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Opinion paper

Anticeramide antibody and butyrylcholinesterase in peripheral neuropathies

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ABSTRACT

Ceramide is a glycosphingolipid, a component of nerve and non neuronal cell membrane and plays a role in maintaining the integrity of neuronal tissue. Butyrylcholinesterase (BChE) is a multifunctional enzyme, its involvement in neurodegenerative diseases has been well established. Anticeramide antibody (Ab-Cer) and enzyme BChE have been implicated in peripheral neuropathies. The present study investigates whether there is an association between Ab-Cer and BChE activities and peripheral neuropathies. Patients included: human immunodeficiency virus associated peripheral neuropathy (HIV-PN, $n = 39$), paucibacillary leprosy (PB-L, $n = 36$), multibacillary leprosy (MB-L, $n = 52$), diabetic neuropathy (DN, $n = 22$), demyelinating sensory motor polyneuropathy (DSMN, $n = 13$) and chronic inflammatory demyelinating polyneuropathy (CIDP, $n = 10$). Plasma Ab-Cer was measured by indirect enzyme linked immune assay (ELISA) and BChE activity in plasma was measured by colorimetric method. Ab-Cer levels were significantly elevated in MB-L and DN as compared to healthy subjects (HS). BChE levels were significantly higher in MB-L and DN as well as in HIV and HIV-PN. There is no significant difference in either Ab-Cer or BChE levels in DSMN and CIDP. Elevated plasma Ab-Cer and BChE levels may be considered significant in the pathogenesis of neuropathies. The variation in concurrent involvement of both the molecules in the neuropathies of the study, suggest their unique involvement in neurodegenerative pathways.

1. Introduction

The pathogenesis of peripheral neuropathy (PN) is known to be orchestrated via multiple pathways. Key pathways could be: auto antibodies to nerve components, cytokines and enzymes involved in nerve damage [1–4]. Studies have shown that auto antibodies to nerve components play a role in peripheral nerve damage in HIV/AIDS [5], leprosy [6] and multiple sclerosis [7].

Ceramide is a glycosphingolipid, a component of nerves and non-neural cell membranes [8]. Ceramide plays a role in a variety of physiological functions including apoptosis, cell growth arrest, differentiation, cell senescence, cell migration and adhesion [9]. Anti-ceramide antibodies (Ab-Cer) have been implicated in a number of pathological conditions including cancer, neurodegeneration, diabetes, and inflammation [10]. Ceramide plays an important role in the neurodegeneration of Alzheimer's disease [11].

The enzyme butyrylcholinesterase (BChE E.C 3.1.1.8) is a serine hydrolase that catalyses the hydrolysis of esters of choline [12]. It is a multifunctional enzyme mainly regulating cholinergic transmission [13]. Its involvement in growth, differentiation and degeneration of nervous tissue has been well established [14]. BChE levels in cerebrospinal fluid samples have been used as a sensitive and specific marker of Alzheimer's disease and their plasma levels have been implicated in leprosy [15–17].

Abbreviations: PN, peripheral neuropathy, anticeramide, autoantibodies; *M. leprae*, *Mycobacterium leprae*; HIV, human immunodeficiency virus; HS, healthy subjects; MB-L, multibacillary leprosy; PB-L, paucibacillary leprosy; DN, diabetic neuropathy; DSMN, demyelinating sensory motor polyneuropathy; CIDP, chronic inflammatory demyelinating polyneuropathy; Ab-Cer, anti-ceramide antibodies; BChE, butyrylcholinesterase; ELISA, enzyme linked immunosorbant assay; OD, optical density; SPSS, statistical package for social sciences; ANOVA, analysis of variance; SD, standard deviation.

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The present study is an exploratory investigation of plasma levels of Ab-Cer and BChE, to determine their association with any peripheral neuropathy: HIV-PN, leprosy, DM, DSMN and CIDP.

2. Methodology

2.1. Patients

Thirty-six paucibacillary leprosy (PB-L), 52 multibacillary leprosy (MB-L), 39 HIV-PN, 22 diabetic neuropathy (DN), 13 demyelinating sensory motor polyneuropathy (SMN), 10 patients with chronic inflammatory demyelinating polyneuropathy (CIDP), 101 HIV positive individuals without neuropathy (HIV) and 113 healthy subjects (HS) were included in the present study.

Leprosy patients were recruited from the Dermatology department of Osmania General Hospital, Hyderabad. Based on clinical examination and slit skin smears they were classified as paucibacillary (PB) and multibacillary (MB) leprosy [18]. All leprosy patients had signs and symptoms of peripheral neuropathy with evidence of sensory and/motor nerve deficit as assessed by sensory testing and voluntary muscle testing.

Patients of HIV ($n = 101$) and HIV-PN ($n = 39$) were recruited from the outpatient of Nireekshana ACET, Hyderabad. All HIV-PN patients were screened for PN using Leeds Assessment of Neuropathic symptoms and signs (S-Lanss) & DN4 questionnaire [19,20]. Out of 39 patients, 34 patients complained of burning, numbness, pins and needles and tingling sensations and the remaining 4 patients showed all three sensations except for burning. The patients suspected of neuropathy were confirmed with electro-neuromyography (ENMG) as distal sensory polyneuropathy. These patients were on antiretroviral treatment, with stavudine (d4T) as one of the combinations. All HIV cases had a confirmed positive report from the Government Integrated Counseling & Testing Centre (ICTC).

Healthy subjects, DN, DSMN and CIDP patients were recruited from Nizam's Institute of Medical Sciences (NIMS) Hyderabad. The classification of neuropathies as DN, DSMN and CIDP was based on filament tests and quantitative sensory testing along with laboratory examinations like complete blood picture, fasting blood glucose and Vitamin B_{12}. CIDP cases additionally included cerebrospinal fluid analysis. All these cases were further confirmed through ENMG studies at NIMS. Samples collected from HS were from healthy blood donors who were screened for HIV and other blood borne infections mandatory according to the National Blood Transfusion guidelines criteria and were found to be negative. Questionnaire on Diabetes Mellitus, TB and other chronic illness were part of the screening programme for blood donation and those without any of the mentioned problems were considered healthy blood donors [21].

2.2. Plasma preparation

5 ml of blood was drawn using universal precautions and transferred to ethylene- diamine- tetra-acetic acid containing tubes. Plasma was separated by centrifugation in a microfuge and quickly frozen at −20 °C for these assays.

2.3. Ceramide enzyme linked immunosorbent assay (ELISA)

2.3.1. Materials

C-2 Ceramide (N-acetyl-D sphingosine; Sigma, USA), alkaline phosphatase conjugated goat anti-human polyclonal antibodies (IgG + IgA + IgM) (Abcam, UK). Para nitrophenylphosphate (pNPP, Bangalore Genei Pvt. ltd., India), polyoxyethylenesorbitan monolaurate (Tween 20), Phosphate Citrate buffer, Tris hydroxylmethyl aminomethane (Tris), flat bottom ELISA plates (Immulon II).

2.3.2. Method

Indirect ELISA technique as described by Vemuri et al. (1996) with minor modifications was used to measure ceramide antibodies [22]. Ceramide stock solution was dissolved in chloroform: methanol (5 mg/ml; 3:1 v/v) with further dilution in ethanol (0.5 mg/ml). Ceramide was dispensed in PBS (pH 9.6) at a concentration of 100 ng/100 μl to coat each well. Since lipids do not disperse homogeneously in aqueous PBS (pH 9.6), it was sonicated at amplitude of 40 for thirty minutes with a pulse of 6 s, which enabled uniform coating. The plates were sealed and incubated at 37 °C overnight. The unbound antigen was washed with PBS (pH 7.4) containing 0.25% Tween 20, 0.1% BSA in a Biorad ELISA microplate washer programmed for five cycle wash. After removal of the unbound antigen the plate was blocked with PBS pH 7.4 containing 2% BSA. The blocking step was followed by 5 wash cycles in wash buffer. The plasma samples were diluted to 1:100 and were added in duplicates and incubated for 1hour at 37 °C, which was followed by a five wash cycle followed by addition of anti-human polyclonal antibody conjugated to alkaline phosphatase (1:20,000 dilution). It was incubated for 2 h followed by five wash cycles using Biorad ELISA microplate washer. The last step was addition of substrate pNPP (1 mg/ml dissolved in 200 mM Tris and 2 mM $MgCl_2$). The time of incubation for color development was 15–30 min after which the reaction was terminated using 1 N NaOH. The optical density was measured at 405 nm using a Biorad ELISA microplate reader.

2.4. Butyrylcholinesterase assay

2.4.1. Materials

Butyrylcholinesterase was kinetically estimated by butyrylthiocholine potassium hexacyanoferrate (III) method. Butyrylthiocholine is hydrolyzed by cholinesterase to produce thiocholine in the presence of potassium hexacyanoferrate (III). The absorbance decrease is proportional to the cholinesterase activity of the sample [23]. The kit contains Reagent I – Buffer reagent & Reagent II – Butyrylthiocholine iodide reagent. 4 parts of Reagent I was mixed with 1 part of Reagent II to make a reagent diluent. 50 ml of 1 X Tris Saline was used. Flat bottom ELISA plates were used for this assay.

2.4.2. Method

15 μL of plasma sample was added to 150 μL of Tris Saline in a dilution plate to make a diluted sample. 100 μL of reagent diluent was added to an ELISA plate. 20 μL of diluted sample was added to it and immediately absorbance was read at 405 nm for every 1 min interval to obtain the optical densities. Delta O.D in linear range was averaged. Delta O.D × 62 = Cholinesterase activity in U/ml.

2.5. Statistical analysis

The optical density (OD) obtained from the samples of each group were expressed as mean ± SE. BChE activity was measured in U/ml. One way ANOVA was carried out with Tukey's post hoc analysis among the groups to analyze the significance of the levels using SPSS 16.1. A p value <0.05 was considered significant.

The study of concordance of Ab-Cer and BChE was also evaluated. Every subject in the study for concordance, had simultaneous evaluations of both Ab-Cer and BChE and Microsoft excel database was created. Concordance is defined as the concomitant occurrence of the two molecules into: both molecules high, both molecules low in an individual of the subject groups. The method of analysis was based on the mean value. High and low levels of