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Feasibility Study of Anti-Neurofilament Antibodies Detection by Indirect Quenching Fluoroimmunoassay

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Abstract

Background: Neurofilaments (NFs) are the main constitutes of intermediate filaments in neurons. They are composed of three subunits with heavey, medium and low molecular weight. Anti-neurofilament antibodies exist in serum of patients with some neurodegenerative diseases.

Materials and Methods: A fluoroimmunoassay has been developed for determining of antibodies against neurofilaments, using an anti-fluorescein serum and fluorescein-labeled NFs. Antibodies raised against bovine spinal cord NFs in rabbit and the labeled NFs are incubated with anti-fluorescein serum at room temperature.

Results: At high levels, binding of anti-neurofilaments (anti-NFs) to labeled NFs prevented subsequent binding of the anti-fluorescein to fluorescein groups, resulting in little change in the signals of the label. Conversely, at low level of anti-NFs the free fraction of the labeled NFs is available to be bound by anti-fluorescein, which markedly reduced fluorescence intensity of label. Thus, the fluorescence intensity of assay mixture directly reflects the amount of anti-NFs antibodies in the serum.

Conclusion: It is concluded that the availability of fluorescein-labeled NFs and antibody directed against fluorescein group permit measurement of anti-NFs antibodies in serum of neurodegenerative patients.

Introduction

Neurofilaments (NFs) are the major intermediate filaments of the most mature neurons and are found exclusively in axons [1]. They are composed of three subunits: NF-L (light), NF-M (medium) and NF-H (heavy), which is 68 kDa, 160 kDa and 200 kDa, respectively [2]. NFs are members of cytoskeleton proteins and together with microtubules and microfilaments increase structural integrity, cell shape, cell and organelle motility. The major function of NFs is control of axonal caliber and impulse conductivity along axon [3, 4]. Abnormal structures of NFs are associated with many human neurodegenerative disorders [5]. Moreover, antibodies against NFs have been found in serum of patient with some neurological disease [6-8]. These anti-neurofilament (Anti-NF) antibodies may be autoantibody and be involve in pathogenesis of disease or can be consequence of neuronal destruction and exposure of neurofilaments to immune system [9]. However, determination of Anti-NFs in serum may be used as a marker for diagnosis of neurodegenerative diseases.

The various methods, such as radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA) and western blot are used for detection of antibodies in biological fluids. RIA is a highly successful analytical method, but radiolabeled materials have limited half life and are health hazardous and often expensive. In addition, separation step of antibody-bound and free fraction of labeled antigen is a practical laborious and potential source of imprecision [10]. These disadvantages have therefore led to search for nonisotopic labels such as enzymes and fluorescence molecules. In ELISA and Western blot, enzymes are used as label. Fluoroimmunoassy (FIA) is one of the immunoassay methods which are used fluorescent labels. Fluorescent labels (fluorochromes) are compounds that absorb radiant energy of one wavelength and emit radiant energy of a longer wavelength [11]. Using fluorescein isothiocyanate (FITC) fluorochrome, indirect quenching non-separation fluorimetric method for a number of molecules have been developed based on the use of antibody directed against fluorescein, which bind to free labeled antigen and decrease its fluorescence. If antibodies against non labeled antigen exist in assay mixture prevent quenching of fluorescence of labeled group [12-14].

The aim of the present study was to evaluate feasibility of indirect quenching fluoroimmnoassay for detection of anti-NFs in serum using fluorescein-labeled NFs and antibody directed against fluorescein group as reagents.

Materials and Methods

This is a basic study that performed in order to evaluation of feasibility of anti-neurofilament antibodies detection in serum.

Chemicals: Fluorescein isothiocyanate, phenylmethyl sulfunylefluride (PMSF), sucrose, Tris-HCl, acrylamide, bis-acrylamide, Triton X-100, 2-mercaptoethanolamine, SDS, glycine, amoniumpersulfate, EDTA, EGTA, DEAE-sephadex A50-120, sephadex G25, bovine serum albumin (BSA), freunds adjuvant (complete and in complete) purchased from sigma (Poole, Dorset, UK).

Preparation of crude NFs: Fresh bovine spinal cord was removed immediately after slaughter of animal in slaughterhouse and transfer to laboratory on ice flask. Crude NFs were prepared according to Tokutake method [15]. Brifely, spinal cord was chopped into the consistency of a mince and 10 g homogenized for 2 min using a glass teflon homogenizer in 2.5 vol (1:2.5 w/v) of 50 mM Tris buffer pH 6.8 contaninig 2 mM EDTA, 2 mM PMSF and 0.5% Triton X-100 (buffer A). The homogenate was passed through screen of 0.2 µm pore size nylon mesh. The filtrate was centrifuge at 13000 g for 15 min at 4 ºC. The supernatant was discarded and the precipitate resuspended in 10 ml buffer A containing 0.9 M sucrose (buffer B) and centrifuge for another 15 min. The pellet was resuspeded in 10 ml 30 mM phosphste buffer pH 7.5 containing 6 M urea, 2 mM EGTA, 0.5 mM PMSF and 1% 2-mercaptoethanolamine (buffer C) and centrifuge at 75000 g for 45 min at 4 ºC. The supernatant was used for further purification of NFs on column chromatography.

Purification of Nurofilaments subunits: DEAE-sephadex A-50 column chromatography (1.5×20 cm) was equilibrated with buffer C. Thirty milliliters of supernatant obtained from previous step loaded onto the column at a flow rate of 9 ml/h. The column was washed with buffer C until no protein was detected by spectrophotometer at 280 nm. Then, neurofilament proteins were eluted using of buffer C containing 0.4 M NaCl. Fractions containing NF proteins were concentrated using sucrose in dialysis bag with cutoff 5 kD. The mixture was dialyzed against phoshphate buffer saline and the protein content measured by the method Lowry et al. [16].

SDS-PAGE and molecular weight analysis of purified NFs: Purifed NFs solution were mixed with equal volume of sample buffer contaning 125 mM Tris-base pH 6.8, 20% (w/v) glycerol, 10% SDS, 5% (v/v) 2-mercaptoethanolamine and 0.001% (w/v) bromophenol blue. Then, mixture was resolved by SDS-PAGE using a buffer containing 25 mM Tris-base pH 8.6, 250 mM glycine and 0.1% SDS. Stacking gel was 5% polyacrylamide in 125 mM Tris-base pH 6.8 containing 0.4% SDS and separation gel was 10% ploy acrylamide in 360 mM Tris-base pH 8.8 containing 0.4% SDS. Elecrophoresis was performed at constant voltage 200 V for 4 h and gel stained with coomassie blue dye. Molecular weight of neurofilament subunits was estimated by comparing their electrophoretic mobility with albumin, β-galactosidase and myosin as markers.

Preparation of Fluorescein-Labeled NFs: According to method of Nargessi et al. fluorescein-labelled NFs were prepared [13]. Purified NFs solution with concentration of 10 mg/ml in 30 mM phoshphate buffer pH 9 was added to equal volume of FITC solution (10 mg/ml in phosphate buffer pH 9) and mixture was stirred overnight at 4 ºC. Separating of labeled NFs from free FITC, was performed by gel filtration on a Sephadex G25 column (1.2×20 cm) and conjugate NFs eluted with phoshphate buffer pH 9 and stored at -20ºC.

Preparation of anti-fluorescein and Anti-NFs antisera: Two male albino rabbits (~2 kg) were subcutaneously immunized with NFs and two others with fluorescein labeled NFs (150 mg protein) emulsified in freund's complete adjuvant in total volume of 1.5 ml. A control rabbit received an emulsion of phosphate buffer in the adjuvant. The animals were boosted after one month and fifteen days next second injection; blood sample was taken from ear vein of animals and serum separated.

Anti-fluorescein dilution curve: Increasing dilutions of the anti-fluorescein serum were made in 100 mM sodium phosphate buffer pH 8.5 [12]. To 100 µl of each dilution was added 100 µl of labeled NFs solution (fluorescence intensity 560 in final volume 2 ml) and mix. After 30 min incubation at room temperature, the total volume increased up to 2 ml by phosphate buffer. Fluorescence intensity of mixture was determined using a Perkin-Elmer spectrophotofluorimeter with the excitation wavelength at 490 nm and emission wavelength at 520 nm. Anti-fluorescein titer was measured by its ability to quenching of labeled-NFs fluorescence.

Anti-NFs dilution curve: Increasing dilutions of anti-NFs serum in phosphate buffer pH 8.5 were prepared and to 100 µl of each dilution was added 100 µl labeled NFs solution (fluorescence intensity 560 in final volume 2 ml). After 15 min incubation at room temperature, 100 µl of 1:40 dilution of anti-fluorescein serum were added and after another 15 min incubation period, the total volume increased up to 2 ml using phosphate buffer. Fluorescence intensity of mixture was determined and anti-NFs antibodies titer was measured by inhibition of quenching of labeled-NFs fluorescence. In all experiments of fluorescence intensity measurement, a correction was made for the background signal, by a blank containing all reagents except labeled NFs. Results are representative of 6 distinct determinations.

Results

Purification of NFs subunits: Figure 1 show electrophoretic pattern of neurofilament proteins from DEAE-sephadex column chromatography. Lane M show molecular weight standards, up to down are myosine, β-galactosidase and bovine serum albumin. Lanes A-C demonstrate SDS-PAGE analysis of eluted NF proteins from DEAE-sephdex column. NF-L appeared approximately as same level with bovine serum albumin (68 kDa), whereas NF-M was upper than β-galactosidase (130 kDa) and NF-H had molecular weight of correspond with myosine (200 kDa) band. Lanes D and E show polyacrylamide gel electrophoresis pattern of crude neurofilament preparation obtained by high-speed centrifugation of spinal cord homogenate.

Anti-fluorescein dilution curve: In order to determine the appropriate dilution of anti-fluorescein serum in forming a mixed-antibody complex, dilution curve for this serum were prepared. Figure 2 shows that the quenching of fluroscence of labeled NFs by anti-fluorescencein serum decreased as the anti-fluorescein serum was diluted. Considering of fluorescence intensity of labeled NFs, a dilution 1:40 of the anti-fluorescein serum that cause appropriate quenching was chosen for anti-NFs dilution curve.

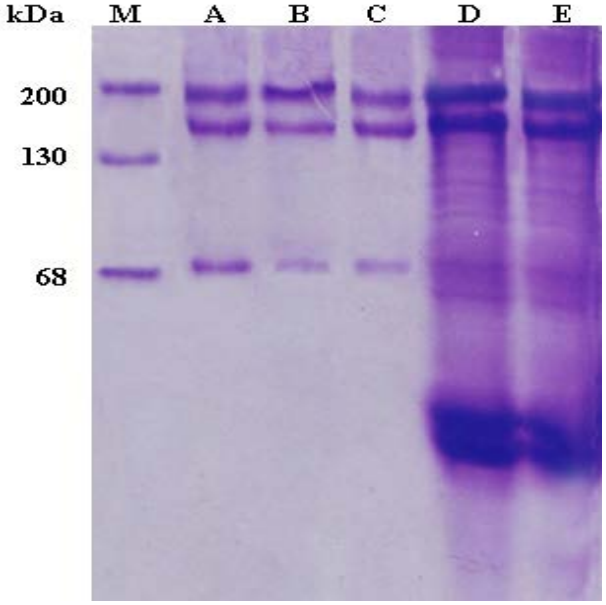

Figure 1. SDS-PAGE analysis of eluted neurofilaments from DEAE-sephadex column. SDS-PAGE was performed as described in materials and methods. Lane M: Protein standards, up to down are myosin (200 kD), β-galactosidase (130 kD) and bovine serum albumin (68 kD). Lanes A-C: neurofilament proteins eluted from DEAE-sephadex column; up to down are NF-H (200 kD), NF-M (160 kD), NF-L (68 kD). Lanes E-F: homogenate supernatant of enriched from NF proteins before chromatography

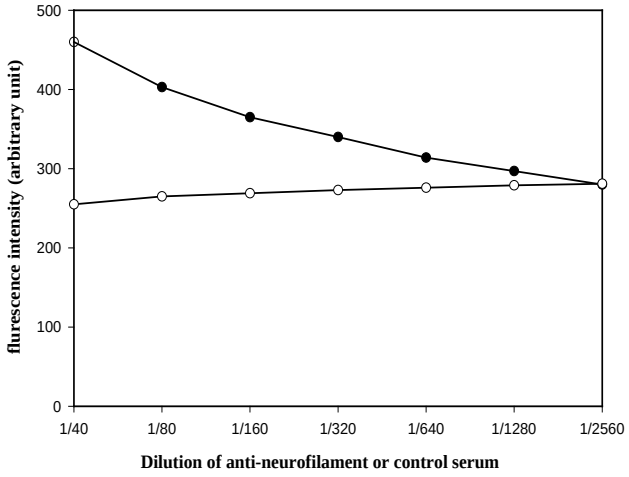

Figure 2. Anti-fluorescein dilution curve. Increasing dilution of the sera from control (○) or immunized rabbits with fluorescein-labeled NFs (●) were incubated with fluorescein-labeled NFs (fluorescence intensity 560 in arbitrary unit) for 30 min in room temperature and fluorescence intensities were measured as described in materials and methods. Each point in curve is representative of 6 distinct determinations

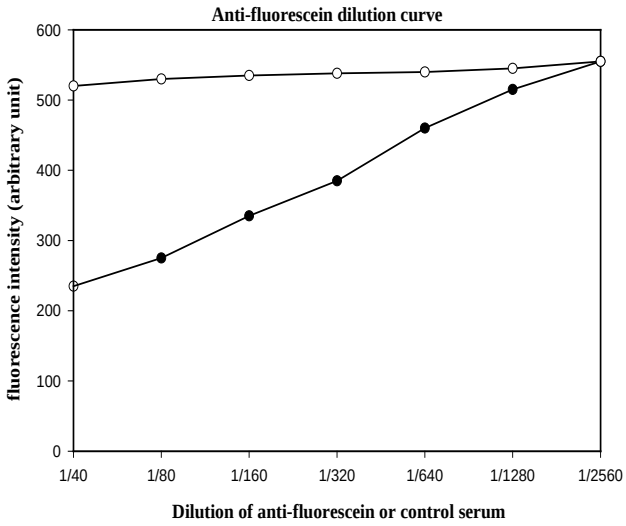

Figure 3. Anti-NFs dilution curve. Increasing dilution of the sera from control (○) or immunized rabbits with NFs (●) were incubated with fluorescein-labeled NFs (fluorescence intensity 560 in arbitrary unit) in presence of dilution of 1/40 of anti-fluorescein for 30 min in room temperature and fluorescence intensities were measured as described in materials and methods. Each point in curve is representative of 6 distinct determinations

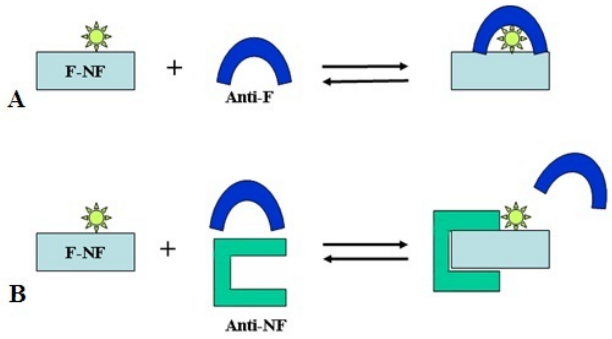

Figure 4. Diagrammatic representation of indirect quenching fluoroimmunoassay. Fluorescence of labeled NFs is quenched by anti-fluorescein serum (A). Addition of anti-NFs serum prevents quenching of fluorescence, probably by steric hindrance due to binding of anti-NFs antibodies to labeled NFs (B). F-NF: fluorescein neurofilament, Anti-F: anti-fluorescein, Anti-NF: anti-neurofilament

Anti-NFs dilution curve: Figure 3 shows that the presence of anti-NFs serum inhibit the quenching of fluorescence of labeled NFs by anti-fluorescein and the fluorescence intensity returned to the quenching level as anti-NFs serum was diluted. This curve will be useable as the standard curve, by which titer of anti-neurofilament antibodies in serum of neurodegenerative patients could be estimated.

Discussion

In the present study we developed one type homogeneous immunoassay method for detection of anti-NFs antibodies titers. This method was previously termed

as indirect FIA [17]. The basis of method is the quenching (decreasing) of fluorescence of fluorescein labeled protein by antibodies against fluorescein group and prevention of this quenching with antibodies to non-labeled protein that are added prior binding of anti-fluorescein [17]. It has been reported that this approach is technically simple, very high precision, short incubation period and in these regards is similar to RIA. Non-isotopic label and omission of separation step also circumvent the limitations of RIA [10]. In order to development of indirect quenching fluoroimmunoassay for estimation of anti-NFs antibodies, we produced anti-fluorescein serum by immunization of two rabbits with fluorescein isothiocyanate labeled NFs and anti-NFs antiserum prepared by immunization of two rabbits with non-labeled NFs. In flurometry protocol, as shown diagrammatically in figure 4, when of anti-fluorescein serum (termed quencher) added to fluorescein-labeled NFs, we observed quenching of its fluorescence (Fig. 4A). The quenching of fluorescence in our results is agreement with reports of other investigators [18, 19]. The prior addition of anti-NFs serum to labeled NFs prevents inhibition fluorescence by anti-fluorescein, probably due to steric hindrance (Fig. 4B). Thus, intensity of fluorescence in mixture will be proportional to anti-NFs antibodies. Meaning that if the amount of anti-NFs in assay mixture be low, fluorescein-labeled NFs remain in free form and their fluorescence can be quenched by anti-fluorescein serum that is added later. The indirect quenching fluoroimmunoassay is appropriate for detection large proteins but no small molecules [17]. Method was applied for detection human albumin, IgG, prolactin and anti-acethylcholine receptor antibodies [12, 14, 17]. The results of present study are in good agreement with those of these reports. In conclusion, if epitope specific and purified antibodies be available, indirect quenching fluoroimmunoassay can be substitute RIA and ELISA, because have not require harmful isotopic reagent and separation step that is necessary in RIA and activity of enzymes that use in ELISA is often modified when they bind to antibody.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of interest

The authors declare no conflict of interest.

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