ACUTE EXPOSURE TO SARIN INCREASES BLOOD BRAIN BARRIER PERMEABILITY AND INDUCES NEUROPATHOLOGICAL CHANGES IN THE RAT BRAIN: DOSE–RESPONSE RELATIONSHIPS

A. ABDEL-RAHMAN,a A. K. SHETTYb,c and M. B. ABOU-DONIA*a,d,e

aDepartment of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA
bDepartment of Surgery (Neurosurgery), Duke University Medical Center, Durham, NC 27710, USA
cMedical Research Service, Veterans Affairs Medical Center, Durham, NC 27705, USA
dDepartment of Neurobiology, Duke University Medical Center, Durham, NC 27710, USA

Abstract—We hypothesize that a single exposure to an LD50 dose of sarin induces widespread early neuropathological changes in the adult brain. In this study, we evaluated the early changes in the adult brain after a single exposure to different doses of sarin. Adult male rats were exposed to sarin by a single intramuscular injection at doses of 1, 0.5, 0.1 and 0.01 × LD50. Twenty-four hours after the treatment, both sarin-treated and vehicle-treated (controls) animals were analyzed for: (i) plasma butyrylcholinesterase (BChE) activity; (ii) brain acetylcholinesterase (AChE) activity, (iii) m2 muscarinic acetylcholine receptor (m2 mAChR) ligand binding; (iv) blood brain barrier (BBB) permeability using [H3]hexamethonium iodide uptake assay and immunostaining for endothelial barrier antigen (EBA); and (v) histopathological changes in the brain using H&E staining, and microtubule-associated protein (MAP)-2 and glial fibrillary acidic protein immunostaining. In animals treated with 1 × LD50 sarin, the significant changes include a decreased plasma BChE, a decreased AChE in the cerebrum, brainstem, midbrain and the cerebellum, a decreased m2 mAChR ligand binding in the cerebrum, an increased BBB permeability in the cerebrum, brainstem, midbrain and the cerebellum associated with a decreased EBA expression, a diffuse neuronal cell death and a decreased MAP-2 expression in the cerebral cortex and the hippocampus, and degeneration of Purkinje neurons in the cerebellum. Animals treated with 0.5 × LD50 sarin however exhibited only a few alterations, which include decreased plasma BChE, an increased BBB permeability in the midbrain and the brain stem but without a decrease in EBA expression, and degeneration of Purkinje neurons in the cerebellum. In contrast, animals treated with 0.1 and 0.01 × LD50 did not exhibit any of the above changes. However, m2 mAChR ligand binding in the brainstem was increased after exposure to all doses of the sarin.

Collectively, the above results indicate that, the early brain damage after acute exposure to sarin is clearly dose-dependent, and that exposure to 1 × LD50 sarin induces detrimental changes in many regions of the adult rat brain as early as 24 hours after the exposure. The early neuropathological changes observed after a single dose of 1 × LD50 sarin could lead to a profound long-term neurodegenerative changes in many regions of the brain, and resulting behavioral abnormalities. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: brain injury, Gulf War syndrome, glial hypertrophy, glial fibrillary acidic protein, microtubule-associated protein-2, neuron degeneration.

Since their return from the Persian Gulf War (PGW), about 30 000 veterans have had a range of unexplained ailments such as chronic fatigue, muscle and joint pain, ataxia, skin rash, headache, loss of concentration, forgetfulness, and irritability (Institute of Medicine, 1995, 1996, 2000). Several studies suggest that the illness of Gulf War veterans is the result of exposure to a psychologically stressful environment and/or a unique combination of multiple chemicals such as pyridostigmine bromide, organophosphorus insecticides, the insect repellent N,N-diethyl-m-toluamide and chemical warfare agents, particularly sarin. It is believed that the chemical warfare agent sarin was released into the atmosphere in certain regions of the Persian Gulf following the destruction of Iraqi arsenals during the war (Committee on Veterans Affairs, 1998; PAC, 1997).

The chemical sarin (methyl isopropyl phosphonofluoridate) is an organophosphorus compound that was developed for use as a chemical weapon during the World War II. Sarin was also used during the Iran–Iraq conflict in the 1980s and the Gulf War (Ivarsson et al., 1992; Defense Science Board, 1994; Committee on Banking, Housing and Urban Affairs, 1994; Brown and Brix, 1998; Institute of Medicine, 2000). The main clinical features associated with acute sarin intoxication include seizures, fasciculations, tremors and hypothermia (Taylor, 1985). Animal studies have shown that acute
exposure to higher doses of sarin causes direct effects on the cholinergic system by inhibition of acetylcholinesterase (AChE) in both the peripheral nervous system (PNS) and CNS (Wood, 1951; Gupta et al., 1991; Gunderson et al., 1992). The consequent rise in acetylcholine (ACh), leads to over-stimulation of cholinergic synapses via nicotinic and muscarinic acetylcholine receptors (nAChR and mAChR; Somani, 1992; Lotti, 2000; Spencer et al., 2000). The nAChR and mAChR mediate diverse cellular responses by distinct signaling mechanisms (Wess, 1996), and excessive accumulation of ACh can lead to activation of ligand-gated ion channels. Several studies suggest that exposure to organophosphates including the sarin can cause differential regulation of both nAChRs and mAChRs (Huff et al., 1994; Katz et al., 1997). In addition to direct cholinergic effects, sarin can induce its effects through other mechanisms which may exert its effects on mAChR, reduce evoked γ-amino butyric acid (GABA) release from neurons, and alter the level of neurotransmitters in many brain regions (Dasheiff et al., 1977; Rocha et al., 1998; Chehabo et al., 1999). Thus, it is likely that alterations in both cholinergic and non-cholinergic pathways would play a key role in the neurotoxicity induced by sarin exposure.

Like soman and other lipophilic organophosphorus compounds, sarin can cross the blood brain barrier (BBB), and cause brain damage leading to neurological symptoms (Veronesi et al., 1990; Marrs, 1993; Taylor, 1996). Sarin exposure also induces progressive, long-term effects in both humans and animal models (Kadar et al., 1995; Savage et al., 1988; Yokoyama et al., 1998). A study by Kadar et al. (1995) has shown that the neuropathological changes observed after a single higher dose of sarin are progressive and the effects increase with time after the exposure, involving brain regions that are initially unaffected. In addition, it has been reported that personnel who were exposed to lower doses of the sarin exhibited persistent neurological and psychiatric abnormalities after 5–10 years of the exposure (Sidell, 1974; Duffy et al., 1979, 1980). A significant neurological dysfunction has also been observed in people exposed to sarin in Japan after 6–8 months of the exposure (Yokoyama et al., 1998). Further, long-term changes in the electroencephalogram have been observed in rhesus monkeys following exposure to a single higher dose (5 μg/kg) or repeated lower dose of sarin (1 μg/kg; Burchfiel et al., 1976; Burchfiel and Duffy, 1982). A recent study also demonstrates increased beta amplitude in marmosets after a single lower dose of the sarin (Pearce et al., 1999).

Since the symptoms reported by veterans of the PGW involved persistent abnormal regulation of functions of the CNS, it is possible that long-term effects following exposure to sarin might have been initiated as early neural damage that persisted and/or exacerbated with time after the exposure. In this study, we tested the hypothesis that a single exposure to sarin at the LD₅₀ dose and fractions thereof induces widespread early neuropathological changes in the adult brain. We rigorously evaluated the early changes in the adult brain after a single exposure to different doses of the sarin. Adult male rats were exposed to the sarin by a single intramuscular injection at a dose of 1, 0.5, 0.1 and 0.01 × LD₅₀. Twenty-four hours after this treatment, both sarin-treated and vehicle-treated (controls) animals were analyzed for plasma butyrylcholinesterase (BChE) and brain AChE activity, m₂ mAChR ligand binding, BBB permeability, and histopathological changes in the brain.

### Experimental Procedures

#### Chemicals

A stock solution of the sarin (1.9 mg/ml in saline) was obtained from the U.S. Army Medical Research and Material Command (Fort Derrick, MD, USA). The chemicals acetylthiocholine iodide and butyrylthiocholine iodide were obtained from the Sigma Chemical (St. Louis, MO, USA). The chemical [⁴¹⁴C]-H]AF-DX384 (2.3-dipropylamino; specific activity 106.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). The polyclonal antibody against glial fibrillary acidic protein (GFAP) was obtained from Dako (Carpenteria, CA, USA). The monoclonal antibodies against microtubule-associated protein-2 (MAP-2) (SMI 52) and endothelial barrier antigen (EBA) (SMI 71) were obtained from Sternberger Monoclonals (Lutherville, MD, USA). The avidin–biotin–peroxidase kits were obtained from Vector Laboratories (Burlingame, CA, USA). All other reagents were of analytical grade and were obtained from commercially available sources.

#### Animals and sarin treatment

Male Sprague-Dawley rats weighing 225–250 g were obtained from Zivic-Miller Laboratories (Allison Park, PA, USA). The animals were randomly assigned to control and treatment groups and housed at 21–23°C with a 12-h light/dark cycle. They were fed with Purina certified rodent chow (Ralston Purina, St. Louis, MO, USA) and tap water ad libitum. The rats were allowed to adjust to their environment for 1 week before the commencement of the chemical treatment. Animal care was in accordance with the Duke University institutional animal care and use committee. For sarin treatment, animals were divided into five groups. Group 1 was comprised of control animals (n = 15), which received a single intramuscular injection of 0.1 ml of normal saline. Animals in Group 2 (n = 20), Group 3 (n = 15), Group 4 (n = 15) and Group 5 (n = 15) were respectively treated with a single intramuscular injection of the sarin at a dose of 1, 0.5, 0.1 and 0.01 × LD₅₀. Based on a series of preliminary experiments on adult rats using intramuscular injections of the sarin in our laboratory, the LD₅₀ dose of the sarin was determined to be 101 μg/kg body weight (data not shown).

#### Sample preparation for biochemical studies

Twenty-four hours following treatment with either saline or sarin, five animals from each group were anesthetized with pentobarbital (100 mg/kg body weight). Blood samples were collected through heart puncture using heparinized syringes. The plasma was separated by centrifugation at 17,000 × g for 5 min. The brains were dissected out and rapidly chilled to obtain the cerebrum, the midbrain, the cerebellum, and the brainstem. The entire dissection procedure was performed rapidly on ice and tissues were snap frozen using liquid nitrogen. Both plasma and dissected brain regions were stored at −80°C for enzyme studies.
Early brain damage after acute sarin exposure to adult rat

The numerical density of dying neurons per mm² area of tissue in H&E-stained sections was measured for layers III and V of the motor cortex, somatosensory cortex, ventral posterolateral (VPL) nucleus of the thalamus, granule cell layer of the dentate gyrus, pyramidal cell layer of CA1 and CA3 subfields of the hippocampus, and Purkinje cell layer of the cerebellum in lobule 2 of the cerebellar vermis. Five sections through each of the above brain regions were employed for measurements in each animal belonging to the following four groups: (a) control animals (n=5); (b) animals treated with 1×LD₅₀ (n=5); (c) animals treated with 0.5×LD₅₀ (n=5); (d) animals treated with 0.1×LD₅₀ (n=5); and (e) animals treated with 0.01×LD₅₀ (n=5). Measurements in sections from various groups were performed blind using experimental codes. The coding was such that animal treatments were not known during measuring; however, sections that came from the same animal were identified. All measurements were performed using a Nikon E600 microscope equipped with eyepiece grid (Southern Micro Instruments, Atlanta, GA, USA). At a magnification of 400× (using 40× objective lens and 10× eyepieces), dying neurons encountered within a unit area of each section were counted.

The unit area selected for measurements varied for different regions of the brain, depending on the availability of the overall area for different layers using eyepiece grid at 400× magnification. The area measured was 0.019 mm² for layer III of the motor and somatosensory cortex, 0.063 mm² for layer V of the motor and somatosensory cortex, 0.013 mm² for the dentate granule cell layer, 0.0063 mm² for the CA1 pyramidal cell layer, 0.013 mm² for the CA3 pyramidal cell layer, 0.063 mm² for the VPL nucleus of the thalamus, and 0.0063 mm² for the Purkinje cell layer of the cerebellum. The counting of dying neurons in all groups involved only those neurons that exhibited dense cosinophilic staining in both soma and proximal dendrites. The density of neurons per unit area was transformed to the numerical density per mm² area of the respective brain region. The mean value for each of the many brain regions (layers III and V of the motor and somatosensory cortex, granule cell layer of the dentate gyrus, CA1 and CA3 pyramidal cell layers of the hippocampus, the dorsal thalamus, and the Purkinje cell layer of the cerebellum) was calculated separately for each animal by using data from five sections before the means and standard errors were determined for the total number of animals included per group.

Histopathological analysis using H&E staining

Twenty-four hours following the treatment, five animals from each group were anesthetized with sodium pentobarbital (100 mg/kg) and perfused through the heart with saline followed by 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M Tris buffer. The brains were removed carefully and post-fixed in the same fixative for 18–24 h. The tissues were then blocked and embedded in paraffin according to the standard histological techniques. Six micrometer-thick coronal sections were cut through different brain regions. From every brain, representative coronal sections (n=5) through the motor and somatosensory cortex, the septal hippocampus, the dorsal thalamus and the cerebellum were stained with hematoxylin and eosin (H&E) for light microscopic observation.

Immunohistochemical staining for MAP-2, EBA and GFAP

Representative brain sections through the motor and somatosensory cortex, the septal hippocampus and the dorsal thalamus from each animal were immunohistochemically stained using antibodies against MAP-2, EBA (SMI-71 and SMI-72, Sternberger Monoclonals, 1:1000 dilution; Rosenstein et al., 1992) and GFAP (Dako, 1:10000 dilution) by employing the avidin–biotin complex staining method (Hsu et al., 1981) using reagents from Vector Laboratories.

Quantitative evaluation of the number of dying neurons in different brain regions

Morphometric analyses of MAP-2-positive and EBA-positive immunoreactivity in different brain regions

Morphometric analyses of MAP-2-positive and EBA (SMI-71) -positive immunoreactive structures in different regions were performed by using Scion Image for Windows, based on NIH Image for Macintosh (Scion Corporation, Frederick, MD, USA). For every brain region, two sections were measured in each animal. All data were collected blind to experimental codes and means were calculated for each animal individually before the means were determined for the five animals per group.

The area occupied by MAP-2-positive immunoreactive structures per unit area of tissue (0.044 mm² in area) was determined for layers III and V of the motor and somatosensory cortex, granule cell layer of the dentate gyrus, CA1 and CA3 pyramidal cell layers of the hippocampus and the dorsal thalamus. The area occupied by EBA-positive immunoreactive structures per unit area of tissue (0.0176 mm² in area) was determined in layer V of the motor and somatosensory cortex, the dentate gyrus, CA1 and CA3 subfields of the hippocampal formation, and the thalamus. For every region, the microscopic image (using 20× objective lens for MAP-2 staining and 10× objective lens for EBA staining) was transferred to the computer screen by focusing the appropriate area of the immunostained section with a Nikon E600 microscope equipped with a digital camera (DAGE-MTI, CCD100) (Atlanta, GA, USA) connected to an

Brain (AChE) and plasma (BChE) activity were determined according to the method of Ellman et al. (1961) but modified for assay in a Molecular Devices UV Max Kinetic Microplate reader (Molecular Devices, Sunnyvale, CA, USA), as previously described (Abou-Donia et al., 1996). Protein concentration was determined by the method of Smith et al. (1985). For the assay of mAChR, tissues were homogenized in 10 mM phosphate buffer (pH = 7.4) and centrifuged at 40,000×g for 10 min and the membranes were suspended in the same buffer at the protein concentration of 1.5–2.5 mg/ml, as described by Hu¡ et al. (1994). The m2 mAChR binding was carried out by using m2-selective ligand, [3H]AFDX 384 at room temperature for 60 min, as described by Slotkin et al. (1999).

Analysis of the BBB permeability using [3H]hexamethonium iodide uptake assay

[3H]hexamethonium iodide uptake assay was performed according to the methods described elsewhere (Petrali et al., 1991; Johnes et al., 2000; Abou-Donia et al., 2001). Twenty-four hours following treatment with either saline or sarin, five animals from each group were anesthetized with sodium pentobarbital (100 mg/kg) and intravenously (i.v.) injected with [3H]hexamethonium iodide (10 μCi, mixed 1:1 with cold hexamethonium iodide) to give a final dose of 0.71 mg/kg body weight (1 μCi/kg). Ten minutes following this injection, the blood was collected from the heart with heparinized syringes and the animals were killed by decapitation. The brains were removed and placed in ice-cold normal saline. The different brain regions (cerebrum, brainstem, midbrain and cerebellum) were rapidly dissected on ice and frozen immediately using liquid nitrogen. The plasma was separated from the whole blood by centrifugation. Both plasma and brain regions were stored at −20°C until further analysis. Region of the cerebrum, the thalamus, the midbrain and the cerebellum (50–100 mg) and 10 μl of plasma were oxidized in a Packard 306B tissue oxidizer (Packard Instrument, Downers Grove, IL, USA), and the extracted tritium was then counted in a liquid scintillation spectrometer (Beckman Instruments, Palo Alto, CA, USA) for 5 min. Counts were recorded as d.p.m./g of tissue or 1 ml plasma. The results are presented as ratio of tissue versus plasma, which was done by dividing d.p.m. of tissue by d.p.m. of plasma. This presentation was incorporated to clearly avoid the background that resulted from blood in the brain capillaries, as the animals were not perfused prior to analysis. This is fully consistent with the presentation of data by Petrali et al., 1991.
IBM Computer. The same intensity of light in the microscope and the same parameters in the digital camera were used to digitize all the samples from different brain regions. Images in the Scion Image are two-dimensional arrays of pixels (picture elements). Pixels are represented by 8-bit unsigned integers, ranging in value from 0 to 255. Scion Image displays zero pixels as white and those with a value of 255 as black. The background and target values were set to 145 and 255, respectively, following digitization of the original gray value image in the computer screen. These values were determined by selecting the background and target areas in several sections from both control and treated animals before commencing the measurements on coded slides for statistical analysis. This scale eliminated the background staining and retained all the target (MAP-2 or EBA-immunopositive) structures in the range (145–255). The binary image of MAP-2 or EBA-positive elements was then generated by selecting a suitable threshold value (which varied from 155 to 165) to include all the MAP-2 or EBA-positive structures without any background. The final binary image was crosschecked with the original gray value image by alternating the two images on the computer screen. Finally, the image was frozen and the area occupied by the MAP-2 or EBA-positive structures in the field was measured by selecting ‘Analyze particles’ command of the Scion Image program. This way, the area of individual particles (i.e. MAP-2 or EBA-immunoreactive structures) in the selected field was measured, and the sum area of all particles was stored for further calculations and statistical analysis. Since spatial calibration of the image was performed in micrometers using ‘Set Scale’ function of the program prior to measurements, the results from area measurements were obtained in square micrometers and converted later into square millimeters.

Data analyses

Mean values between different groups of animals were compared separately for each of the above parameters using one-way analysis of variance (ANOVA) with Student’s Newman–Keuls multiple comparisons post-hoc test.

RESULTS

Clinical signs

All animals injected with 1×LD₅₀ of sarin developed excessive salivation, severe tremors, seizures, and convulsions within 5–10 min, and subsequently exhibited prolonged convulsions lasting approximately 3 h. Since all animals treated with 1×LD₅₀ exhibited closely comparable level of convulsions, the intensity and the duration of convulsions were not correlated with the extent of BBB permeability and the degree of neuropathology. Out of 20 rats treated with 1×LD₅₀ sarin, four animals (20%) died within the first 1–3 h following sarin administration; all other animals in this group survived the end point employed in this study (i.e. 24 h after the exposure). Animals treated with 0.5, 0.1 and 0.01×LD₅₀ did not exhibit signs of cholinergic toxicity with the exception of animals that received 0.5×LD₅₀ dose. In this latter group, three animals out of 15 treated (20%) exhibited mild clinical signs 25–60 min after sarin treatment.

Effects on BChE activity in plasma and AChE activity and m2 mAChRs in brain regions

The BChE activity in the plasma and AChE in the cerebral cortex, the brainstem, the midbrain and the cerebellum were assayed and the results are presented in Fig. 1. Animals treated with 1 and 0.5×LD₅₀ exhibited a significant inhibition in plasma BChE (30–41% of control, \( P<0.05 \); Fig. 1). However, the overall extent of BChE inhibition in plasma of animals exposed to 0.01–0.1×LD₅₀ was not significant, as the effect was highly variable between animals within these groups. A slightly higher degree of BChE inhibition in plasma of animals exposed to 0.5×LD₅₀ compared to that observed following 1×LD₅₀ likely reflects a variability between animals within the two groups.

Animals treated with 1×LD₅₀ also exhibited a significant inhibition of AChE in all of the above brain regions (31–44% of control, \( P<0.05 \); Fig. 1). Evaluation of m2 mAChRs revealed that animals treated with 1×LD₅₀ exhibited a significant decrease in m2 mAChR ligand binding in the cerebral cortex (39% of control,
P < 0.05; Fig. 2); however, the receptor ligand binding was significantly increased in the brainstem (253% of control, P < 0.001; Fig. 2) and the cerebellum (138% of control, P < 0.001; Fig. 2). Animals treated with 0.5 U LD₅₀ showed a significant decrease in the cerebellum (53% of control, P < 0.05; Fig. 2) but, a significant increase in the brainstem (237% of control, P < 0.01; Fig. 2). In contrast, animals treated with either 0.1 or 0.01 U LD₅₀ exhibited a significant increase in only the brainstem (253% and 313% of control, P < 0.05; Fig. 2).

**Alterations in the BBB**

We evaluated the effects of a single intramuscular injection of sarin at 1.0, 0.5, 0.1, or 0.01 U LD₅₀ dose on BBB permeability 24 h after the treatment. The data are presented in Fig. 3. The [³H]hexamethonium iodide was used to monitor changes in the permeability of the BBB within the cerebrum, the brainstem, the midbrain and the cerebellum. Exposure to lower doses of sarin (0.1 or 0.01 U LD₅₀) did not induce significant alterations in the permeability of the BBB (Fig. 3). In contrast, exposure to higher doses of sarin (1 and 0.5 U LD₅₀) lead to a significant increase in BBB permeability. With 1 U LD₅₀ exposure, BBB permeability was dramatically increased in the cerebrum, the midbrain, the brain stem and the cerebellum (144–183% of control, P < 0.01; Fig. 3). With 0.5 U LD₅₀ exposure, BBB permeability was significantly increased in only the midbrain and the brain stem (138–168% of control, P < 0.01; Fig. 3).

The alterations in BBB permeability were also assessed by EBA immunostaining that shows BBB protein in brain capillaries and also in smaller vessels invading the brain parenchyma (Fig. 4). Earlier studies have demonstrated that a dramatic reduction in EBA staining is indicative of an alteration in the BBB (Jensen et al.,

![Graph showing alteration in BBB permeability](image-url)
Animals treated with a single intramuscular injection of $1 \times LD_{50}$, exhibited dramatically decreased EBA immunoreactive elements at 2–24 h after sarin treatment. This was particularly evident in the cerebral cortex and the hippocampus (Fig. 4). Occasionally, decreased EBA immunostaining was also observed in animals treated with $0.5 \times LD_{50}$ dose of sarin. In contrast, animals treated with either 0.1 or $0.01 \times LD_{50}$ dose of sarin did not exhibit significant changes in the EBA immunostaining compared to control animals. Quantification of the area of EBA (or SMI-71) immunoreactive elements per unit area of the motor cortex, the somatosensory cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus and the dorsal thalamus at 24 h following sarin treatment demonstrated that only the animals treated with $1 \times LD_{50}$ showed a dramatic decrease in EBA immunostaining in all of the above regions at 24 h after the exposure (Fig. 5). The decrease in EBA immunoreactive structures was 49% in the motor cortex ($P < 0.05$), 61% in the somatosensory cortex ($P < 0.01$), 46% in the dentate gyrus ($P < 0.001$), 43% in the CA1 subfield ($P < 0.01$), 38% in the CA3 subfield ($P < 0.001$) and 38% in the VPL nucleus of the thalamus ($P < 0.001$; Fig. 5). In order to determine the earliest time at which BBB shows changes after the $1 \times LD_{50}$ sarin exposure, we quantified EBA staining in an additional group of animals ($n = 5$) killed at 2 h after $1 \times LD_{50}$ exposure. This showed comparable reductions to that observed at 24 h after the exposure (i.e. 56% in the motor cortex, $P < 0.05$; 43% in the somatosensory cortex, $P < 0.01$; 42% in the dentate gyrus, $P < 0.001$; 39% in the CA1 subfield, $P < 0.001$; 38% in the CA3 subfield, $P < 0.001$; 38% in the VPL nucleus of the thalamus, $P < 0.001$).

Fig. 4. Expression of EBA in the cerebral cortex and the hippocampus at 2 h following sarin exposure. (A) shows EBA staining pattern in a coronal section of brain through the cortex and the hippocampus from a control animal. (B) shows a coronal section of the brain from an animal killed 2 h after a single intramuscular injection of $1 \times LD_{50}$ of sarin. Note that EBA expression is dramatically decreased in the sarin-treated animal, suggesting that $1 \times LD_{50}$ sarin alters the BBB as early as 2 h after the exposure.

Fig. 5. Histograms show the extent of EBA immunoreactive elements, in mm$^2$ per unit area (0.176 mm$^2$) of the motor cortex, the sensory cortex, the dentate gyrus, CA1 and CA3 subfields, and the VPL nucleus of the thalamus. Animals were treated with a single intramuscular injection of 1, 0.5, 0.1 and $0.01 \times LD_{50}$ of sarin and analyzed for EBA staining at 24 h after the exposure. Values represent means and standard errors ($n = 5$ per group). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. 
Fig. 6. Alterations in different layers (layers I–VI) of the motor cortex at 24 h following sarin exposure, visualized with H&E staining. A1 is an example from a control rat. A2 is an example from a rat that was treated with 1×LD₅₀ sarin. A3 is an example from a rat treated with 0.5×LD₅₀ sarin. A4 is an example from a rat treated with 0.1×LD₅₀ sarin. A5 is an example from a rat treated with 0.01×LD₅₀ sarin. A number of degenerating neurons are clearly visible in layers II, III and V of the cerebral cortex in rats treated with 1×LD₅₀ sarin (A2). Note that animals treated with 0.5, 0.1 and 0.01×LD₅₀ did not exhibit degenerating neurons. Scale bar = 200 μm.
Fig. 7. Alterations in the superficial layers (layers I–III) of the motor cortex at 24 h following sarin exposure. A1–A5, H&E staining; B1–B5, EBA immunostaining; C1–C5, MAP-2 immunostaining. A1, B1 and C1 are examples from a control rat. A2, B2 and C2 are examples from a rat that was treated with 1×LD₅₀ sarin. A3, B3 and C3 are examples from a rat treated with 0.5×LD₅₀ sarin. A4, B4 and C4 are examples from a rat treated with 0.1×LD₅₀ sarin. A5, B5 and C5 are examples from a rat treated with 0.01×LD₅₀ sarin. A number of degenerating neurons are clearly visible in rats treated with sarin only at 1×LD₅₀ (arrows in A2). A dramatic reduction in EBA immunostaining is also obvious in rats treated with sarin at 1×LD₅₀. In addition, animals treated with 1×LD₅₀ sarin exhibit significantly reduced MAP-2-positive structures with characteristically wavy appearance of dendrites. In contrast, animals treated with 0.5×LD₅₀ exhibit only a slight reduction in MAP-2 immunoreactivity, and animals treated with 0.1 and 0.01×LD₅₀ do not exhibit any of the above changes. Scale bar = 100 µm.
32% in the dentate gyrus, $P < 0.001$; 51% in the CA1 subfield, $P < 0.01$; 29% in the CA3 subfield, $P < 0.001$; and 36% in the dorsal thalamus, $P < 0.001$). Thus, reductions in EBA expression occurs as early as 2 h after 1×LD_{50} sarin exposure and persists at the same level at least until 24 h after the exposure.

Additionally, we quantified the area of EBA immunoreactive elements per unit area of the midbrain and the brain stem following 1.0 and 0.5×LD_{50} sarin, as [^{3}H]hexamethonium iodide uptake assay suggested an increased BBB permeability in these regions. This analysis revealed a significant change in EBA staining pattern following exposure to a dose of 1×LD_{50} in comparison to controls within both brainstem (mean ± S.E.M.: controls, 8182 ± 568; 1×LD_{50} sarin, 5013 ± 1148; $P < 0.05$) and midbrain (controls, 10648 ± 449; 1×LD_{50} sarin, 8765 ± 313; $P < 0.05$). However, with a dose of 0.5×LD_{50} sarin, the area of EBA immunoreactive elements per unit area remained similar to control values in both brainstem and midbrain ($P > 0.05$). Thus, animals intoxicated with 0.5×LD_{50} sarin show an increased BBB permeability in only brainstem and midbrain with [^{3}H]hexamethonium uptake assay but not with EBA analysis, suggesting only a minor or transient leakage of the BBB in these regions with 0.5×LD_{50} dose.

**Histopathological changes in the brain**

Evaluation of brain sections stained with H&E clearly revealed significant neuronal degeneration in rats treated with 1×LD_{50} and occasional neurodegeneration in animals treated with 0.5×LD_{50}, in comparison to animals treated with 0.1 and 0.01×LD_{50}, or vehicle alone. Degenerating neurons were characterized by eosinophilic staining of both cell body and proximal dendrites. In contrast, the healthy neurons in the same section exhibited hematoxylin-stained nuclei with a lightly eosin-stained perinuclear cytoplasm. The brain regions where neuronal degeneration was most obvious include the motor and somatosensory areas of the cerebral cortex, the dorsal thalamus, dentate gyrus, the CA1 and CA3 subfields of the hippocampus and the cerebellum. Other areas of the brain showed only occasional dying neurons in some animals.

**Alterations in the cerebral cortex**

In animals treated with 1×LD_{50}, both superficial and deeper layers of the motor and somatosensory cortex exhibited degenerating neurons in H&E-stained sections. In the superficial layer (layers I–III; Figs. 6 and 7), degenerating neurons were conspicuous in all the layers. The majority of degenerating neurons in these layers were of the pyramidal type with prominent eosinophilic apical dendrites (Figs. 6 and 7). In deeper layers of the cortex (layers IV and V), degenerating neurons were mostly observed in the layer V (Figs. 6 and 8). These are larger pyramidal neurons with prominent apical and basal dendrites emanating from a larger pyramidal-shaped cell body (Fig. 8). Only a few degenerating neurons were observed in animals treated with 0.5×LD_{50} in both superficial and deeper layers of the motor and somatosensory cortex (layers II and V, Figs. 6, 7 and 8). Further, the overall cytoarchitecture of the cortex of animals treated with 0.5×LD_{50} was comparable to animals treated with 0.1 and 0.01×LD_{50} and vehicle-treated control animals (Figs. 6, 7 and 8). The adjacent sections stained for MAP-2 substantiated the above finding in animals treated with 1×LD_{50} dose by exhibiting a greatly reduced MAP-2 staining of dendrites in both superficial and deeper layers of the cortex (Figs. 7 and 8). Animals treated with 0.5×LD_{50} also showed a slight reduction in MAP-2 immunostaining (Figs. 7 and 8). MAP-2 immunostaining in animals treated both 0.1 and 0.01×LD_{50} or vehicle showed normal distribution of MAP-2-positive dendrites and neuronal perikarya within both superficial and deeper layers of the cerebral cortex (Figs. 7 and 8). Evaluation of GFAP immunoreactivity at 24 h after the exposure showed no changes in the different treated groups, in comparison to the control animals (data not illustrated).

**Extent of neuron loss and reductions in MAP-2 immunoreactivity within the motor and somatosensory cortex and the thalamus**

Quantification of dying neurons per mm² area of the motor and somatosensory cortex and the VPL nucleus of the thalamus revealed significant neuronal cell death in all of the above regions in animals treated with 1×LD_{50} of sarin ($P < 0.001$; Fig. 9) in comparison to control animals. Further comparison revealed that treatment with 0.5, 0.1, or 0.01×LD_{50} of sarin does not result in significant neuronal cell death at 24 h after the exposure (Fig. 9). Thus, acute exposure to a larger dose of sarin (1×LD_{50}) leads to a significant early neuronal cell death in the motor and somatosensory cortex, and the dorsal thalamus. Quantification of the area of MAP-2-immunoreactive elements per unit area of the motor cortex, the somatosensory cortex and the VPL nucleus of the thalamus at 24 h following sarin treatment (1, 0.5, 0.1 and 0.01×LD_{50}) demonstrated the same trend observed for neuronal cell death. The only exception is the VPL nucleus of the thalamus, which exhibited no significant differences at all doses in comparison to the control animals. Animals treated with 0.5, 0.1 and 0.01×LD_{50} of sarin did not exhibit a significant reduction in MAP-2-positive structures in all of the above regions (Fig. 9). However, animals treated with 1×LD_{50} exhibited 44% reduction in MAP-2-positive elements in the motor cortex ($P > 0.05$) and 34% reduction in the somatosensory cortex ($P > 0.05$; Fig. 9).

**Alterations in the hippocampal formation**

Neuronal degeneration was obvious in the dentate gyrus, and the CA1 and CA3 subfields of the hippocampal formation following exposure to sarin at a dose of 1×LD_{50} (Figs. 10–13). In the dentate gyrus, degenerating neurons were observed in both the granule cell layer and the dentate hilus (Figs. 10 and 11). However, MAP-2
Fig. 8. Changes in the deeper layers (layers IV and V) of the motor cortex at 24 h following sarin exposure. A1–A5, H&E staining; B1–B5, EBA immunostaining; C1–C5, MAP-2 immunostaining. A1, B1 and C1 are examples from a control rat. A2, B2 and C2 are examples from a rat that was treated with 1×LD₅₀ sarin. A3, B3 and C3 are examples from a rat treated with 0.5×LD₅₀ sarin. A4, B4 and C4 are examples from a rat treated with 0.1×LD₅₀ sarin. A5, B5 and C5 are examples from a rat treated with 0.01×LD₅₀ sarin. A number of degenerating neurons are clearly visible in rats treated with sarin at 1×LD₅₀ (arrows in A2). The latter group also exhibits a dramatic reduction in EBA (B2) and MAP-2 immunoreactivity (C2); in addition, dendrites appear thicker, swollen and fragmented. However, animals treated with 0.5×LD₅₀ exhibit only a slight reduction in MAP-2 immunoreactivity (C3), and animals treated with 0.1 and 0.01×LD₅₀ did not exhibit any changes.

Scale bar = 100 µm.
immunostaining of the granule cell layer and the molecular layer did not show any significant differences between animals belonging to different treated groups. In the CA1 subfield of the hippocampus, degenerating neurons were clearly observed in the stratum pyramidale of animals treated with 1 × LD₅₀ of sarin (Figs. 10 and 12). Evaluation of MAP-2 immunostaining showed greatly decreased expression of MAP-2-positive dendrites in animals treated with 1 × LD₅₀ of sarin (Fig. 12). In the latter group, alterations in MAP-2 expression of dendrites were particularly conspicuous in the CA1 stratum radiatum; the MAP-2 dendrites appeared fragmented and thinner (Fig. 12).

In the CA3 subfield of the hippocampus, the degenerating neurons were conspicuous in the stratum pyramidale of animals treated with 1 × LD₅₀ (Figs. 10 and 13). The MAP-2 staining of adjacent sections demonstrated a reduced density of MAP-2-positive dendrites in animals belonging to groups that received 1.0 or 0.5 × LD₅₀ sarin compared to control animals (Fig. 13). Evaluation of GFAP immunoreactivity in different regions of the hippocampal formation at 24 h after the exposure showed no changes in different treatment groups in comparison to the control animals (data not illustrated).

**Extent of neuron loss and reductions in MAP-2 immunoreactivity in the hippocampal formation**

Quantification of dying neurons per mm² area of the granule cell layer of the dentate gyrus and pyramidal cell layer of subfields CA1 and CA3 (Fig. 14) demonstrated that in the dentate granule cell layer, animals treated with 1 × LD₅₀ of sarin exhibited a significant number of dying neurons compared to control animals (P < 0.01; Fig. 14). In CA1 and CA3 subfields, the same trend was observed in that only animals treated with 1 × LD₅₀ exhibited a significant increased in dying neurons (P < 0.001; Fig. 14). Quantification of the area of MAP-2-immunoreactive elements per unit area of the pyramidal cell layer of subfields CA1 and CA3 exhibited a significant reduction in MAP-2-positive structures in animals treated with 1 × LD₅₀ dose of sarin (53–42% reduction, respectively, P < 0.001; Fig. 14). The CA3 region also showed a reduced MAP-2 staining in animals receiving 0.5 × LD₅₀ sarin (22% reduction, P < 0.05).

**Alterations in the cerebellum**

In the cerebellum, the most conspicuous damage in animals treated with 1 and 0.5 × LD₅₀ of sarin was in the Purkinje cell layer (Fig. 15) compared to animals treated with 0.1 or 0.01 × LD₅₀ sarin or control animals. Evaluation of GFAP immunoreactivity at 24 h after the exposure showed no changes in the cerebellar white matter and the cerebellar cortex of different treatment groups in comparison to the control animals (data not shown). Quantitative analysis of Purkinje cells in lobule 2 of the cerebellar vermis showed that animals treated with sarin at 1 and 0.5 × LD₅₀ exhibited a significant number of dying neurons in comparison to control animals (Fig. 16; P > 0.001).

**DISCUSSION**

The present study was designed to investigate the early neuropathological changes in the adult rat brain following acute exposure to different doses (1, 0.5, 0.1 and 0.01 × LD₅₀) of sarin. Analyses of the early effects of different doses of sarin on the brain is of considerable significance for assessing the extent of early brain damage that could occur when people are acutely exposed to either higher doses of sarin (i.e. following direct exposure during war or terrorist attacks) or lower doses of sarin (i.e. following indirect exposure). The results of this combined biochemical and neuropathological study suggest that a single exposure to sarin at 1 × LD₅₀ can cause extensive brain damage by 24 h mainly involving the cerebral cortex, the hippocampus (dentate gyrus and the CA1 and CA3 subfields) and the cerebellum. This was evidenced by: (i) a significant inhibition of plasma BChE, regional brain AChE, and m2 mAChR ligand binding; (ii) a dramatic increase in the BBB permeability associated with a drastic decrease in the EBA immunostaining; and (iii) a diffuse neuronal cell death coupled with decreased MAP-2 expression within dendrites of surviving neurons. Various clinical signs such as saliva-
Fig. 10. Alterations in different regions of the hippocampus at 24 h following sarin exposure, visualized with H&E staining; A1 is an example from a control rat. A2 is an example from a rat that was treated with 1 LD₅₀ sarin. A3 is an example from a rat treated with 0.5 LD₅₀ sarin. A4 is an example from a rat treated with 0.1 LD₅₀ sarin. A5 is an example from a rat treated with 0.01 LD₅₀ sarin. A number of degenerating neurons are clearly visible in different regions of the hippocampus in rats treated with 1 LD₅₀ sarin (A2). Note that animals treated with 0.5, 0.1 and 0.01 LD₅₀ did not exhibit degenerating neurons. DH, dentate hilus; GCL, granule cell layer; SLM, stratum lacunosum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 200 μm.
Fig. 11. Alterations in the dentate gyrus at 24 h following sarin exposure. A1-A5, H&E staining; B1-B5, MAP-2 immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat treated with 1×LD₅₀. A3 and B3 are examples from a rat treated with 0.5×LD₅₀. A4 and B4 are examples from a rat treated with 0.1×LD₅₀. A5 and B5 are examples from a rat treated with 0.01×LD₅₀. Note a large number of degenerating neurons in the dentate granule cell layer (GCL) and the dentate hilus (DH) of rats treated with 1×LD₅₀ (arrows in A2). However, MAP-2 expression in all treated animal groups appears comparable to control animals. Scale bar = 100 μm.
Fig. 12. Alterations in the CA1 subfield of the hippocampus at 24 h following sarin exposure. A1-A5, H&E staining; B1-B5, MAP-2 immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat treated with 1×LD₅₀ sarin. A3 and B3 are examples from a rat treated with 0.5×LD₅₀. A4 and B4 are examples from a rat treated 0.1×LD₅₀. A5 and B5 are examples from a rat treated 0.01×LD₅₀. A large number of degenerating pyramidal neurons are clearly visible in the stratum pyramidale (SP) of rats treated with 1×LD₅₀ (arrows in A2). In addition, the latter group exhibits a significantly reduced MAP-2 immunoreactivity (B2) associated with alterations in the pattern of MAP-2 expression in dendrites (B1). Scale bar, 100 μm.
Fig. 13. Changes in the CA3 subfield of the hippocampus at 24 h following sarin exposure. A1–A5, H&E staining; B1–B5, MAP-2 immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat treated with $1\times LD_{50}$. A3 and B3 are examples from a rat treated with $0.5\times LD_{50}$. A4 and B4 are examples from a rat treated with $0.1\times LD_{50}$. A5 and B5 are examples from a rat treated $0.01\times LD_{50}$. A large number of degenerating neurons are clearly visible in the stratum pyramidale (SP) of CA3 subfield of the rat treated with $1\times LD_{50}$ (arrows in A2). In addition, the latter group exhibits a reduced MAP-2 immunoreactivity (B2) in comparison to the control group (B1). Scale bar = 100 μm.
tion, severe tremors, seizures, and convulsions clearly precede these changes in the brain. The above changes in the brain following 1×LD₅₀ sarin exposure are significant and could lead to considerable motor and sensory abnormalities, ataxia, and learning and memory deficits, as observed with exposure to other organophosphates including soman (Gardner et al., 1984; McDonald et al., 1988; Veronesi et al., 1990).

Interestingly, animals treated with 0.5, 0.1 and 0.01×LD₅₀ did not exhibit motor convulsions and the above-mentioned neuropathological changes except in the cerebellum, where animals treated with 0.5×LD₅₀ showed some Purkinje neuron loss. Thus, it appears that the initiation of acute brain damage produced by sarin is related to the sarin-induced seizures, which is consistent with the results obtained after acute soman exposure (Burchfiel and Duffy, 1982; McDonough et al., 1987; Singer et al., 1987; Nieminen et al., 1997; Kadar et al., 1992). On the other hand, the degeneration of Purkinje cells in the cerebellum with 0.5×LD₅₀ dose of sarin reveals that degenerative changes in the cerebellum following sarin exposure can occur in the absence of seizures, suggesting that cerebellum is quite vulnerable to even lower doses of the sarin. Taken together, the above results reflect differential sensitivity of different regions of the brain to lower doses of the sarin.

Seizures and BBB disruption

In this study, the sarin-induced alteration in the BBB was evaluated using [³H]hexamethonium iodide uptake assay and immunolabeling that recognizes a BBB-related antigen, EBA (Rosenstein et al., 1992; Sternberger and Sternberger, 1987). The BBB plays a significant role in maintaining the homeostasis of the CNS microenvironment (Joo, 1996; Jensen et al., 1998). Further, the cerebrovasculature is a site of injury in a number of neurological diseases and is involved in toxicant-induced injury to the nervous system (Jacobs, 1982; Aschner and Gannon, 1994; Uno et al., 1996). A study by Nitsch and Klatzow (1983) suggested that cerebrovascular alterations can also occur following seizures. Indeed, BBB damage has been observed in many animal models of experimental seizures (McLeod et al., 1984; Bolwig, 1989; Grange-Messent et al., 1999). However, the alteration in BBB permeability is not directly dependent on AChE inhibition (Ashani and Catravas, 1981) but prolonged changes in one molecule could produce a transient change in the physiological state of the BBB (Petralli et al., 1991). Our results demonstrate dramatic increases in BBB permeability (associated with a drastic reduction in the expression of EBA in many regions of the brain including the cerebral cortex and the hippocampus at 24 h after 1×LD₅₀ sarin exposure. This is clearly indicative of a marked alteration in the BBB after 1×LD₅₀ sarin exposure. Moreover, the changes in the BBB in animals receiving 1×LD₅₀ were clearly associated with seizures. Alterations in the EBA staining pattern could be due to a number of alterations ranging from an overt tissue disruption and subsequent remodeling of the vasculature to more subtle changes such as degradation of the antigen or post-translational alterations influencing epitope availability (Sternberger and Sternberger, 1987; Mori et al., 1992). In contrast, animals intoxicated with the 0.5×LD₅₀ dose did not convulse but showed BBB damage in the brainstem and the midbrain with [³H]hexamethonium uptake assay but not with EBA analysis. This suggests that there is only a minor or transient leakage of the BBB in these regions with 0.5×LD₅₀ dose. The decrease in plasma BChE with 0.5×LD₅₀ dose in this study and the previous findings that a significant inhibition of the cholinesterase in brain capillary walls by cholinesterase inhibitors can alter the BBB permeability (Grauer et al., 2001; Skultetyova et al., 1998) also support the above interpretation. No significant changes in BBB permeability and EBA staining were observed in animals receiving 0.1 and 0.01×LD₅₀ sarin. Animals belonging to the latter groups also did not exhibit seizures. Thus, a significant and prolonged disruption of the BBB (evidenced by contemporaneous increases in BBB permeability and decreases in EBA immunoreactive elements) after sarin exposure is clearly dose-dependent and
Fig. 15. Alterations in the cerebellum at 24 h following sarin exposure. Values represent means and standard errors (n=5 per group). A1–A5. H&E staining of lobule 2 of the cerebellar vermis. A1 shows an example from a control rat. A2 illustrates an example from a rat treated with 1×LD₅₀ sarin. A3 shows an example from a rat treated with 0.5×LD₅₀ sarin. A4 and A5 show examples from rats treated with 0.1 and 0.01×LD₅₀ sarin, respectively. A large number of degenerating Purkinje neurons are clearly visible in the Purkinje cell layer of rats treated with either 1 or 0.5×LD₅₀ (arrows in A2 and A3). Scale bar = 100 µm.
appears to be coupled to the development of seizures after the sarin exposure.

Pattern of brain injury induced by sarin

The results of this study indicate that an acute sarin exposure at 1×LD₅₀ can result in extensive damage to the adult brain, particularly involving the motor and somatosensory cortex, the hippocampus, and the cerebellum. The rats displaying the latter neuropathology also had seizures and BBB damage. The pattern of neuropathological change depends upon a number of factors such as dosage, duration of the exposure, age of the animal, and species (Storm-Mathisen, 1970; McLeod et al., 1984; McDonough et al., 1989; Veronesi et al., 1990). The neuropathological changes observed in surviving rats treated with 1×LD₅₀ sarin in this study is somewhat similar to those observed in rats following lethal doses of soman, a military nerve agent, and following subchronic exposure to anticholinesterases (Storm-Mathisen, 1970; McLeod et al., 1984; McDonough et al., 1984; McDonough et al., 1989; Veronesi et al., 1990). The neuropathological changes observed in surviving rats treated with 1×LD₅₀ sarin in this study is somewhat similar to those observed in rats following lethal doses of soman, a military nerve agent, and following subchronic exposure to anticholinesterases (Storm-Mathisen, 1970; McLeod et al., 1984; McDonough et al., 1984; McDonough et al., 1989; Veronesi et al., 1990). The neuropathology in distinct brain regions as described above is likely the result of ischemia or a direct neurotoxic action attributed to the seizures associated with nerve agents (Millis et al., 1988; Carpentier et al., 1990). Thus, the pattern of brain injury induced by 1×LD₅₀ sarin has similarities to those described after 1×LD₅₀ soman and other anticholinesterases.

Extent of alterations in MAP-2 expression after sarin exposure

An important neuronal component, MAP-2, is enriched in dendrites and cell bodies of neurons (Tucker et al., 1988; Gavin, 1997). The MAP-2 plays a key role as a structural protein necessary for the maintenance of cytoskeleton integrity and in neuronal growth, plasticity, and regeneration (Johnson and Jope, 1992), and also aids in dendritic remodeling and post-synaptic changes that occur after certain lesions. The present results suggest that acute 1×LD₅₀ sarin exposure induces a significant decrease in the expression MAP-2 in dendrites of neurons belonging to the cerebral cortex and CA1 subfield of the hippocampus. These changes were clearly evident by 24 h after the injury. However, with lower doses of sarin, such changes were not observed. Further, in control animals and animals treated with lower doses of sarin, MAP-2-positive dendrites in the cerebral cortex and CA1 subfield of the hippocampus appeared as long, branching processes. After 1×LD₅₀ sarin injury, MAP-2-positive dendrites not only reduced in number but also exhibited a fragmented appearance with swelling occurring along the length of individual dendrites. Several studies suggest that degradation of MAP-2 following exposure to neurotoxic chemicals in the cerebral cortex and the hippocampus is a result of global ischemia (Inuzuka et al., 1990; Matesic and Lin, 1994), or excitotoxic cascade of biochemicals, which could initiate a massive cell depolarization involving glutamate release and activation of N-methyl-D-aspartate receptors (Fink et al., 1993). Activation of calcium calmodulin kinase II is another possibility, which may result in abnormal phosphorylation of phosphoproteins such as MAP-2 and thereby impair their normal structure and function of neurons (Folkerts et al., 1998). Thus, in the present study, a significantly decreased MAP-2 staining following 1×LD₅₀ sarin could be due to abnormal phosphorylation of phosphoproteins in the surviving neurons. Abnormal phosphorylation of MAP-2 can occur after depolymerization of microtubules within dendrites and lead to cytoskeletal alterations (Folkerts et al., 1998). Further, with time, the early cytoskeletal alterations observed after 1×LD₅₀ sarin exposure can lead to disruption of neuron and synaptic structure, axonal transport, and ultimately motor and cognitive processes.

Relationship between neuronal injury and neurochemical changes

The initiation of sarin-induced seizures and convulsions appeared to be due to cholinergic and non-cholinergic effects. The cholinergic system in the CNS plays an important role in learning and memory and has a regulatory role in certain neurobehavioral functions (Lyeth et al., 1990; Levey et al., 1991; Nieminen et al., 1997). Previous studies have reported that sarin may exert its cholinergic effects directly by the activation of limbic AChRs and accumulation of endogenous ACh following ChE inhibition in the PNS or CNS, which is sufficient to cause severe seizures followed by neurodegenerative processes (Taylor, 1985, 1996). Our data suggest that acute treatment with sarin at 1.0 and 0.5×LD₅₀ causes a significant inhibition in the plasma BChE as well as the brain AChE. This inhibition may be responsible for the differential regulation of the receptors activated directly by ACh.

We also measured m2 muscarinic receptors in this study because effects on mAChRs can play a role in the toxicity of certain organophosphate compounds, as these receptors are coupled to different G-proteins to induce cellular signaling from the cell surface. The m2 mAChR is coupled to Gi protein, and hence activation...
of m2 mAChRs can lead to inhibition of the adenylate cyclase (Abdallah et al., 1992; Huff et al., 1994). It has been also suggested that the toxicity of organophosphate anticholinesterases have actions on both nAChRs and mAChRs when their concentrations in circulation rise above micromolar levels. However, at nanomolar concentrations many organophosphates as well as the nerve gas agents such as soman and sarin can selectively affect a small population of mAChRs (Bakry et al., 1988; Silveira et al., 1990; Katz et al., 1997). Thus, a differential regulation of m2 muscarinic receptors in different regions may lead to a differential response leading to inhibition of the adenylate cyclase (Headley and Grillner, 1990). In this context, the sarin-induced reductions in mACh receptors in the cerebrum, and increases in mACh receptors in the midbrain, brain stem and the cerebellum, could transduce a differential response in these brain regions. The decrease of mAChRs in the cortex is consistent with the receptor plasticity observed following exposure to organophosphate compounds (Jett et al., 1994). However, the increase in other brain regions in this study is intriguing. The increase in binding parameters is typically related to the absence of the natural ligand or to the effect of chronic treatment with an antagonist. However, in this study, the level of natural ligand has been increased with sarin exposure and also there was no treatment with antagonists. In this context, it appears that the differential effect of sarin exposure on mACh receptors in different regions of the brain reflects region-specific response of the mACh receptors to the inhibition of AChE.

Sarin may also exert its effects through other cholinergic mechanisms, which can interact directly with mACh receptors (Rocha et al., 1998; Chebabo et al., 1999), or pre-synaptic muscarinic receptors by reducing action potential-dependent release of GABA in the post-synaptic neuron (Chebabo et al., 1999). Alternatively, sarin may exert its effect through non-cholinergic mechanisms, which can lead to neuronal cytotoxicity similar to what is observed after the soman exposure (John and Brian, 1993; Somani, 1992; Fernando et al., 1984). Taken together, alterations in the expression of mACh receptors observed in this study after 1×LD₅₀ sarin exposure could lead to a number of physiological shifts in the brain which in turn can lead to behavioral abnormalities.

CONCLUSIONS

This novel study evaluated the early changes in the adult brain after a single exposure to different doses of the sarin. The results demonstrate that a single exposure to sarin at 1×LD₅₀ causes seizures and leads to an extensive brain damage by 24 h involving the cerebral cortex, the hippocampus (dentate gyrus, and CA1 and CA3 subfields) and the cerebellum. This was characterized by a significant inhibition of brain regional AChE and m2 mAChR ligand binding in the cerebrum, a dramatic increase in the BBB permeability with a severe decrease in EBA expression, a diffuse neuronal cell death coupled with a decrease in MAP-2 expression within dendrites of surviving neurons. The above changes in the brain following 1×LD₅₀ sarin exposure are considerable and could lead to a substantial motor and sensory abnormalities, ataxia, and learning and memory deficits. However, animals treated with 0.5, 0.1 and 0.01×LD₅₀ exhibited neither seizures nor the above-mentioned combination of neuropathological changes. This may suggest that the early brain damage after acute exposure to sarin is clearly dose-dependent and brain pathology is clearly apparent with only 1×LD₅₀ exposure. However, this study does not rule out long-term changes after lower doses of the sarin exposure, as pathological changes in the brain are usually not pronounced with milder injury during the early post-injury period. Thus, analyses of the long-term effects of different doses of the sarin on morphological, neurochemical, and behavioral alterations in the brain remain interesting and important issues for future studies.

Acknowledgements—This work was supported from the United States Army Medical Research and Material Command under the Contract number: DAMD 17-99-1-9020 to M.B.A. and a VA Merit Review Award to A.K.S. The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REFERENCES


Committee on Banking, Housing and Urban Affairs, US Senate, 1994. US Chemical and Biological Warfare-Related Dual Use Exports to Iraq and Their Possible Impact on the Health Consequences of the Persian Gulf War. U.S. Senate, Washington, DC.


Institute of Medicine, 1996. Interactions of Drugs, Biological, and Chemicals in US Military Forces. National Academy, Washington, DC.

Institute of Medicine, 2000. Clearing the Air; Asthma and Indoor Air Exposures. National Academy, Washington, DC.


(accepted 24 January 2002)