin may impair the TCR-induced signal transduction pathway at step(s) proximal to the Ca^{2+} response.

Although sarin exposure for 5 or 10 days did not inhibit the total brain ChE activity, there were small but significant decreases in the enzyme activity in some brain areas, including hippocampus (Henderson *et al.*, 2002), which, together with the hypothalamus, are the two most important brain regions for neuroimmune modulation (Blalock, 1994; Lathe, 2001). High concentrations of sarin have been detected in hippocampi and hypothalami of mice administered with [³H]sarin (Little *et al.*, 1998). Therefore, it is possible that sarin affects the ChE activity in the brain areas critical for neuroimmune communication. However, major changes in the synaptic levels of acetylcholine may not be required for immunosuppression. Indeed, levels of sarin/organophosphates that do not produce overt changes in the brain ChE activity affect several brain functions in treated animals (Chebabo *et al.*, 1999; Jones *et al.*, 2000).

Many neuroactive substances, including opiates (Nelson and Lysle, 2001) and nicotine (Sopori *et al.*, 1998; Sopori, 2002), affect the immune system through the CNS. However, the mechanism by which these substances affect the neuroimmune interaction is not clear. The neuroendocrine and immune sys-

tems are intimately related during development, maturation, and the aging process (reviewed in Heijnen and Kavelaars, 1999). The two systems communicate bidirectionally through hormones, cytokines, and neurotransmitters via several pathways (Blalock, 1994). The HPA axis communicates with the periphery through the release of anterior and posterior pituitary hormones and leads to the production of glucocorticoids from the adrenals, which inhibit immune responses (Turnbull and Rivier, 1999). Another pathway involves the ANS, which connects the CNS directly to visceral target tissues via the sympathetic and parasympathetic nerves (Felten and Felten, 1994; Borovikova *et al.*, 2000), and these nerve fibers are in direct contact with T cells in the white pulp of the spleen (Felten and Felten, 1994). Experiments to ascertain whether sarin affected T cell responses through activation of the HPA axis revealed that CON animals had high serum CORT levels. This might result from a stress response due to restraining the animals for nose-only exposures. Surprisingly, however, despite immunosuppression, sarin-treated animals had dramatically lower serum CORT levels compared to the CON animals. It is possible that sarin decreased the serum CORT levels by moderating the restraint-induced stress in these animals. However, our preliminary results (not shown) indicate that other cholinergic agents administered via non-stress-inducing routes also decrease serum CORT levels. The mechanism of this decrease is not clear at present and may result from impairment of the adrenal function by cholinergic agents. Whether the decrease in serum CORT levels is a characteristic of cholinergic toxicity remains to be proven. However, despite high serum CORT levels, CON animals showed significantly higher AFC and T cell proliferative responses than sarin-treated animals. These results suggest that sarin-induced immunosuppression does not result from overproduction of CORT (i.e., hyperactivation of the HPA axis). Moreover, changes in serum CORT levels are not necessarily associated with immunosuppression. Thus, inhibition of T cell proliferation by acute nicotine administration was unrelated to increases in serum CORT levels (Mellon and Bayer, 1999), and blockers of the glucocorticoid receptor did not affect the immune changes associated with sympathectomy (Kruszewska *et al.*, 1998).

Pretreatment of rats with the ganglionic blocker CHL, which inhibits the nicotinic acetylcholine receptors on the ganglion and blocks the ANS transmission, abrogated the effects of sarin on T cell proliferation and the Ca^{2+} response. The sympathetic nervous system innervates primary and secondary lymphoid tissues and modulates a wide variety of innate and adaptive immune responses (Madden *et al.*, 1995). Besides, lymphocytes and other immune cells express functional adrenergic and cholinergic receptors (Schauenstein *et al.*, 2001). Chemical sympathectomy has been shown to affect T-dependent antibody responses, interleukin-2 production, cytotoxic T cell activity, and delayed-type hypersensitivity responses (Madden *et al.*, 1989; Kruszewska *et al.*, 1998). Moreover, in animal models of autoimmune disease, ablation of the sympathetic

nervous system increases the severity of the disease (Chelmicka-Schorr *et al.*, 1988, 1993). Thus, the sympathetic ANS plays an important role in the regulation of neuroimmune interaction. Our results with subclinical sarin exposure suggest that sarin-induced immunosuppression is mediated primarily through the ANS; however, whether these effects are mediated through the sympathetic and/or parasympathetic pathways remains to be determined.

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