The Effects of 2,5-Hexane Dione on Remyelination in the Peripheral Nervous System of the Mouse

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Abstract. We compared the development and resolution of a demyelinating lesion (produced by the intraneural injection of lysophosphatidyl choline) in mice exposed to a known neurotoxic agent (2,5-hexane dione) with similar events in control animals. A prolongation of the pro-myelin stage was observed in the hexane dione-treated animals: the suppression of remyelination became progressively more marked with the length of exposure to hexane dione. Prolongation of the pro-myelin stage is an indication of a disturbance in some component of the complex interaction between Schwann cell and axon, and presumably reflects the neurotoxicity of 2,5-hexane dione.

Key Words: Demyelination, 2,5-hexane dione, Mouse, CFLP, Peripheral nerve.

INTRODUCTION

Any successful program of repair that occurs in a peripheral nerve fiber following injury includes a phase of debris removal and a phase when axon-Schwann cell associations (both structural and functional) are re-established. When cellular activity within these phases is synchronized, as occurs after the induction of primary demyelination by the intraneural injection of lysophosphatidyl choline (LPC) (1), a more detailed analysis of factors involved in their regulation is possible. In previous studies we manipulated certain sequences experimentally by administering antimitotic and other drugs at known stages during post-LPC repair. From the results we have been able to draw some conclusions regarding the role and significance of Schwann cell mitosis in this process (2, 3). However, in these studies, active experimental intervention occurred after the instigation of repair. We are currently examining the effects of agents which alter the metabolic status of either neuron or satellite cell, given before and during the induction of demyelination and its subsequent repair. A metabolic disequilibrium insufficient to produce any morphological change in normal nerve (within the time scale of the experiment) might be revealed as alterations in the usual pattern of repair if that nerve is further stressed by the super-imposition of a demyelinating lesion.

In this paper we compare certain features of cellular repair following LPC-induced demyelination in the sciatic nerves of mice exposed to the known neurotoxic agent 2,5-hexane dione (HD) with the "standard" repair observed in unexposed animals. HD is the γ-diketone metabolite of the industrial solvent methyl n-butyl ketone, and its neurotoxicity has been well documented both clinically and experimentally in a range of laboratory animals. The primary mechanism of toxic action is unknown, but 2,5-hexane dione is capable of cross-linking proteins (4), and it has been suggested that a dose-dependent inhibition of glycolytic enzymes along the axons may be the local damaging consequence. Neuropathological studies have revealed focal accumulations of neurofilaments in large myelinated fibers, axonal swellings on the proximal sides of nodes of Ranvier, focal demyelination and remyelination, and axonal degeneration and axonal transport defects (5, 6). LPC produces a localized

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primary demyelination after intraneural injection. The major morphological, electrophysiological, and biochemical features of the demyelination and subsequent remyelination have been correlated in several studies (7, 8).

MATERIALS AND METHODS

One hundred forty-eight CFLP adult female mice were used in this study.

Hexane dione-LPC

All animals were fed ad lib with Standard Mouse Chow. Hexane dione (Aldrich Chemical Co.) was administered in the drinking water (0.5% or 2% v/v) at either concentration for the duration of the experiments. All animals were observed regularly and weighed prior to sacrifice. One hundred thirty-two animals received intraneural injections of lysophosphatidylcholine (LPC) (10 mg/ml in physiological saline) into the left sciatic nerve. Animals were injected in groups of 12 at 1, 3, 6, 12, and 15 weeks (wk) after starting the oral hexane dione regime at the 0.5% level, and one wk after starting at the 2% level. Three mice from each group were sacrificed at 1, 4, 11, and 28 days post-injection of LPC, and the injected and contralateral sciatic nerves were removed, processed, and examined light- and electron-microscopically. Details of the method of preparation of LPC, of the technique of intraneural injection, and of subsequent tissue processing have been published previously (1, 3).

Controls (mice on normal drinking water) received an intraneural injection of LPC 10 mg/ml and were killed in groups of three at similar time intervals. Both injected and contralateral sciatic nerves were removed and processed for microscopy.

Quantitation

Although all specimens were examined light- and electron-microscopically, the lesion selected for detailed quantitative analysis was that seen at 11 days post-injection. At earlier stages, i.e., one and four days, the cellular response is insufficiently synchronized to allow for easy morphological analysis. Photographs of each of the 11-day lesions were taken at random. Once the exposure setting had been selected and focus controls had been adjusted, the operator did not view the field through the binoculars, but merely moved the stage to ensure non-overlap of consecutive micrographs. Since the hexane dione was administered orally there was no reason to assume any non-uniformity of its access to the nerve fiber, and it was therefore not thought necessary to count all demyelinated fibers. Up to 450 fibers were counted in each sample. Micrographs were prepared at a final magnification of ×2,500.

Fiber counts were made of the following categories:

A. Pro-myelinated axons—single axons of any caliber (but usually >1.5 μm) enclosed within the cytoplasmic processes of typically one Schwann cell, and lacking both a myelin sheath and any intracellular debris from the earlier demyelination;

B. Axons surrounded by regions of still-compacted myelin, or myelin showing early LPC-type alterations (1);

C. Demyelinated axons surrounded by Schwann cells containing whorls of lamellar myelin debris, lipid droplets, etc;

R. Remyelinating axons (i.e., axons, generally greater than 1.5 μm in diameter, surrounded by more than one lamella of myelin);

D. Nuclei of axon-associated Schwann cells, for all categories of affected axons. Care was taken not to include nuclei of other intratubal cells which were not axon-associated, e.g., macrophages or lymphocytes.
All grids and micrographs subsequently prepared from the sections on them were coded, and counted before decoding. In 11 samples, counting was performed on sections from blocks separated by 2 mm from each other. In four randomly selected samples, the counting process was repeated at a later date on adjacent sections.

Transverse sections from all contralateral nerves (HD/LPC and LPC only) were examined ultrastructurally. Photographs were taken at random of one-month postoperative nerves, and coded micrographs were prepared at a final magnification of ×2,500.

Axonal areas were measured on photographic prints of low-magnification electron micrographs using a digitizing "bit-pad" (Summographics) linked to a Commodore 3032 computer, and converted to diameters. Random fields were photographed from each animal, and every myelinated axon measured. Nodes and paranodes were omitted. Approximately 250 fiber areas were measured in each nerve. Three animals were used in each group.

Estimation of Potential Anti-Mitotic Activity of HD

Animals were injected i.p. with ³H-methyl thymidine (s.a., 5 Ci/m.mol) at a dosage of 1 μCi/g body wt. Animals were killed 24 hours (h) later, and pieces of small intestine (c.a., 40 mg wet wt) and 1-cm lengths of the sciatic nerves were removed. The tissues were treated with 60% HClO₄ and H₂O₂ at 80°C for 24 h in tightly-closed vials for digestion of water-soluble products. After cooling, 10 ml of scintillation cocktail ("Biofluor", New England Nuclear) was added, and the radioactivity measured in a Beckman Scintillation counter and the counts corrected to d.p.m. Some groups of mice were treated with hydroxyurea (1 mg/g body wt in saline) intraperitoneal injection three h before administration of ³H-thymidine.

RESULTS

HD-treated mice did not exhibit any loss of weight or obvious clinical deficiencies during the course of the experiment. Some of the longer-term mice developed brownish discoloration of the interscapular fur, and all treated mice were fluorescent when viewed under an ultraviolet lamp. This fluorescence was of the fur and skin, and was slightly patchy.

The processes of demyelination and remyelination that occur in a peripheral nerve fiber following intraneural injection of LPC have been described both qualitatively and quantitatively in several previous papers (1, 7). In the present study, the initial characteristic pattern of myelin degradation within Schwann cell cytoplasm was similar in both experimental and control animals. Although no quantitative analysis of four-day animals was undertaken, the process of demyelination appeared to be slower in some of the hexane dione-exposed animals, in that a number of affected fibers in these mice were surrounded by myelin undergoing changes more typical of the earliest stages of the response. By 11 days post-injection, remyelination was well established in all of the controls: up to 87% of affected axons were surrounded by their myelin sheaths, and few axon-associated Schwann cells contained remnants of myelin debris (Table 1). In experimental animals, remyelination occurred in a similar percentage of affected fibers in mice exposed to HD (0.5% or 2%) for one wk before the induction of demyelination. Remyelination was observed in fewer affected fibers in animals exposed to hexane dione for longer periods before injection of LPC. Thus, after 12 wk of exposure, less than 46% of fibers had begun remyelination, and after 15 wk of exposure this category included only 8.5% of the total counted.

In nerves exposed to HD (0.5–2%) for one wk before LCP injection, the morphology of the 11-day lesion was indistinguishable from that observed in control animals. However, when nerves from animals exposed to HD for longer periods

TABLE 1
Percentage Distribution of Pro-myelin and Remyelinating Axons Obtained from Analysis of Transverse Sections of Sciatic Nerves of Hexane Dione-treated and Control Mice Eleven Days and Twenty-eight Days after Intraneural Injection of LPC

<table>
<thead>
<tr>
<th>Exposure is HD, weeks</th>
<th>Days after LPC</th>
<th>A*</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11</td>
<td>23.9 ± 10.2</td>
<td>0</td>
<td>0.3</td>
<td>14.6 ± 0.9</td>
<td>77.6 ± 10.5 (3)†</td>
</tr>
<tr>
<td>1‡</td>
<td>12§</td>
<td>14.8 ± 2.8</td>
<td>0</td>
<td>0.1</td>
<td>12.5 ± 0.6</td>
<td>84.6 ± 2.7 (3)</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>87.4</td>
<td>0</td>
<td>1.2</td>
<td>29.9</td>
<td>11.4</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>18.8</td>
<td>0</td>
<td>0.85</td>
<td>19.7</td>
<td>80.3 (3)</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>43</td>
<td>3.6</td>
<td>24</td>
<td>26</td>
<td>29</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>12.8</td>
<td>84</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>30.5</td>
<td>1.3</td>
<td>0</td>
<td>15.9</td>
<td>68.2 (2)</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>51.3 ± 8.6</td>
<td>0.6</td>
<td>8.3 ± 3.6</td>
<td>21.5 ± 1.9</td>
<td>37.9 ± 10.5 (3)</td>
</tr>
<tr>
<td>15</td>
<td>11</td>
<td>76.1 ± 10.4</td>
<td>0.9</td>
<td>14.8 ± 13.1</td>
<td>21.2 ± 4.6</td>
<td>8.1 ± 0.6 (3)</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>12.2 ± 1.9</td>
<td>0</td>
<td>0.9</td>
<td>12.3 ± 1.6</td>
<td>86.8 ± 2.8 (3)</td>
</tr>
<tr>
<td>0</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.1 ± 1.6</td>
<td>(3)</td>
</tr>
<tr>
<td>6</td>
<td>28</td>
<td>0.49 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>6.7 ± 1.9</td>
<td>(3)</td>
</tr>
<tr>
<td>12</td>
<td>28</td>
<td>1.6 ± 0.7</td>
<td>0</td>
<td>0</td>
<td>7.4 ± 1.6</td>
<td>(3)</td>
</tr>
<tr>
<td>15</td>
<td>28</td>
<td>2.7 ± 0.9</td>
<td>0</td>
<td>0</td>
<td>6.0 ± 0.7</td>
<td>(3)</td>
</tr>
</tbody>
</table>

Expressed as percentage of total profiles per sample (means ± SD).

* Categories: A, pro-myelin; B, axons surrounded by regions of still-compact myelin or myelin showing acute LPC change; C, demyelinated axons surrounded by debris-containing Schwann cells; D, axon-associated Schwann cell nuclei; R, remyelinating axons.

† ( ) = number of animals.
‡ Number indicates the duration of exposure to oral 2,5-hexane dione in weeks.
§ This group was analyzed 12 days after injection of LPC.

All animals were exposed to 2,5-hexane dione at the 0.5% level except the group indicated. These animals were exposed to 2,5-hexane dione at the 2% level.
were examined, several differences emerged, becoming more marked with increasing time of exposure. In general, bundles exhibited varying degrees of endoneurial edema: in several lesions, floccular proteinaceous material was present in the endoneurium. There was evidence of previous Wallerian-type degeneration in a small proportion of fibers, particularly among the unmyelinated bundles. These changes ranged from infrequently-found collapsed and empty basal lamina tubes to basal lamina tubes containing bundles of processes of Schwann cell cytoplasm with or without axon sprouts. Many demyelinated axons shared their basal lamina tubes with several other cells. The intratubal population included Schwann cells with or without debris, macrophages, and, less frequently, small lymphocytes. It was not uncommon to find that the axon was closely surrounded by the processes of a debris-free Schwann cell (i.e., in pro-myelin association), but that debris-laden cells remained within the basal lamina tube. In some instances, these latter cells penetrated the basal lamina and were partly in the endoneurium. Cellular debris was also observed lying free in the spaces between intratubal cells: this was usually seen where the intratubal cells included macrophages (Fig. 1). These complex groupings of axons and intratubal cells were a feature of lesions in which there was the most marked depression of remyelination.

There was some evidence of Schwann cell degeneration. Swollen vacuolated processes of effete Schwann cell cytoplasm associated with normal Schwann cells in configurations similar to those described by Raine in chronic relapsing EAE were occasionally seen (9).

Where remyelination had occurred, there was some evidence of dysmyelination (e.g., outpushings of the sheath, redundant whorls of compact myelin lying in the abnormal Schwann cytoplasm, and irregular loopings of the mesaxon). Since there was no evidence of either persistent dysmyelination or loss of myelinated axons one month after injection, it must be assumed that the aberrant remyelination was a transitory phenomenon, and that either successful remodeling of the affected sheaths occurred, or the incompetent Schwann cells were eliminated and replaced by others. An interesting and somewhat unexpected result of the study was the involvement of cells other than Schwann cells in the process of demyelination. There was evidence of "stripping" of myelin by insinuating processes of debris-containing macrophages in all of the 15-wk nerves when they were examined 11 days after injection of LPC. The more usual finding, however, was that of debris-laden intratubal macrophages associated with demyelinated axons. The origin of the debris in these cells is unclear, but there are three possible sources: direct uptake during macrophage-mediated demyelination, uptake from the lumen of the basal lamina tubes following exocytosis by Schwann cells, or uptake from the lumen of the basal lamina tubes following degeneration of Schwann cells.

\[ ^{3}H \text{-Thymidine Uptake} \]

\[ ^{3}H \text{-Thymidine uptake in the gut samples was high in control and HD-exposed animals at both six and 12 wk (Table 2). This was significantly depressed in the presence of hydroxyurea (HU). Although counts recovered from the nerves were predictably lower than those from the gut, the results demonstrated: 1. that HU administered intraperitoneally was effective in reducing }^{3}H \text{-thymidine uptake in the nerve, and 2. that HD did not exert any anti-mitotic effect.} \]
Fig. 1. Electron micrograph of a transverse section of two demyelinated axons (a) from a 15-wk HD-exposed mouse, 11 days after injection of LPC. The axons are associated with processes of Schwann cell cytoplasm (s) and debris-laden intratubal macrophages (m). Macrophage processes extend through breaks in the Schwann cell basal laminae into the endoneurium (arrows). ×7,000.
### TABLE 2

**3H-Thymidine Uptake in Mice on 2,5-Hexane Dione Regime and in Controls With or Without Hydroxyurea**

<table>
<thead>
<tr>
<th>Animal group</th>
<th>n*</th>
<th>LPC-treated sciatic nerve</th>
<th>Contralateral sciatic nerve</th>
<th>Gut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control: 6-wk</td>
<td>2</td>
<td>446 (404:484)</td>
<td>135.2 (93.5:176.9)</td>
<td>1820.6 ± 216.9 (4)</td>
</tr>
<tr>
<td>Control: 6-wk + hydroxyurea</td>
<td>3</td>
<td>290.4 ± 92.6 (3)†</td>
<td>76 ± 51.8 (3)</td>
<td>880.9 ± 215.3 (6)††</td>
</tr>
<tr>
<td>Hexane dione: 6-wk</td>
<td>3</td>
<td>377.2 ± 173.4 (3)</td>
<td>195.1 ± 59.8 (3)</td>
<td>1823.8 ± 669 (6)</td>
</tr>
<tr>
<td>Hexane dione: 6-wk + hydroxyurea</td>
<td>3</td>
<td>303.3 ± 68.6 (3)</td>
<td>212.1 ± 26.1 (3)</td>
<td>844.4 ± 68.9 (5)†††</td>
</tr>
<tr>
<td>Control: 12-wk</td>
<td>2</td>
<td>—</td>
<td>94.7 (154.5:34.9)</td>
<td>2193.3 ± 661 (4)</td>
</tr>
<tr>
<td>Hexane dione: 12-wk</td>
<td>3</td>
<td>—</td>
<td>168.6 ± 61.5 (3)</td>
<td>2120.8 ± 374.2 (6)</td>
</tr>
</tbody>
</table>

* n = number of mice.
† Means ± SD (no. of samples).
Analysis of variance against non-hydroxyurea control: † p > 0.05, †† p < 0.01, ††† 0.05 > p > 0.01.
Morphometric Analysis

The axonal diameters of myelinated fibers were measured (Fig. 2). No significant difference between the values for normal control animals and those for HD-treated animals (one- and 15-wk) was found. Similarly, no significant difference in the relationship of myelin sheath thickness to axon diameter was found in the three groups.

DISCUSSION

This experimental study was undertaken in an attempt to answer the question: Will covert alterations in the axon-Schwann cell relationship (produced by agent A) be revealed when that relationship is further stressed by the metabolic requirements of responding to an additional traumatic event (produced by agent B)? To test this proposal we have used a substance with known neurotoxic effects, namely 2,5-hexane dione (agent A) and we have compared the development and resolution of a

![Axon diameter (μm)](image)

**Fig. 2.** Distribution of axon diameters in myelinated fibers of control (——) and hexane dione-treated animals after one-wk (----) and 15-wk (-----) exposure. The populations represent the pooled results from three animals, with a total of 503 measurements for the controls, 625 measurements for one-wk exposure, and 745 for 15-wk exposure to hexane dione.
demyelinating lesion induced by the intraneural injection of LPC (agent B) in HD-conditioned mice with similar events in control animals. It was not our intention to analyze in any detail the mechanism of action of HD. Although the clinicopathological changes that occur in HD-exposed rats are quite marked (10), they have been reported as being less dramatic, although qualitatively similar, in mice (11). Using CFLP mice and dosage levels and exposure times similar to those of other workers, we have not seen changes typically associated with HD intoxication, other than finding an occasional degenerating fiber, and the yellow-brown discoloration of inter-scrapular fur exhibited by some of our longer-term mice. These differences presumably reflect variations in strain susceptibility, similar to those which have been described in experimental hexachlorophene intoxication (12, 13).

Analysis of the HD-LPC lesions revealed that, as the period of exposure to HD was increased, the efficiency of repair decreased, so that in nerves exposed to HD for 15 wk, only 8% of the affected fibers had begun remyelination, whereas in control nerves remyelination was established in 80% of fibers, when comparisons of 11-day post-LPC nerves were made. (The effect was limited to the site of the demyelinating lesion, and there was no evidence to suggest that more fibers than are usually affected by a standard intraneural injection of LPC were involved, and random demyelination in nerve outside the lesion was not seen.)

There are a number of possible reasons for this diminished response. It could reflect: 1. a toxin-induced interference with some aspect of axon-Schwann cell interaction; 2. the consequences of direct intoxication of the Schwann cell; and 3. the result of independently-mediated actions of the toxin on neuron and satellite cell. The increased number of pro-myelin fibers and of axons associated with debris-containing Schwann cells at 11 days post-injection is reminiscent of our previous findings in LPC-demyelinated nerves treated with the anti-mitotic bifunctional alkylating agent mitomycin C (2). In order to exclude the possibility that the delay in repair seen in the present study might be attributable to HD producing a generalized depression of nuclear DNA synthesis, rather than to any specific effects it might exert upon the cellular elements of the nervous system, we examined the uptake of $^3$H-thymidine in nerve and gut in control and experimental animals with and without hydroxyurea. Our findings confirmed that the experimental system is sufficiently sensitive to detect anti-mitotic activity and demonstrated that $^3$H-thymidine incorporation was unaffected by exposure to HD.

There was no evidence of any pathological alteration in Schwann cell cytoplasm such as have been described by Powell et al (11) in either control or HD-LPC long-term mice. Moreover, direct Schwann cell intoxication might be expected to result in some loss of Schwann cells or impairment of the repair program at an earlier stage than the achievement of the pro-myelin state. It must, however, remain a possibility.

The results are of interest, since they reveal an effect of the neurotoxic agent at concentrations that do not otherwise produce detectable morphological changes in the nerve (at least during a relatively short time interval). Visual inspection of the contralateral nerves and of fields proximal and distal to the zone of demyelination/ remyelination in injected nerves did not reveal any obvious structural changes in our samples during the 15-wk period, other than an occasional swollen paranode. Morphometric analysis confirmed this impression. Thus, over the time when the altered response to LPC was readily apparent in HD-conditioned mice, the change in non-LPC-affected nerve was minimal. This, of course, correlated with the lack of clinical findings.
This experimental system may be of predictive value in assessing an agent for its ability to so disturb some component of a peripheral nerve fiber that the normal relationship between axon and Schwann cell is temporarily lost; such an effect may be potentially neurotoxic. It may also be a suitable procedure for investigating further the action of known neurotoxic agents.

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