Neurofilament Distribution is Altered in the 
Mnd (Motor Neuron Degeneration) Mouse

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ANNE MESSER, PH.D., AND JOSEPH E. MAZURKIEWICZ, PH.D.

Abstract. Motor neuron degeneration (Mnd) is a genetic neurodegenerative disease of the mouse that is characterized by a progressive increase in motor dysfunction, moving from hind to fore limbs, leading to paralysis. An immunocytochemical analysis of the neurofilament distribution in spinal motor neurons in Mnd mice from all stages of the disease, including the presymptomatic, was performed using antibodies to different neurofilament subunits with different degrees of phosphorylation. Perikarya that stained with antibodies to phosphorylated neurofilaments were present in Mnd and control spinal cords, but the number of stained perikarya in Mnd was not significantly different from controls. There was a marked redistribution of neurofilaments within the cytoplasm of some motor neurons in Mnd cords. In Mnd but not controls, the immunoreaction product appeared margined, leaving areas in the cytoplasm absent of immunostaining. These areas were observed in all stages of the disease, but less predictably in presymptomatics. Both the size of the areas and the number of motoneurons containing these areas appeared to increase with the severity of the disease. The number of anterior horn neurons in the hind limb region of lamina IX in spinal segment L4 of Mnd was lower than in controls, suggesting there is a loss of neurons in Mnd.

Key Words: Amyotrophic Lateral Sclerosis; Immunocytochemistry; (Mnd); Motor neuron disease; Motor neuron degeneration mouse; Mouse, mutant; Neurofilaments.

INTRODUCTION

Although the etiology is still unknown for human motor neuron disease, a number of characteristics about this disease group are known: (a) the disease presents as a local weakness of muscles that follows a progressive degenerating course to include most muscles of the body; (b) the primary pathology is located in the anterior horn neuron, not within myelin sheaths of neurons or the muscles (1–3); (c) many of the human motor neuron diseases are late in onset (reviewed in 4, 5); and (d) a clear morphologic pattern of the early pathology of the human disease is not obtainable.

There are two extensively studied hereditary or spontaneously occurring animal models that exhibit diseases resembling human motor neuron diseases: the wobbler mutant in the mouse and the hereditary canine spinal muscular atrophy (HCSMA) mutant of the dog (4). Both diseases show selective degeneration of the motor neuronal perikarya. However, the wobbler mutant shows clinical symptoms at just three to five weeks of age, only the cervical region of the spinal cord is involved, and no upper motor neuron involvement at any stage of the disease has been demonstrated (5). The canine model exhibits three different phenotypes, each showing a different

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progression of the disease (6). Homozygous animals have a predictable onset of disease at sixteen weeks of age. The variability in the onset and progression of the disease in heterozygous offspring, however, necessitates waiting two to four years before determining if the dog is of the intermediate phenotype, or up to seven years if it is the chronic phenotype. Nonetheless, the importance of this model has been underscored by a number of studies that examined early pathologic changes in these animals (7, 8).

The Motor neuron degeneration (Mnd) mouse (9) affords another opportunity to examine pathology leading to movement impairment. This mutant exhibits a clinical picture resembling human motor neuron disease. The strength of the model lies in a relatively predictable time of onset and pattern of progression of the disease. The disease in Mnd/Mnd mice is late in onset, beginning by the age of 6.5 months with weakness of the hindlimbs; it progresses over a course of three months to almost complete hind- and forelimb involvement. The late stages of the disease are characterized by severely impaired movement that results from paralysis of the limbs. Both upper and lower motor neurons are affected in the severe stage. Histopathologic analysis has revealed that motor neurons but not muscle or myelin sheaths are affected (10).

The following studies were undertaken to further characterize the alterations in the spinal cord motor neurons of the Mnd/Mnd mice. Neurofilament distribution was analyzed to determine if this cytoskeletal element was abnormally distributed as has been suggested for a number of other neurodegenerative diseases. Further analysis of the predictability of the onset and progression of the Mnd/Mnd disease is also discussed.

MATERIALS AND METHODS

Animals

Mnd/Mnd mice and control mice came from the breeding colony maintained at the Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY. The Mnd mice are of the C57BL/6.KB2/MSR sub-line (9), with a further inbreeding of an Mnd/Mnd sub-line which shows consistent onset of disease at about six months. All mutants used in these studies were bred from homozygotes. Control mice were age matched C57BL/6. The Mnd disease has been classified into stages based on the signs displayed (see Symptomology below and 9). Presymptomatic animals and animals exhibiting three major stages of the disease were examined: mild, moderate, and severe. On a sliding scale of severity of symptoms within the moderate category, animals could be further classified as being closer to mild or to severe stages, thus establishing two additional stages: mild/moderate and moderate/severe.

Animals were used in three separate studies: (a) a behavioral study in which 22 newborn mice were analyzed for behavioral characteristics over their entire postnatal lifetime; (b) an immunocytochemical study in which 7 severe stage, 3 moderate/severe stage, 4 moderate stage, 2 mild/moderate stage, and 3 presymptomatic Mnd/Mnd spinal cords were examined. The number of age-matched controls for each stage was 3, 3, 3, 2, and 4, respectively; and (c) a quantitative study, in which 4 moderate and 3 age-matched controls for moderates were examined. Tissue in the latter two studies were used for immunocytochemistry with neurofilament antibodies.

Symptomology

In the initial studies of the development of the Mnd animal model, behavioral tests were established to provide a consistent method for evaluating the motor dysfunction (8). While effectively serving the purpose for which it was designed, the original procedure was tedious and involved. Continuous use of this procedure over an extended period of time suggested
that only five of the tests were necessary to accurately define the stage of the disease based on symptomology. A simpler method of analysis was thus pursued. Twenty-two mice were followed by a single observer (ELW) over their postnatal lifetime, and a simple table was devised which defined the major stages of the disease (Table 1).

**Tissue Preparation**

Mice were anesthetized with either Nembutal or Metofane, and perfused intracardially with either 4% formaldehyde in 150 mM phosphate buffer, pH 7.4, or Zamboni's fixative (11). Animals were perfused with 50 ml of solution over a ten minute (min) period. The animals were wrapped in plastic wrap and kept at 4°C overnight (12). The cords were then exposed and immersion-fixed in the same fixative as used for the perfusion. In the immunocytochemical study, segments of lumbosacral and cervical cord were removed and prepared for Vibratome sectioning. Fifty to one hundred μm thick sections were obtained and placed in vials of 50 mM Tris buffer, pH 7.2-7.4, at room temperature. In the quantitative study the cord was precisely dissected. The fourth lumbar segment was obtained as described below. Vibratome sections (25 μm in thickness) were placed into individual wells of 96 well cluster plates (Corning), covered with 50 mM Tris buffer and stored until used.

**Dissection of the Cord to Obtain the L4 Segment**

In the mouse, the lumbar region of the spinal cord is located between vertebral segments T12 and L2. The ventral root contributions to the murine sciatic nerve originate from spinal segments L3, L4, and L5 (13). As is typical for lumbar spinal cord, the spinal segment is significantly rostral to the site of exit of the spinal nerve from the vertebral column. Determination of the actual spinal cord segment is best performed by tracing the roots back to their dorsal attachments. This can be accomplished by a precise dissection of the spinal roots using a dissecting microscope at ×50 magnification. Of special note is that the L3 root gives a branch to the L2 root as the L2 root passes to innervate anterior thigh muscles (13). For this reason a precise dissection must be performed so that there is no confusion as to which root is actually L3. Once the L3 root is correctly identified, it can be traced back to its dorsal rootlets. There is a distinctive pattern of the dorsal rootlets for L3: a small gap exists between the most caudal L3 rootlet and the most rostral L4 rootlet. No gap is present between the end of the L4 rootlets and the beginning of the L5 rootlets. Thus, while it is possible to estimate the division between these two rootlet groups by pulling the roots, identification of sections from the presumed L4 or the presumed L5 segment needs to be confirmed after the segment has been sectioned.

For the present study, identification of the L4 segment was performed as follows: the sciatic nerve was dissected free of its location within the posterior thigh region, then followed through its course beneath the hip bone until the point where it lies next to the vertebral column. A dorsal laminectomy of the vertebral column was carefully performed to expose the spinal cord. The lateral portion of one side of the vertebral column was then removed in small pieces with iris scissors to expose the roots. Care was taken since the roots could be easily torn. The L3, L4, and L5 roots were isolated intact with the sciatic nerve. The pia surrounding the cord was carefully removed with fine tweezers. Each root contributing to the sciatic nerve was then traced back to the points of attachment of its rootlets, the rootlet pattern of L3 being used as a landmark. The L4 segment was defined as the segment of cord between the dorsal rootlets spaying from the L4 root. Since the caudal L4 dorsal rootlets blend with the rostral rootlets of L5, an exact end point of L4 is difficult to establish; however, a good approximation of its location can be determined by pulling on the root. Confirmation that a section used was actually from the L4 region was made using a projecting microscope to trace gray matter patterns and comparing them with gray matter outlines typical of L4 (13).

**Immunocytochemistry**

In the second study, the 50–100 μm sections were placed into individual wells of 96 well cluster plates and incubated with 50 mM Tris buffered saline (TBS) containing 1% bovine
<table>
<thead>
<tr>
<th>Age range (months)</th>
<th>Hindleg flexion</th>
<th>Gait</th>
<th>Grasps cage with hindpaws</th>
<th>Grasps cage with forepaws</th>
<th>Bipedal posture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>5.5–6.5</td>
<td>slight change</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Moderate</td>
<td>6.5–8.5</td>
<td>some flexion</td>
<td>some misses</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Severe</td>
<td>greater than 8.5</td>
<td>extreme flexion</td>
<td>ambulates with difficulty</td>
<td>difficult or unable; flexion in forepaws</td>
<td>unable to assume</td>
</tr>
</tbody>
</table>
serum albumin (BSA), 2% fetal calf serum (FCS) or horse serum (HS), and 0.1% Triton-X 100 for 45 min. The tissue was then incubated with one of the following mouse monoclonal antibodies (MAb) for 24–96 hours (h) at 4°C: SM131 (1/1,000), SM132 (1/1,000), RMS21 (neat), RMO108.1 (1/100), RMO93.1 (1/100), RMD09.5 (neat). All dilutions were made with 50 mM TBS.

For the quantification study, in order to eliminate the possibility of counting the same motor neuron twice in successive sections, every third or fourth section of the serially sectioned L4 segment was used for immunostaining. This procedure was followed since in the mouse, motor neurons range in size from 12–37 μm (14). It is unlikely that the cell cytoplasm would span more than three tissue sections (important for the highly phosphorylated neurofilament counting procedure) or that a nucleus would span more than two sections (important for the counting of total number of nuclei). Sections were incubated for 30 min in three changes each of 50 mM TBS/1% BSA/2% HS/0.1% TX-100 pH 7.4 at room temperature, followed by incubation with MAb RMO24 (1/750) at 4°C for 14–16 h. As a spot check for counting the total number of neurons in a section, adjacent or nearly adjacent sections were incubated with RMD09.5 (1/100) and processed as before.

In both studies, following the incubation in the primary antibodies, the sections were processed using a modified ABC (Vectastain) (Vector Labs, Inc., Burlingame, CA) immunoperoxidase procedure. Sections were washed in TBS, then incubated with biotinylated horse anti-mouse IgG (1/200) at room temperature for one h. Sections were washed again in TBS, then incubated for two h with ABC following the manufacturer's suggestions. Color was developed by incubation in a solution containing 0.05% diaminobenzidine (DAB) in Tris-buffer, pH 7.6, with 0.01% H2O2. Controls for the antibody reaction consisted of sections to which no primary antibody was added, or sections incubated with an antibody that results in a distinctly different known immunostaining pattern. Monoclonal antibody RMD09.5 was used in place of RMO24. RMD09.5 was raised against the dephosphorylated form of the antigen that was used to generate RMO24 (15).

Monoclonal antibodies RMS21, RMO108.1, RMO24, RMO93.1, and RMD09.5 were kindly provided by Dr. Virginia Lee, Department of Pathology, University of Pennsylvania School of Medicine. The details of the generation and specificity of these MAb can be found in Lee et al (15). Monoclonal antibodies SM131 and SM132 were purchased from Sternberger-Meyer Immunochemicals Inc., Jarrettsville, MD.

Analysis of Neuronal Cell Bodies Immunopositive for Phosphorylated Neurofibrillary (NF) Epitopes

Following immunocytochemical reaction, the sections were mounted on glass microscope slides in glycerol/PBS (1:1; v:v). The slides were then labeled with tape, shuffled, and randomly assigned a letter. Two categories of cells were counted: (a) stained perikarya (slightly to intensely), and (b) unstained perikarya. Unstained perikarya could be detected by closing the condenser aperture until the cells were distinctly seen. Only neurons containing nuclei were counted.

In the L4 segment of mouse spinal cord, lamina IX is split by lamina VII into a medial region containing axial muscle motor nuclei and a lateral region containing limb muscle motor nuclei. The lateral region is characterized by the presence of larger, easily identified multipolar motor neurons, and since this region contains the neurons most likely to be involved in the pathology of motor units that is observed, morphometric analysis was performed on this region only. While it is possible there could have been a small contribution to number counts from cells in lamina VII, this was considered to be of minimal effect, since such a contribution was likely to occur for both the Med and control sections. A reticle was used in the counting to provide consistent boundaries for the lamina IX region. Accuracy of counting of the total number of neurons per section was cross-checked by a random selection of adjacent or nearly adjacent sections to those used for the RMO24 study, and incubating them with RMD09.5 instead. Neurons were then counted in another blind analysis. Since RMD09.5 should stain all neuronal perikarya, a cross-check of the unstained neurons in the RMO24 sections vs
stained neurons in the RMD09.5 sections was possible. Concordance in counting was observed.

Cellular shrinkage has been observed in degenerative diseases, and as a control for nuclear shrinkage in the Mnd/Mnd tissue, nuclear diameters were measured in the lateral region of lamina IX of the sections stained with MAb RMD09.5. Eight to ten randomly selected nuclei were measured for each horn of four sections each of the four Mnd and three controls. The diameter of prominent nuclei showing distinct boundaries and containing a nucleolus was measured. Measurements were made at ×1,000 magnification using an ocular micrometer. A blind analysis was performed.

Data were analyzed as follows: (a) subsequent to the completion of a blind study, data and slides were regrouped by animal number into diseased and control categories; (b) the number of immunopositive and the number of unstained perikarya in the four sections of each cord (four moderate Mnd/Mnd and three age-matched controls) was obtained; (c) the total number of neurons was obtained by summing the number of stained and unstained perikarya for each cord; and (d) the means for the number of immunopositive cells, total number of cells, and nuclear diameters were obtained by summing the total per animal, and then calculating the mean of those sums.

A normal distribution was assumed since the sections were known to be only from L4. Two-tailed t-tests were performed to test if there was a difference between the control and Mnd/Mnd lateral IX motor nucleus region (area directly responsible for hindlimb innervation) with respect to: (a) the number of cells staining for phosphorylated neurofilament, (b) the total number of cells, and (c) nuclear diameter. A 95% confidence level was used to accept or reject the null hypothesis.

RESULTS

Behavioral Testing

Five signs were determined to be readily assessable characteristics of the Mnd disease:

1. Hindleg Signs: Normal mice, when lifted by the tail, reflexively extend their hindlegs to form an approximate 120 degree angle with the body. Loss of this reflex was the first sign of the Mnd disease. Mice which had lost this reflex (first detectable at 5.5–6.5 months of age) were categorized as being in the mild stage of the disease. Loss of this reflex progressed with age, until the hindlegs were flexed when the mouse was lifted by the tail. This was classified as the moderate stage of the disease, and occurred from 6.5–8.5 months of age. During the moderate stage, the hindlimb flexion became more pronounced and intractable, until at approximately 8.5 months of age the hindlegs were held practically locked in flexion. Mice exhibiting this extreme hindlimb flexion were categorized as severe. In addition, in the severe stage the hindpaws also showed pronounced flexion.

2. Gait: Mice in the mild stage of the disease exhibited little or no gait changes. The moderate stage was characterized by noticeable gait abnormalities, which became more pronounced as the mouse aged. Mice in the severe stage had extreme difficulty in ambulating.

3. Cage Rung Grasping with Hindpaws: When held by the tail and placed on a metal cage top, control mice and mice in the mild stage of the disease grasped the cage rungs with all four paws. At the beginning of the moderate stage, mice failed to grasp the cage rungs with hindpaws about half the time or less, and toward the end of this stage, mice failed 80 to 100% of the time. Severely affected mice were unable to grasp the rungs; moreover, the hindpaws appeared to be locked in flexion.

4. Cage Rung Grasping with Forepaws: The forepaw changes were similar to those described for hindpaws; however, the forepaw deficits appeared approximately two
to four weeks later (approximately 7.0–7.5 months of age) than the hindpaw abnormalities (approximately 6.5 months of age).

5. Bipedal Activity: Control mice assumed a bipedal posture when drinking from the waterspout in a cage, eating food from the cage reservoir, or when exploring. Very late in the moderate stage of the Mnd disease, mice had difficulty assuming this posture. In the severe stage, they were unable to assume bipedal posture, thus necessitating modifications in the waterspout and food reservoir.

The signs progressed on a continuum, which could be divided into three major stages. Each stage corresponded to a relatively narrow age range. In general, mice 5.5–6.5 months of age showed mild signs, defined as a marked loss of the hindleg extension reflex. Mice 6.5–8.5 months showed moderate signs: they exhibited hindleg flexion when lifted by the tail and with increasing frequency failed to grasp the rungs of the cage as the stage progressed. Mice 8.5 months and older exhibited severe signs characterized by maximally tight hindleg flexion and complete failure to grasp the cage rungs. They also showed similar though less marked involvement of the forepaws, leading to failure to grasp the cage rungs.

The moderate stage progressed over a two month period, and three phases of the stage were easily distinguished. Mice showing the moderate stage characteristic of hindleg flexion, but not the marked ambulation difficulties, were classified as mild/moderate. Mice showing hindleg flexion and frequent inability to grasp the cage rungs were classified as moderate. Mice showing hindleg flexion, inability to grasp the cage rungs, pronounced ambulation problems as usually seen in the severe stage, but when given enough time (15–30 seconds) were still able to attain bipedal posture for exploration when the cage top was removed, were classified as moderate/severe.

A retrospective study of the animal records for the neurofilament studies reported here indicated that in 85% of the cases, the symptoms of the mice employed were as predicted, based on the age of the mouse. In the 15% which did not show all of the expected symptoms at the age chosen, the progression of the disease was slower than expected.

General Observations for Antibody Localization

The immunostaining pattern for all of the antibodies used on Vibratome sections of mouse tissue in this study was similar to the staining patterns reported for these antibodies when used on paraffin sections of tissue from other mammalian species. Monoclonal antibodies SMI32 and RM09.5, antibodies which react with non-phosphorylated neurofilament epitopes, stained all neuronal cell bodies and neuropil. SMI31, RMO108.1, RMO24, and RMO93.1, antibodies that react with neurofilament epitopes that are phosphorylated to some extent (slightly to highly), stained all distal axons, although to varying densities, presumably related to the degree of phosphorylation of the neurofilament epitopes with which they react. They stained some neuronal cell bodies in both normal and Mnd/Mnd tissue. RMS21, an antibody raised to an epitope in the core of the NF-L subunit, stained most cell bodies densely, but axons were variably stained. This antibody stains neuronal elements in other species weakly or not at all (15).

Distribution of Phosphorylated Neurofilament

Initially, SMI31 was used to determine if neuronal cell bodies in spinal cords of Mnd/Mnd mice were immunopositive for phosphorylated neurofilaments. It has been reported that an abnormal number of perikarya containing highly phosphorylated neurofilament epitopes were present in a variety of neurodegenerative diseases,

Fig. 1. Light micrographs of spinal motor neurons immunostained with antibodies to different neurofilament epitopes. (A) Non-diseased, control animal stained with MAb RMDO9.5 (dephosphorylated NF-H); (B) pre-symptomatic Mnd mouse stained with MAb RMDO9.5; (C) Mnd mouse with severe signs stained with MAb SMI31 (highly phosphorylated NF-H and NF-M); (D) Mnd mouse with moderate–severe signs stained with MAb RMO108 (slightly phosphorylated NF-M). (A) Dense, uniform staining for neurofilaments observed in all non-diseased mice. (B) The open arrows indicate incipient spaces in the cytoplasm of otherwise densely stained nerve cell bodies. This illustrates that the neurofilament distribution in some Mnd motor neurons is abnormal even before overt symptoms appear. (C) The solid arrows indicate nerve cell bodies that were unstained with an antibody to highly phosphorylated neurofilament epitopes. The cells stand out in negative relief due to the positive staining of surrounding axons and synaptic endings in the neuropil. Two densely stained neurons are shown, one having extreme margination of the neurofilaments (open arrow). The larger unstained circular structure is the nucleus. (D) Two neurons densely stained with antibody to lightly phosphorylated neurofilament epitopes, also displaying a marginated distribution of
TABLE 2

<table>
<thead>
<tr>
<th>Immunopositive cells</th>
<th>Total number of cells</th>
<th>Range</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>20.33 ± 10.41</td>
<td>12–32</td>
<td>239.7 ± 16.8</td>
</tr>
<tr>
<td>Mnd</td>
<td>5.25 ± 4.57</td>
<td>0–11</td>
<td>199.5 ± 20.7*</td>
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* Two-tailed t-test @ p = 0.05.
MAb RMO24 was used.

and this was possibly related to the disease process (16–18). Perikarya immunopositive for highly phosphorylated neurofilament epitopes were present in the Mnd/Mnd spinal cords of animals at all stages of the disease. Perikaryal staining varied from slight to intense. Figure 1C illustrates staining with SMI31 in neurons from a severe stage animal. Stained perikarya were also observed in the control spinal cords, and with densities similar to those present in Mnd/Mnd cords. Qualitative and semi-quantitative analysis suggested that there was a greater number of stained perikarya in the mutant cord. However, there was great variability in the number of stained perikarya in the different sections of control and mutant tissue used in this initial study, possibly due to the selection method in which the sections were taken from within the entire lumbar sacral region of the cord. The question was re-examined using a defined segment of the spinal cord, and monoclonal antibody RMO24, an antibody to phosphorylated neurofilament, which had been demonstrated to have no cross-reactivity with other cytoskeletal elements (15). The change in antibody was deemed necessary because of reports that there could be cross-reactivity of SMI31 with MAP2 or tau (19, 20).

The study utilizing RMO24 was performed with tissue sections taken from spinal segment L4, chosen because it was the segment deemed most likely to contain motor neurons involved in the degeneration. This assumption was based on an assessment of the particular hindlimb muscles most likely to be involved with the symptomology observed at the moderate stage. As with SMI31, there were perikarya immunopositive with RMO24 in both Mnd and normal spinal cords. Quantitative analysis revealed there was no significant difference (at the p = 0.05 level) between the number of perikarya immunopositive for phosphorylated neurofilaments in spinal lamina IX limb region in Mnd and normal spinal cord (Table 1). However, there was a significant difference between the total number of cells in lamina IX limb region of Mnd and control spinal cord segment L4 (Table 2). A lower number of cells was observed in Mnd cord suggesting a loss of anterior horn neurons in Mnd mice. Nuclear shrinkage was not observed. For Mnd/Mnd the mean nuclear diameter was 14.06 ± 1.07 μm (n = 65), while for the age-matched controls the mean nuclear diameter was 13.67 ± 2.37 μm (n = 55).

Intracellular Distribution of Neurofilaments in Spinal Motor Neurons

Motor neurons from control mice, when immunostained with MAb to non-phosphorylated neurofilaments, contained homogeneous immunoreaction product that

←

neurofilaments (open arrows). Note the immunopositive proximal and distal axons that criss-cross all of the sections. Bar = 20 μm.
filled the entire cell body and extended into cellular processes. In some instances the reaction product appeared filamentous. Figure 1A illustrates staining of control perikarya with MAb RMD09.5. However, when these MAb were applied to tissue from Mnd/Mnd mice, there was a change in the distribution of neurofilament epitopes within the perikarya of some motor neurons. The reaction product appeared marginated leaving a large area in the cytoplasm absent of immunostaining. This redistribution of reaction product was observed in all cases of Mnd/Mnd stained with all of the antibodies used in this study. Figure 1B–D illustrates selected examples of this phenomenon with different antibodies and different stages of the disease. Control mice did not show this change. In the earlier stages of the disease few of the large motor neurons contained the region, and they were small (Fig. 1B). In the severe stage animals, most of the large motor neurons contained these regions, and the regions were quite prominent, qualitatively appearing to occupy up to a third of the visible cytoplasm (Fig. 1C). The morphologies of the unstained areas ranged from ill-defined regions of decreased immunoperoxidase activity to large, well-defined circular or ovoid regions entirely devoid of reaction product (Fig. 1B–D).

All three mutant spinal cord regions examined (sacral, lumbar, and cervical) contained motor neurons with margined neurofilament epitopes. Although the number of motor neurons containing margined neurofilaments was not counted, it appeared there were more in the lumbosacral region of moderates than of the cervical region of animals at this stage. It also appeared there were more motor neurons with larger, well-defined unstained regions in severe stage lumbosacral cord than in moderate stage lumbosacral cord.

DISCUSSION

These studies were designed to further characterize alterations in spinal motor neurons of the Mnd/Mnd mouse, with particular emphasis on the distribution of neurofilaments. In the process of the studies, the behavioral tests that were used for evaluating the motor dysfunction in Mnd were refined such that the classification system could be used to more easily and precisely define the stages of the disease based on the relationship of the symptomology to the age of the animal.

It is possible to reasonably predict the symptoms an Mnd/Mnd mouse will have based on its age. The degeneration that occurs during the six to nine month period is predictably progressive, enabling studies that examine a certain stage. Since all progeny are homozygous for the Mnd gene, this predictability also allows analysis of presymptomatic animals with the assurance that any animal chosen would have shown symptoms beginning by 6.5 months of age. Consequently, results obtained from studying the presymptomatic animal may reveal pathologic alterations occurring before clinical appearance of the disease.

While designed to quantify the number of neuronal perikarya in Mnd that were immunopositive for highly phosphorylated neurofilament epitopes, this study also provided a preliminary assessment of the total number of neurons in the lateral lamina IX region. The blind analysis revealed that the number of anterior horn neurons in lateral lamina IX (limb region) of spinal segment L4 was lower in moderate stage Mnd/Mnd than in age and strain matched controls, suggesting there is a loss of neurons in the mutants. A finding of decreased number of anterior horn neurons is consistent with reports in human motor neuron disease as well as the wobbler mouse animal model. In an initial report on the hereditary canine spinal muscular atrophy model, a reduction in the number of motor neurons in the lumbar ventral horn in
an autopsied pup was noted, and when coupled with other pathologic findings such as evidence of denervation of muscle fibers leading to fiber type grouping, suggested that along with a progressive dysfunction of motor neurons, there might also be a disappearance of neurons (7, 21). However, Cork et al (7) later suggested that rather than a loss, in HCSMA, spinal neurons, including motor neurons, may instead fail to reach normal size and/or undergo atrophy. For Mnd further analysis is necessary to confirm the initial assessment of motor neuron loss in spinal cord.

There was a marked redistribution of neurofilaments within the cytoplasm of anterior horn neurons in Mnd/Mnd spinal cords. In mutants but not controls, the immunoreaction product appeared marginated, leaving areas in the cytoplasm absent of immunostaining. Based on this altered neurofilament distribution, it is possible to distinguish moderate and severe Mnd/Mnd mice from their age-matched controls. Mild and some presymptomatic animals also contain neurons with cytoplasmic regions devoid of neurofilament immunoreactivity. In these early stages of the disease, there appears to be variability in the density of staining in these areas, some regions being more lightly stained than in controls. This suggests there is a gradual rearrangement of the entire neurofilament cytoskeleton since the panel of antibodies that was used was directed against epitopes on all three neurofilament subunits (NF-H, NF-M, and NF-L), these epitopes were in different states of phosphorylation, and none stained the regions in the cytoplasm.

The unstained areas were observed within sacral, lumbar, and cervical regions indicating that this is a consistent response in the Mnd/Mnd cord. By qualitative observation at the moderate stage, there were more motor neurons within the sacral and lumbar regions that contained these areas than in cervical regions, suggesting an increase in the number of neurons containing these areas might correlate with the progression of the behavioral degeneration from hindlimbs to forelimbs. The size of the areas was conspicuously larger in the severe stage motor neurons relative to the mild stage, again suggesting an increase in size of the areas may correlate to the severity of the disease.

The contents of these unstained regions is speculative at present. It would seem likely that the clear areas are devoid of neurofilament because of the lack of immunoreactivity with such a varied panel of antibodies. It could be argued that an antibody might not recognize its epitope if the neurofilament protein within these areas is altered; however, every epitope would have to be so altered. Previous electron microscopic analysis (22) revealed the presence of accumulations which were comprised of aggregations of 25 nm diameter tubular profiles, suggestive of microtubules, perhaps associated with lysosomes. Ubiquitin deposits have been immunolocalized to the same area in the cytoplasm of many motor neurons in Mnd/Mnd spinal cord (23), and may represent ubiquitinated proteins in the aggregates. In human motor neuron disease tissue, abnormal inclusions that were positive with antibodies to ubiquitin but negative for neurofilament immunoreactivity have also been observed (24). It appears that some proteins other than cytoskeletal proteins can be ubiquitinated and form inclusions.

The abnormal distribution of neurofilament epitopes is a consistent finding in human motor neuron disease tissue as well as in other animal models of that disease (1, 4). These abnormalities are expressed either as large accumulations of neurofilaments in axons, with or without swellings, or as an increase in the number of neuronal perikarya that stain positively with antibodies against phosphorylated neurofilament epitopes (17, 25). The Mnd/Mnd mouse does not appear to express these swellings filled with neurofilaments, rather the neurofilament distribution seems to be one of

relocation only. In the wobbler mouse mutant axonal swellings and perikaryal neurofilament accumulations are also infrequent (26). While there was immunoreactivity in motor neurons of Mnd with several antibodies that react with phosphorylated neurofilament epitopes, the number of such cells was not significantly greater than in controls. Early studies of motor neuron disease tissue implied that the immunolocalization of phosphorylated neurofilaments was a somatofugal phenomenon (27, 28). However, recent studies of this distribution in the most common motor neuron disease, amyotrophic lateral sclerosis (ALS) (17, 25, 29), have demonstrated a significant number of neuronal perikarya in normal human nervous tissue that also stain positively for phosphorylated neurofilaments. Leigh et al (29) found no specific pattern to the labelling of neuronal cell bodies with anti-phosphorylated neurofilament MAb in normal versus motor neuron disease anterior horn cells. In fact, they reported that the labelling was more frequent in normal spinal gray matter than in human motor neuron disease cords. In addition, in a large study in which brains from normal individuals with no known neurologic disorder and brains from patients diagnosed as having either Alzheimer's disease or schizophrenia were immunostained for phosphorylated neurofilaments, a large number of immunonegative perikarya were found in normal as well as diseased tissue (30). Those authors strongly suggested that phosphorylation of neurofilaments may occur in normal perikarya, and the staining may not be related to neurofilament accumulation. Further, Lee et al (15) suggest there is an orderly progression of neurofilament phosphorylation, starting in the perikarya, following synthesis and assembly, with the eventual transport along axons. In that study, antibodies to neurofilament subunits with different degrees of phosphorylation were shown to stain perikarya of normal neurons; the lesser the degree of phosphorylation, the greater the immunoreactivity: i.e. antibodies to slightly phosphorylated neurofilaments were more likely to stain perikarya than those directed against highly phosphorylated subunits.

The common observation in all of these studies is that in motor neuron diseases, the distribution of elements of the neurofilament cytoskeleton is altered, and the consequences of that alteration could wreak havoc on normal cell function. A major requirement for normal function of motor neurons is axonal transport, and an alteration in cytoskeleton would presumably result in an alteration of such transport. This hypothesis has been proposed for a number of neurodegenerative diseases, both human and non-human (1, 31). Accumulation of neurofilaments in the perikarya or in swellings of proximal axons, no matter the mechanism (aberrant phosphorylation, aberrant ubiquitination or cross-linking to other cellular elements via ubiquitin interaction, or another as yet unknown cause), could lead to an impairment in axonal transport leading to a general neuronal degeneration.

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