Immunization With Neurofilament Light Protein Induces Spastic Paresis and Axonal Degeneration in Biozzi ABH Mice

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Abstract

Axonal damage is the major cause of irreversible neurologic disability in patients with multiple sclerosis. Although axonal damage correlates with antibodies against neurofilament light (NFL) protein, a major component of the axonal cytoskeleton, the possible pathogenic role of autoimmunity to axonal antigens such as NFL has so far been ignored. Here we show that Biozzi ABH mice immunized with NFL protein develop neurologic disease characterized by spastic paresis and paralysis concomitant with axonal degeneration and inflammation primarily in the dorsal column of the spinal cord. The inflammatory central nervous system lesions were dominated by F4/80+ macrophages/microglia and relatively low numbers of CD4+ and CD8+ T-cells. In splenocyte cultures, proliferation to NFL was observed in CD4+ T-cells accompanied by the production of the proinflammatory cytokine interferon-γ. Elevated levels of circulating antibodies recognizing recombinant mouse NFL were present in the serum, and immunoglobulin deposits were observed within axons in spinal cord lesions of mice exhibiting clinical disease. These data provide evidence that autoimmunity to NFL protein induces axonal degeneration and clinical neurologic disease in mice, indicating that autoimmunity to axonal antigens, as described in multiple sclerosis, may be pathogenic rather than acting merely as a surrogate marker for axonal degeneration.

Key Words: Animal model, Axonal damage, Autoimmunity, Multiple sclerosis, Neurofilament light, Spastic paresis

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the brain and spinal cord characterized by demyelination and axonal pathologic changes (1, 2). Although it is now generally accepted that myelin damage results from a synergistic autoimmune attack of myelin-reactive T-cells and antibodies to myelin oligodendrocyte protein, for example (3), the mechanism of axonal damage is still unclear. Elucidation of this event is crucial to preventing the irreversible neurologic deficits in MS associated with axonal loss (4–6). Damage to axons may occur via several mechanisms, including a direct autoimmune attack. However, the role of autoimmunity to neuronal antigens in axonal damage is only supported by circumstantial evidence, i.e. the presence of antibodies against neurofilaments (NFs) and tubulin, in the cerebrospinal fluid (CSF) (7, 8) and serum (9–11) of patients with MS. These cytoskeletal proteins, critical for maintaining axonal caliber (12), are released into the CSF after axonal damage (13, 14) and may, as has been shown with myelin antigens, drain to the cervical lymph nodes (15). Subsequently, they may provoke an autoimmune response as has been described for β-tubulin (16). In patients with MS, the level of antibodies against the light subunit of NFs (NF-L) correlates with the degree of cerebral atrophy (17), suggesting that autoimmunity to neuronal antigens may indeed play a role in inducing axonal damage. Furthermore, axon-reactive B-cells have been detected in lesions in the brain and isolated from the CSF of patients with MS (18, 19).

Autoimmunity to neuronal antigens may also play a pathogenic role in other neurologic diseases, such as paraneoplastic neurologic degeneration (20), sporadic amyotrophic lateral sclerosis (21), and, more recently, Alzheimer disease, in which antibodies have been found within degenerating neurons (22). Antibodies to NF proteins have also been detected in cases of rheumatoid arthritis complicated by peripheral neuropathy (23). Despite suggestive evidence in humans, animal models confirming a role for antineuronal responses in neurodegeneration are scarce. Until recently, attempts to develop these models for paraneoplastic syndromes have been unsuccessful (24). Now it is known that mice develop brain inflammation after passive transfer of paraneoplastic Ma1-reactive T-cells (25). In addition, mice immunized with the neuronal tau protein, amyloid-β, or β-synuclein peptides develop experimental autoimmune encephalomyelitis (EAE) (26–28), emphasizing the pathogenic role of these antineuronal autoimmune responses.

Although various reports demonstrate the presence of autoimmunity to NF-L in neurologic diseases, the pathogenic...
role of these responses to NF-L is unclear. To investigate the possible role of autoimmunity to NF-L, Biozzi ABH mice were immunized with recombinant mouse NF-L (rmNF-L) in adjuvant. We now show for the first time that immunization with rmNF-L protein causes not only neurologic disease (i.e. spasticity and paralysis) but also axonal degeneration. The axonal damage was associated with the presence of activated macrophages, CD4+ and CD8+ T-cells, and immunoglobulin deposits. This result strongly indicates that autoimmunity to axonal proteins, rather than being merely a surrogate marker, may be a key pathogenic mechanism in neurodegeneration. This new animal model will be important in elucidating the role of autoimmunity to neuronal antigens in chronic neurologic disease and may prove to be a valuable tool for the development of novel therapies for neuroprotection.

MATERIALS AND METHODS

Mice

Biozzi ABH (H-2^{k}) mice were bred from stock at the Biomedical Primate Research Centre (Rijswijk, The Netherlands). Male and female mice of 6 to 12 weeks of age were used for immunization. Animals were kept at standard laboratory conditions and were fed ad libitum. An animal welfare committee reviewed and approved all experiments (according to the Dutch law).

Immunization and Assessment of Clinical Signs

Mice were immunized with rmNF-L (29, 30) or recombinant mouse myelin oligodendrocyte glycoprotein (rmMOG) as described previously (31, 32). Briefly, for each mouse, 200 μg of protein was dissolved in PBS and emulsified (1:1) with complete Freund’s adjuvant (CFA), prepared by adding 48 μg of Mycobacterium tuberculosis and 6 μg of Mycobacterium butyricum to 150 μL of incomplete Freund’s adjuvant (IFA) (Difco Laboratories, Detroit, MI). On postsensitization days (PSD) 0 and 7, the mice were immunized subcutaneously with a 300-μL emulsion divided over two sites on the flanks. A control group received CFA without antigens. Pertussis toxin (200 ng dissolved in PBS) derived from Bordetella pertussis (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) was given intraperitoneally immediately and 24 hours after immunization. From PSD 10 to 50, mice were weighed daily and examined for neurologic signs (i.e. flaccid paralysis and spastic paresis) by visual assessment. Scores were as follows: 1, paralysis or spasticity of the tail; 2, impaired righting reflex; 3, paralysis or spastic paresis of one limb; 4, paralysis or spastic paresis of two limbs; and 5, moribund. Animals exhibiting signs that were less severe than typically observed were scored 0.5 less than the indicated grade. Spasticity of the limbs was also monitored using a purpose-build strain gauge as has been described by Baker et al (33).

T-Cell Proliferation Assay

T-cell responses to rmNF-L, rmMOG, Biozzi ABH spinal cord homogenate, or ovalbumin (OVA) were measured in a proliferation assay. Spleens from rmNF-L-immunized animals were isolated, and a single cell suspension was obtained by passing the tissue through a 100-μm cell strainer. Mononuclear cells were isolated on a Lympholyte-M gradient (Cedarlane, Hornby, ON, Canada) by density centrifugation. Cells were washed twice and seeded (4 × 10^5/well) in RPMI 1640 supplemented with 2% normal mouse serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and 5 × 10^{-5} mol/L 2-mercaptoethanol (Gibco [Invitrogen], Breda, The Netherlands) in the presence of 0.1, 1, 10, or 100 μg/mL rmNF-L, OVA, or spinal cord homogenate. As a positive control, concanavalin A (5 μg/mL) was used. Cells were cultured for 3 days, and proliferation was measured by incubating for the last 18 hours with [^3]H]thymidine (Amersham Biosciences, Roosendaal, The Netherlands) at 1 μCi/well. Incorporated [^3]H]thymidine was expressed as mean counts per minute (cpm). Stimulation indices were calculated by dividing the mean cpm of antigen-stimulated cells by the mean cpm of control cells without antigens.

Fluorescence-Activated Cell Sorter Analysis of Proliferating Cells

Splenocytes were carboxyfluorescein succinimidyl ester (CFSE)-labeled by incubation with carboxyfluorescein diacetate succinimidyl ester (Fluka Biochemika, Buchs, Switzerland) and cultured in the absence or presence of 10 μg/mL rmNF-L or OVA for 3 days. Cells were stained with anti-CD4-PE and anti-CD8-PerCP (BD Biosciences, Alphen aan den Rijn, The Netherlands), and the percentage of cells with diluted CFSE in the CD4+ or CD8+ compartment was determined using flow cytometry (FACSaria, BD Biosciences). Data were analyzed using FACSDiva software.

Cytokine Analysis

Culture supernatants from stimulated splenocytes were collected on day 3. ELISAs were performed to assess the production of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-10 (Biosource, Etten-Leur, The Netherlands), and IL-4 (BD Biosciences). The assays were performed according to the manufacturer’s procedures.

ELISA for Neurofilament Light Antibody Levels

Serum was obtained from peripheral blood of normal mice and mice immunized with CFA only or with NF-L/CFA. To study the presence and the isotypes of NF-L antibodies, Microlon plates were coated overnight at 4°C with 5 μg/mL rmNF-L in PBS and subsequently blocked with 2% bovine serum albumin in PBS. Plates were incubated with sera (1:100 in PBS/1% bovine serum albumin) and, after washing, with alkaline phosphatase-conjugated secondary antibodies directed to total mouse immunoglobulins (rabbit anti-mouse Ig-alkaline phosphatase; DakoCytomation, Glostrup, Denmark), IgG1, IgG2b, IgG3, IgM (all goat anti-mouse; Serotec, Oxford, UK), or IgG2c isotypes (goat anti-mouse; Bethyl Laboratories, Inc., Montgomery, TX). Bound antibodies were subsequently
visualized with p-nitrophenyl phosphate in Tris buffer (Sigma-Aldrich Chemie) and absorbance was measured at 405 nm.

**Histology and Electron Microscopy**

Brain, spinal cord, and sciatic nerve with attached muscle were removed, fixed in 5% buffered formal saline, and routinely processed for paraffin embedding. The degree of inflammation was assessed by hematoxylin and eosin stain. In addition, mice were perfused with 4% paraformaldehyde, and spinal cord blocks (1–2 mm) were fixed for 4 hours in 4% paraformaldehyde in 0.15 M Sörenson’s phosphate buffer, followed by 2 hours in 2.5% glutaraldehyde (Agar Scientific, Stansted, UK). Tissue blocks were subsequently processed for resin embedding (Araldite CY212; Agar Scientific), semithin 1–2 μm sections were cut, stained with uranyl acetate and lead citrate, and viewed on an electron microscope (Jeol 1010 TEM).

**Immunohistochemistry**

Frozen sections (8 μm) from brains and spinal cords were fixed with acetone, blocked with 10% goat serum, and, incubated with primary antibodies against F4/80 (Serotec), CD4 (clone YTS 191.1.2), or CD8 (clone YTS 169AG; ImmunoTools, Friesoythe, Germany). After washing, sections were incubated with biotinylated goat anti-rat Ig (Southern Biotech, Birmingham, AL) followed by ABC amplification (Vector Laboratories, Burlingame, CA). To stain for the presence of Ig isotypes in the spinal cord, sections were blocked with 10% goat serum and incubated with biotinylated goat anti-mouse IgG1, IgG2a, or IgG2b (Jackson ImmunoResearch Europe Ltd., Suffolk, UK), followed by ABC amplification. In addition, unfixed spinal cord sections were extensively washed (three times for 1 hour with PBS/0.05% Tween) before fixation to wash off all antibodies that were not complexed to specific antigen. Bound antibodies were visualized using 3-amino-9-ethylcarbazole (Sigma-Aldrich Chemie), and sections were counterstained with hematoxylin.

**Statistical Analysis**

Data are expressed as mean ± standard error and were analyzed using either the Mann-Whitney U test, analysis of variance, or Kruskal-Wallis test. As posttests, Newman-Keuls or Dunn’s multiple comparison tests were used. p < 0.05 was regarded as statistically significant.

**RESULTS**

**Immunization With Recombinant Mouse Neurofilament Light Induces Neurologic Disease**

To examine the immunogenic and pathogenic potential of NF-L protein, Biozzi ABH mice were immunized with rmNF-L in CFA. Of the 28 animals, 15 developed weight loss and neurologic disease (Fig. 1). Three types of neurologic disease were observed: 1) flaccid paralysis of hindlimbs, as in classical EAE; 2) spastic paresis (i.e. hyperextension of the hindlimb); and 3) forelimb weakness without involvement of the hindlimbs. Animals immunized with CFA alone did not develop neurologic signs (Table).

**TABLE.** Characteristics of Neurologic Disease in Biozzi ABH Mice Immunized With rmNF-L in CFA or with CFA Alone

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Clinical Signs</th>
<th>Incidence</th>
<th>Onset*</th>
<th>Disease Score†</th>
</tr>
</thead>
<tbody>
<tr>
<td>rmNF-L</td>
<td>Spastic paresis</td>
<td>7/15</td>
<td>29.3 ± 5.7</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Forelimb dysfunction‡</td>
<td>4/15</td>
<td>38.0 ± 8.4</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>Paralysis</td>
<td></td>
<td>4/15</td>
<td>25.8 ± 6.3</td>
<td>3.5 ± 0.0</td>
</tr>
<tr>
<td>Total signs</td>
<td></td>
<td>15/28 (54%)</td>
<td>31.7 ± 7.5</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>CFA</td>
<td>None</td>
<td>0/6</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Mice were immunized with recombinant mouse neurofilament light (rmNF-L) in complete Freund’s adjuvant (CFA) or with CFA alone as described in the Materials and Methods section. The mice were examined for clinical signs for 50 days after immunization, and the numbers of animals exhibiting spasticity and paralysis of the limbs were recorded.*

†*Mean maximal neurologic score ± SD of animals exhibiting clinical disease.*

‡*Animals with forelimb dysfunction did not display overt spasticity.*

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Immunized animals develop autoimmune T-cell responses to recombinant mouse neurofilament light (rmNF-L). Splenocyte proliferation to rmNF-L was measured by \[^{3}H\]thymidine incorporation. Splenocytes from rmNF-L-immunized mice (n = 7) showed a dose-dependent proliferation to rmNF-L, in contrast to splenocytes from mice immunized with CFA alone (n = 3) (A; **, p < 0.01, Mann-Whitney U test). Carboxyfluorescein succinimidyl ester (CFSE) labeling combined with CD4 and CD8 staining shows that CD4\(^{+}\) but not CD8\(^{+}\) cells were proliferating to rmNF-L (B; n = 5, **, p < 0.01, analysis of variance followed by Newman-Keuls multiple comparison test). Representative fluorescence-activated cell sorter plots of ovalbumin (OVA) (C)- and rmNF-L (D)-stimulated, CFSE-labeled splenocytes of one animal show the divided CD4\(^{+}\) T-cells to rmNF-L as several peaks left of the undivided CD4\(^{+}\) population (numbers refer to divided CD4\(^{+}\) or CD8\(^{+}\) cells as a percentage of the total population of CD4\(^{+}\) or CD8\(^{+}\) cells). Culture supernatant were collected after 3 days and assessed for interferon (IFN)-\(\gamma\) (E, n = 7), tumor necrosis factor (TNF)-\(\alpha\) (F, n = 7), interleukin (IL)-10 (G, n = 3), and IL-4 (H, n = 6) production. Cytokines were secreted dose dependently upon stimulation with rmNF-L but not OVA (*, p < 0.05; ***, p < 0.001, compared with OVA, Mann-Whitney U test).
Mice immunized with rmNF-L exhibiting flaccid paralysis had a tendency to develop disease earlier (PSD 25.8 ± 6.3) (Table) than those with spastic signs of the hindlimbs (PSD 29.3 ± 5.7) or with forelimb involvement (PSD 38.0 ± 8.4). In 4 animals the clinical spastic paresis or paralysis resolved after 5 to 6 days and did not reoccur during the observation period (14–21 days).

To quantitatively measure the spasticity we determined the resistance to hindlimb flexion using a strain gauge. This system has been successfully used to measure spastic limbs in later stages of EAE, induced with spinal cord homogenate (33). Compared with normal mice, rmNF-L-immunized animals with spastic paresis were not significantly different in resistance to hindlimb flexion. This finding is consistent with the observation that spasticity was not always present (e.g. when mice were picked up their limbs relaxed) and that spasticity increased upon intentional movement, such as when mice attempted to walk. Mice with paralyzed limbs showed decreased resistance to hindlimb flexion compared with controls (data not shown).

**Recombinant Mouse Neurofilament Light-Immunized Mice Develop Autoimmune T-Cell Responses to Recombinant Mouse Neurofilament Light**

To determine whether the rmNF-L-immunized animals developed autoimmune responses to NF-L, T-cell proliferation assays were performed using splenocytes. All animals, irrespective of their clinical status, developed a dose-dependent proliferative response of splenocytes to rmNF-L (Fig. 2A), with a maximum stimulation index of 14.9 ± 2.3 at 10 µg of rmNF-L. In contrast, splenocytes from mice immunized with CFA alone did not proliferate to rmNF-L. The splenocytes from rmNF-L-immunized mice also proliferated to spinal cord homogenate, which contains native mouse NF-L, although these responses were not as high as those observed with rmNF-L (data not shown). No proliferation was observed at similar concentrations of OVA or a control recombinant mouse protein (i.e. rmMOG), whereas all splenocyte cultures proliferated in response to concanavalin A (data not shown).

To characterize the phenotype of the T-cell proliferative response to rmNF-L, splenocytes were labeled with CFSE and cultured in the presence or absence of rmNF-L or OVA. In response to rmNF-L, 2.4% of the total CD4+ population had diluted CFSE indicative of proliferation, compared with 0.6% for OVA or when cultured without antigen (p < 0.01; Fig. 2B–D). In contrast, in the CD8+ compartment, no differences were observed between rmNF-L-stimulated splenocytes and controls. T-cell proliferation to rmNF-L was accompanied by the production of cytokines. The most abundant cytokine present in the supernatants of splenocyte cultures stimulated with rmNF-L was IFN-γ, which was produced dose dependently. The level of IFN-γ was significantly higher in NF-L-stimulated cultures (430 ± 101 pg) compared with OVA stimulation (28 ± 6 pg) (p < 0.001; Fig. 2E). The production of TNF-α and IL-10 was also higher in rmNF-L-stimulated splenocytes compared with OVA (both p < 0.05; Fig. 2F, G). Although IL-4 was present in the supernatant of rmNF-L-stimulated splenocytes, the difference compared with OVA-stimulated cells was not significant (Fig. 2H). In summary, rmNF-L-immunized mice developed a significant CD4+ T-cell response to rmNF-L concomitant with the production of high levels of IFN-γ.

**Autoantibodies to rmNF-L Are Present in rmNF-L-Immunized Mice**

We next studied whether rmNF-L-immunized animals also produced autoantibodies to NF-L. Compared with CFA-immunized controls, all mice immunized with rmNF-L developed significantly elevated levels of antibodies (total Ig) against rmNF-L (p < 0.05; Fig. 3A). Although some animals immunized with only CFA developed IgG1 responses to rmNF-L, mice immunized with rmNF-L produced significantly higher levels of IgG1 (p < 0.01), elevated levels of IgG2c and IgG3 but not IgM antibodies to rmNF-L were found (Fig. 3B). Also, elevated levels of IgG2c and IgG3 but not IgM antibodies to rmNF-L were found (B) * p < 0.05; ** p < 0.01; *** p < 0.001, analysis of variance followed by Dunn’s multiple comparison test.

**FIGURE 3.** Immunized animals develop autoantibodies to recombinant mouse neurofilament light (rmNF-L). Antibodies to rmNF-L were determined in sera of normal mice (NMS), CFA-immunized mice, and rmNF-L-immunized mice using an ELISA. Animals immunized with rmNF-L had significantly higher levels of total Ig, IgG1, and IgG2b antibodies to rmNF-L (A). Also, elevated levels of IgG2c and IgG3 but not IgM antibodies to rmNF-L were found (B) * p < 0.05; ** p < 0.01; *** p < 0.001, analysis of variance followed by Dunn’s multiple comparison test.
IgG2b, IgG2c, and IgG3 (all p < 0.001) antibodies (Fig. 3B). The levels of IgM antibodies directed to rmNF-L in sera of mice immunized with rmNF-L were not significantly higher than those in sera of animals immunized with only CFA or in normal mouse serum. In all mice, antibodies to the irrelevant protein OVA were absent (data not shown). Although mice exhibiting neurologic disease had a tendency to produce higher amounts of antibody compared with asymptomatic mice, no clear association could be found between antibody levels or isotypes and the severity and duration of disease.

**Mice With Clinical Disease Have Inflammation and Axonal Damage**

In mice exhibiting clinical neurologic disease, lesions of cellular infiltration and axonal degeneration were observed in both the grey and the white matter of the spinal cord (Fig. 4A–C). The lesions in the white matter were primarily observed in the dorsal column, in some mice extending to the corticospinal tract, but lesions were also observed in the lateral and ventral columns. Lesions typically showed Wallerian degeneration consisting of vacuoles in which axons had degenerated, leaving myelin sheaths containing only axonal debris (Fig. 4B). In some cases the axon was absent as observed from “empty” myelin sheaths. Swollen axons containing an accumulation of mitochondria, indicative of axonal injury (34), were also observed (Fig. 4F). Generally, lesions contained less myelin, although demyelinated axons were not observed, suggesting that the demyelination occurred simultaneously with, or later than, axonal degeneration. Phagocytes within the lesions contained myelin debris (Fig. 4B) or were observed surrounding damaged axons with intact myelin sheaths (Fig. 4G). Lesions in the grey matter consisted of more compact groups of inflammatory cells, often associated with blood vessels (Fig. 4C). No lesions were observed in central nervous system tissues of asymptomatic mice or in brains of affected mice (data not shown). Biozzi ABH mice immunized with rmMOG with similar disease duration showed predominantly inflammation (perivascular cuffs and submeningeal inflammation) as well as myelin degeneration. However, compared with rmNF-L-immunized mice, only limited axonal degeneration in the spinal cord was observed (Fig. 4D, E). This indicates that immunization with a neuronal/axonal protein induces more axonal damage than immunization with a myelin protein. The different clinical manifestations of disease seem to associate with typical pathologic changes. Mice in which the forelimbs were affected were noted to have lesions in the dorsal column of the cervical cord, whereas those with hindlimb paresis had lesions in the dorsal column throughout the cord but most predominantly in the lumbar regions.

Because pathologic abnormalities were observed in axons of the dorsal column, which transmit sensory information from muscle and skin to the brainstem, we also examined the sciatic nerve and muscle. Preliminary examination of the sciatic nerve (Fig. 4H, I) and surrounding muscle tissue (Fig. 4J, K) revealed the presence of inflammatory cells, suggesting that the lesions of inflammation and axonal degeneration were present throughout the ascending sensory nerve tract. This finding is further supported by the presence of cellular infiltrates in dorsal roots (Fig. 4L).

Immunohistochemistry was performed to further study the phenotype of infiltrating cells in the spinal cord. Most infiltrated cells in the spinal cord were activated F4/80+ macrophages (Fig. 4L, M). In addition, in the dorsal horns, F4/80+ cells with a ramified morphology were present, possibly representing activated microglia (Fig. 4N). Compared with the abundant macrophages in the lesions, relatively low numbers of CD4+ (Fig. 4O) and CD8+ (Fig. 4P) T-cells were present in both the grey and the white matter of rmNF-L-immunized mice exhibiting neurologic disease. To investigate the role of antibodies in the

**FIGURE 4.** Mice with neurologic disease develop inflammation and axonal degeneration in the spinal cord and sciatic nerve. (A) Semithin resin section of a mouse with spastic paresis reveals extensive vacuolation (arrowheads) in the dorsal column of the spinal cord (toluidine blue; original magnification: 25×). (B) Axons in this area show signs of Wallerian degeneration (arrowheads, enlargement of upper rectangle in A; original magnification: 400×); note that phagocytes contain myelin debris (arrows). (C) In the grey matter, groups of inflammatory cells are associated with blood vessels (arrows, enlargement of lowest rectangle in A; original magnification: 400×). Biozzi ABH mice immunized with rmMOG had inflammation in the spinal cord (D, arrowheads; original magnification: 25×), myelin degeneration (arrows in E, enlargement of rectangle in D; original magnification: 400×), but only limited axonal degeneration (arrow in D). Electron microscopy of the spinal cord of affected recombinant mouse neurofilament light (rmNF-L)-immunized mice shows accumulated mitochondria in a swollen axon (F, arrowhead; asterisk indicates normal axon) and a phagocyte engulfing a damaged axon with its myelin sheath (G; original magnification: 3,000×). The sciatic nerve of asymptomatic mice appears normal (H, hematoxylin and eosin stain; original magnification: 200×), whereas inflammation is present in the sciatic nerve of affected mice (I; original magnification: 200×). Surrounding muscle tissue in mice without clinical disease appears normal (J; original magnification: 200×), in contrast with mice exhibiting disease, in which cellular infiltrates can be seen (K; original magnification: 200×). F4/80+ macrophages are present in high numbers in the dorsal column of a mouse with neurologic disease (L, original magnification: 25× and M, enlargement of the right rectangle in L; original magnification: 400×). In the grey matter, cells with a ramified morphology, resembling microglia, express the F4/80 antigen (N, enlargement of the left rectangle in L, original magnification: 400×). T-cells expressing the CD4 (O, arrowheads; original magnification: 200×) and CD8 (P, arrowheads; original magnification: 200×) antigen are present in lesions. After extensive washing of unfixed sections, IgG1 is still complexed to axons in the dorsal column of rmNF-L-immunized mice with clinical disease (Q; original magnification: 400×), whereas IgG1 is complexed to myelin sheaths and not to axons in rmMOG-immunized mice (R; original magnification: 400×).
pathologic condition, spinal cord sections were stained for mouse immunoglobulin isotypes. Biozzi ABH mice make high levels of IgG1 and IgG2b, but do not produce IgG2a (35). In the spinal cord of rmNF-L-immunized mice with neurologic disease, IgG1 and IgG2b antibodies were observed not only in the inflammatory lesions, as expected due to the loss of blood-brain barrier integrity but also within axons and nerve cell bodies. Such staining was not seen in asymptomatic rmNF-L-immunized or control mice (data not shown). IgG2a could not be detected in the spinal cord of affected rmNF-L-immunized mice, indicating that the immunoglobulin staining was specific. To determine whether the observed antibodies in the spinal cord were bound to their target antigen, the unfixed snap-frozen sections from rmNF-L and rmMOG-immunized mice were extensively washed before fixation, assuming that when immunoglobulin was not complexed to a specific antigen, the immunoglobulin should be washed out. After washing, IgG1 staining was reduced in neuronal cell bodies, whereas axonal profiles in lesioned dorsal column areas remained positive (Fig. 4Q). In rmMOG-immunized mice Ig in neither neuronal cell bodies nor axons were present. Instead, thin parallel lines of punctuate staining were observed near inflammatory lesions, representing immunoglobulin complexes on myelin sheaths (Fig. 4R). In conclusion, these results show that antibodies complexed to axons are only observed in the lesioned dorsal column of mice immunized with rmNF-L and not in lesions of mice immunized with rmMOG.

**DISCUSSION**

Axonal damage has been identified as the major cause of irreversible neurologic disability in patients with MS. The mechanism for the development of axonal damage is still unknown, but autoimmunity to neuronal antigens has been proposed to play a role (36). This study demonstrates that autoimmunity to the neuronal antigen NF-L is pathogenic and induces clinical neurologic signs in mice that are also observed in MS. In this study we have shown that 1) immunization of Biozzi ABH mice with rmNF-L induces autoimmune T- and B-cell responses to NF-L, 2) 54% of the animals immunized with rmNF-L develop spastic or paralytic disease, and 3) animals with clinical neurologic disease have severe inflammation and axonal degeneration indicative of Wallerian degeneration. These results suggest that NF-L protein is not only immunogenic but that autoimmunity to NF-L could play a role in inducing axonal damage and clinical neurologic symptoms. Although this report describes autoimmunity to NF-L in an experimental system, such autoimmunity to neuronal antigens may be applicable to human neurodegenerative disorders such as MS in which antibodies to NF-L have been described (7–11).

To our knowledge, our study is the first to show that immunization with the intracellular neuronal protein NF-L induces clinical neurologic disease associated with axonal degeneration and inflammation. However, neurologic disease induced with neuronal antigens has been reported before, using tissue homogenates of the ventral horn of the spinal cord or purified motor neurons (37, 38). The specific autoantigens were, however, not defined. Of note is the fact that several of the rmNF-L-immunized mice in our study showed clinical signs similar to those of guinea pigs immunized with spinal cord ventral horn homogenate, suggesting that (one of the) disease-inducing proteins in the ventral horn may be NF-L. Also, immunization with tau protein has been reported to induce neurologic disease, that is, limb paralysis (28). Compared with myelin protein-induced EAE, tau-immunized C57BL/6 mice developed disease relatively late (PSD 41–149). This result is consistent with our findings of late-onset disease in Biozzi ABH mice after NF-L immunization (PSD 21–50). However, tau is not only expressed by neurons, but also by astrocytes and oligodendrocytes (39) and thus disease may not be specifically targeted to neurons alone.

The Biozzi ABH mice immunized with rmNF-L showed axonal degeneration predominantly in the dorsal column of the spinal cord. In rodents, the dorsal column consists of ascending sensory fibers and descending corticospinal fibers (40). In humans, degeneration of these tracts is known to occur in hereditary spastic paraplegia and in MS (41, 42). Corticospinal fibers are important for the control of movement through their terminations on spinal motor neurons that innervate the trunk and limb muscles. Damage to these fibers is known to result in spasticity (43), a symptom also frequently observed in hereditary spastic paraplegia and MS (44). The spastic model in mice described here thus provides an ideal platform for the development of new compounds for treating spasticity in these neurologic disorders.

Exactly how and where the pathogenic events result in axonal damage in the rmNF-L-immunized mice is still unclear. The observation that axons in the spinal cord show signs of Wallerian degeneration indicates that the primary lesion may be not in the spinal cord but rather in the peripheral nerve or dorsal root ganglia. In support of this, inflammation in the sciatic nerve and the dorsal roots was observed in affected animals. The axonal damage observed in the mice may be initiated by CD4+ T-cells, which may activate microglia and macrophages to produce cytokines and reactive oxygen species. In vitro, CD4+ T-cells proliferated and secreted high levels of IFN-γ in response to rmNF-L, pointing to a proinflammatory Th1-mediated disease, similar to that observed in myelin-induced EAE (45).

Alternatively, axonal damage may occur due to pathogenic antibodies recognizing NF-L protein in axons. In rmNF-L-immunized mice, immunoglobulin was found complexed to axons in the lesioned dorsal column of the spinal cord. Recently, a pathogenic role has been described for antibodies against amphiphysin, a protein associated with synapses, in stiff-person syndrome. Passive transfer of high-titer antibodies isolated from a patient with this syndrome induced spasms and stiffness in rats, which had also received encephalitogenic T-cells to open up the blood-brain barrier (46). Like amphiphysin, NF-L is also an intracellular protein; therefore, antibodies must enter the cell first to bind...
their antigen. This could be achieved through retrograde axonal transport (47, 48) or by internalization via the cell body (49). Once inside axons, antibodies may interfere with the cytoskeleton, as has been described for tubulin antibodies (50, 51). This mechanism would explain why axonal degeneration in rmNF-L-immunized mice was observed predominantly in the sensory and corticospinal fibers: these axons are the longest in the nervous system and are therefore the most reliant on an intact cytoskeleton.

Whether T-cells, antibodies, or combinations of both are responsible for the clinical manifestation observed in rmNF-L-immunized mice remains to be determined. To address this issue, we performed a preliminary study in which rmNF-L-activated splenocytes from rmNF-L-immunized mice were adoptively transferred into naive mice. Although one of six mice developed very mild disease (i.e. hindlimb weakness), we observed no apparent pathologic changes in the spinal cord. However, it cannot yet be firmly established that NF-L-reactive T-cells alone are insufficient to induce disease, because naive Biozzi ABH mice appeared to be refractory to disease induced after adoptive transfer of proteolipid protein peptide specific T-cells (S. Amor and D. Baker, unpublished data, 1995). Studies in other mouse strains may therefore be more suitable to address this issue.

With regard to establishing the pathogenicity of NF-L-reactive antibodies in disease, it is apparent that high titers of antineuronal antibodies, as described by Sommer et al (46), are required to induce experimental neurologic disease. To obtain sufficient antibodies as well as determine the relevance for antibodies to NF-L in human neurodegenerative diseases, our current studies are focused on purifying NF-L immunoglobulin from humans for adoptive transfer into mice. Determining the pathogenicity of antineuronal antibodies and/or T-cells will not only be important for MS, in which antineuronal antibodies are present in the serum and CSF (11, 17) and, more critically, inside axons (19). The outcome may also be relevant for other neurologic diseases, such as amyotrophic lateral sclerosis, Alzheimer disease, and Parkinson disease, in which antineuronal antibodies are observed (21, 22). Unraveling the role of autoimmunity to neuronal antigens in neurodegenerative disorders will crucially expand the possibility for treating these otherwise chronic disorders.

In summary, this study shows that immunization with a single neuronal protein, NF-L, induces neurologic disease in Biozzi ABH mice associated with inflammation and axonal degeneration predominantly of the dorsal column. These results indicate that, in humans, autoimmunity to NF-L may not be solely a surrogate marker of axonal pathology but could also play a crucial role in the progressive neurodegeneration observed in MS and other neurologic diseases.

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