Organophosphorus Ester-Induced Chronic Neurotoxicity

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ABSTRACT. Organophosphorus compounds are potent neurotoxic chemicals that are widely used in medicine, industry, and agriculture. The neurotoxicity of these chemicals has been documented in accidental human poisoning, epidemiological studies, and animal models. Organophosphorus compounds have 3 distinct neurotoxic actions. The primary action is the irreversible inhibition of acetylcholinesterase, resulting in the accumulation of acetylcholine and subsequent overstimulation of the nicotinic and muscarinic acetylcholine receptors, resulting in cholinergic effects. Another action of some of these compounds, arising from single or repeated exposure, is a delayed onset of ataxia, accompanied by a Wallerian-type degeneration of the axon and myelin in the most distal portion of the longest tracts in both the central and peripheral nervous systems, and is known as organophosphorus ester-induced delayed neurotoxicity (OPIDN). In addition, since the introduction and extensive use of synthetic organophosphorus compounds in agriculture and industry half a century ago, many studies have reported long-term, persistent, chronic neurotoxicity symptoms in individuals as a result of acute exposure to high doses that cause acute cholinergic toxicity, or from long-term, low-level, subclinical doses of these chemicals. The author attempts to define the neuronal disorder that results from organophosphorus ester-induced chronic neurotoxicity (OPICN), which leads to long-term neurological and neurobehavioral deficits. Although the mechanisms of this neurodegenerative disorder have yet to be established, the sparse available data suggest that large toxic doses of organophosphorus compounds cause acute necrotic neuronal cell death in the brain, whereas sublethal or subclinical doses produce apoptotic neuronal cell death and involve oxidative stress.
<Key words: acetylcholinesterase, apoptosis, brain, necrosis, nervous system, neurobehavioral, neuropathological, neuropsychological, OPICN, OPIDN, organophosphorus compounds>

ORGANOPHOSPHORUS COMPOUNDS are chemicals that contain both carbon and phosphorus atoms.[1] They are derivatives of phosphoric (H_3PO_4), phosphorus or phosphonic (H_3PO_3), and phosphinic (H_3PO_2) acids (Fig. 1). The biological action of organophosphorus compounds is related to their phosphorylating abilities. This is dependent on the electrophilicity (positive character) of the phosphorus atom, which is determined by its substituent groups. Steric factors of substituents also play a major role in determining the biological activity of these chemicals. Lipid solubility is important because it enhances the ability of these compounds to cross biological membranes and the blood-brain barrier, leading to increased biological activity. Organophosphorus compounds are an economically important class of chemical compounds with numerous uses, such as in pesticides, industrial fluids, flame retardants, therapeutics, and nerve gas agents.

Most modern synthetic organophosphorus compounds are tailor-made to inhibit acetylcholinesterase (AChE), an enzyme essential for life in humans and other animal species. Tetraethylpyrophosphate was the 1st organophosphate synthesized as an AChE inhibitor in 1854.[2] Later, dimethyl and diethyl phosphorofluoridates were synthesized.[2] During World War II, organophosphorus compounds were developed primarily as agricultural insecticides, and later as chemical warfare agents. The majority of organophosphorus insecticides are organophosphorothioates; nerve agents are organophosphonates or organophosphonothioates; industrial chemicals are typically organophosphates[3] (Table 1).

Biologically, organophosphorus compounds are neu-

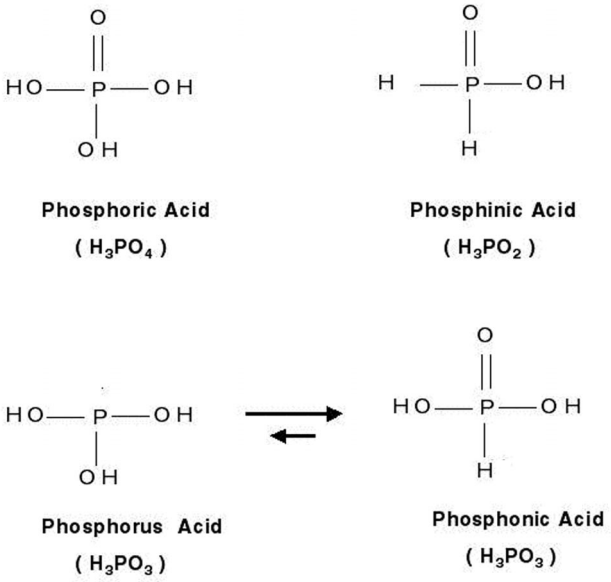

Fig. 1. Chemical structures of phosphoric acid, phosphinic acid, phosphorus acid, and phosphonic acid.

rotoxic to humans and other animals via 3 distinct actions: (1) cholinergic neurotoxicity, (2) organophosphorus ester-induced delayed neurotoxicity (OPIDN), and (3) organophosphorus ester-induced chronic neurotoxicity (OPICN).

Cholinergic Neurotoxicity of Organophosphorus Compounds

Organophosphorus compounds cause cholinergic neurotoxicity by disrupting the cholinergic system that includes AChE and its natural substrate, the neurotransmitter acetylcholine.[3] Acetylcholine is released in response to nerve stimulation and binds to post-synaptic acetylcholine receptors, resulting in muscle contraction or gland secretions. Its action is rapidly terminated by hydrolysis with AChE via the serine hydroxyl in the catalytic triad of AChE.[2] The 3-dimensional structure of AChE reveals an active center located at the base of a narrow gorge about 20 Å in depth.[4] The active center includes the following sites (Fig. 2): (a) the catalytic triad: Glu 334, His 447, and Ser 203; (b) an aceyl pocket: Phe 295 and Phe 297; (c) a choline subunit: Trp 86, Glu 202, and Tyr 337; and (d) a peripheral site: Trp 286, Tyr 72, Tyr 124, and Asp 74.

Organophosphorus ester inhibition of AChE. Organophosphorus esters inhibit AChE by phosphorylating the serine hydroxyl group at the catalytic triad site (Fig. 3). The phosphoric or phosphonic acid ester formed with the enzyme is extremely stable and is hydrolyzed very slowly.[1] If the phosphorylated enzyme contains methyl or ethyl groups, the enzyme is regenerated in several hours by hydrolysis. On the other hand, virtually no hydrolysis occurs with an isopropyl group (e.g., sarin) and the return of AChE is dependent upon synthesis of a new enzyme. Phosphorylated AChE undergoes aging—a process that involves the loss of an alkyl group, resulting in a negatively charged monoalkyl enzyme (Fig. 4).[3] Organophosphorus compounds undergo detoxification by binding to other enzymes that contain

Table 1.—Compounds Cited in the Text and Their IUPAC Designations

Compound	IUPAC designation
Chlorpyrifos	*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate
Cyclosarin (GF)	*O*-cyclohexyl methylphosphonofluoridate
DFP	*O,O*-diisopropyl phosphorofluoridate
DEET	*N,N*-diethyl-m-toluamide
Diazinon	*O,O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate
Fenthion	*O,O*-dimethyl *O*-4 methylthio-m-tolyl phosphorothioate
Malathion	*S*-1,2-*bis*(ethoxycarbonyl)ethyl *O,O*-dimethyl phosphorodithioate
Methamidophos	*O,S*-dimethyl phosphoramidothioate
Permethrin	3-phenoxybenzl (1*RS*)-*cis-trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
Quinalphos	*O,O*-diethyl *O*-quinoxalin-2-yl phosphorothioate
Tabun (GA)	*O*-ethyl *N,N*-dimethylphosphoamidocyanidate
Sarin (GB)	*O*-isopropyl methylphosphonofluoridate
Soman (GD)	*O*-2,2-trimethypropyl methylphosphonofluoridate
TOCP	Tri-ortho-cresylphosphate
VX	*O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonothioate
VR	*O*-isobutyl *S*-2-diethylaminoethyl methylphosphonothioate

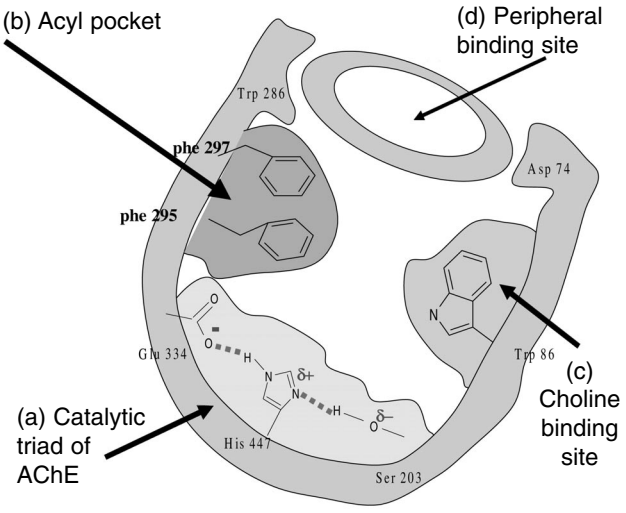

Fig. 2. Active center gorge of mammalian acetylcholinesterase (AChE).

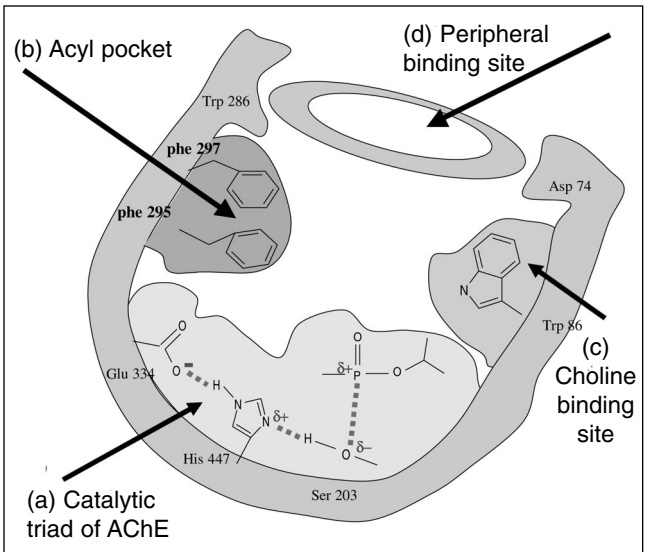

Fig. 3. Sarin phosphonyl enzyme: isopropyl methylphosphonyl acetylcholinesterase (AChE).

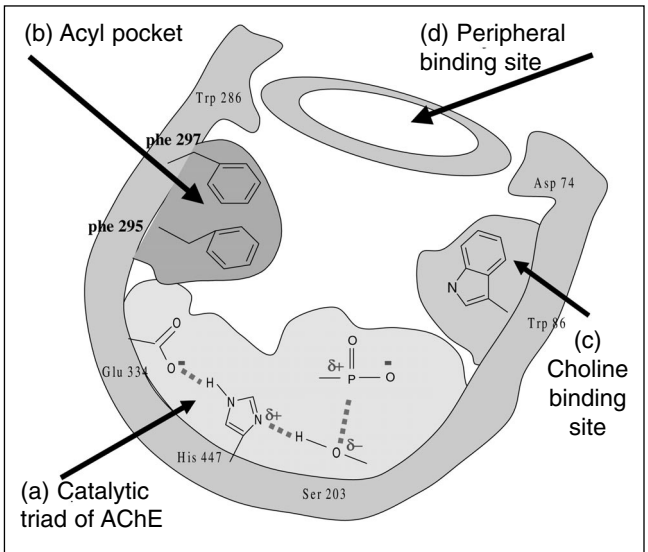

Fig. 4. Sarin aged phosphonyl enzyme: methylphosphonyl acetylcholinesterase (AChE).

the amino acid serine. These enzymes include plasma butyrylcholinesterase (BChE)[5,6] and paraoxonase.[7,8]

Inhibition of AChE results in the accumulation of acetylcholine at both the muscarinic and nicotinic receptors in the central nervous system (CNS) and the peripheral nervous system (PNS). Excess Acetylcholine initially causes excitation, and then paralysis, of cholinergic transmission, resulting in some or all of the cholinergic symptoms, depending on the dose size, frequency of exposure, duration of exposure, and route of exposure, as well as other factors such as combined exposure to other chemicals and individual sensitivity and susceptibility.

Human exposure. Human exposure—mostly via inhalation—to the organophosphorus nerve agent sarin was recently documented in 2 terrorist incidents in Japan. At midnight on June 27, 1994, sarin was released in Matsumoto City.[9] Of the 600 persons who were exposed, 58 were admitted to hospitals, where 7 died. Although miosis was the most common symptom, severely poisoned patients developed CNS symptoms and cardiomyopathy. A few victims complained of arrhythmia and showed cardiac contraction. The 2nd terrorist attack by sarin was in the Tokyo subway trains, at 8:05 A.M. on March 20, 1995, when a total of 5,000 persons were hospitalized and 11 died.[10] Patients with high exposure to sarin in the Tokyo subway incident exhibited marked muscle fasciculation, tachycardia, high blood pressure (nicotinic responses), sneezing, rhinorrhea, miosis, reduced consciousness, respiratory compromise, seizures, and flaccid paralysis.[11] Patients with mild exposure complained of headaches, dizziness, nausea, chest discomfort, abdominal cramps, and miosis. Interestingly, patients had pupillary constriction, even when their cholinesterase activity was normal. Furthermore, inhibition of red blood cell AChE activity was a more sensitive indicator of exposure than serum BChE activity.[12] The absence of bradycardia and excessive secretions—which are common in dermal or ingestion exposures—suggested that the major route of exposure to the sarin gas in these instances was via inhalation. The patients were treated with atropine eye drops for marked miosis, and with pralidoxime iodide (2-PAM).

Organophosphorus Ester-Induced Delayed Neurotoxicity (OPIDN)

Characteristics of OPIDN. OPIDN is a neurodegenerative disorder characterized by a delayed onset of prolonged ataxia and upper motor neuron spasticity as a result of a single or repeated exposure to organophosphorus esters.[13–16] The neuropathological lesion is a central-peripheral distal axonopathy caused by a chemical transection of the axon (known as Wallerian-type degeneration), followed by myelin degeneration of distal portions of the long and large-diameter tracts of the CNS and PNS.[17] Incidents of OPIDN have been documented for over a century. The earliest recorded cases were attributed to the use of tri-*o*-cresyl phosphate (TOCP)-containing creosote oil for treatment of pulmonary tuberculosis in France in 1899.[13,15,16] In 1930, TOCP was identified as the chemical responsible for an estimated 50,000 cases of OPIDN in the Southern and Midwestern regions of the United States.[13,15,16] More recently, Himuro et al.[18] reported that a 51-yr–old man who was exposed to sarin during the Tokyo subway incident and survived its acute toxicity, then died 15 mo later. Neuropathological alterations and neurological deficits observed in this individual were consistent with the dying-back degeneration of the nervous system characteristic of OPIDN. This incident indicated that humans are more sensitive than experimental animals to sarin-induced OPIDN, inasmuch as it required

26–28 daily doses of LD_{50} (25 μg/kg, i.m.) sarin to produce OPIDN in the hen.[19] OPIDN has been divided into 3 classes: *Type I* is caused by the pentavalent phosphates and phosphonates, as well as their sulfur analogs; *Type II* is produced by the trivalent phosphites;[20] and *Type III* is induced by phosphines.[21,22] All 3 OPIDN types are produced by organophosphorus compounds and characterized by central-peripheral distal axonopathy. Type II differs from Type I in terms of the susceptibility of rodents and the presence of neuropathological lesions in neuronal cell bodies.[20] Type III OPIDN is not accompanied by inhibition of the neurotoxicity target esterase (NTE), thus casting further doubt on this enzyme as the target for OPIDN.[21,22]

Mechanisms of OPIDN. Early studies on the mechanisms of OPIDN centered on the inhibition of the esterases AChE[23] and BChE[24] by organophosphorus esters. Subsequent studies eliminated both enzymes as targets for OPIDN.[25] Johnson[26] proposed an NTE—an enzymatic activity preferentially inhibited by organophosphorus compounds capable of producing OPIDN as its target. Despite numerous studies since the introduction of this concept 35 yr ago, the NTE hypothesis has not advanced our understanding of the mechanism of OPIDN because: (a) evidence for the involvement of NTE in the development of OPIDN is only correlative; (b) it has not been shown how inhibition and aging of NTE leads to axonal degeneration; (c) NTE, which is present in neuronal and non-neuronal tissues and in sensitive and insensitive species, has no known biochemical or physiological function; (d) some organophosphorus pesticides that produce OPIDN in humans do not inhibit or age NTE;[27–30] and (e) phosphines that produce Type III OPIDN do not inhibit NTE.[21,22] However, the most convincing evidence against this hypothesis is the recent finding that NTE-knockout mice are sensitive to the development of OPIDN,[31–33] indicating that this enzyme is not involved in the mechanisms of OPIDN.

Protein kinases as targets for OPIDN. Because research on esterases did not increase our understanding of the mechanisms of OPIDN, we have been studying the involvement of protein kinase-mediated phosphorylation of cytoskeletal proteins in the development of OPIDN. These studies were prompted by the following observations: (a) Since organophosphorus compounds are effective phosphorylating agents, it is reasonable to expect that they would interfere with normal kinase-mediated phosphorylation of a serine or threonine group at the target protein. (b) The earliest ultrastructural alterations in OPIDN are seen mostly as aggregation and accumulation of cytoskeletal proteins, microtubules, and neurofilaments, followed by their dissolution and disappearance. (c) The structural and functional status of cytoskeletal proteins are affected significantly by protein kinase-mediated phosphorylation.

Anomalous hyperphosphorylation of cytoskeletal elements is associated with OPIDN, a neurodegenerative disorder characterized by distally located swellings in large axons of the CNS and PNS, with subsequent axonal degeneration. Central to our hypothesis is the observation that increased aberrant protein kinase-mediated phosphorylation of cytoskeletal proteins could result in the destabilization of microtubules and neurofilaments, leading to their aggregation and deregulation in the axon.[34] Protein kinases are able to amplify and distribute signals because a single protein kinase can phosphorylate many different target proteins. Several protein kinases are turned on by 2nd messengers. For example, calcium/calmodulin-dependent protein kinase II (CaM kinase II) is inactive until it is bound by the calcium-calmodulin complex that induces conformational changes and causes the enzyme to unfold an inhibitory domain from its active site.[35] We have demonstrated substantial increases in the autophosphorylation,[36,37] enzymatic activity,[38] protein levels, and mRNAs of CaM kinase II in hens treated with diisopropylphosphorofluoridate (DFP).[39] These aberrant alterations have resulted in increased phosphorylation of the following cytoskeletal proteins: tubulin, neurofilaments, microtubule associated proteins-2 (MAP-2), and tau proteins.[39–42] Increased activity of CaM kinase II can affect the stability of cytoskeletal proteins through post-translational modification. Phosphorylation of these proteins interrupts their interaction, polymerization, and stabilization, leading to their degeneration.[39,43,44]

On the other hand, early studies identified transcription factors as critical phosphoproteins in signaling cascades. Immediate early genes control gene expression and therefore affect long-term cellular responses. We have demonstrated that the transcription of c-fos is elevated in OPIDN, perhaps through the activation of cAMP (adenosine monophosphate) response element binding (CREB), which is phosphorylated by CaM kinase II. Subsequent to c-fos activation,[45] we observed altered gene expression of CaM kinase II,[43] neurofilaments,[46] glial fibrillary acidic protein (GFAP), and vimentin.[47] Our results also showed an increase in medium (NF-M) and a decrease in low (NF-L) and high (NF-H) molecular weight neurofilaments in the spinal cords of hens treated with DFP. [46] This imbalance in the stoichiometry of neurofilament proteins interferes with their interaction with microtubules and promotes neurofilament dissociation from microtubules, leading to the aggregation of both cytoskeletal proteins.[48] Immunohistochemical studies in nervous system tissues from TOCP- and DFP-treated hens demonstrated aberrant aggregation of phosphorylated neurofilament, tubulin, and CaM kinase II.[49]

Organophosphorus Ester-Induced Chronic Neurotoxicity (OPICN)

Various epidemiological studies have demonstrated that individuals exposed to a single large toxic dose, or

to small subclinical doses, of organophosphorus compounds have developed a chronic neurotoxicity that persists for years after exposure and is distinct from both cholinergic and OPIDN effects.[50] This disorder has been variously referred to in the literature as: "chronic neurobehavioral effects,"[11] "chronic organophosphate-induced neuropsychiatric disorder (COPIND),[50] "psychiatric sequelae of chronic exposure,"[51] "central nervous system effects of chronic exposure,"[52] "psychological and neurological alterations,"[53] "long-term effects,"[54] "neuropsychological abnormalities,"[55] "central cholinergic involvement in behavioral hyperreactivity,"[56] "chorea and psychiatric changes,"[57] "chronic central nervous effects of acute organophosphate pesticide intoxication,"[58] "chronic neurological sequelae,"[59,60] "neuropsychological effects of long-term exposure,"[61] "neurobehavioral effects,"[62] and "delayed neurologic behavioral effects of subtoxic doses."[63] Our review of the literature indicated that these studies describe a nervous system disorder induced by organophosphorus compounds which involves neuronal degeneration and subsequent neurological, neurobehavioral, and neuropsychological consequences. We will next define and describe this disorder, and refer to it as "organophosphorus ester-induced chronic neurotoxicity" or OPICN.

Characteristics of OPICN. OPICN is produced by exposure to large, acutely toxic—or small subclinical—doses of organophosphorus compounds. Clinical signs, which continue for a prolonged time ranging from weeks to years after exposure, consist of neurological and neurobehavioral abnormalities. Damage is present in both the PNS and CNS, with greater involvement of the latter. Within the brain, neuropathological lesions are seen in various regions, including the cortex, hippocampal formation, and cerebellum. The lesions are characterized by neuronal cell death resulting from early necrosis or delayed apoptosis. Neurological and neurobehavioral alterations are exacerbated by concurrent exposure to stress or to other chemicals that cause neuronal cell death or oxidative stress. Because CNS injury predominates, improvement is slow and complete recovery is unlikely.

OPICN following large toxic exposure to organophosphorus compounds. Several studies have reported that some individuals who were exposed to large toxic doses of organophosphorus compounds, and who experienced severe acute poisoning and subsequent recovery, eventually developed the long-term and persistent symptoms of OPICN. Many of the adverse effects produced by organophosphorus compounds are not related to AChE inhibition.[64] Individuals with a history of acute organophosphate exposure reported an increased incidence of depression, irritability, confusion, and social isolation.[65] Such exposures resulted in decreased verbal attention, visual memory, motoricity, and affectivity.[66] Rosenstock et al.[58] reported that even a single exposure to organophosphates requiring medical treatment was associated with a persistent deficit in neuropsychological functions. A study of long-term effects in individuals who experienced acute toxicity with organophosphorus insecticides indicated dose-dependent decreases in sustained visual attention and vibrotactile sensitivity.[59] In another study, one-fourth of the patients who were hospitalized following exposure to methamidophos exhibited an abnormal vibrotactile threshold between 10 and 34 mo after hospitalization.[67]

Callender et al.[60] have described a woman with chronic neurological sequelae following acute exposure to a combination of an organophosphorothioate insecticide, pyrethrin, piperonyl butoxide, and petroleum distillates. Initially, she developed symptoms of acute cholinergic toxicity. One month after exposure, she experienced severe frequent headaches, muscle cramps, and diarrhea. After 3.5 mo, she developed numbness in her legs, tremors, memory problems, anxiety-depression, and insomnia. One year following exposure, she developed weakness, imbalance, and dizziness, and was confined to a wheelchair. Her symptoms were all characteristic of OPIDN. Twenty-eight months after exposure, she developed "delayed sequelae of gross neurologic symptoms," consisting of coarse tremors, intermittent hemiballistic movements of the right arm and leg, flaccid fasciculations of muscle groups, muscle cramps, and sensory disturbances.

Some victims of the Tokyo subway sarin incident, who developed acute cholinergic neurotoxicity, also developed long-term, chronic neurotoxicity characterized by CNS neurological deficits and neurobehavioral impairments.[11] Six to 8 mo after the Tokyo poisoning, some victims showed delayed effects on psychomotor performance, the visual nervous system, and the vestibule-cerebellar system.[68] It is noteworthy that females were more likely than males to exhibit delayed effects on the vestibular-cerebellar system. Three years after the Matsumoto attack in Japan, some patients complained of fatigue, shoulder stiffness, weakness, and blurred vision.[9] Others complained of insomnia, bad dreams, husky voice, slight fever, and palpations. Colosio et al.[62] reviewed the literature on the neurobehavioral toxicity of pesticides, and reported that some individuals who were acutely poisoned with organophosphorus compounds developed long-term impairment of neurobehavioral performance. They also concluded that these effects were only "an aspecific expression of damage and not of direct neurotoxicity."

OPICN following subclinical exposures to organophosphorus compounds. Reports on OPICN in individuals following long-term, subclinical exposures, without previous acute poisoning, have been inconsistent, mostly because of difficulty in the quantitative determination of exposure levels, but also because of problems with selection of controls. Several studies of workers

exposed to low subclinical doses of organophosphorus insecticides failed to show neurobehavioral alterations between pre- and post-exposure measurements.[69–75] It has been suggested that the levels of exposure of subjects in these reports might have been below the threshold level needed to cause neurobehavioral deficits, and that studies of prolonged low-level exposures may eventually reveal neurobehavioral deficits.[62] Consistent with this opinion are the reports of impairment in neurobehavioral performance in individuals exposed to low-levels of organophosphorus insecticides. Professional pesticide applicators and farmers who had been exposed to organophosphorus pesticides showed elevated levels of anxiety, impaired vigilance and reduced concentration.[76] Kaplan et al.[77] reported persistent long-term cognitive dysfunction and defects in concentration, word finding, and short-term memory in individuals exposed to low subclinical levels of the organophosphorus insecticide chlorpyrifos. A significant increase in hand vibration threshold was reported in a group of pesticide applicators,[78] and significant cognitive and neuropsychological deficits have been found in sheep dippers who had been exposed to organophosphorus insecticides.[72,73] Male fruit farmers who were chronically exposed to organophosphorus insecticides showed significant slowing of their reaction time.[79] Female pesticide applicators exhibited longer reaction times, reduced motor steadiness, and increased tension, depression, and fatigue compared with controls.[74] Workers exposed to the organophosphorus insecticide quinalphos during its manufacture exhibited alterations in CNS function that were manifested as memory, learning, vigilance, and motor deficits, despite having normal AChE activity.[80] Rescue workers and some victims who did not develop any acute neurotoxicity symptoms nevertheless complained of a chronic decline in memory 3 yr and 9 mo after the Tokyo attack.[81] Pilkington et al.[55] reported a strong association between chronic low-level exposure to organophosphate concentrates in sheep dips and neurological symptoms in sheep dippers—suggesting that long-term health effects may occur in at least some sheep dippers exposed to these insecticides over their working lives.

Neurological and neurobehavioral alterations. Although the symptoms of OPICN are a consequence of damage to both the PNS and CNS, they are related primarily to CNS injury and resultant neurological and neurobehavioral abnormalities. Studies on the effects of exposure to organophosphorus compounds over the past half century have shown that chronic neurological and neurobehavioral symptoms include headache, drowsiness, dizziness, anxiety, apathy, mental confusion, restlessness, labile emotions, anorexia, insomnia, lethargy, fatigue, inability to concentrate, memory deficits, depression, irritability, confusion, generalized weakness and tremors.[51,52,82,83] Respiratory, circulatory, and skin problems may be present as well in cases of chronic toxicity.[1] It should be noted that not every patient exhibits all of these symptoms. Gershon and Shaw[51] reported that most of the symptoms that develop after organophosphate exposure resolve within 1 yr. Jamal[50] conducted an extensive review of the health effects of organophosphorus compounds and concluded that either acute or long-term, low-level exposure to these chemicals produces a number of chronic neurological and psychiatric abnormalities that he called "chronic organophosphate-induced neuropsychiatric disorder," or COPIND. Jamal recommended a multifaceted approach to the evaluation of the toxic effects of chronic, subclinical, repeated, low-level exposures to organophosphorus compounds; included were structural and quantitative analyses of symptoms and clinical neurological signs. In the present article, our concept of OPICN encompasses structural, functional, physiological, neurological, and neurobehavioral abnormalities, including neuropsychiatric alterations or COPIND. OPICN may be caused by an acute exposure that results in cholinergic toxicity, or by exposure to subclinical doses that do not produce acute poisoning.

Neuropathological alterations. Petras[84] investigated the neuropathological alterations in rat brains 15–28 days following intramuscular injections of large, acutely toxic doses (79.4–114.8 μg/kg) of the nerve agent soman. He reported that the brain damage in all 4 animals that developed seizures was comparable to damage present in 3 of the 4 animals that exhibited only limb tremor. Neuropathological lesions were characterized by axonal degeneration, seen in the cerebral cortex, basal ganglia, thalamus, subthalamic region, hypothalamus, hippocampus, fornix, septum, preoptic area, superior colliculus, pretectal area, basilar pontine nuclei, medullary tegmentum, and corticospinal tracts. Although the mechanism of soman-induced brain injury was not known, Petras noted that the lesions did not resemble those present in experimental fetal hypoxia[85] or OPIDN.[17] These results are consistent with later findings obtained after acute soman exposure,[86,87] exposure to the nerve agent sarin,[88] and neuronal necrosis induced by the organophosphorus insecticide fenthion.[89] Petras also indicated that soman-treated rats did not need to experience a seizure to develop lesions. Abdel-Rahman et al.[90] demonstrated neuropathological alterations in rat brain 24 hr after administration of an intramuscular LD_{50} dose (100 μg/kg) of sarin. Neuronal degeneration was present in the cerebral cortex, dentate gyrus, CA1 and CA3 subfields of the hippocampal formation, and the in Purkinje cells of the cerebellum. Neuronal degeneration of hippocampal cells is consistent with organophosphorus compound-induced alterations in behavior, and cognitive deficits such as impaired learning and memory.[91–94] Furthermore, chronic exposure to organophosphorus compounds resulted in long-term cognitive

deficits, even in the absence of clinical signs of acute cholinergic toxicity.[95,96] Shih et al.[97] demonstrated that lethal doses ($2 \times LD_{50}$) of all tested nerve agents (i.e., tabun, sarin, soman, cyclosarin, VR, and VX) induced seizures accompanied by neuropathological lesions in the brains of guinea pigs, similar to those lesions reported for other organophosphorus compounds in other species.[98–103] Recent reports have indicated that anticonvulsants protected guinea pigs against soman- and sarin-induced seizures and the development of neuropathological lesions.[104,105] Time-course studies also have reported that sarin-induced brain lesions exacerbated over time and extended into brain areas that were not initially affected.[88,106] Kim et al.[107] found that that an intraperitoneal injection of 9 mg/kg ($1.8 \times LD_{50}$) DFP in rats protected with pyridostigmine bromide and atropine nitrate caused tonic-clonic seizures, followed by prolonged mild clonic epilepsy accompanied by early necrotic and delayed apoptotic neuronal degeneration. Early necrotic brain injury in the hippocampus and piriform/entorhinal cortices was seen between 1 and 12 hr after dosing. On the other hand, typical apoptotic terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling (TUNEL)-positive cell death began to appear at 12 hr in the thalamus. Daily dermal administration of $0.01 \times LD_{50}$ of malathion for 28 days caused neuronal degeneration in the rat brain that was exacerbated by combined exposure to the insect repellent DEET and/or the insecticide permethrin.[108]

Correlation between neuropathological lesions and neurological and neurobehavioral alterations. Neuropathological changes—the hallmark of OPICN—could explain the neurological, neurobehavioral, and neuropsychological abnormalities reported in humans and animals exposed to organophosphorus compounds. A subcutaneous dose of 104 µg/kg soman induced status epilepticus in rats, followed by degeneration of neuronal cells in the piriform cortex and CA3 of the hippocampus.[103] Similar results have been reported in a variety of species.[98,109,110] In another study, only those mice treated with a subcutaneous dose of 90 µg/kg of soman which developed long-lasting convulsive seizures exhibited the neuropathological alterations.[111] Twenty-four hours after dosing, numerous eosinophilic cells and deoxyribonucleic acid (DNA) fragmentation (TUNEL-positive) cells were observed in the lateral septum, the endopiriform and entorhinal cortices, the dorsal thalamus, the hippocampus, and the amygdala. Animals that had only slight tremors and no convulsions did not show any lesions.[111] Guinea pigs given a subcutaneous dose of 200 µg/kg soman ($2 \times LD_{50}$) developed seizures and exhibited neuropathological lesions in the amygdala; the substantia nigra; the thalamus; the piriform, entorhinal, and perirhinal cortices; and the hippocampus between 24–48 hr following injection.[104] Male guinea pigs developed epileptiform seizures after receiving $2 \times LD_{50}$ subcutaneous doses of the following nerve agents: tabun (240 µg/kg), sarin (84 µg/kg), soman (56 µg/kg), cyclosarin (114 µg/kg), VX (16 µg/kg), or VR (22 µg/kg). The seizures were accompanied by necrotic death of neuronal cells, with the amygdala having the most severe injury, followed by the cortex and the caudate nucleus.[97]

An intraperitoneal injection of 9 mg/kg ($1.8 \times LD_{50}$) DFP caused severe early (15–90 min) tonic-clonic limbic seizures, followed by prolonged mild clonic epilepsy.[107] Necrotic cell death was seen 1 hr after DFP administration, primarily in the CA1 and CA3 subfields of the hippocampus and piriform/entorhinal cortices, and manifest as degeneration of neuronal cells and spongiform of neuropils. Whereas the severity of hippocampal injury remained the same for up to 12 hr, damage to the piriform/entorhinal cortices, thalamus, and amygdala continued to increase up to 12 hr. Furthermore, apoptotic death of neuronal cells (TUNEL-positive) was seen in the thalamus at 12 hr, and peaked at 24 hr. Rats that survived $1 \times LD_{50}$ sarin (95 µg/kg) exhibited persistent lesions, mainly in the hippocampus, piriform cortex, and thalamus.[88] Furthermore, brain injury was exacerbated over time; at 3 mo after exposure, other areas that were not initially affected became damaged. A recent study has described the early neuropathological changes in the adult male rat brain 24 hr after exposure to a single intramuscular dose of 1.0, 0.5, 0.1, or 0.01 $\times LD_{50}$ (100 µg/kg) sarin.[90] Sarin at $1.0 \times LD_{50}$ caused extensive severe tremors, seizures, and convulsions accompanied by damage involving mainly the cerebral cortex, the hippocampal formation (dentate gyrus, and CA1 and CA3 subfields) and the cerebellum. Damage was evidenced by (a) a significant inhibition of plasma BChE, brain region AChE, and M2 M-acetylcholine receptor ligand binding; (b) an increase in permeability of the blood-brain barrier; and (c) diffuse neuronal cell death coupled with decreased MAP-2 expression within the dendrites of surviving neurons. The $0.5 \times LD_{50}$ sarin dose did not cause motor convulsions, and only moderate Purkinje neuron loss. The 0.1 and $0.01 \times LD_{50}$ doses of sarin caused no alterations at 24 hr after dosing. These results indicate that sarin-induced acute brain injury is dose-dependent.

In animals treated with $1 \times LD_{50}$ sarin, both superficial layers (I–III) and deeper layers (IV–V) of the motor cortex and somatosensory cortex showed degeneration of neurons. In the deeper layers of the cortex, neuron degeneration was seen in layer V. Pyramidal neurons in layers III and V of the cortex are the source of the axons of the corticospinal tract, which is the largest descending fiber tract (or motor pathway) from the brain controlling movements of various contralateral muscle groups. Thus, sarin-induced death of layers III and V neurons of the motor cortex could lead to considerable motor and sensory abnormalities, ataxia, weakness, and

loss of strength. Furthermore, disruption of the hippocampal circuitry because of the degeneration of neurons in different subfields can lead to learning and memory deficits. Lesions in the cerebellum could result in gait and coordination abnormalities. Because the severely affected areas (e.g., the limbic system, corticofugal system, and central motor system) are associated with mood, judgment, emotion, posture, locomotion, and skilled movements, humans exhibiting acute toxicity symptoms following exposure to large doses of organophosphates may also develop psychiatric and motor deficits. Inasmuch as the damaged areas of the brain do not regenerate, these symptoms are expected to persist long-term.[112–114] These findings are in agreement with a recent study by Kilburn[115] which evaluated the neurobehavioral effects of chronic low-level exposure to the organophosphorus insecticide chlorpyrifos in 22 patients. Kilburn demonstrated, for the 1st time, an association between chlorpyrifos sprayed inside homes and offices and neurophysiological impairments in balance, visual fields, color discrimination, hearing reaction time, and grip strength. These patients also had psychological impairment of verbal recall and cognitive function, and two-thirds of them had been prescribed antidepressant drugs. In addition, the patients exhibited severe respiratory symptoms, accompanied by airway obstruction. Other chlorpyrifos-induced neurotoxicity incidents in humans have been reported.[116] These results are consistent with the report that daily dermal application of 0.1 mg/kg chlorpyrifos to adult rats resulted in sensorimotor deficits.[117] Also, maternal exposure to a daily dermal dose of 0.1 mg/kg chlorpyrifos during gestational days 4–20 caused an increased expression of GFAP in the cerebellum and hippocampus of offspring on postnatal day 30.[118] A major component of astrocytic intermediate neurofilaments, GFAP is up-regulated in response to reactive gliosis resulting from insults such as trauma, neurodegenerative disease, and exposure to neurotoxicants.[119]

Mechanisms of OPICN. Recent studies have shown that large toxic doses of organophosphorus compounds cause early convulsive seizures and subsequent encephalopathy, leading to the necrotic death of brain neuronal cells, whereas small doses produce delayed apoptotic death. Pazdernik et al.[103] have proposed the following 5 phases that result in organophosphorus compound-induced cholinergic seizures: (1) initiation, (2) limbic status epilepticus, (3) motor convulsions, (4) early excitotoxic damage, and (5) delayed oxidative stress. The mechanisms of neuronal cell death in OPICN that appear to be mediated through necrosis or apoptosis, which may involve increased AChE gene expression, are discussed below.

Necrosis. The large toxic doses of organophosphorus compounds which induce early seizures activate the glutamatergic system and involve the Ca^{2+}-related excitotoxic process,[120,121] possibly mediated by the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors.[122,123] De Groot[104] hypothesized that accumulated acetylcholine, resulting from acute inhibition of AChE by organophosphorus compounds, leads to activation of glutamatergic neurons and the release of the excitatory L-glutamate amino acid neurotransmitter. This in turn produces increased depolarization and subsequent activation of the NMDA subtype of glutamate receptors—and the opening of NMDA ion channels—resulting in massive Ca^{2+} fluxes into the post-synaptic cell and causing neuronal degeneration. Thus, glutamate-induced neuronal degeneration during seizures may occur as a result of lowering of the threshold for glutamate excitation at NMDA receptor sites.

Activation of nitric oxide synthase, following stimulation of NMDA receptor sites, increases the level of nitric oxide, which functions as a signaling or cytotoxic molecule responsible for neuronal cell death.[124] As a retrograde messenger, nitric oxide induces the release of several neurotransmitters, including excitatory amino acid L-glutamate[125] which alters neurotransmitter balance and affects neuronal excitability. The production of nitric oxide is enhanced in AChE-inhibitor–induced seizures.[126,127] Kim et al.[107] demonstrated the involvement of nitric oxide in organophosphate-induced seizures and the effectiveness of nitric oxide synthesis inhibitors in preventing such seizures.

Apoptosis. Neuronal degeneration caused by apoptosis or programmed cell death may have physiologic or pathologic consequences. Elimination of precancerous, old, or excess cells is carried out by apoptosis without injury to surrounding cells as seen in necrosis.[128] Small doses of organophosphorus compounds cause delayed neuronal cell death that involves free radical generation (i.e., reactive oxygen species [ROS]). Organophosphates that cause mitochondrial damage/dysfunction also cause depletion of ATP and increased generation of ROS, which results in oxidative stress.[129,130] ROS can cause fatal depletion of mitochondrial energy (ATP), induction of proteolytic enzymes, and DNA fragmentation, leading to apoptotic death.[129,131,132] These results are consistent with the DNA damage detected in the lymphocytes in peripheral blood in 8 individuals, following residential exposure to the organophosphorus insecticides chlorpyrifos and diazinon.[133]

The brain is highly susceptible to oxidative stress-induced injury for several reasons: (a) its oxygen requirements are high; (b) it has a high rate of glucose consumption; (c) it contains large amounts of peroxidizable fatty acids; and (d) it has relatively low antioxidant capacity.[131,132] A single sublethal dose of $0.5 \times LD_{50}$ sarin, which did not induce seizures, nevertheless caused delayed apoptotic death of rat brain neurons in the cerebral cortex, hippocampus, and Purkinje cells of the cerebellum 24 hr after dosing.[90,134] Furthermore,

rats treated with a single 0.1 × LD_{50} dose of sarin, and which did not exhibit brain histopathological alterations 1, 7, or 30 days after dosing, nevertheless showed apoptotic death of brain neurons in the same areas mentioned above, 1 yr after dosing.[90,135] These results are consistent with the sensorimotor deficits exhibited by sarin-treated animals 3 mo after exposure; the animals showed continued deterioration when tested 6 mo after dosing.

Increased AChE gene expression. Recent studies have suggested that AChE may play a role in the pathogenesis of OPICN similar to that reported for Alzheimer's Disease.[136,137] We have demonstrated that sarin induced the AChE gene in the same regions of the brain that underwent neuronal degeneration.[138] AChE has been shown to be neurotoxic in vivo and in vitro; it accelerates assembly of amyloid peptide in Alzheimer's fibrils, leading to cell death via apoptosis.[139] Some studies have demonstrated increased AChE expression in apoptotic neuroblastoma SK-N-SH cells after long-term culturing.[139] Brain AChE has been shown to be toxic to neuronal (Neuro 2a) and glial-like (B12) cells.[137] There are also reports that transgenic mice overexpressing human AChE in brain neurons undergo progressive cognitive deterioration.[140] These results suggest that sarin may provoke an endogenous cell suicide pathway cascade in susceptible neurons (e.g., in the caspase-3 pathway), resulting in the release of AChE into adjacent brain tissues. The aggregation of AChE initiates more apoptotic neuronal death. Amplification of this cascade thus may result in the progressive neuronal loss that is the hallmark of sarin-induced chronic neurotoxicity. It is noteworthy that a common symptom of both OPICN and Alzheimer's Disease is memory deficit, suggesting that the aging process may be accelerated following exposure to organophosphorus compounds in OPICN.

Other factors. The occurrence and severity of OPICN is influenced by factors such as environmental exposure to other chemicals, stress, or individual genetic differences. For example, cholinotoxicants such as organophosphates or carbamates—which do not have a positive charge and are capable of crossing the blood-brain barrier—act at the same receptors and thus exacerbate OPICN. Individuals with low levels of the plasma enzymes BChE[5,6,141] or paraoxonase[7,8] that act as the 1st line of defense against neurotoxicity (by removing organophosphates from circulation through scavenging or hydrolysis) are vulnerable to the development of persistent OPICN. All of these factors may be involved in development of the phenomenon known as "chemical sensitivity" or "multiple chemical sensitivity."[142] Thus, prior chemical exposure, stress, or genetic factors might make individuals predisposed or susceptible to CNS injury upon subsequent exposure to other chemicals.

Combined exposure to other chemicals that cause oxidative stress can intensify OPICN which results from exposure to organophosphorus compounds.[108] Furthermore, stress that also causes oxidative stress decreases the threshold level required to produce neuronal damage and results in increased OPICN following combined exposure to stress and organophosphates. Thus, OPICN may explain the reports that Persian Gulf War veterans showed a higher than normal propensity toward persistent neurological complaints such as memory and attention deficits, irritability, chronic fatigue, muscle and joint pain, and poor performance on cognition tests.[143–147] A large number of these personnel were exposed to low levels of sarin during the demolition of Iraqi munitions at Khamisiya,[148,149] as well as to other chemicals and to stress.[150,151] Also, OPICN may explain the recent report that Persian Gulf War veterans are at an almost 2-fold greater risk of developing amyotrophic lateral sclerosis (ALS) than other veterans,[152] which is consistent with Haley's[153] suggestion that the increase in ALS is "a war-related environmental trigger." Furthermore, OPICN induced by low-level inhalation of organophosphates present in jet engine lubricating oils and the hydraulic fluids of aircraft[154] could explain the long-term neurologic deficits consistently reported by crewmembers and passengers, although organophosphate levels may have been too low to produce OPIDN.[155]

Prognosis

Previous reports have indicated that, subsequent to exposure to organophosphorus compounds, an individual could develop acute cholinergic neurotoxicity, followed by OPICN. In a few cases, OPIDN may occur with or without the development of cholinergic neurotoxicity, with OPICN developing later. Furthermore, OPICN may occur following long-term, low-level exposure to organophosphorus compounds, and without the development of acute neurotoxicity. Because the long-term, persistent effects of OPICN result from neuronal degeneration of the PNS and CNS, induced by organophosphates, it is unlikely that improvement is the consequence of the regeneration of brain neurons, inasmuch as such repair is not typical of the CNS. Clinical improvement may occur, however, through repair of the PNS. Also, reversible changes in the CNS that might be present initially (e.g., edema) could later subside, giving the *appearance* of repair. Furthermore, if damage is not too extensive, other neurons having the same function could meet the added demands and maintain normal activity. When the CNS is severely damaged, however, neither of these repair mechanisms is possible and some loss of function will likely occur.

Conclusions

Herein we have described the long-term, persistent neurodegenerative disorder induced by exposure to

organophosphorus compounds. We define this effect as organophosphorus ester-induced chronic neurotoxicity, or OPICN. Numerous cases documenting this disorder have been reported since the extensive use of these chemicals in industry and agriculture began more than 50 yr ago. Although largely characterized by chronic neurobehavioral alterations, OPICN involves other molecular, neurochemical, neurophysiological, neuropathological, neuropsychological, and neurological changes. The term "neurotoxicity" encompasses all of these, and adequately describes this neurodegenerative disorder.

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