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**Research Article**

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# Antibodies to Neurofilament Protein in Retinitis Pigmentosa

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## Abstract

Antibodies reactive with heterologous neural tissue were detected by indirect immunofluorescence microscopy in the sera of 17 of 34 patients with retinitis pigmentosa, one of 30 normal control sera, and a variable percentage of sera derived from subjects with diverse ocular and neurological diseases. These antibodies were also found both in disease-free first degree relatives and in spouses of patients with retinitis pigmentosa. Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis of human spinal cord components followed by immunoblots with sera under study revealed that the serum antibody was specific for the high molecular weight protein subunit of neurofilaments. No correlation was found between the presence of these antibodies and other immunological and clinical parameters in retinitis pigmentosa. These findings suggest that release of piled-up neurofilaments from damaged neurones in retinitis pigmentosa triggers B lymphocytes autoreactive to neurofilament antigens.

## Introduction

Retinitis pigmentosa is a syndrome characterized by progressive degeneration of retinal photoreceptors with concomitant increasing visual loss (1). The etiology of retinitis pigmentosa remains unknown, although genetic factors are thought to play a role in a large proportion of patients (2). Previous immunological studies revealed evidence of T lymphocyte reactivity to retinal extracts from patients with retinitis pigmentosa, which promoted the concept of an autoimmune pathogenesis in this syndrome (3, 4). Further evidence of altered immune function was yielded by the demonstration of defective gamma interferon production by cells derived from patients with retinitis pigmentosa (5). Our own previous studies of the syndrome clearly defined two patient populations, one with apparently intact immune function, and the other with aberrant cell-mediated immunity characterized by diminished T lymphocyte rosette formation with transformed B lymphocytes, and defective mitogen-dependent lymphokine production (6). In this report we describe the detection of serum antibodies reactive with heterologous neurofilament proteins in patients with retinitis pigmentosa. Examination of spinal cord

neurofilament preparations by an immunoblot technique showed that these antibodies were specific for the high molecular weight protein subunit of human neurofilaments (H-NF), which has an apparent molecular weight of 200,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

## Methods

Peripheral venous blood samples were obtained with informed consent from 34 patients with retinitis pigmentosa. Serum was obtained by centrifugation and stored in aliquots at -20°C until tested. Results of cell-mediated immune function and other tests of 20 of these patients have been previously reported (6). The total patient population included 18 females and 16 males, age 5-77 yr (mean, age 36 yr). Disease inheritance patterns were defined by family history: 23 patients had sporadic disease, three had autosomal dominant disease, seven had autosomal recessive disease and one had X-linked retinitis pigmentosa. Tunnel vision (defined for the purposes of this study as >50% loss of visual field) was employed as the index of disease severity, and was found in 14 of the 24 patients for whom the information was available.

Sera were also obtained from 30 normal, healthy volunteers age 22-40 yr, mean age, 29 yr. A number of disease control groups were studied; these included eight patients with idiopathic uveitis or retinitis, 35 patients with Alzheimer's disease/senile dementia Alzheimer-type, 10 patients with multiple sclerosis, 11 patients with amyotrophic lateral sclerosis, and 10 patients with a history of multiple cerebrovascular accidents. Finally, sera were obtained for investigation from 37 disease-free first-degree relatives of 19 patients with retinitis pigmentosa and 8 spouses of probands.

### *Detection of anti-neurofilament protein antibodies*

TISSUE PREPARATION. All sera were screened for reactivity with heterologous neural tissue obtained from rat spinal cord. Sprague-Dawley rats were killed by decapitation, and the spinal cords were expelled whole by pressure of normal saline injected caudally. Lengths of cord were snap-frozen in liquid nitrogen-cooled 2-methyl-butane. Longitudinal cryostat sections were prepared from this tissue; 4-μm sections were fixed in methanol for 10 min at -20°C before fluorescence procedures, and 8-μm sections were fixed in acetone for 10 min at -20°C before silver impregnation procedures. Sections of human spinal cord obtained within 6 h of death from nonneurological cause were similarly processed.

INDIRECT IMMUNOFLUORESCENCE MICROSCOPY. Methanol-fixed sections were washed for 10 min with stirring in 0.15 M phosphate-buffered saline (PBS), pH 7.2. The tissues were then sequentially incubated with serum under test, diluted 1:10 in PBS, and fluorescein isothiocyanate (FITC)-labeled F(ab)<sub>2</sub> fraction of goat antiserum to human IgG F(ab)<sub>2</sub>, diluted 1:30 (fluorescein:protein [F/P] ratio, 2 μg: 1 mg; Cappel Laboratories, Cochranville, PA), with intervening washes in PBS. The sections were subsequently mounted in 70% glycerol in PBS, pH 7.8, and examined for fluorescence using a microscope (Universal; Carl Zeiss, Inc., Thornwood, NY) fitted for epi-illumination with a xenon 75-W light source and appropriate filter system. Photographs were taken using an automatic camera (Olympus Corp. of America, New Hyde Park, NY) with Tri-X film (Eastman Kodak Co., Rochester, NY). Antibody titers in sera giving positive results were determined by doubling dilution.

Control experiments included substitution of PBS for serum (con-

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1. Abbreviations used in this paper: FITC, fluorescein isothiocyanate; H-NF, high molecular weight protein subunit of human neurofilaments.

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jugate control). Positive control fluorescence was achieved by sequential reaction of tissue with monoclonal mouse anti-neurofilament 200,000-mol-wt subunit, diluted 1:20 (Enzo Biochem, Inc., New York, NY) and FITC-labeled goat antiserum to mouse IgG diluted 1:30 (Cappel Laboratories, F:P ratio, 2  $\mu$ g:1 mg).

All slides were coded before examination for fluorescence.

**SILVER IMPREGNATION.** Histochemical localization of neurofilaments in the rat spinal cord sections was achieved by silver impregnation as described by Luna (7). These sections were viewed under the Universal microscope by transmitted light from a halogen 200-W light source.

**REACTION OF SERA WITH CULTURED MURINE NEURONAL CELLS.** Of the sera examined for reactivity with rat spinal cord, 19 (10 positive, 9 negative) were selected, coded, and examined independently by indirect immunofluorescence microscopy for reactivity with cultured neurones, as described elsewhere in full (8).

### Characterization of antibodies to neural antigens

**IMMUNOGLOBULIN CLASS.** Positive sera were examined for reactivity with rat spinal cord as described above, except that immunoglobulin class-specific conjugates were employed (FITC-labeled goat anti-human IgG [ $\gamma$ -chain-specific] and anti-human IgM [ $\mu$ -chain-specific] both diluted 1:20 [Tago, Inc., Burlingame, CA]).

**ANTIBODY SPECIFICITY. Preparation of spinal cord neurofilaments.** A segment of human spinal cord was obtained at autopsy within 6 h of death from nonneurological cause. The dura was dissected off and a crude suspension containing neurofilaments was prepared from the remaining tissue by use of a modification of the method described by Liem (9). In brief, the tissue was minced, then homogenized in 0.85 M sucrose in a buffer containing 0.1 M NaCl, 10 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM EDTA, and 10  $\mu$ M phenylmethylsulfonyl fluoride (PMSF), pH 6.5. The homogenate was centrifuged at 9,000 g for 15 min in a centrifuge (model RC-5B; Sorvall Instruments, DuPont Co., Wilmington, DE) using an SS-34 rotor, to achieve flotation of myelinated axons. These axons were retrieved and demyelinated by homogenization in 1% Triton X-100 in the above buffer, followed by stirring of this homogenate overnight at 4°C. The homogenate was then centrifuged at 100,000 g for 2 h through a 0.85-M sucrose gradient in a centrifuge (model L265B; Beckman Instruments, Inc., Fullerton, CA) using an SW28 rotor. The pellet was resuspended in the above buffer at a protein concentration of 0.8 mg/ml and stored in aliquots at -20°C until use. The protein concentration of the preparation was determined by a protein assay kit obtained from Bio-Rad Laboratories (Richmond, CA), using purified transferrin as a standard. This crude preparation was designated "neurofilament preparation."

**Electrophoresis and immunoblot procedures.** The neurofilament preparation was subjected to SDS-PAGE in a 5-20% linear gradient of acrylamide using a discontinuous buffer system (10), and the gels were stained with Coomassie Blue or silver (11) or electroblotted onto nitrocellulose as previously described (12). The nitrocellulose transblots were cut into strips and processed in the usual fashion by blocking remaining binding sites with 3% Hipure Liquid Gelatin (Norland Products, Inc., North Brunswick, NJ) in PBS for 1 h. Individual transblot strips were then serially reacted with either monoclonal mouse anti-neurofilament 200,000-mol-wt subunit (diluted 1:200 in PBS; Enzo Biochem, Inc.), monoclonal mouse anti-neurofilament 160,000-mol-wt subunit (Boehringer Mannheim Biochemicals, Indianapolis, IN; diluted 1:50 in PBS) or human serum (diluted 1:50 to 1:100 in PBS) for 1 h. After intervening washes with PBS (3  $\times$  10 min) the strips were reacted with either peroxidase-labeled goat anti-mouse IgG or peroxidase-labeled goat anti-human Ig ( $\alpha$ ,  $\gamma$ , and  $\mu$ -chain-specific) (1:500 in PBS, Cappel Laboratories). After the final washes in PBS (3  $\times$  10 min) the blots were developed with 4-chloro-1-naphthol (HRP reagent; Bio-Rad Laboratories) as described by the manufacturer.

### Analysis of data

Possible associations between the presence of serum antibodies to neurofilament proteins in patients with retinitis pigmentosa and other factors

including disease severity, genetic form of disease, and abnormal cell-mediated immune function were examined by  $\chi^2$  analysis of data, with Yates' correction for continuity where appropriate. Association with patient age was examined by Student's *t* test for two means.

## Results

**Reaction of sera with rat spinal cord.** 17 of the 34 sera from patients with retinitis pigmentosa were found by indirect immunofluorescence microscopy to react with rat neural tissue. In contrast, only one of 30 normal sera, and none of eight sera from patients with other ocular diseases gave positive results. A variable proportion of neurological disease control sera also reacted with rat spinal cord, as shown in Table I. In addition, positive results were obtained with 12 of 37 sera from first-degree relatives of patients with retinitis pigmentosa, and with two of the eight sera from spouses of probands.

The distribution of fluorescence was that of spinal cord axons, most readily visualized in the area of the white matter (Fig. 1). The pattern obtained was similar to that produced by reaction of tissue with monoclonal antiserum to neurofilament 200,000-mol-wt subunit (Fig. 2) and was also comparable to that of neurofilaments visualized by silver impregnation (Fig. 3). Background fluorescence was minimal, and the conjugate controls were uniformly negative. Positive sera gave fluorescence at dilutions of 1:10 to 1:160.

**Reaction of sera with human spinal cord.** Immunofluorescence microscopy of human tissue was complicated by strong background fluorescence, which severely hampered recognition of axonal fluorescence. Attempts to prevent the unwanted fluorescence by use of nonfixed tissue or other fixatives were unsuccessful.

**Reaction of sera with mouse neuronal cells.** Double-blind independent study of 19 sera (10 positive, 9 negative), reacted with cultured mouse neuronal cells, revealed concordance of results in 18 of the 19 tested.

**Immunoglobulin class.** Examination of 12 positive sera from retinitis pigmentosa patients by indirect immunofluorescence microscopy of rat spinal cord using  $\gamma$ -chain- and  $\mu$ -chain-specific fluorescein conjugates revealed that antibodies reactive with axonal antigen were of the IgG class in all sera; in addition, two sera also contained IgM antibody, which gave the same distribution of fluorescence.

**Table I. Serum Antibodies to Neurofilaments in Retinitis Pigmentosa and Other Diseases**

Disease	No. sera tested	No. positive*	Percent positive
Retinitis pigmentosa	34	17	50
Idiopathic retinitis/uveitis	8	0	0
Alzheimer's disease/senile dementia Alzheimer-type	35	6	17
Multiple sclerosis	10	0	0
Amyotrophic lateral sclerosis	11	4	36
Multiple cerebrovascular accident	12	0	0
Normal controls	30	1	3

\* Sera were examined for reactivity with rat spinal cord by indirect immunofluorescence microscopy.



Figure 1. Immunofluorescence microscopy of neurofilaments. Cryostat section of rat spinal cord was serially reacted with serum, diluted 1:10, from patient with retinitis pigmentosa, and FITC-F(ab')<sub>2</sub> goat anti-human IgG F(ab')<sub>2</sub> diluted 1:30.  $\times 400$ .

**Serum antibody specificity.** Human spinal cord neurofilament preparations yielded several bands on SDS-PAGE, including those with apparent molecular weights of 200,000, 160,000, and 68,000 corresponding to high, middle, and low molecular weight neurofilament protein subunits (13) (Fig. 4 A). Immunoblotting with monoclonal antibodies to the 200,000-mol-wt and 160,000-mol-wt subunits of neurofilaments revealed reaction of the antibodies with the protein bands at expected molecular weights (Fig. 4 B, lanes 1 and 2). Blots performed using retinitis pigmentosa sera-positive by immunofluorescence microscopy on rat spinal cord revealed that the specificity of the serum antibody was for the high molecular weight subunit (apparent 200,000) of neurofilaments (Fig. 4 B, lanes 3 and 4). Similar examination of a positive Alzheimer's serum revealed the same antibody specificity (Fig. 4 B, lane 5). In a separate experiment, myosin (the 200,000-mol-wt standard) was electrophoresed alone on SDS-PAGE, and reacted with sera by immunoblotting: this gave negative results, indicating no reaction between serum antibodies and myosin contamination of the neurofilament preparation. Finally, 14 control sera-negative by

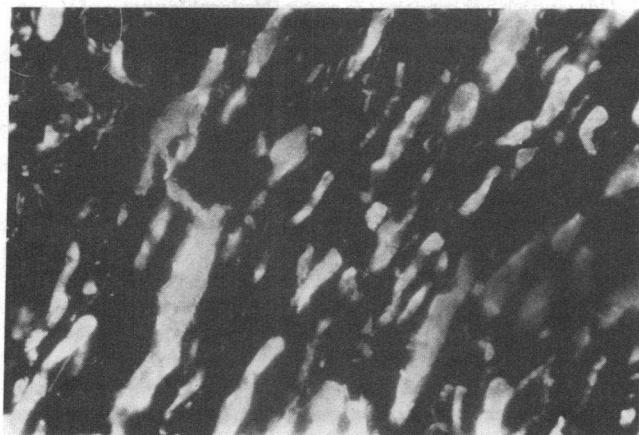


Figure 2. Immunofluorescence microscopy of neurofilaments. Cryostat section of rat spinal cord was serially reacted with monoclonal mouse anti-neurofilament 200,000 molecular weight subunit diluted 1:20, and FITC-goat antiserum to mouse IgG diluted 1:30.  $\times 400$ .

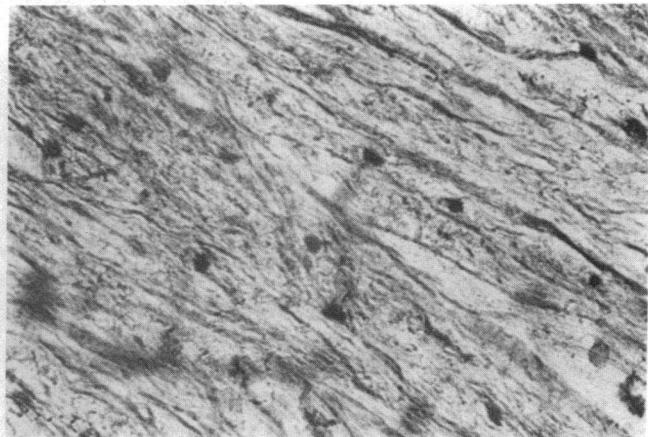


Figure 3. Bodian silver impregnation of cryostat section of rat spinal cord.  $\times 400$ .

immunofluorescence microscopy were examined by the immunoblot method. No reactivity with any of the three neurofilament subunits was detectable in any of these sera.

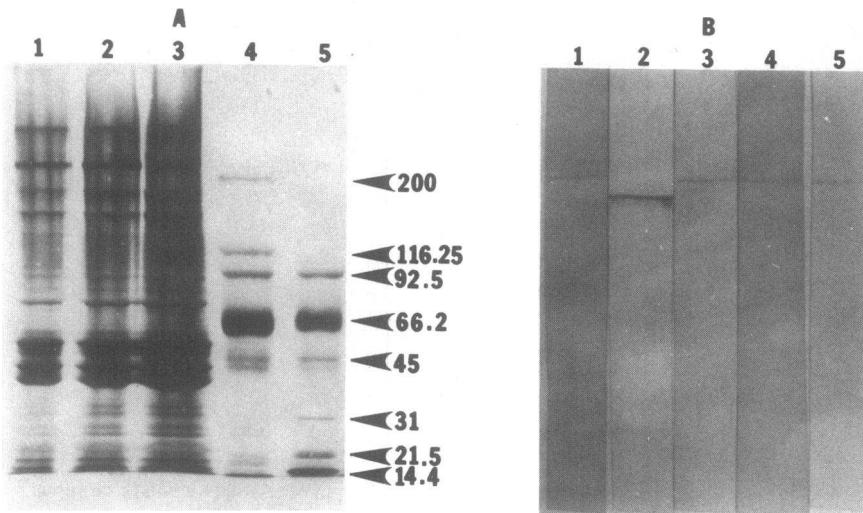
**Associations with other disease factors.** Statistical analysis revealed no association between presence of serum anti-neurofilament antibodies and other clinical factors including patient age, disease severity, genetic form of disease, and cell-mediated immune status (interactive T cell numbers, mitogen-dependent lymphokine production) (data on file).

## Discussion

A high proportion of patients with retinitis pigmentosa had serum antibodies reactive with heterologous axonal antigens and with specificity for the high molecular weight subunit of human neurofilaments. The antibodies were mostly of the IgG class, were present at low titers, and were detected only very rarely in healthy subjects or in patients with other ocular diseases.

Anti-neurofilament antibodies have been previously detected in the sera of patients with neurological disorders such as Alzheimer's disease and amyotrophic lateral sclerosis, with a frequency comparable to that reported here (8, 14, 15). Such antibodies have also been found in a high percentage of patients with subacute spongiform encephalopathies, such as Creutzfeldt-Jakob disease and kuru (8, 14, 15). These diseases, in which the etiologic agent is an unconventional or "slow" virus, are remarkable for their predilection for the nervous system, their long incubation period, and their apparent failure to elicit an immune response (16). Similar diseases in sheep and other animals include scrapie; in the context of the present study it is of particular interest to note the recent report of the induction of progressive retinal photoreceptor degeneration in hamsters inoculated with the scrapie agent (17). In retinitis pigmentosa, as in the natural and experimentally transmitted slow viral diseases, the major specificity of the serum anti-neurofilament antibody was for the H-NF (13, 15, 18). In those and other studies, H-NF was referred to as the "200-K neurofilament protein," because of its apparent molecular weight on SDS-PAGE: direct molecular mass determination has shown that this is an overestimation (19).

The data presented here are, in part, in conflict with those recently reported by Stefansson et al. (20). Those authors found serum antibodies to the 200,000 apparent molecular weight



**Figure 4.** (A) Silver stained SDS-PAGE of human spinal cord neurofilament preparation. (Lanes 1, 2, 3) neurofilament preparation at 16, 24, and 32 µg total protein, respectively. (Lanes 4 and 5) high and low molecular weight standards, respectively (molecular  $\times 10^{-3}$ ); myosin, 200,000 mol wt;  $\beta$ -galactosidase, 116,250 mol wt; phosphorylase B, 92,500 mol wt; bovine serum albumin, 66,200 mol wt; ovalbumin, 45,000 mol wt; carbonic anhydrase, 31,000 mol wt; soybean trypsin inhibitor, 21,500 mol wt; lysozyme, 14,400 mol wt (Bio-rad Laboratories). (B) SDS-PAGE immunoblots of neurofilament preparation. Transblots were reacted as follows: lane 1 was serially reacted with monoclonal mouse anti-neurofilament 200,000-mol-wt subunit, diluted 1:200, and peroxidase-labeled goat anti-mouse IgG was diluted 1:500; lane 2 was serially reacted with peroxidase-labeled goat anti-mouse IgG was diluted 1:500; lane 3 was serially reacted with peroxidase-labeled goat anti-human Ig, diluted 1:100, and peroxidase-labeled goat anti-human Ig was diluted 1:500. The blots were developed with 4-chloro-1-naphthol.

monoclonal mouse anti-neurofilament 160,000-mol-wt subunit, diluted 1:50, and peroxidase-labeled goat anti-mouse IgG was diluted 1:500; lanes 3 and 4 were serially reacted with sera from retinitis pigmentosa patients, diluted 1:50, and peroxidase-labeled goat anti-human Ig; lane 5 was serially reacted with serum from a patient with Alzheimer's disease, diluted 1:100, and peroxidase-labeled goat anti-human Ig was diluted 1:500. The blots were developed with 4-chloro-1-naphthol.

neurofilament subunit in 99% of both healthy subjects and patients with neurological diseases such as those included as disease controls in the present study. These results contrast particularly with our negative findings in normal control sera. These differences cannot be reconciled on the basis of differences in sensitivity of antibody detection, since the immunoblot techniques used were comparable and the same serum dilutions were employed. However, there were differences in both the source and methods of preparation of neurofilament proteins. In the present study, the degree of concordance of results obtained by two independent immunohistological methods and by immunoblot technique strongly substantiates previously reported evidence of an association between the presence of anti-neurofilament antibodies and disease states (8, 14, 15).

Serum antibodies reactive with cytoskeletal elements have been found in a wide variety of diseases (reviewed in 21). Intermediate filament antibodies were detected predominantly in rheumatic and viral diseases (21). For example, anti-vimentin antibodies and anti-cytokeratin antibodies were found in systemic lupus erythematosus, rheumatoid arthritis, and Sjogren's syndrome (22, 23); antibodies to vimentin were also detected in chronic active hepatitis (24). The transient appearance of antibodies to intermediate filaments has been described in several viral diseases including infectious mononucleosis (25), measles, mumps, and varicella (26). The majority of the antibodies described in these studies were of the IgM class, whereas the anti-neurofilament antibodies detected in the subacute spongiform encephalopathies and in the present study of retinitis pigmentosa were predominantly of the IgG class.

The trigger mechanism that results in production of antibodies to intermediate filaments in these diverse diseases remains unidentified. It is possible that, in general, such antibodies represent essential homeostatic mechanisms for removal of damaged or infected cells, and in certain instances the same antibodies may participate in further pathogenic processes. It is not known whether anti-neurofilament antibodies play a role in the disease process of retinitis pigmentosa. However, it is of interest to note that in mice, antibodies raised to the high molecular weight subunit of neurofilaments failed to recognize the retinal ganglion

cells and axons unless such cells were experimentally damaged (27). Furthermore, this reaction was demonstrable long before degeneration of neurones occurred.

The etiology of retinitis pigmentosa remains unknown. We have shown that a substantial percentage of patients possess serum antibodies directed against the high molecular weight component of neurofilaments. One other group of diseases in which this antibody specificity is found in such a large proportion of patients is comprised of the subacute spongiform viral encephalopathies. Interruption of axonal transport with pooling of neurofilaments and neural cell lysis has been suggested to be the stimulus for increased production of such antibodies in many central nervous system diseases (28). It is possible that similar processes may occur in retinal neurones in retinitis pigmentosa.

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