Ultrastructure of the ciliary muscle treated by organophosphate pesticide in Beagle dogs

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Organophosphate compounds, which are potent irreversible inhibitors of carboxylic esterases, including cholinesterase, have been extensively used as pesticides throughout the world, and many unfortunate incidents of acute intoxication have been reported (Grob and Harvey, 1953; Goldman and Teitel, 1958; Healy, 1959; Dinman, 1964; Quinby, 1964; Crowley and Johns, 1966; DePalma, Kwalick, and Zukerberg, 1970; Namba, Greenfield, and Grob, 1970; Ishikawa, 1973a). Eye symptoms include spasm of the ciliary muscle and constriction of the pupil (Namba, Nolte, Jackrel, and Grob, 1971). Recent evidence of chronic intoxication (Namba and others, 1971; Ishikawa, 1971) due to environmental exposure to the compounds has also indicated a severe effect on refraction, i.e. permanent myopia (Ishikawa and Ohto, 1973).

In the present investigation, ultrastructural changes in the ciliary muscle in Beagle dogs treated by long-term oral administration of the organophosphate compound (Ethylthiometon) have been studied. Refractive change was also recorded. The data were compared with normal controls. Ultrastructural changes in the ciliary muscles as well as the development of myopia seemed to be related to the dosage of Ethylthiometon.

Material and methods

Ten pedigree Beagle dogs were used in this study. Five were treated with oral administration of Ethylthiometon* and five others served as controls. In order to produce a chronic condition, the dose of Ethylthiometon was about one tenth of the lethal dose (50 mg./kg.). This value was, however, obtained from mongrel dogs (Honma, 1970). The doses were 5 mg./dog/day for two dogs (No. 539 and 606), 10 mg./dog/day for two dogs (No. 600 and 604), and 15 mg./dog/day for one dog (No. 537). The capsules containing Ethylthiometon were given orally every day, 5 days a week, except holidays from November 1, 1970, to November 1, 1972. Empty capsules were given to the controls. All the

* Ethylthiometon: Chemical formula and toxicity are listed below.

\[
\text{C}_2\text{H}_5\text{O}_\overset{\text{P-S-CH}_2\text{CH}_2\text{SC}_2\text{H}_5}{\text{S}}\text{C}_2\text{H}_5\text{O}
\]

Lethal doses 50 mg./kg.: 14·1 to mice and 6 to mongrel dogs (Honma, 1970)

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dogs were about 6 months old and weighed 5 to 9.4 kg. at the start of the trial. All increased their weight by 7.1 to 10.6 kg. by November 1, 1972.

Refraction in dioptres was measured before the trial and then once a month using the co-incidence optometer (Chin, Ishikawa, Lappin, Davidowitz, and Breinin, 1968) (Hartinger-JENA) 30 minutes after the topical administration of 3 drops of 1 per cent. cyclopentolate hydrochloride over a 5-second period. The dog was placed in a comfortable box and the Vernier adjustment was done by the same examiner at a point on the retina approximately two disc diameters temporal to the disc. Horizontal and vertical meridians of both eyes were measured. In addition, corneal curvature, ocular axial length, depth of the anterior chamber, thickness of the lens, and ocular tension were determined. Other general studies included a haemogram and chemical analyses as well as a measurement of pseudo and true cholinesterase. These details have been reported by our co-investigators (Tokoro, Suzuki, Nakano, and Otsuka, 1973; Hikita, Miyata, and Ishikawa, 1973; Mukuno and Imai, 1973).

Eight dogs (see Table with asterisks) were killed within 10 days after November 1, 1972, by intravenous Nembutal anesthesia (20 mg./kg.) for electron microscopic study.

The anterior segment of each globe was removed immediately after enucleation. Small blocks of the longitudinal portion of the ciliary body were dissected in 2 per cent. osmium tetroxide in 0.2 M cacodylate buffer (pH 7.4) at 4°C. The blocks were fixed for 2 hrs, dehydrated in graded alcohols, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi HU-12A electron microscope.

Results

(1) CHANGE IN REFRACTION

All dogs treated by Ethylthiometon showed myopia 12 months after administration, and this myopia progressed until the cessation of the drug. Initial and final measurements of refraction are listed in the Table. In general, higher doses (10 to 15 mg./dog) tended to produce higher myopia. In the controls, however, the refraction moved towards myopia with increase in age as seen especially in dogs No. 756 and 754. There was 1.25 dioptres difference in mean values of refraction between the treated and non-treated groups. It was therefore interesting to investigate further morphological comparison between the treated and non-treated dogs, and an electronmicroscopic study was started.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dog no.</th>
<th>Body weight (kg)</th>
<th>Dose of Ethylthiometon (mg./dog day)</th>
<th>Total doses (mg.)</th>
<th>Refraction in dioptres after topical use of 1 percent. cyclopentolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial Final</td>
<td></td>
<td></td>
<td>Initial Right eye Left eye Final Right eye Left eye</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H V H V H V H V</td>
</tr>
<tr>
<td>Treated</td>
<td>539(*)</td>
<td>7.8 10-1</td>
<td>5</td>
<td>2300</td>
<td>-0.25 -0.50 +0.25 -0.25 -2.00 -2.00 -1.75 -1.75</td>
</tr>
<tr>
<td></td>
<td>606(*)</td>
<td>5.0 8-6</td>
<td>5</td>
<td>2300</td>
<td>+0.25 -0.25 +0.25 -0.25 +0.50 -0.50 -1.00 -1.00</td>
</tr>
<tr>
<td></td>
<td>604(*)</td>
<td>7.0 9-1</td>
<td>10</td>
<td>4800</td>
<td>+0.50 +0.25 +0.25 +0.75 +1.00 +1.00 +1.00 +1.00</td>
</tr>
<tr>
<td></td>
<td>537(*)</td>
<td>8.1 9-9</td>
<td>15</td>
<td>6900</td>
<td>-0.50 -0.75 -0.50 -0.75 -2.00 -2.00 -2.00 -2.00</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>6.78 9.04</td>
<td>9</td>
<td>4140</td>
<td>-0.10 -0.25 0.00 +0.01 -2.00 -2.50 -1.90 -2.35</td>
</tr>
<tr>
<td>Control</td>
<td>653</td>
<td>8.0 8.8</td>
<td>none</td>
<td>none</td>
<td>+0.75 +0.75 +1.00 +1.00 +0.50 -0.50 -0.25 -0.25</td>
</tr>
<tr>
<td></td>
<td>756(*)</td>
<td>6.6 7.1</td>
<td>none</td>
<td>none</td>
<td>-0.25 -0.25 0.00 -0.00 -0.75 -0.75 -1.00 -1.00</td>
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<tr>
<td></td>
<td>631(*)</td>
<td>8.7 8.4</td>
<td>none</td>
<td>none</td>
<td>+0.50 +0.50 +0.75 +0.75 +1.00 +1.00 +1.00 +1.00</td>
</tr>
<tr>
<td></td>
<td>754(*)</td>
<td>7.0 7.6</td>
<td>none</td>
<td>none</td>
<td>0.00 0.00 -0.75 -0.75 -1.00 -1.00 -1.00 -1.00</td>
</tr>
<tr>
<td></td>
<td>661</td>
<td>9.4 10-6</td>
<td>none</td>
<td>none</td>
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</tr>
<tr>
<td></td>
<td>Mean</td>
<td>7.94 8.50</td>
<td>0</td>
<td>0</td>
<td>+0.40 +0.40 +0.45 +0.45 -0.45 -0.45 -0.65 -0.65</td>
</tr>
</tbody>
</table>

(*): Electronmicroscopic study carried out
H = horizontal meridian  V = vertical meridian
(2) Fine Structure of Normal Ciliary Muscle (Dogs No. 756, 631, and 754)

Fig. 1 shows normal ciliary muscle obtained from an untreated dog (No. 756). The longitudinal portion of ten muscle cells is presented. The fine structure of the ciliary muscles in the dog is basically similar in appearance to that previously reported in man (Shiose, 1967; Ishikawa, 1962; Zypen, 1967), monkey (Uga, 1967; Hirano, 1965), and cat (Hirano, 1969). The cell organelles, e.g. mitochondria (M), Golgi complex, granular and agranular endoplasmic reticula (E), myofilaments, and so-called dense bodies (D), were almost identical in shape, size, and distribution to those observed by previous authors. The only relevant features of the present study are that the myofilaments fill a large part of the cytoplasm except where there are cell organelles and that the normal endoplasmic reticulum shows occasionally concentric lamellar structures (Fig. 1 (inset) and Fig. 7 (arrow)). These findings were the same as in other dogs (No. 631 and 754).

Fig. 1 Normal fine structure of ciliary muscle. Longitudinal portion of approximately ten muscle cells is presented. The cell organelles, e.g. mitochondria (M), endoplasmic reticulum (E), myofilaments, and so-called dense bodies (D), are almost identical in shape, size, and distribution to those observed by past investigators. The myofilaments fill a large part of the cytoplasm except where there are cell organelles. Bar 5μ.

Fig. 1 (inset) Smooth endoplasmic reticulum in normal muscle cell. Occasionally, smooth endoplasmic reticulum shows concentric lamellar structures. Bar 1μ.

(3) Fine Structural Changes of Treated Ciliary Muscle

(a) Low dosage group (Dogs No. 539 and 606)

Figs 2 to 5 illustrate marked changes in the ciliary muscle cells of the treated dog with about 1 D myopia (left eye of No. 606). The cytoplasm in some muscle cells was occupied
exclusively with unique membranous structures (UMS) resembling mesh-work or the layered coats of an onion. These findings were seen in about one-third of all the muscle cells examined while the other cells seemed to be normal. The affected cells were closely intermingled with normal cells. The intensity of distention or enlargement of these UMS differed from one cell to another. More extensive changes in the UMS are shown in Fig. 5. The cell was almost occupied by these distended membranous structures and myofilaments (MF) were pushed out to the periphery of the cells.

At higher magnifications (Figs 3, 4, and 5), the UMS were composed of numerous paired membranous structures. The outer spaces of each pair of membranes (arrows), where they were filled with an amorphous mass, were highly distended and became occasionally cystic. However, the interspaces between the membranes were closely apposed and contained a small amount of cytoplasm, which was continuous with the peripheral cytoplasm (Fig. 5, arrows). Some contained a lysosomal body (L) (Fig. 3) or mitochondria (Fig. 4) at their centres. Even in affected cells, some of the mitochondria showed a normal appearance. Another dog (No. 539) showed almost similar changes.
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**FIG. 3** Concentric lamellar appearance of UMS, composed of numerous paired membranes. The outer space of each doubled membrane (arrows) is distended and contains an amorphous mass. The interspace between the membranes is closely apposed. One of these structures has four lysosomal bodies (L) at the centre. Bar 1μ

**FIG. 4** Lamellar and mesh-work appearance of UMS. Outer space of paired membrane (arrows) is more distended and becomes cystic. The mitochondria are preserved and one of them is observed in the centre of the UMS. Bar 1μ.
FIG. 5 Mesh-work appearance of UMS. The cell is almost filled with distended membranous structures, and myofilaments (Mf) are pushed out to the periphery. The interspace between each paired membrane (arrows) is continuous with the peripheral cytoplasm. Bar 1 μ

(b) High dosage group (Dogs No. 600, 604, and 537)

Figs 6 and 7 demonstrate further destructive changes in the ciliary muscle of the high dosage group with maximum myopia (right eye of No. 604). The cytoplasm in approximately half the cells was occupied with a large amount of amorphous mass (A) and had scarcely any definite organelles. Only a few vacuolar systems (V), mitochondria (M), and UMS (Fig. 6 (arrow), and Fig. 7) were preserved. The myofilaments and dense bodies were almost extinguished from the muscle cells and, if preserved, were in disarray. A few preserved mitochondria (M) showed an almost normal appearance. Here again, normal cells existed among these highly affected cells. Other dogs (No. 600 and 537) showed approximately the same changes, but No. 604 and the left eye of No. 537 were the most severely affected. In spite of the severe destruction observed within the muscle cells, the cell membrane of the muscle cell and the axon and end-plate of the nervous system showed no remarkable change. These observations indicated that Ethylthiometon produced definite intracellular changes in the ciliary muscle, even with lesser development of myopia, with a dose of 5 mg./dog under the experimental conditions. These changes were not seen in the controls.
FIG. 6 Striking changes in ciliary muscle of treated dog (high dosage), showing about 4 D myopia (No. 604). The cytoplasm, in approximately half the cells is filled by amorphous masses (A) and show scarcely any organelles. Only a few vacuolar systems (V), mitochondria (M), and UMS (arrows) are preserved. The myofilaments and dense bodies are almost extinguished from the muscle cells. Bar 5μ

Discussion

The present study showed a marked destructive change in the muscle cells of the ciliary body in all treated dogs, but this was not seen in the controls. Beagle dogs have been used as a standard animal for the study of chronic toxicity. Especially in the dogs which developed over 4 D of myopia (No. 604 and 537) the affected cells could no longer be called smooth-muscle cells since they were almost devoid of the myofilaments essential to muscle. These changes could not be due to artefact because of the fixation procedures since there were still normal muscle cells among highly affected cells, and even in the affected cells, the residual mitochondria retained their normal appearance. Therefore, the changes must have been induced by Ethylthiometon.

One of the interesting observations in the present study is the existence of unique membranous structures (UMS) seen in the treated group. A close resemblance is observed between this structure, and normal endoplasmic reticulum (see Fig. 1 (inset) and Fig. 7 (arrow)); they are composed of paired membranes. This may indicate that the UMS in the affected cell are a modification of normal endoplasmic reticulum. However, its cytopathological or toxicological mechanism is a matter of dispute.

It has been reported that proliferation (Loker, Scallen, and Dietert, 1970; Kovacs, Blaschek, and Gardell, 1970) or aggregation (Esau and Gill, 1971) of the endoplasmic
Highly affected muscle cells among normal cells (N). Two affected cells (A) are almost amorphous in their cytoplasm with slight preservation of the vacuolar system (V) and the ums. Myofilaments and so-called dense bodies are almost extinguished and, if preserved, are in disarray. There are two normal-looking muscle cells (N) among the affected cells with a concentric lamellar structure of the endoplasmic reticulum (arrow). Bar 1 μm.

Reticulum can be induced by phenobarbiturate (Loker, 1970) or spironolacton (Kovacs and others, 1970) in the hepatocytes. In corneal oedema (Kanai and Kaufman, 1971a; Kanai, Waltman, Polack, and Kaufman, 1971b; Yukioka, 1968; Goldman and Kuwabara, 1968), the endoplasmic reticulum in the corneal epithelium was observed to be distended. The ums in the present study, however, differ from those reported in the past in their morphological features; the former are composed of numerous paired membranes and cystic distention occurs in the outer space of each paired membrane producing a concentric lamellar or meshwork appearance. In oedematous conditions, such as brain oedema (Koizumi and Shiraishi, 1970) and papilloedema (Schutta and Hedges, 1971), the fluid accumulation occurred not in the intracellular space but in the extracellular space. In the present study, an amorphous mass occupied a large part of the cytoplasm in the high dose group. These findings may suggest that accumulation or retention of the fluid occurred in the intra-cellular space of the muscle cell and the cystic enlargement of the outer space of the ums may be a prestige of the intracellular accumulation of the fluid.

Acute transient myopia, possibly due to oedema in the ciliary muscle is known to be caused by the various drugs, e.g. acetazolamide (Muirhead and Scheie, 1960), the sulphonamides (Diallo and Chiappore, 1970), and the parasympathomimetics (Grant, 1962),
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but its histopathological basis in the ciliary muscle especially in chronic conditions has not been reported. The myopia seen in the present study may be produced by pathological changes in the ciliary muscle. Changes in the corneal curvature was not the major factor producing myopia. This has been discussed elsewhere (Ishikawa, 1973).

Organophosphate compounds require microsomal oxidation to become actively toxic; consequently they increase microsomal enzyme activity and finally destroy the microsomes (Street, 1969). This may account for the destructive change in the microsomes within the ciliary muscle cells. Furthermore, the high peaks of the residues of both phosphorus and sulphur, probably Ethylthiometon and/or dissolved substances within the cells of the ciliary muscle and hepatocytes, were seen only in treated dogs. This was tested by an electron probe microanalyser (Andersen, 1967). These peaks were not seen in the controls (Ishikawa, 1973). This data may support the idea that the ciliary muscles have a special ability to react to and take up organophosphate compounds possibly because of their rich cholinergic innervation (Hikita and others, 1973). Ethylthiometon is in extensive use in Japan because of its selective toxicity to insects. These compounds, used as pesticides in farms and orchards, must be suspected of being trigger substances producing myopia in man.

Normal cells were observed among affected cells and they were intermingled. These findings would indicate that there may be exist two kinds of cell groups in the ciliary muscle (Uga, 1967) which differ substantially in their sensitivity to cholinesterase inhibitors. The possible co-existence of two such types is an interesting finding but needs further elucidation.

Summary

The fine structure of the ciliary muscle of Beagle dogs which developed myopia during oral administration of Ethylthiometon was studied. A dosage of 5 to 15 mg./dog/day was administered for 2 years. In the low dosage group (5 mg.), the cytoplasm of the muscle cells was occupied by unique membranous structures (UMS) resembling a mesh-work or the layered coats of an onion. This finding was found in about one-third of the muscle cells examined. In the high dosage group (10 to 15 mg.), the cytoplasm of the muscle cells became amorphous, and myofilaments and other organelles were almost extinguished. A close structural resemblance between the normal endoplasmic reticulum and UMS suggests that the latter might be a modification of the normal endoplasmic reticulum. Amorphous changes in the cytoplasm possibly indicate an intracellular accumulation of fluid. These changes were considered to be due to Ethylthiometon since they were not seen in the controls. The myopia seen in the high dosage group was considered to be mainly secondary to changes within the ciliary muscle.

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