Tauopathy-like Abnormalities and Neurologic Deficits in Mice Immunized With Neuronal Tau Protein

Hanna Rosenmann, PhD; Nikolaos Grigoriadis, MD, PhD; Dimitrios Karussis, MD, PhD; Moran Boimel, BSc; Olga Touloumi, BSc; Haim Ovadia, PhD; Oded Abramsky, MD, PhD

**Background:** A possible role of autoimmunity in Alzheimer disease pathogenesis has recently attracted increasing attention. Vaccination with amyloid-β peptide was reported to cause marked reduction in amyloid deposition, but it also induced encephalitis. Not much is known regarding neurofibrillary tangle–related autoimmune effects.

**Objective:** To use the main component of tangles—microtubule-associated tau protein—to test the feasibility of active induction of a neuroautoimmune disorder in mice.

**Design:** Prospective, randomized controlled animal study.

**Setting:** University medical center research laboratory.

**Subjects:** Female C57BL/6 mice.

**Interventions:** Inoculation with recombinant human tau protein emulsified in complete Freund adjuvant and with pertussis toxin.

**Main Outcome Measures:** Clinical, immunologic, pathologic, and behavioral evaluations were performed.

**Results:** Vaccination with tau protein induced histopathologic features of Alzheimer disease and tauopathies, indicated by the presence of neurofibrillary tangle–like structures, axonal damage, and gliosis. Also, mononuclear infiltrates without demyelination in the central nervous system, accompanied by neurologic deficits (such as a limp tail and limb paralysis), were observed. Anti–tau antibodies were detected in the serum of tau-immunized mice.

**Conclusions:** These results provide a link between tau autoimmunity and tauopathy-like abnormalities and indicate potential dangers of using tau for immunotherapy. This experimental autoimmune tauopathy-like model is due to a pathogenic immune response against an intraneuronal antigen and is not related to myelin antigens.

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The possible involvement of autoimmune mechanisms in the pathogenesis of Alzheimer disease (AD) has recently attracted attention. Vaccination with amyloid-β (Aβ) peptide caused a marked reduction in brain amyloid abnormalities and memory deficits in mice models and in patients with AD. However, these beneficial therapeutic effects of Aβ immunization were accompanied by neuroinflammation that finally led to cessation of the clinical trials.

Little is known regarding the possible autoimmunity potential of the other major component of neuropathologic abnormalities in AD, neurofibrillary tangles (NFTs). The NFTs are aggregates of hyperphosphorylated tau microtubule-associated protein, expressed predominantly in neurons and to some extent in glia. Tau aggregation is also the major histopathologic hallmark of other tauopathies (without plaques), such as frontotemporal dementia and Pick disease, and it correlates with the severity of dementia in neurodegenerative diseases. Rosenmann et al report the presence of anti–NFT antibodies in elderly people, with a prominent IgM isotype in patients with AD, suggesting that an NFT-directed autoimmune mechanism is involved in the pathogenesis of AD. This prompted us to use the main component of tangles—microtubule-associated tau protein—to examine whether injection of this neuronal protein can induce an autoimmune response in the central nervous system (CNS).

The feasibility of the development of such tau-mediated immune effects may shed some light on the possible involvement of autoimmunity in the pathogenesis of AD and tauopathies. It may also indicate an additional potential danger of therapeutic immunization targeting tau and NFTs. Moreover, possible encephalitogenic properties will be of interest because tau is a neuronal protein. And to date, mostly myelin-related rather than...
neuronal antigens are known to induce encephalomyelitis or neurodegeneration.6,7

**METHODS**

**IMMUNIZATIONS**

Female C57BL/6 mice were inoculated with 50 µg of recombinant human tau protein (Invitrogen Corp, Carlsbad, Calif) (close to 100% homology with mice) emulsified in complete Freund adjuvant (CFA) supplemented with Mycobacterium tuberculosis. Pertussis toxin (PT) was administered intraperitoneally the same day and 48 hours later. An additional tau injection in CFA was administered 1 week later. Mice immunized only with CFA supplemented with M tuberculosis and PT served as controls. For comparison of CNS abnormalities we also immunized mice with myelin oligodendrocyte glycoprotein (MOG) 33-54 peptide for induction of experimental autoimmune encephalomyelitis (EAE) using a similar immunization protocol. Clinical evaluation was performed using the EAE clinical score for paralysis (scale of 0-6). The study was conducted as 2 sets of independent experiments using different batches of tau protein.

**NEUROPATHOLOGIC EXAMINATION**

While anesthetized, the animals were perfused transcardially with 4% paraformaldehyde, and their brains and spinal cords were excised and embedded in paraffin. Animals immunized with MOG were humanely killed during the acute phase after the initiation of paralytic signs. Animals immunized with MOG were humanely killed mostly during the acute phase and some in the chronic phase. In parallel, CFA-PT– and tau-immunized animals that were not clinically affected were also humanely killed at the same time as the affected animals.

**ANTIBODIES**

The following antibodies were used: anti–AT8 and anti–AT100 mouse monoclonal antibodies (Innogenetics, Gent, Belgium), which recognize tau phosphorylated at S202/T2058 and T145/1460 (close to 100% homology with mice) emulsified in complete Freund adjuvant (CFA) supplemented with Mycobacterium tuberculosis and PT. These slides were then treated with other primary antibodies against CNPase, tubulin beta III isoform, and glial fibrillary acidic protein (astrocytes), or AT8 or AT100. Briefly, paraffin sections were deparaffinized and hydrated in xylene and alcohol solutions and then were rinsed with Tris-buffered saline. Citrate buffer (pH 6) was used for antigen retrieval. After incubation of the section in blocking buffer for 1 hour, sections were treated with a first primary antibody against AT8 or AT100, then with goat anti–mouse IgG conjugated to rhodamine (Jackson ImmunoResearch Laboratories Inc, West Grove, Pa). These slides were then treated with other primary antibodies against CNPase, tubulin beta III isoform, and glial fibrillary acidic protein and, finally, incubated with goat anti–mouse IgG antibody or goat anti–rabbit conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories Inc). The slides were mounted using 4',6-diamidino-2-phenylindole (Vector Laboratories) and were evaluated using a fluorescent microscope (Zeiss Axioplan 2; Carl Zeiss MicroImaging GmbH, Gottingen, Germany) by 2 independent observers (N.G. and O.T.). In several adjacent sections, both primary antibodies were omitted in either single or double immunohistochemical assays and served as negative controls.

Microglial cells were stained with biotinylated lectin Lycopersicon esculentum and were visualized using the labeled streptavidin biotin technique (LSAB2 System Peroxidase; Dako Cytomation). The peroxidase reaction was visualized using 3,3-diaminobenzidine and hydrogen peroxide. Sections were then dehydrated in graded ethanol and covered with a rapid embedding agent (Entelan; Merck, Darmstadt, Germany). Images were visualized using a microscope (Zeiss Axioplan 2) and were digitized via a digital camera (DS-SMc-L1; Nikon, Tokyo, Japan).

**QUANTIFICATION**

In tau- and CFA-PT–immunized animals, the burden of lectin-positive microglia was determined in 5 randomly selected microscopic fields per section, each measuring 22 500 µm², defined by an ocular morphometric grid. The adjacent serial sections were used for the evaluation of AT8- and AT100-positive cells. A total of 5 randomly selected sections per tissue and per animal were evaluated. A semiquantitative assessment of activated microglia was performed according to a grading scale: 0 indicates no microglial cells; 1 (+), 1 to 10 microglial cells; 2 (+ +), 11 to 20 microglial cells; and 3 (+ + +), more than 20 microglial cells. For evaluation of the AT8 and AT100 burden, scores of the areas studied represented subjective assessments of staining intensity and numbers of labeled cells according to the following scores, as used by Simic et al: 0 indicates no labeling observed; 1 (+), small number of cells that are only weakly labeled; 2 (+ +), moderate number of cells that are clearly labeled; and 3 (+ + +), large number of intensely labeled cells present. The semiquantitative assessment of AT8- and AT100-positive cells and glial cells was performed by 2 independent observers (N.G. and O.T.). In cases in which significant discrepancies were obvious between the 2 observers, the evaluation was repeated by a third observer. The χ² Fisher exact test was used to compare the different groups.

**IMMUNOLOGIC ASSAYS AND BEHAVIORAL STUDIES**

The presence of anti–tau antibodies in serum was detected by means of enzyme-linked immunosorbent assay. For conf-
mation, a Western blot analysis was performed, and membranes were incubated with the serum of tau-immunized mice diluted 1:100 as the primary antibody compared with the serum of CFA-PT mice. For testing the in vitro proliferative responses of lymphocytes to antigens, draining lymph nodes and spleens were excised from immunized mice 22 days after immunization and were assayed as described elsewhere.6 For evaluation of spatial memory we used the radial 8-arm spatial maze13 and the Morris water maze.14

RESULTS

IMMUNIZATION WITH TAU PROTEIN INDUCES NEUROLOGIC DEFICITS

Immunization with tau protein induced clinical symptoms reminiscent of EAE, as was evaluated using the EAE severity score (Figure 1). However, distinct from EAE in tau-immunized animals, clinical symptoms appeared relatively late after immunization (days 41-149). In the first experiment, 3 of 5 tau-immunized animals developed EAE-like symptoms 41 to 46 days after immunization (Figure 1A). The animals presented with a limp tail followed by hind leg paresis (scores of 2-3) for at least 5 days and were humanely killed at day 55. In the second experiment, 3 of 6 mice immunized with tau developed clinical symptoms on days 57, 85, and 149 after immunization (tail and hind leg paralysis with mild forelimb weakness: scores of 2-4) and were humanely killed on days 70, 100, and 156, respectively (Figure 1B). All mice immunized with MOG developed EAE on days 11 to 14, with tail paralysis followed by hind leg paresis (scores of 2.5-4). None of the 14 negative controls immunized with CFA-PT developed any abnormality.

TAU-RELATED ABNORMALITIES IN TAU-IMMUNIZED ANIMALS

Tau-related abnormalities were evident with Gallyas staining after tau immunization because cytoplasmic argyrophilic deposits were detected in neurons and glial cells in the brainstem and spinal cord (Figure 2). Tau-related pathologic findings were more prominent in clinically affected tau-immunized mice. Some tau abnormalities were also detected in MOG-EAE mice, notably in glial cells and not in neurons, but much less intensive than in tau-immunized animals. Further characterization of the tau-related abnormalities was performed using immunohistochemical analyses using anti–AT8 and anti–AT100 antibodies. Cytoplasmic AT8 immunoreactivity was detected in neuronal and glial cells of the spinal cord in tau-immunized animals, and AT100 nuclear localization was evident in neurons and glial cells in the brainstem and spinal cord (Figure 3). Quantification of the tau pathologic burden revealed that in tau-immunized mice vs CFA-PT controls, 49.3% of examined fields had AT8-positive cells, with 9.3% showing numerous intensely labeled cells, vs 90.7% without any AT8-positive cells and 9.3% showing only weak labeling (P<.001) (Figure 3A). Similarly, AT100-positive cells were detected in 64.7% of the fields of the tau-immunized mice (44.0% with a small, 13.3% with a moderate, and 7.3% with a large number of positive cells) relative to only 35.3% of the fields in CFA-PT controls showing mostly weak labeling of cells (P<.001) (Figure 3B). Confirmation of the presence of tau-related abnormalities in neurons and oligodendrocytes was performed by means of double labeling of AT8 with tubulin beta III antibody for neurons and CNPase staining for oligodendrocytes (Figure 4); tau abnormalities were not detected in astrocytes when double stained with AT8 or AT100 (data not shown).

AXONAL DAMAGE AND INFLAMMATION WITHOUT DEMYELINATION IN TAU-IMMUNIZED ANIMALS

Severe inflammation indicated by severe astrocytosis, activated microglia, and mononuclear cell infiltrates (Figure 5) and axonal injury and loss (Figure 6) were prominently evident in the spinal cord of clinically affected tau-immunized animals. On the contrary, although some gliosis was present in nonaffected mice, neither infiltrates nor axonal injury was detected. The injured axons were in close contact with the infiltrates (Figure 6B). Quantification of the microglial burden revealed the following: 76.5% of the examined fields exhibited activated microglia (49.0% with 1-10 positive cells, 20.8% with 11-20 positive cells, and 6.7% with >20 positive cells per field) rela-
tive to only 20% of the fields showing 1 to 10 activated microglia per field in CFA-PT controls (P<.001). Pathological examination of the MOG-EAE mice revealed a characteristic picture of infiltrates, axonal damage, reactive gliosis, and demyelination. No evidence of demyelination was detected in clinically affected and nonaffected tau-immunized mice using Luxol fast blue staining. No infiltrates or axonal degeneration in the CNS of CFA-PT controls or clinically nonaffected mice were noticed.

Figure 2. Tau abnormalities in the spinal cords of tau-immunized mice. A, More prominent Gallyas- (upper 2 panels), AT8- (middle 2 panels), and AT100- (lower 2 panels) positive neurons (arrows) and glia (arrowheads) in clinically affected tau-immunized vs myelin oligodendrocyte glycoprotein-experimental autoimmune encephalomyelitis (MOG-EAE) mice (limited to glia) or complete Freund adjuvant–pertussis toxin (CFA-PT) controls (no reaction). B, Higher magnification of tau aggregate formation in clinically affected animals. Gallyas-positive (white arrow) and Gallyas-negative (arrowheads) neurons and neutrophil threads (black arrows) in an area with mononuclear infiltrates (upper panel). AT8 (middle panel) (arrows) and AT100 (lower panel) (arrows) immunoreactivity with cytoplasmic and nuclear localization, respectively. Arrowheads indicate negative cells counterstained with hematoxylin-eosin.

Anti–tau antibodies were detected in the serum of all mice immunized with the recombinant tau protein 22 days after induction. The titer of anti–tau antibodies was in most cases close to 1:2430 (Figure 7A). The presence of anti–tau antibodies was confirmed by Western blot analysis in the serum of tau-immunized mice, whereas no such an-
Tibodies were detected in CFA-PT controls (Figure 7B). All immunized animals developed anti–tau antibodies, whereas only half of them developed neurologic symptoms, with no correlation between antibody titers and clinical disease development or time at onset. Tau-immunized animals showed only a weak (stimulation index=1.9) in vitro proliferative response against tau protein (in lymph node and spleen lymphocytes). Presumably, the time during which T cells are activated against tau may be delayed and reach a peak at a later phase. Similarly, no response to myelin antigens (proteolipid protein and MOG) could be detected in tau-immunized mice.

**IMPAIRED PERFORMANCE IN MAZES BY TAU-IMMUNIZED AND CFA-PT MICE**

Behavioral studies using the Morris water maze and an 8-arm maze showed impaired performance of tau-immunized compared with naive animals. However, CFA-PT–immunized mice showed reduced cognitive performance, which could be attributed to the fact that inoculation with CFA is known to cause granulomas and arthritis that can impair locomotion in mazes in CFA-injected animals.

The results of this study show that vaccination with tau protein in CFA and PT induced a neurologic disease characterized by AD and tauopathy-like histopathologic features and neurologic deficits. The NFT-like structures detected in tau-immunized animals were present in neurons and in oligodendrocytes, similar to histopathologic changes in AD and in various tauopathies where also glial cells are affected.

To our knowledge, this is the first study indicating that immune mechanisms targeting tau may induce tauopathy-like features, providing a possible link between tau autoimmunity and AD-like abnormalities. Moreover, the present results indicate that humoral and cellular immune responses are raised against tau protein. At least for mice that developed clinical signs, it may be suggested that cellular immune responses are involved because mononuclear infiltrates were detected in the CNS. This should be confirmed by means of transfer experiments though. On the other hand, anti–tau antibodies can also mediate axonal damage, similar to that suggested for MOG anti-

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**Figure 3.** AT8 (A) and AT100 (B) burden in tau-immunized mice. A semiquantitative assessment of activated microglia was performed using the following grading scale: 0, no labeling observed; 1+, small number of cells that are only weakly labeled; 2+, moderate number of cells that are clearly labeled; and 3+, large number of intensely labeled cells present. For AT8 and AT100, $P<.001$ by $\chi^2$ Fisher exact test. CFA-PT indicates complete Freund adjuvant–pertussis toxin.
bodies in EAE. Because the axonal damage in tau-immunized mice occurs in close contact with cellular infiltrates (Figure 6), we assume that local disruption of the blood-brain barrier facilitates the passage of serum anti–tau antibodies. Such a mechanism was shown recently using T-encephalitogenic cells and anti–amphiphysin antibodies. The possible involvement of the peripheral anti–tau antibodies in the degeneration of neurons requires their crossing through the blood-brain barrier, as was previously shown with Aβ immunization. However, although the mechanism by which anti–Aβ antibodies react with amyloid plaques (reducing the burden of these plaques) involves extracellular binding of the antibodies to the senile amyloid plaques, the potential interference of anti–tau antibodies with intraneuronal tau demands an intracellular interaction. Such binding to intracellular antigens may occur similarly to the binding of anti–human antibodies (in paraneoplastic neurologic syndromes) to nuclear and cytoplasmic components, possibly through retrograde axonal transport or by internalization via the cell body. Moreover, the localization of tau protein also to the plasma membrane may facilitate the internalization of anti–tau antibodies. Because myelin

Figure 4. Tau abnormalities in neurons and oligodendrocytes. Identification of tubulin beta III (A and D) or 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (G and J) double stained with AT8 (B and E and H and K, respectively) and the corresponding 4',6-diamidino-2-phenylindole–positive nuclei of the cells (C, F, I, and L) in clinically affected tau-immunized (A-C and G-I) and complete Freund adjuvant–pertussis toxin (D-F and J-L) animals. Cells positive for either tubulin beta III (neurons) or CNPase (oligodendrocytes) and AT8 are indicated with arrows and were evident in tau-immunized animals. In contrast, similarly double-stained cells could hardly be identified in complete Freund adjuvant–pertussis toxin–treated animals (AT8-negative neurons or oligodendrocytes) (arrowheads).
was unaffected, the possibility that myelin-related autoimmune processes after tau immunization could account for the clinical paralytic signs is unlikely.

As stated by Mor and Cohen, the only way to discover the pathogenic potential of a candidate protein is active immunization with the particular protein; even use of the major histocompatibility complex class II motif to select candidate epitopes for the induction of T-cell autoimmunity did not seem to be operative. Although many myelin antigens have been shown to be encephalitogenic in experimental animals, other myelin peptides were devoid of pathogenicity. Interestingly, Aβ, a nonmyelin antigen, was recently reported to induce EAE, but no report of tau-induced immune disease exists in the literature.

Figure 5. Glial cell activation in the spinal cord of tau-immunized mice. Gliarial fibrillary acidic protein–positive astrocytes (A) and lectin Lycopersicon esculentum–positive microglia (B) (arrows) are more evident in clinically affected tau-immunized mice relative to complete Freund adjuvant–pertussis toxin (CFA-PT) controls. Myelin oligodendrocyte glycoprotein–experimental autoimmune encephalomyelitis (MOG-EAE) mice exhibited glial activation in the acute stage and less in the chronic stage.

The induction of CNS abnormalities and clinical disease through tau-immunization in the present study may be explained by (1) the use of full-length tau protein, which contains all relevant B- and T-cell epitopes; (2) the use of PT, which may contribute to blood-brain barrier permeability; and (3) the long clinical follow-up for detection of late-onset clinical symptoms.

The present findings can be further supported by the recent article by Kitazawa et al indicating that lipopolysaccharide-induced inflammation in the CNS can exacerbate tau abnormalities in the hippocampus of AD Tg mice. The neuroanatomic localization of the abnormality primarily to the spinal cord detected by us seems similar to some of the mutant tau Tg mice reported for tauo-
pathy, where tau abnormalities were detected in the spinal cord.\textsuperscript{24,25} In summary, the present results show the feasibility of induction of a CNS disease mediated by autoimmune

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\caption{Infiltrates and axonal damage in the spinal cords of tau-immunized mice. A, Bielschowsky staining for axonal injury, counterstained with Nissl (6 panels). Axonal loss (area between asterisks) and injured axons (arrows) were detected in close proximity to infiltrates (asterisks) only in tau-immunized affected animals. Findings were similar in myelin oligodendrocyte glycoprotein–experimental autoimmune encephalomyelitis (MOG-EAE) mice. B, Close contacts are shown (4 panels) between injured axons (ovoids and spheroids) and mononuclear cells (arrows). CFA-PT indicates complete Freund adjuvant–pertussis toxin.}
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reactivity against tau protein with histopathologic features at least partially resemblance of AD and tauopathy. The prominent tau-related abnormalities in this model, together with the delayed onset of clinical neurologic deficits, possess similarities to late-onset neurodegenerative CNS diseases (AD and tauopathies), as contrasted with the earlier onset of demyelinating CNS diseases induced with myelin antigens. In addition, the encephalitogenic potential of tau protein should be taken into account in future therapeutic strategies in which vaccination with this protein or its derivatives might be used.

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Correspondence: Hanna Rosenmann, PhD, Department of Neurology, Hadassah University Hospital, Ein Kerem, Jerusalem 91120, Israel (rosenmann@hadassah.org.il).

Author Contributions: Dr Rosenmann had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Rosenmann, Karussis, Ovadia, and Abramsky. Acquisition of data: Rosenmann, Grigoriadis, Boimel, and Touloumi. Analysis and interpretation of data: Rosenmann and Grigoriadis. Drafting of the manuscript: Rosenmann. Critical revision of the manuscript for important intellectual content: Rosenmann, Grigoriadis, Karussis, Boimel, Touloumi, and Ovadia. Study supervision: Rosenmann and Abramsky.

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