STUDIES ON THE DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS COMPOUNDS-(III)

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Accepted January 16, 1985

Abstracts—TOCP (Tri-orthocresyl phosphate), an organophosphorus compound, has been implicated in producing neuropathy in the male S. D. rats. Repeated subcutaneous doses of TOCP (600 mg/kg) for up to 6 weeks produced ataxia, most striking at 50 days after final injection, followed by gradual recovery. Ultrastructurally, the internal structure of affected nerve fibers was primarily composed of altered smooth endoplasmic reticulum, tubular membrane system, and mitochondria, although myelin sheath was found to be essentially normal. In the histopathological examination, axonal and myelin degeneration was disclosed in the gracile nucleus and in the gracile fasciculus of the cords as well as in the sciatic nerves. The localization and degree of these changes were considered to be “dying back”, showing systemic neuropathy. In addition, muscular lesion showed small group atrophy, corresponding to Type I fiber atrophy.

Key words: Delayed neuropathy, TOCP, rat, dying back

INTRODUCTION

It has been known that organophosphorus compounds are extensively used as agricultural pesticides and for industrial purposes. The toxic effects of these compounds have been said to be attributable to the inhibition of acetylcholinesterase in the nervous system of insects and certain species of animals. On the other hand, a variety of organophosphorus compounds have been introduced to cause delayed neurotoxicity in man and animals. One of these compounds is TOCP (Tri-orthocresyl phosphate).
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Since the review of the toxicological property of TOCP by Smith and Lillie (1931), poisoning of this compound has been extensively studied, and the quality of this toxicity has been confirmed to be "dying back" neuropathy.

Based on the results of our previous studies in adult hens (Itoh et al., 1981 and 1984), the animals have developed ataxia 10-20 days after exposure to TOCP, which eventually led to paralysis. Axonal degeneration accompanied by demyelination has been predominantly observed in the spinal cords and peripheral nerves of afflicted animals both in light and electron microscopies. Quite a few experiments in rodents, however, have been carried out to confirm the delayed neurotoxicity following administration of organophosphorus compounds. The only literature has been made by Majno and Karnovsky (1961) who introduced periperal neuropathy in the rats exposed to Mipafox (N,N'-Diisopropylphosphoro-diamidic fluoride). And there have been no attempts to establish delayed neurotoxicity in rodents after exposure to TOCP.

This report describes the clinical conditions and morphological lesions observed in the rats following subcutaneous administration of TOCP.

MATERIALS AND METHODS

**Animals**:

Thirty male Sprague–Dawley rats of 7 weeks of age weighing approximately 100 gr. were purchased from Clea Japan Inc. The animals were individually housed in wire mesh cages, and were kept in a room controlled at the temperature of 24±1°C and relative humidity of 50±10% with a 12-hr. light and dark cycle. They were fed a commercial laboratory pelleted diet (CE-2, Clea Japan Inc.) and tap water ab libitum, and the body weight of these animals were weekly determined. In this experiment, the rats were divided into two groups which consisted of 10 and 20 animals of control and dosed groups, respectively.

**Administration**:

TOCP was suspended in polyethylene glycol-400 and subcutaneously administered into the flank of the rats at the dosage of 600 mg/kg body weight twice a week for 6 weeks (12 doses). The rats were daily observed on their general appearance, onset, and severity of neurological signs. Sixty days after the final administration, 10 dosed animals out of 20 were sacrificed and both electron and light microscopic examinations were carried out. The remaining 10 animals were maintained for another 100-day period under observation and sacrificed. Control group was also killed 200 days after initiation of the experiment.

At the respective time of the investigation, the rats were anesthetized with ether, and were perfused for 15 minutes through the right and left carotid arteries with the respective fixatives. Then they were dissected and the cerebrum, cerebellum, spinal cords, sciatic nerves, and gastocnemius muscle were excised.

In electron microscopy, a 2% glutaraldehyde solution in phosphate buffer, kept at 4°C, was perfused in 3 animals. The excised tissues were placed in the same solution for 2 hours, and were post-fixed in a 2% OsO$_4$ solution for 2 hours. After that, these were
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dehydrated in graded alcohols and embedded in Epon 812, and ultrathin sections were
doubly stained with uranyl acetate and lead citrate, and were examined with a Hitachi
HS-9 electron microscope.

Similar procedures were conducted in the light microscopy using a 10% buffered
formalin solution. After 15 minutes perfusion fixation, excised tissues were placed in
the same solution, and were dehydrated, embedded in Paraplast and sectioned at 4 μm.
Sections were stained with hematoxylin and eosin (H. E.), Klüver-Barrera, Bodian,
Masson-trichrome and immunohistochemical staining, myoglobin (PAP method).

RESULTS

1) Clinical Symptoms and Mortality

The rats when given in repeated subcutaneous doses of TOCP showed salivation,
diarrhea and rough hair which were attributable to acute cholinergic intoxication of
this compound. These symptoms lasted for several days after administration, and were
observed even at the time of the next injection. Before 1-2 weeks of the final administra-
tion, the rats began to develop a sign being diagnosed to be an incipient neuropathy
characterized by ataxic movement. This sign was found to be progressive gradually,
and the rats developed weakness of the hind legs. Around 50 days after the final
administration, the rats sometimes kept their hind legs stretched while walking. In
this condition, ataxia became more evident characterized by difficulty in moving their
hind legs, and after that, they often laid down their lower abdomen on the bottom of
the cages. Although occasional fasciculation of skeletal muscles was observed, the rats
were well tolerated, and showed a tendency of recovery without having developed
paralysis thereafter. At the termination of the experiment, all clinical symptoms
completely disappeared in 7 animals and minimal ataxia was observed in the remaining
3 animals.

On the other hand, the body weight gain of the dosed animals was significantly
reduced during the entire period of the experiment, as compared to that of the control
(Fig. 1). In this experiment, no rats died.

2) Electron Microscopy

The ultrastructural investigation was carried out in the sciatic nerves of the rats
sacrificed 60 days after the final injection of TOCP and remarkable alterations were
encountered in the axons. The internal structure of the axons took the form of
increased electron density, and the lesions were characterized by increased prominence
of smooth endoplasmic reticulum (Photo 1) which in part were found to be dilated
showing form of channels and vacuoles. These lesions were associated with aggrega-
tion and disruption of neurofilaments and neurotubules together with a focal conden-
sation of these elements (Photo 2). Moreover, degenerative mitochondria and occa-
sional giant mitochondria were striking (Photo 3). These lesions were well disclosed in
relatively large fibers in diameter.

In contrast to these axonal alterations, myelin sheaths were found to be essentially
normal, while occasional vacuolic spaces were associated with minimal fragmentation of
Fig. 1. Body weight gain of rats exposed to TOCP.

Fig. 2. Schematic diagram of the lesions in the nervous system of rats exposed to TOCP.
laminar structure (Photos 4 and 5). And it was remarkable that the Schwann cells were not involved in these alterations.

3) Light Microscopy

The histopathological examinations were carried out in the cerebrum, cerebellum, brain stem, medulla, spinal cords, sciatic nerves, and gastrocnemius of the rats sacrificed at 100 and 200 days (terminal) of the experiment. As illustrated in Fig. 2, the lesions were seen in the brain stem, medulla, spinal cords, sciatic nerves, and gastrocnemius showing rather varying degree from case to case.

As shown in Photo 6 the gracile nucleus was markedly affected characterized by nerve cell degeneration and axonal swelling. Occasional loss of nerve cells was also prominent. In the spinal cords, degeneration, swelling and loss of axons were associated with minimal fragmentation and destruction of myelin sheaths in the gracile fasciculus where minimal glial proliferation was also seen. These lesions were more pronounced in the medulla and relatively moderate in the cervical cord (Photo 7), although minimal changes were recognized in the lower portion of this pathway (Photo 8). There were no remarkable changes in the other pathways of the cords except occasional swollen axons were involved within the anterior funiculus. In addition, nerve cells were essentially normal throughout the entire spinal cords, while equivocal swelling and loss of nerve cells were encountered in the anterior horn of the lumbar spinal cord.

As to the sciatic nerves, swollen axons were accompanied by slight fragmentation.
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of myelin sheaths (Photo 9). And the gastrocnemius muscle of the rats sacrificed at the termination of the experiment revealed several foci of eosinophilic, small and angulated fibers, showing figures of small group atrophy (Photo 10). Based on the results of immunohistochemical staining of myoglobin, affected fibers were confirmed to be Type I fibers (Photo 11) and the histogram of the muscular fibers in diameter given in Fig. 3 showed increased numbers of small fibers, indicating atrophy of Type I fibers.

Photo 1. Sciatic nerve fibers from rat, 60 days after exposure to TOCP.
The internal structure of the axons takes the form of increased electron density characterized by increased prominence of smooth endoplasmic reticulum. (×3000)
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Photo. 2. Sciatic nerve fibers of rat, 60 days after exposure to TOCP.
The axonal lesions are characterized by dilated smooth endoplasmic reticulum showing the form of channels and vacuoles. Aggregation and disruption of neurofilaments and neurotubules are associated. (×6000)

Photo. 3. Sciatic nerve fibers from rat, 60 days after exposure to TOCP.
Degenerative mitochondria and occasional giant mitochondria are seen within condensed axons. (×3000)
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Photo. 4. Sciatic nerve fibers from rats, 60 days after exposure to TOCP. Occasional vacuolic spaces are seen in the myelin sheaths. (×3000)

Photo. 5. Sciatic nerve fibers from rat, 60 days after exposure to TOCP. Myelinic sheaths are essentially normal, although minimal fragmentation of laminar structure is prominent. (×3000)
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Photo 6. Gracile nucleus from rat, 60 days after exposure to TOCP.
In the gracile nucleus, marked nerve cell degeneration and axonal swelling are accompanied by the loss of nerve cells. Minimal proliferation of glia cells is also seen. H. E. ×50

Photo 7. Cervical spinal cord from rat, after exposure to TOCP (Terminal sacrifice). The gracile funiculus shows degeneration of myelin and axons associated with glia cell proliferation. H. E. ×25
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Photo. 8. Lumber spinal cord from rat after exposure to TOCP (Terminal sacrifice). Degeneration of myelin and axons is occasionally seen in the gracile funiculus. H. E. ×25

Photo. 9. Sciatic nerve fibers from rat, 60 days after exposure to TOCP. The longitudinal section of the sciatic nerves shows moderate degree of axonal swelling. Bodian ×100
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Photo 10. Gastrocnemius muscle from rat after exposure to TOCP (Terminal sacrifice).
There are several foci of eosinophilic, small and angulated muscular fibers showing figures of small group atrophy. Masson trichrome × 50

Photo 11. Gastrocnemius muscle from rat after exposure to TOCP (Terminal sacrifice).
The immunohistochemical examination of myoglobin shows that the majority of degenerated fibers correspond to Type I fibers. Myoglobin (PAP) × 50
DISCUSSION

This report describes the sensitivity of the rats to dying-back neuropathy induced by TOCP administration, and the distribution and morphology of neuropathologic lesions both in the central and peripheral nervous system were examined.

As previously described by Smith and Lillie (1931), the clinical picture in man was typical of delayed neurotoxicity following ingestion of TOCP. Symptoms of this neuropathy did not commence until 8 days after the acute poisoning. Involvement of upper and lower limbs occurred later still and there was progressive deterioration for about 60 days. The predominant distal weakness was characteristic, as was the finding of only minor sensory loss. Also, it has been introduced by Smith and Spalding (1959) that those patients exposed to TOCP showed distal weakness alone and recovered well, but it commonly took at least one year. On the other hand, Mipaflox poisoning has been reported by Bidstrup and Bonnell (1953) and patients showed paralysis 3 weeks after intoxication. There was, however, no description concerning recovery in their report.

In the cat study made by Cavanagh (1946b), single subcutaneous injection of TOCP produced neurological symptoms characterized by loss of dragging their hind legs. These symptoms were recognized 2 weeks after administration, and the animals recovered sufficiently within 17 weeks, although some cases died within 4 weeks.

Clinical signs of neuropathy in rats have been reviewed by Majno and Karnovsky (1961) using subcutaneous injection of Mipaflox. The initial sign became manifested 4-5 weeks after exposure and typical neuropathy developed within 3-4 months. However, they also did not give any results of recovery in their rats.

In contrast, Fullerton and Barnes (1966) have stated that weekly oral administration of acrylamide in rats caused weakness of legs after 6 doses of 100 mg/kg and a few cases recovered 3 weeks later. Hexachlorophene study in rats has been carried out by Towfighi et al. (1973), and dietary administration of 300 ppm hexachlorophene produced neurological signs within 8 weeks. These two compounds were well introduced to cause neuropathy in rats, but the onset and recovery of the clinical symptoms were considered to be somewhat different from those induced by certain organophosphorus compounds.

On the other hand, quite a number of studies have been carried out in hen exposed to TOCP. Smith and Lillie (1931), Baron et al. (1962), and Glees (1961) stated that hens developed weakness of their legs 8-12 days after single oral dose of TOCP. After 3 weeks, ataxia became more evident and many cases died, probably due to difficulty in rising to a standing position resulting in disability to consume their diet and water. These results were supported by our previous studies (Itoh et al., 1981 and 1984). There were, however, no experiments showing recovery in hen after exposure to organophosphorus compounds.

In this study, the rats given TOCP showed ataxia 1-2 weeks before the final dose and distal weakness became more prominent after 50 days. This sign showed a tendency of reduction thereafter, and complete recovery was noted in some cases at the
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termination of the experiment. Although the time of onset seemed to be rather
different from that in man (Smith and Spaldig, 1959), neurological symptoms and their
recovery were similar to those in human cases.

Majno and Karnovsky (1961) stated that the growth curve of rats exposed to
Mipaflox remained indistinguishable from that of the control, although the body weight
gain of the rats showed significant reduction in this study. This difference might be
due to different amounts of compounds administered.

Studies on electron microscope gave characteristic results in the sciatic nerves from
rats sacrificed 60 days after final administration, and lesions were predominantly
confirmed within the large nerve fibers in diameter. The internal structure showed an
increased electron density characterized by increased numbers of smooth endoplasmic
reticulum, neurotubules, and neurofilaments. These lesions were associated with dis-
ruption of neurofilaments and dilated smooth endoplasmic reticulum as well as enlarged
and degenerated mitochondria. Vial (1959) and Schlole (1964) have stated that a
proliferation of vesicular elements together with disruption and dissolution of neurofila-
ments was disclosed in the cases of Wallerian degeneration. Also, these findings were
similar to those results given by Blakemore and Cavanagh (1969) in the spinal cords of
the rats exposed to p-Bromophenylacethylurea, and were said to be predominantly seen
in large and long nerve fibers.

In the myelinic sheaths of our rats, occasional vacuolic spaces were accompanied by
minimal fragmentation of laminar structure. Prineas (1969a) has described that myelin
destruction was most marked in the peripheral nerves of the cats exposed to TOCP, and
was invariably associated with severe rarefaction or other degenerative changes within
the axoplasm showing increased numbers of endoplasmic reticulum, neurotubules, and
neurofilaments. In addition, these lesions were seen within large-diameter fibers.
Our rats, however, revealed only minor changes in the myelinic sheaths. Prineas
(1969b) stated in the study on cats exposed to acrylamide, that axonal degeneration in
TOCP neuropathy was said to be quite different from that observed in acrylamide
neuropathy, showing a marked accumulation of neurofilaments as the earliest change
and degenerative changes in mitochondria within the gracile nucleus. However, there
were no descriptions concerning myelinic changes and he concluded that there were at
least two distinct pathophysiological mechanisms leading to degeneration of axons in
dying-back polymyopathies. On the other hand, Bischoff (1967 and 1970) has pointed
out that both axons and myelinic sheaths were affected in the nerve fibers of hens
exposed to TOCP. Also, Towfighi et al. (1973) confirmed marked myelinic degeneration
with occasional lesions in the axon of the rats exposed to hexachlorophene. In con-
trast, Bouldin and Cavanagh (1979) in the cats exposed to DFP (di-isopropyl fluoro-
phosphate) and Itoh et al. (1984) in the hens exposed to TOCP have reported that the
initial lesions were manifested in the axon rather than myelin.

Because these investigations were carried out under different conditions, it could
not be concluded which lesions of axons and myelin would be initiated. However, the
results given here suggested that incipient lesions would be appreciated in the axons
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rather than myelin under the conditions of neuropathy induced by TOCP.

In the histopathological examinations, changes were not remarkable in the cerebrum and cerebellum. In the brain stem, however, the gracile nucleus was found to be markedly affected showing degeneration and loss of nerve cells and axons. The spinal cords also revealed axonal and myelinic degenerations associated with minimal glial proliferation in the gracile fasciculus. These changes were markedly pronounced in the medulla and relatively moderate in the upper levels of the spinal cords as compared to those in the lower levels of the cords where changes were found to be diminished. Accordingly, it was concluded that lesions in the spinal cords were the "dying-back" degeneration, while lesions were not remarkable in the other pathways involving the other ascending and descending tracts.

According to Cavanagh and Patangia (1965), the lesions were also said to be manifested in the gracile nucleus and dorsal funiculi of the cats exposed to TOCP. In addition, they observed lesions in the spino-cerebellar tracts and anterior funiculus as described in the previous studies in hens (Barnes and Denz, 1965, Itoh et al., 1981 and 1984). Abou-Denia et al. (1974) also showed similar lesions in hens exposed to leptophos [phenylphosphonothioic acid O-(4-bromo-2, 5-dichlorophenyl) O-methylester] to those described by Cavanagh (1964a). In addition, the study made by Bouldin and Cavanagh (1979) in the cats exposed to DFP showed fiber degeneration being prominent within the fasciculus gracilis and spino-cerebellar tracts. Similar results were confirmed by Blakemore and Cavanagh (1969) in the rats exposed to p-Bromophenylacetylurea (BPAU). The histopathological investigations of human cases, however, have not yielded a definite picture being observed to be minor demyelination in the white matter of the spinal cords (Aring, 1942).

The neuroglial response has been already given by Baron et al. (1962), Blakemore and Cavanagh (1969), and Itoh et al. (1981 and 1984) showing changes of activation.

Many literature has reviewed muscular lesions in dying back neuropathy (Abou-Denia, 1976, Baron et al. 1962 and Smith and Lillie, 1931). Vora et al. (1962) have also described lesions in human cases due to an intoxication of TOCP. In our previous study (Itoh et al., 1984), Type I fibers of hens exposed to TOCP were selectively affected as compared to Type II fibers, showing figures of small group atrophy.

In the present investigation with gastrocnemius, Type I fibers were also selectively affected. And the distribution of muscular fibers in diameter showed increased numbers of small fibers indicating atrophy of Type I fibers. This result was well correlated to the clinical sign. Type I fibers are known to be tonic muscle and atrophy of these fibers may cause gait disturbance. Also, the change of Type I fibers might correspond to the lesions of anterior horn cells, while minimal and equivocal changes were recognized in these cells.

Aldridge and Barnes (1961), Aldridge et al. (1969), Aldridge and Johnson (1971), and Davies et al. (1960) have extensively investigated the relationship between the incidence of neuropathy and chemical structure on many organophosphorus compounds, giving the results of no–simple relation among them. Recently, Johnson (1975 and 1978) has
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reviewed the enzymatic activity of neurotoxic esterase in the nervous system of some experimental animals exposed to certain organophosphorus compounds. His results, however, did not give any clear cut reason why some selected compounds produce the neuropathy.

To date, the pathogenesis of the chemical-induced “dying back” polyneuropathy has not yet been confirmed.

As mentioned above, administration of TOCP produced delayed neuropathy in rats, and the morphological lesions were considered to be dying-back. Clinical signs as well as distribution and localization of the lesions were similar to those in human cases, although the rats showed less sensitivity than other species of mammals after exposure to TOCP.

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