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The activity and expression of NTPDase is altered in lymphocytes of multiple sclerosis patients

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ABSTRACT

Background: Multiple sclerosis (MS) is a demyelinating neurological disease, which is presumed to be a consequence of infiltrating lymphocytes that are autoreactive to myelin proteins. ATP and adenosine contribute to fine-tuning immune responses and NTPDase (CD39) and adenosine deaminase (ADA) are important enzymes in the control of the extracellular levels of these molecules at the site of inflammation. We evaluated the activity and expression of NTPDase and adenosine deaminase (ADA) activity in lymphocytes from patients with the relapsing–remitting form of MS (RRMS).
Methods: This study involved 22 patients with RRMS and 22 healthy subjects as a control group. The lymphocytes were isolated from blood and separated on Ficoll density gradients and after isolation the NTPDase and ADA activities were determined.
Results: The NTPDase activity and expression were increased in lymphocytes from RRMS patients when compared with the control group ($p<0.05$). In addition, a decrease in ADA activity was observed in lymphocytes from these patients when compared to the control group ($p<0.05$).
Conclusions: The regulation of ATP and adenosine levels by NTPDase and ADA activities may be important to preserve cellular integrity and to modulate the immune response in MS.

1. Introduction

Multiple sclerosis (MS) is the most common chronic demyelinating disease of the central nervous system (CNS) and predominantly affects young adults, leading to permanent disability in a large proportion of patients [1,2]. It is now well accepted that MS is an immunologically mediated disease directed against CNS myelin or oligodendrocytes and that both cell and humoral immune mechanisms may contribute to demyelination and axonal damage [3,4]. However, the importance of these findings in the understanding of the pathogenesis and mechanisms involved in MS remains under investigation.

Over the last decade, it has been established that purinergic signaling contributes to the fine-tuning of inflammatory and immune responses [5,6]. The overwhelming evidence indicates that extracellular ATP acting through specific cell surface receptors is involved in proinflammatory functions such as stimulation and proliferation of lymphocytes and cytokine release [5,7]. On the contrary, its breakdown product, adenosine, exhibits potent anti-inflammatory and immunosuppressive action by inhibiting proliferation of T cells and secretion of cytokines [8–10].

Extracellular ATP and adenosine levels and the ensuing purinergic signaling can be dynamically controlled during inflammation by the action of enzymes expressed in immune cells [5]. NTPDase (CD39) is the membrane-bound enzyme involved in the breakdown of ATP and ADP to AMP which is sequentially hydrolyzed by 5′-nucleotidase to adenosine [11–13]. CD39 was first described as a B lymphocyte activation marker, however this ectonucleotidase is also expressed on natural killer (NK) cells, monocytes and activated T cells [5,14]. CD39 has powerful functions in the immune system including cytokine expression, cell–cell adhesion and cell proliferation and apoptosis via modulation of ATP levels [15]. Most importantly, alterations in the activity of this enzyme can be very important in immune diseases [16].

Adenosine deaminase (ADA) is considered to be a key enzyme in purine metabolism, catalyzing the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine respectively, closely regulating extracellular adenosine concentrations [17]. ADA is present in all cell types, but high ADA activity is present in the

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thymus, lymphoid tissues and peripheral lymphocytes. It has been demonstrated that this enzyme plays an important role in lymphocyte function and is essential for the normal growth, differentiation and proliferation of T lymphocytes [17,18]. The observation that ADA deficiency leads to severe combined immunodeficiency syndrome points to the physiological importance of controlling extracellular adenosine levels in the immune system [19].

Due to the fact that NTPDase and ADA control the levels of two potent and counteractive immunomodulatory molecules, ATP and adenosine, and that abnormal level of these enzymes may be associated with an autoimmune pathology; the aim of the present study was to evaluate NTPDase and ADA activities in lymphocytes from patients with the relapsing–remitting form of MS (RRMS).

2. Material and methods

2.1. Chemicals

Nucleotides and Trizma base were from Sigma (St. Louis, MO). Ficoll–Histopaque (Lymphoprep™) was purchased from Nycomed Pharma (Oslo, Norway). Antibodies for the flow cytometry analysis, R-Phycoerythrin (R-PE)–conjugated mouse anti-human monoclonal antibody against CD39 and fluorescein isothiocyanate (FITC)–conjugated mouse anti-human monoclonal antibody against CD45, were purchased from BD PharMingen Technical Data Sheet (San Jose, CA). All other reagents used in the experiments were of analytical grade and the highest purity.

2.2. Patients

The sample consisted of 22 MS patients and 22 healthy subjects as a control group. The diagnosis of MS was based on the McDonald criteria [20] and all patients had the RRMS form. All patients were receiving current immunosuppressive therapy or other medications. The general characteristics of the patients are shown in Table 1. All subjects gave written informed consent to participate in this study and the Human Ethics Committee of the Health Science Center from the Federal University of Santa Maria approved the protocol under number 23081.007854/2007-44. Twelve milliliters of blood were obtained from each patient during the remission phase of the disease and used for lymphocyte preparation and other biochemical determinations. The same procedure was carried out for the control group.

2.3. Isolation of lymphocytes from human blood

Lymphocytes were isolated from human blood collected with EDTA and separated on Ficoll–Histopaque density gradients as described by Böyum [21].

2.4. NTPDase enzyme assays

After lymphocyte isolation, NTPDase activity was determined as described by Leal et al. [22] where the reaction medium contained 0.5 mmol/l $CaCl_2$, 120 mmol/l NaCl, 5 mmol/l KCl, 6 mmol/l glucose and 50 mmol/l Tris–HCl buffer at pH 8.0, with a final volume of 200 µl. Twenty microliters of the intact mononuclear cells suspended in saline solution were added to the reaction medium (2–4 µg of protein) and pre-incubated for 10 min at 37 °C and incubation proceeded for 70 min. The reaction was initiated by the addition of substrate (ATP or ADP) at a final concentration of 2.0 mmol/l and stopped with 200 µl of 10% trichloracetic acid (TCA). The released inorganic phosphate (Pi) was assayed by the method of Chan et al. [23] using malachite green as colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the enzyme preparation after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate and the specific activity is reported as nmol Pi released/min/mg of protein.

Table 1
General characteristics of MS patients and healthy subjects.

	Control	MS patients
N	22	22
Women	17	18
Men	5	4
Age women (median, range)	40.6 ± 1.9, 28–51	45.6 ± 2.1, 26–57
Age men (median, range)	41.6 ± 3.8, 32–52	53.7 ± 5.7, 39–67
Disease duration (years)	–	5.89 ± 1.0
Use of interferon β (*n*)	–	17

Variables such as age and duration of disease are presented as mean ± SEM.

2.5. Flow cytometry analysis for CD39

Peripheral blood cells were incubated with anti CD39 and anti CD45 (20 µl/10^6 cells) for 25 min, erythrocytes were lysed with reagent FACS (fluorescent activated cell sorter) lysis and incubated again for 15 min in the dark. Cells were washed twice in PBS buffer (pH 7.4) containing 0.02% (W/V) sodium azide and 0.2% (W/v) BSA. The cells were then resuspended in PBS buffer (pH 7.4) and immediately analyzed by FACSCalibur flow cytometer using Cell quest software (Becton Dickinson, San Jose, CA) without fixation.

2.6. ADA enzyme assay

Adenosine deaminase activity was measured spectrophotometrically in lymphocytes by the method of Giusti et al. [24]. The reaction was started by addition of the substrate (adenosine) to a final concentration of 21 mmol/l and incubations were carried out for 1 h at 37 °C. The reaction was stopped by adding 106 mmol/l/0.16 mmol/l phenol-nitroprusside/ml solution. The reaction mixtures were immediately mixed to 125 mmol/l/11 mmol/l alkaline-hypochlorite (sodium hypochlorite) and vortexed. Ammonium sulphate of 75 umol/l was used as ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 650 nm. The specific activity is reported as U/l.

2.7. Protein determination

Protein was measured by the Coomassie blue method according to Bradford [25] using serum albumin as standard.

2.8. In vitro effects of drugs used in the treatment of patients with acute MS on NTPDase and ADA activities

The in vitro effects of interferon β, paracetamol and clonazepam on NTPDase and ADA activities were evaluated. Isolated lymphocytes from healthy subjects were incubated with different concentrations of these drugs in the medium reaction as previously described. All concentrations of interferon β, paracetamol and clonazepam used in vitro were based on the mean plasma values of the medications [26–29].

2.9. Statistical analysis

Data were analyzed statistically by the Student's *t* test for independent samples and one-way ANOVA followed by Duncan's multiple range test. Correlation was evaluated by Pearson's test. A $p<0.05$ was considered to represent a significant difference in all analyses used. All data were expressed as mean ± SEM.