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Seizure-related Encephalopathy in Rats Intoxicated with Diisopropylfluorophosphate

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ABSTRACT *: The incidence and distribution of necrotic and apoptotic neural cells, and activated astrocytes in the brain of rats intoxicated intraperitoneally with diisopropylfluorophosphate were investigated. Pyridostigmine bromide (0.1 mg/kg) and atropine methylnitrate (20 mg/kg) were pretreated intramuscularly 30 min and 10 min, respectively, prior to diisopropylfluorophosphate (4~10 mg/kg) administration. Diisopropylfluorophosphate induced severe limbic seizures, early necrotic and delayed apoptotic brain injuries, and rapid astrocytic responses. The necrosis, which was closely related to seizure intensity, was observed as early as 1 hr after intoxication predominently in hippocampal pyramidal cells, cerebellar Purkinje cells and neurons in pyriform/entorhinal cortices, showing malacia of neurophils. In contrast, apoptosis started to appear 12 hr after intoxication in neurons in thalamus, amygdala and neocortex, and ephendymal cells surrounding the 4th ventricle. Since marked apoptosis was induced in rats exhibiting relatively-low seizure intensity, the degree of necrosis and apoptosis was shifted to each type of injury according to the seizure intensity. Activated astrocytes, observed within 1 hr along the limbic system, were suggested to affect the neural injury patterns by producing high level of nitric oxide. However, the distribution of activated astrocytes was not in parallel with those of necrotic or apoptotic injuries, implying that the astrocytic responses resulted from seizure activity rather than neural injuries. Furthermore, astrocytes in malacic tissues disappeared during the severe limbic seizures. Therefore, it would be one of the cautionary notes on the expression of glial fibrillary acidic protein in astrocytes as a biochemical marker of brain injuries following acute exposure to organophosphates.*

Key Words *: Diisopropylfluorophosphate, Brain injuries, Necrosis, Apoptosis, Terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling (TUNEL), Glial fibrillary acidic protein (GFAP), Immunohistochemistry*

I. INTRODUCTION

In addition to diverse cholinergic symptoms which may cause acute death, organophosphates induce brain injuries following epileptiform seizures (Dunn and Sidell, 1989). Some features of neural and cardiac injuries in animals intoxicated with pinacolylmethylphosphonofluoridate (soman) have been demonstrated (Kim *et al.*, 1999; McDonough *et al.*, 1995; Tryphonas and Clement, 1995). It has also been reported that excitatory amino acids increased during seizures following soman intoxication (Lallement *et al.*, 1991, 1992), and the excitatory amino acids and their receptor agonists (excitotoxins) were proposed to play a causative role in the injury of central nervous system (Choi *et al.*, 1987; McDonough and Shih, 1993; Shih *et al.*, 1991).

Interestingly, the involvement of nitric oxide in cholinergic models of epilepsy was confirmed, although controversial results have been achieved with nitric oxide synthase inhibitors (Bagetta *et al.*, 1992; Starr and Starr, 1993). In a previous paper, we also demonstrated a role of nitric oxide in diisopropylfluorophosphate-induced seizures (Kim *et al.*, 1997). In the central nervous system, the activation of nitric oxide synthase, following stimulation of *N*-methyl-D-aspartate receptor, produces nitric oxide which functions as a signalling or cytotoxic molecule (Dawson *et al.*, 1991, 1993; Lancaster, 1992).

Meanwhile, nitric oxide has been reported to induce

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apoptotic death of neural cells (Bolanos *et al.*, 1997; Bonfoco *et al.*, 1995). Recent reports showed that soman intoxication rapidly activated astrocytes and microglia (Zimmer *et al.*, 1997), and induced early astrocytic swelling and later astrocytosis in discrete brain regions (Tryphonas and Clement, 1995), which may produce high concentration of nitric oxide. Moreover, selective potentiation of *N*-methyl-D-aspartate-induced neuronal injury following induction of astrocytic nitric oxide synthase with cytokines was observed (Hewett *et al.*, 1994). Thus, high concentraion of nitric oxide produced by inducible nitric oxide synthase of astrocytes might be related to the brain injuries, especially neural apoptosis. Furthermore, it was demonstrated that suppression of cerebrospinal nitrite/nitrate content with L-N^G-nitroarginine, a nitric oxide synthase inhibitor, selectively attenuated the delayed apoptosis, but not necrosis, of neural cells, implying the nitric oxide-mediated neural apoptosis following seizures (Kim *et al.*, 1999).

Such observations led us to investigate the relationship between the features of neural injury and astrocytic response, since the increased level of GFAP following reactive astrogliosis has been used as a biomarker of neuropathy (O'Callghan, 1991, 1993). The present study describes the distribution and time-course of apoptotic (TUNEL-positive) and necrotic (TUNEL-negative) neural cells, and activated astrocytes in discrete brain regions of rats following seizures with various intensity induced by diisopropylfluorophosphate.

II. MATERIALS AND METHODS

1. Materials

Various chemicals and reagents, including pyridostigmine bromide, atropine methylnitrate and monoclonal mouse anti-cow GFAP antibody, were from Sigma Chemical Co. (St. Louis, MO), except diisopropylfluorophosphate which was procured from Adrich Chemical Co. (Milwakee, WI). *In Situ* Apoptosis Detection Kit-Peroxidase (ApopTag TUNEL assay kit) and GFAP immunohistochemistry kit (Vectastain *Elite* avidin-biotin-peroxidase kit) were obtained from Oncor Co. (Gaithersberg, MD) and Vector Laboratories (Burlingame, CA), respectively.

2. Seizure induction

Adult Sprague-Dawley female rats (200~300 g) were intoxicated intraperitoneally with 4~10 mg/kg (0.8~2× LD_{50}) of diisopropylfluorophosphate. Pyridostigmine bromide (0.1 mg/kg) and atropine methylnitrate (20 mg/kg), which are centrally inactive (Shih *et al.*, 1991), were pretreated intramuscularly 30 min and 10 min, respectively, before diisopropylfluorophosphate challenge to reduce the mortality and eliminate peripheral signs for the clear demonstration of seizure activity (Kim *et al.*, 1997). The intensity of seizures was evaluated using 5-point scores (De Sarro *et al.*, 1993; Kim *et al.*, 1997) as follows; 0, no response; 1, myoclonic jerks of the contralateral forelimb; 2, mouth and facial movements (i.e., facial myoclonus, clonus of the jaw and vibrissae) and head nodding with or without mild forelimb clonus; 3, severe forelimb clonus; 4, rearing and severe forelimb clonus; 5, rearing and falling. All compounds were dissolved in saline, except diisopropylfluorophosphate which was diluted in distilled water, and administered in a volume of 1 ml/kg.

3. Cell death-type determination

Whole brain was removed after fixation by *in situ* intracardial perfusion with 10% neutral formalin solution containing 2 IU heparin/ml under ether anesthesia. For the identification of apoptotic injury, paraffin-embedded brain sections (4 μm in thickness) were stained immunohistochemically using TUNEL technique of DNA fragments with diaminobenzidine as chromogenic substrate, and counterstained with methyl green (Kim *et al.*, 1999; Rink *et al.*, 1995). The injury type of morphologically-dead cells was determined based on the staining patterns of nucleus; brown staining as apoptosis vs purple or blue as necrosis (Kim *et al.*, 1999). Live cells were stained green by counterstaining. The degree of brain injury was evaluated using 5-point scores based on the approximate percentage of tissue involvement according to the grading system of McDonough *et al.* (1995) with slight modification (Kim *et al.*, 1999); 0, no lesion; 1, minimal (1~10%); 2, mild (11~25%); 3, moderate (26~45%); 4, severe (46~60%); 5, extreme (> 60%).

4. GFAP immunohistochemistry

For the identification of astrocytes, paraffin-embedded brain sections were stained immunohistochemically for GFAP using a Vectastain *Elite* ABC kit with diaminobenzidine as chromogenic substrate (Fix *et al.*, 1996; Hur *et al.*, 1999; Zimmer *et al.*, 1997). In brief, after deparaffinization, H_2O_2 treatment to quench endogenous peroxidase activity and blocking with normal horse serum, the sections were incubated in primary monoclonal mouse anti-cow GFAP antibody for 30 min (1 : 500 dilution), followed by each 30-min incubation with biotinylated goat anti-mouse IgG and avidin-biotin-peroxidase complex. All incubations were performed in a humidity chamber at 37°C. For color development, the sections were stained with 0.05% diaminobenzidine with 0.1% H_2O_2, and counterstained with methyl green. The intensity of astocytic response was evaluated using arbitrary scores as follows (Fix *et al.*, 1996); -, no response; +, slight increase; ++, mild increase; +++, moderate increase; ++++, prominent increase; +++++, extreme increase.

The experiments were conducted according to the *"Guide Principles in the Use of Animals in Toxicology"* which had been adopted by the Society of Toxicology in 1989.

III. RESULTS

A high dose of diisopropylfluorophosphate (9 mg/

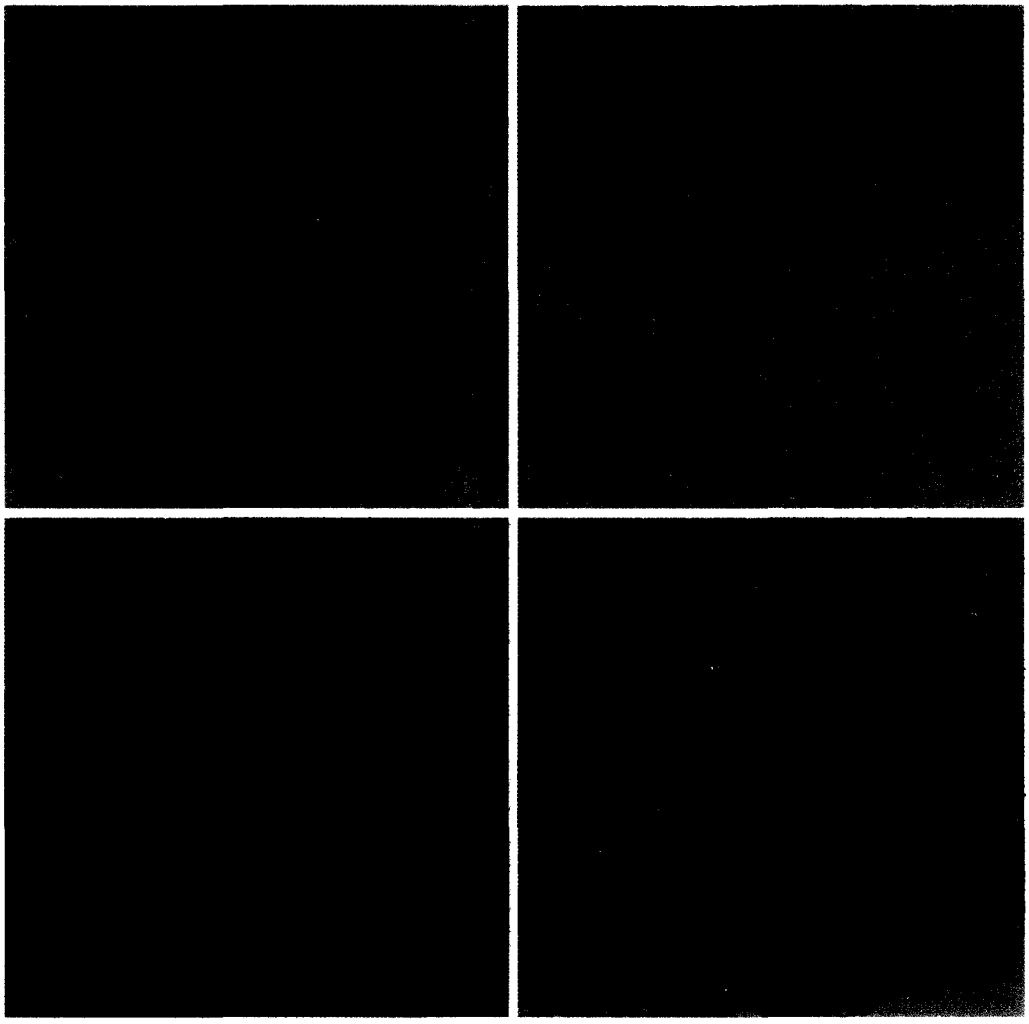

Fig. 1. Representative necrotic (TUNEL-negative) brain injuries induced by diisopropylfluorophosphate (9 mg/kg, $1.8 \times LD_{50}$), exhibiting dark degeneration of neural cells and severe malacia of neuropils. A, hippocampus (24 hr); B, cerebellum (24 hr); C, entorhinal cortex (12 hr); D, pyriform cortex (24 hr).

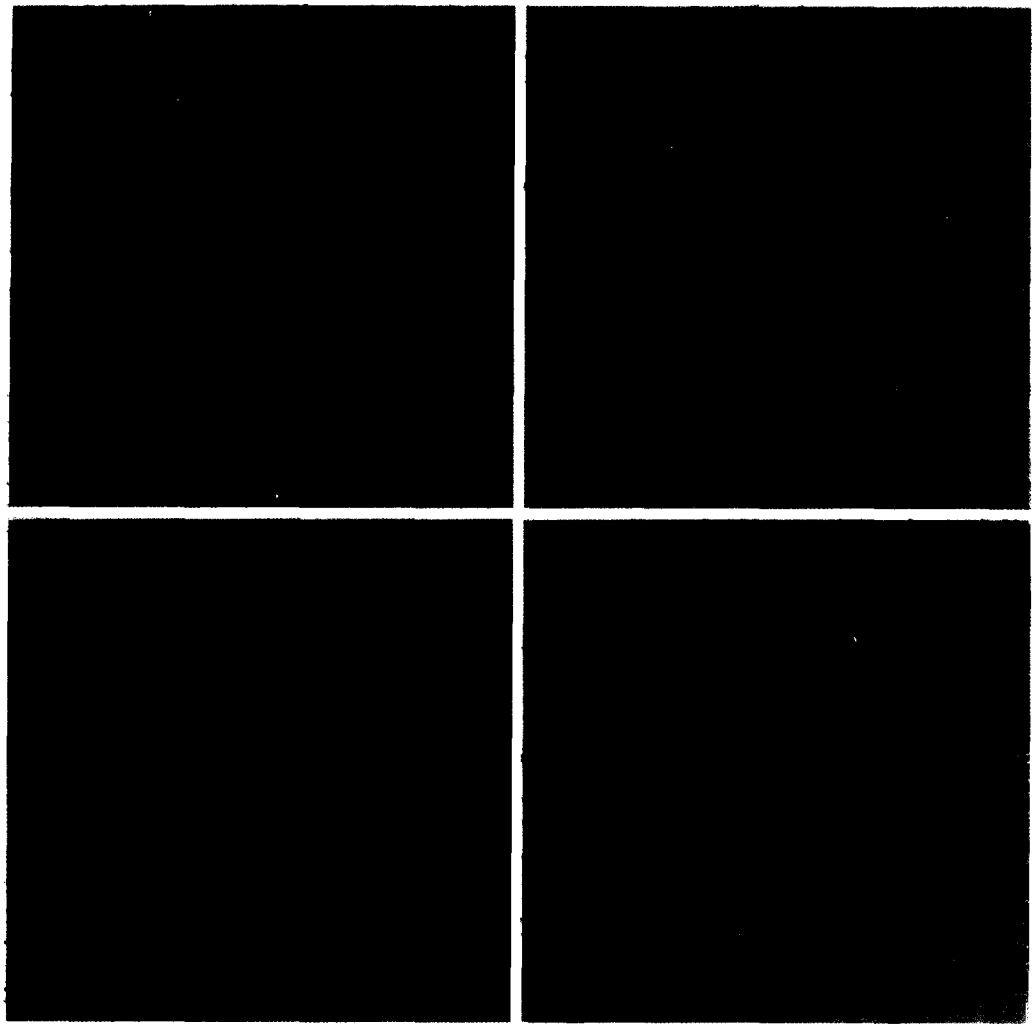

Fig. 2. Representative apoptotic (TUNEL-positive) brain injuries induced by diisopropylfluorophosphate (9 mg/kg, 1.8×LD_{50}), showing shrinkage of neural cells leading to a pericellular halo. A, neocortex (72 hr); B, thalamus (24 hr); C, entorhinal cortex (72 hr); D, 4th ventricle (24 hr).

kg, 1.8×LD_{50}) induced severe limbic seizures, exhibiting early (15~90 min) tonic-clonic seizures (intensity 3.5~4.0) and later prolonged mild clonic epilepsy. Such seizures led to necrotic and apoptotic brain injuries (Figs. 1 and 2). Interestingly, the type of injuries was observed to be somewhat different in discrete brain regions. Necrotic (TUNEL-negative) injury was characterized morphologically as dark degeneration of neural cells and malacia of neurophils. Dark degeneration was observed predominantly in hippocampal pyramidal cells in CA1 - CA3 regions, cerebellar Purkinje cells and pyriform/entorhinal cortical neurons, and the malacia was most severe in hippocampus and pyriform/entorhinal cortices (Figs. 1A~1D). Moreover, the malacia spread to all hippocampal formation (CA1 - CA4 and dentate gyrus) and to neocortex and amygdala in rats exhibited high intensity of seizures. In contrast, typical apoptotic (TUNEL-positive) cells were observed most predominantly in thalamus (Fig. 2B), and also in amygdala, though wherein mixed type of injury was shown. Interestingly, neurons in less-severely affected cortices, including neocrotex and entorhinal cortex, underwent time-dependent apoptotic change (Figs. 2A and 2C). Also, ephendymal cells surrounding the 4th ventricle were found to be TUNEL-positive (Fig. 2D). On the other hand, no remarkable apoptosis was found in hippocampus and pyriform cortex, exhibiting predominantly malacic necrosis, although a few samples were proved to be partially TUNEL-positive.

Table 1. Time-course of neural injuries, necrosis and apoptosis, induced by diisopropylfluorophosphate (9 mg/kg, 1.8×LD_{50})

Brain region	Time after poisoning (hr)				
	1	4	12	24	72
Necrosis					
Hippocampus					
pyramidal cell layer	2.9	3.1	2.8	2.9	2.9
granular layer of dentate	1.8	2.0	2.3	2.1	2.0
Thalamus					
dorsolateral nucleus	1.5	2.0	2.6	2.7	2.7
Cortices					
pyriform cortex	2.7	3.1	4.1	4.6	4.0
entorhinal cortex	1.7	2.1	3.2	3.5	3.3
Apoptosis					
Hippocampus					
pyramidal cell layer	0.0	0.0	1.0	0.7	0.8
Thalamus					
dorsolateral nucleus	0.0	0.0	2.1	4.1	4.3
Cortices					
pyriform cortex	0.0	0.0	1.0	1.7	1.9
entorhinal cortex	0.0	0.0	1.5	2.5	2.7

More interestingly, the degree of necrotic injury in hippocampus was determined within 1 hr and maintained constant (Table 1), while that of necrosis in thalamus, pyriform/entorhinal cortices and amygdala increased further after 4 hr, demonstrating an additional delayed effect. However, such a necrotic change in most regions examined terminated within 12 hr, as an early event. In comparison, apoptotic change started to appear at 12 hr in most regions including thalamus and cortices, as a delayed event, indicative of the involvement of a time-consuming process.

For further analyses of the relationship between seizure intensity and the features of brain injures, i.e., necrosis in hippocampus and apoptosis in thalamus, rats were intoxicated with various doses (4, 6, 8 or 10 mg/kg) of diisopropylfluorophosphate. As a result, necrotic brain injury was seen in rats showing seizure intensity higher than 2.0, displaying a good relationship with seizure intensity (Table 2). Interestingly, apoptosis was induced in rats exhibiting relatively-low seizure intensity, reaching a peak level at 4.0 of seizure intensity. However, the degree of apoptosis rather reduced in rats showing extremely severe seizures (seizure intensity > 4.0). Such trends in brain injuries were determined by the degree of central impact following organophosphate poisoning; necrotic injury in severe impact vs apoptotic shift in relatively-mild one. However, it is of interest to note that there are discrete regions showing predominantly necrotic (hippocampus, cerebellum and pyriform cortex), apoptotic (thalamus and ventricles) or mixed-type (amygdala, entorhinal cortex and neocortex) injury.

Table 2. Seizure-related changes in hippocampal necrosis and thalamic apoptosis of rats (n = 6) 24 hr after intoxication with diisopropylfluorophosphate (4~10 mg/kg)

Brain injuries	Seizure intensity					
	1.0	2.0	3.0	3.5	4.0	5.0
Hippocampal necrosis	0.0 ±0.0	0.3 ±0.1	1.3 ±0.2	2.3 ±0.6	3.4 ±0.6	4.8 ±0.5
Thalamic apoptosis	0.0 ±0.0	0.7 ±0.4	2.6 ±0.6	3.7 ±0.6	4.1 ±0.6	3.2 ±0.7

Although astrogliosis has been used as a marker of neural injury, activated astrocytes were detected along the limbic system as early as 1 hr after diisopropylflu-

Table 3. Time-course of astrocytic response (GFAP expression) after intoxication with diisopropylfluorophosphate (9 mg/kg, 1.8×LD_{50})

Brain region	Time after poisoning (hr)				
	1	4	12	24	72
Hippocampus					
alveus & fimbria	+++	+++	++++	++++	++++
oriens layer	+++	+++	++++	++++	++++
pyramidal cell layer	-	-	+	+	+
lacunosum moleculare	+++	++++	+++++	+++++	++++
granular layer of dentate	-	-	+	++	++
hilus of dentate	+++	++++	++++	++++	++++
Thalamus					
dorsolateral nucleus	-	-	+	+	+
Cortices					
pyriform cortex	+	++	++	+	+
entorhinal cortex	+	+	+	+	+

orophosphate (9 mg/kg, 1.8×LD_{50}) intoxication (Table 3). The most remarkable expression of GFAP was found in hippocampal lacunosum moleculare, followed by hilus of dentate, oriens layer, and alveus and fimbria (Figs. 3A and 3B). In comparison, the GFAP expression in pyriform cortex was transient at 4~12 hr, and reduced thereafter (Fig. 3F) (Zimmer *et al.*, 1997). Interestingly, however, the distribution of activated astrocytes was not in parallel with that of malacic tissue injury in hippocamal pyramidal layer, pyriform/entorhinal cortices and neocortex (Figs. 3A, 3C, 3E and 3F), or that of apoptotic neural cells in thalamic dorsolateral nucleus (Fig. 3D), inconsistent with the previous reports indicating increased astrocytosis at the sites of tissue injury (O'Callaghan, 1993; Zimmer *et al.*, 1997).

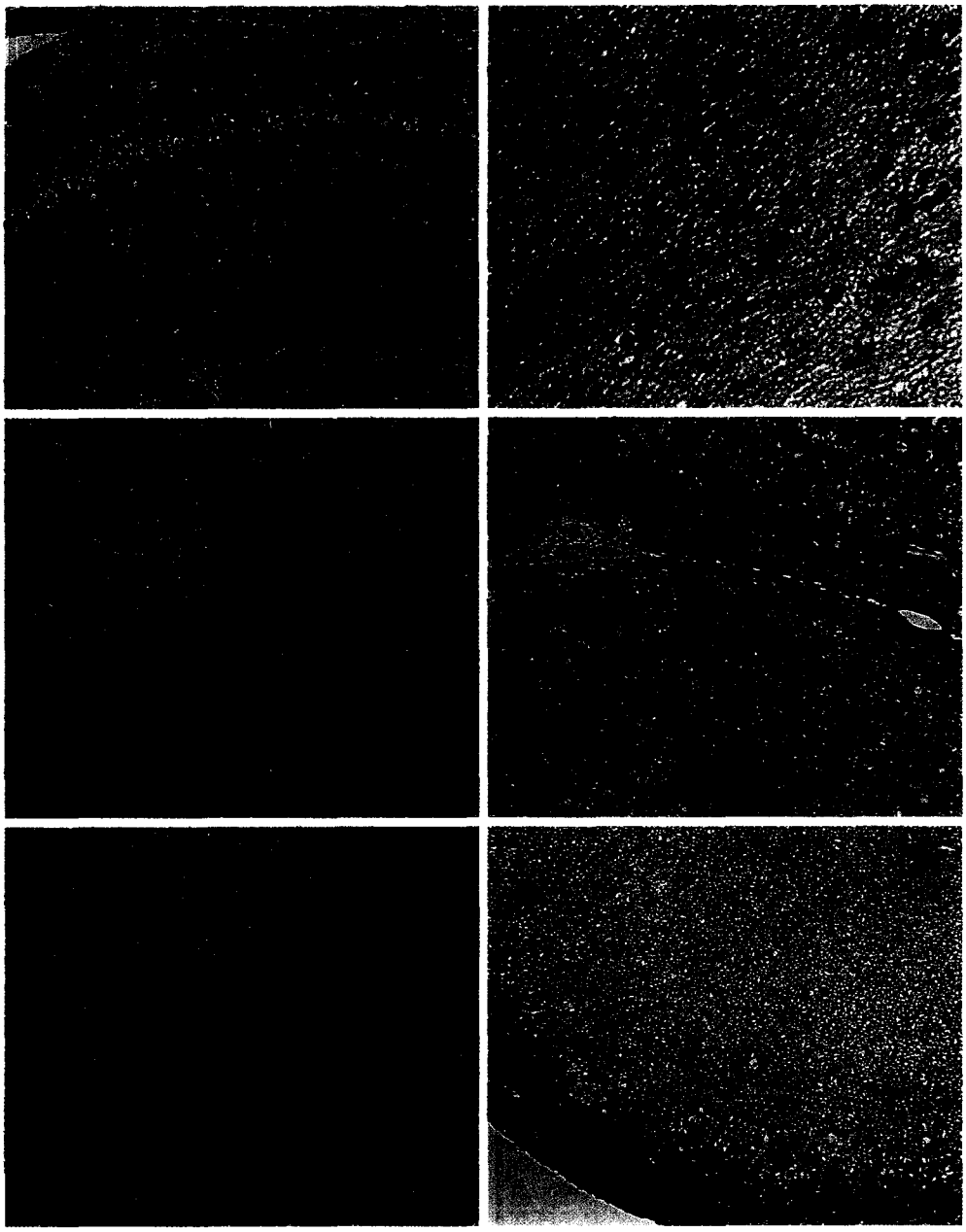

Fig. 3. Representative astrocytic responses to diisopropylfluorophosphate (9 mg/kg, 1.8×LD_{50}) intoxication. A, hippocampal oriens layer and lacunosum moleculare (1 hr); B, hippocampal fimbria (24 hr); C, neocortex (24 hr); D, thalamic dorsolateral nucleus (24 hr); E, entorhinal cortex (24 hr); F, pyriform cortex (24 hr).

IV. DISCUSSION

It is well known that reactive astrogliosis occurs in response to injury of central nervous system and as a concomitant neuropathological process (O'Callaghan, 1991, 1993). Such a change is accompanied by a dramatic increase in the level of GFAP, which has been used as a hallmark of activated astrocytes at the sites of central nervous system injury. However, there were a few activated astrocytes in malacic tissues in the present study (Fig. 3). Such a finding might be explained by an observation that vacuolation of neurophils induced by soman was followed by neuronal necrosis as well as astrocytic degeneration (Tryphonas and Clement, 1995). In spite of the similar time-courses of GFAP expression in pyriform cortex between the present study (Table 3 and Fig. 3F) and a previous report (Zimmer *et al.*, 1997), which reached a peak level at 8 hr and decreased thereafter, the level of GFAP expression induced by diisopropylfluorophosphate was much lower than that by soman. Such a discrepancy might be due to the difference in the intensity of insults produced by 77.7 μg/kg (0.7× LD_{50}) of soman and 9 mg/kg (1.8×LD_{50}) of diisopropylfluorophosphate, suggestive of a rapid degeneration of astrocytes that underwent a more severe insults from a high dose of diisopropylfluorophosphate.

Nitric oxide has been proposed as a mediator of neural cell injury induced by excitotoxins including *N*-methyl-D-aspartate (Bonfoco *et al.*, 1995; Dawson *et al.*, 1991, 1993; Gunasekar *et al.*, 1995). It has been reported that some excitotoxins can induce necrosis or apoptosis, according to the difference in the concentration of themselves or reactive radicals, nitric oxide and oxygen radicals, produced following *N*-methyl-D-aspartate receptor stimulation (Ankarcrona *et al.*, 1995; Bonfoco *et al.*, 1995; Gunasekar *et al.*, 1995; Lafon-Cazal *et al.*, 1993). Reaction of nitric oxide with superoxide anion forms highly reactive peroxynitrite and hydroxyl radicals, the ultimately cytotoxic molecules (Beckman *et al.*, 1990; Xia *et al.*, 1996). Cerebral insults trigger toxic activation of *N*-methyl-D-aspartate receptors and also activate microglia to produce diverse cytokines, some of which induce nitric oxide synthase in astrocytes (Lee *et al.*, 1993; Taupin *et al.*, 1993). Also, neuronal activity up-regulates astroglial gene expression (Steward *et al.*, 1991). Recently, it was reported that soman intoxication rapidly activated astrocytes in discrete brain regions (Zimmer *et al.*, 1997) and induced early astrocytic swelling and later astrocytosis in hippocampus, thalamus and amygdala (Tryphonas and Clement, 1995), and that excitotoxic or ischemic brain insults led to the intense nitric oxide synthase immunostaining in astrocytes (Wallace and Fredens, 1992).

It is known that neural cells are different in their susceptibility to excitotoxins; nitric oxide synthase-containing cells are relatively resistant to excitotoxic insults (Koh and Choi, 1988). Interestingly, the neuronal cells in *N*-methyl-D-aspartate receptor-rich regions, hippocampus and cerebellum, underwent predominantly necrosis, and the type of brain injuries, necrosis and apoptosis, was shifted to each other according to the degree of seizure intensity. *N*-methyl-D-aspartate receptor stimulation produces nitric oxide as well as superoxide by activating constitutive nitric oxide synthase, and reactive astrocytes also produce high level of nitric oxide following activation of inducible nitric oxide synthase. Excessive production of peroxynitrite and hydroxyl radicals might cause severe insults, resulting in necrosis. These observations may explain in part the distribution of necrotic and apoptotic cells.

In an our previous report (Kim *et al.*, 1999), cerebrospinal nitrite/nitrate content increased 7~8 folds of normal level after diisopropylfluorophosphate poisoning, which may be closely related to brain nitric oxide synthase activity (Slater *et al.*, 1996). Furthermore, the suppression of cerebrospinal nitrite/nitrate content by L-N^G-nitroarginine, a nitric oxide synthase inhibitor, selectively attenuated the delayed apoptosis, implying the nitric oxide-mediated process. However, in spite of the relationship between increased level of nitric oxide and neural apoptosis, the distribution of nitric oxide-producing astrocytes was not in parallel with that of apoptotic cells. Noteworthy, cerebral nitric oxide may diffuse easily into surrounding tissues, ventricles and cerebrospinal fluid, while diffusion of nitrite/nitrate might be limited by brain-cerebrospinal fluid interface, as inferred from the large difference in nitrite/nitrate content between brain tissues (52 μM) and cerebrospinal fluid (7.8 μM) (Slater *et al.*, 1996). This may explain ephendymal cell apoptosis by diffusible nitric oxide (Fig. 2D), and delayed

increase in nitrite/nitrate level in cerebrospinal fluid (Kim *et al.*, 1999). Based on the time-courses of rapid activation of astrocytes, lagged time in cerebrospinal nitrite/nitrate accumulation and delayed onset of apoptotic neural injury, it is proposed that nitric oxide produced and diffused from the rapidly activated astrocytes in limbic system, especially hippocampus, might led to the delayed apoptosis in the regions relatively remote from the sites of marked astrocytosis (Kim *et al.*, 1999).

Additionally, it is important to note that the neural apoptosis was prominent in the area less-severely affected, and there was either vascular response in the regions showing neural apoptosis or choroid plexus close to the apoptotic ephendymal cells (Fig. 2). Interestingly, the topographical distribution of vascular leakage following soman intoxication was similar to that of the present apoptotic neural injury (Carpentier *et al.*, 1990), implying a relationship between "vasogenic" edema and apoptotic change; seizures induced transient opening of blood-brain barrier in the anatomically-defined brain regions including thalamus and amygdala, the areas exhibiting apoptotic changes. In contrast, hippocampus, cerebellum and pyriform/entorhinal cortices, the "cytotoxic" regions exerted poor permeability of blood-brain barrier during soman-induced seizures (Carpentier *et al.*, 1990), exhibited predominantly necrosis (Kim *et al.*, 1999; Lallement *et al.*, 1997). Therefore, it is not excluded that the neural apoptosis may be related to vascular responses, leading to a transient increase in permeability of blood-brain barrier, during central seizures (Carpentier *et al.*, 1990; Petrali *et al.*, 1991; Pont *et al.*, 1995).

Our studies clearly show the role of nitric oxide as both a seizure modulator and a cytotoxic mediator following diisopropylfluorophosphate intoxication (Kim *et al.*, 1997, 1999). It is suggested that high level of nitric oxide produced by activated astrocytes may influence the cell injury patterns, as inferred from that the early astrocytic responses, especially in hippocampus, resulted from seizure activity rather than neural injuries (Table 3 and Fig. 3A; Steward *et al.*, 1991; Zimmer *et al.*, 1997). Although it is well known that GFAP expression markedly increases in the region of neuropathy, the distribution of activated astrocytes was not in parallel with those of necrotic or apoptotic injuries. Rather, astrocytes in malacic tissues disappeared during the severe limbic seizures. Therefore, it would be one of the cautionary notes on the expression of GFAP in astrocytes as a biochemical marker of brain injuries following acute exposure to organophosphates.

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