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Nicotinic receptor activation negatively modulates pro-inflammatory cytokine production in multiple sclerosis patients

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ABSTRACT

Acetylcholine (ACh) and its receptors of muscarinic and nicotinic types are involved in the modulation of immune and inflammatory responses. In present work we have characterized the nicotinic receptors expression in PBMC of RR-MS patients and healthy donors (HD) and their ability to modulate pro-inflammatory cytokines. Here we report that the IL-1β e IL-17 levels are significantly increased in serum of RR-MS patients in respect to HD and that the PBMC stimulation with PHA caused a significant increase in pro-inflammatory cytokine levels both in RR-MS and HD subjects, with higher increase of protein release in RR-MS patients than in HD. The PBMC treatment with PHA plus nicotine produced a significant decrease of IL-1β e IL-17 both as transcript and as protein, confirming that the PBMC of the patients respond to the cholinergic stimulation more than PBMC of HD. By real time PCR and western blot analysis we have also demonstrated that in particular α7 receptor subtype appeared expressed at comparable levels both in RR-MS patients and HD. The PHA stimulation results to inhibit the α7 subunit expression while the nicotine causes a significant increase in α7 transcripts but only in MS patients. The data obtained highlight the role of α7 receptor subtype in the modulation of anti-inflammatory cytokines also in MS. Moreover the ability of nicotine to up-regulate the expression of α7 receptor subtype in RR-MS patients, indicates that nicotinic receptor stimulation may contribute to down-modulate the inflammation occurred in MS by a positive feedback control of its expression.

1. Introduction

The role of the cholinergic system in the pathogenesis of human diseases and its involvement in the modulation of the inflammatory states in cancers and in several autoimmune pathologies is largely emerging [1]. Different brain degenerative disorders are characterized by cholinergic deficiency that may be responsible of the cognitive disabilities and in the same time contribute to the dis-regulation of the inflammatory processes in the brain [2,3].

At least the status of the knowledge of cholinergic system activity in multiple sclerosis (MS) is very poor. Several observations, obtained in autoimmune encephalomyelitis (EAE) animal models, have demonstrated that acetylcholine (ACh) reduces the inflammatory state, as indicated by a consistent reduction of CNS lymphocyte infiltrates in brain. Moreover, chronic administration of acetylcholinesterase (AChE) inhibitors ameliorates clinical symptoms [4] as well as nicotine exposure significantly delays and attenuates inflammatory and autoimmune responses. Considering these evidences, the ACh presents in inflammatory sites, probably synthesized by immune cells, might play a relevant role in modulating inflammatory states also in MS. Although the data obtained in EAE model, currently there is not any evidence on the cholinergic system activity in MS patients. Recently, our group have demonstrated that the ACh levels appear significantly decreased both in serum and in cerebrospinal fluid of Relapse Remitting-Multiple Sclerosis (RR-MS) patients compared with subjects affected by neurological non-inflammatory diseases (OND), suggesting that a dis-regulation of acetylcholine levels may influence the inflammatory state in RR-MS patients [5].

Cholinergic receptors are expressed in immune cells and their stimulation by ACh directly produced by immune system cells, may contribute to modulate immune response in autocrine/paracrine manner [6,7].

The cholinergic anti-inflammatory effects are in general mediated by nicotinic receptors. In fact it has been demonstrated that nicotinic receptors inhibit, in the periphery, the proliferation of the auto-reactive T cells and alters the cytokine profile of T helper cells [8,9]. In CNS the nicotine exposure reduces number of dendritic cells, infiltrating

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monocytes and resident microglial cells and down-regulates the expression of MHC class II [10,11]. Moreover, in particular α7 nicotinic receptors are expressed also in microglial cells where their activation plays a role in the modulation of inflammation also in the brain, by affecting the release of pro-inflammatory agents [12].

In RR-MS patients the decreased levels of ACh are correlated to higher levels of pro-inflammatory cytokines [5]. In order to evaluate if the effects of the cholinergic system dis-regulation on pro-inflammatory cytokine production may be counteracted by nicotinic receptor stimulation, in the present work we have evaluated the ability of the nicotinic agonist nicotine, to modulate the synthesis and production of IL-1β and IL-17 in peripheral blood mononuclear cells (PBMC) of RR-MS patients and in healthy donors (HD).

Considering the relevant role of α7 receptor subtypes in the anti-inflammatory responses [8], we have also evaluated the expression of this nicotinic receptor subunit in RR-MS patients and HD and the ability of nicotine stimulation to modulate the expression of this receptor subtype.

The data obtained may contribute to increase the knowledge on role of nicotinic receptors in the modulation of the inflammatory state in MS patients.

2. Material and methods

2.1. Subjects

RR-MS patients followed at U.O. of Neurology Villa Serena Hospital (Citta Sant' Angelo, Pescara, Italy) were enrolled. Definition of RR-MS course was established considering clinical [13–15] and a Kurtzke's EDSS score ≤5.5 [16]. The diagnosis of RR-MS was confirmed by brain magnetic resonance imaging (MRI) with gadolinium. Pregnant or lactating patients as well as patients treated with immunosuppressive drugs were excluded. Patients with known sensitivity to gadolinium chelates or for inability to undergo MRI were not considered. Moreover patients with recent vaccination were also excluded. Before inclusion in the study, all patients were screened for infectious conditions. Healthy donors (HD) from the Transfusion Blood Bank Services of Chieti, Italy, frequency matched for age were enrolled. Mean age, mean disease duration, mean EDSS are shown in Table 1. None of the participants were smokers or vegetarians, none were known to be taking vitamin B12 or folates (including multivitamins) or prescribed drugs known to affect circulating homocysteine or nitric oxide concentrations. The study was approved by the Ethics Committee of Chieti e Pescara (Italy) and all patients and controls included, signed an informed consent.

2.2. Cell culture

Venipuncture was performed in the morning between 08:00 and 10:00. Serum was immediately stored at −20 °C after separation and a consecutive code number was assigned to each sample to ensure that all assays were performed in a blinded condition. Peripheral blood samples were collected into endotoxin-free EDTA tubes (Vacutainer, Becton Dickinson, NJ, USA) and transported to the laboratory for processing within 1 h of collection. PBMC were isolated by Ficoll–Hypaque density gradient centrifugation and re-suspended in complete culture media consisting of RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 25 mM Hepes buffer, 50 U/ml penicillin, and 50 mg/L streptomycin. PBMC (2×10^6) were immediately placed in polypropylene culture tubes (Bibby Sterilin Italia, Italy) in a volume of 1 ml of complete culture medium and incubated at 37 °C in 95% air and 5% CO_2 cell culture incubator for 24 h. Nicotine and phytohemagglutinin (PHA) were dissolved in culture medium and added to cell cultures at the final concentration of 10 μM and 20 μg/ml respectively. An equal volume of media was added to control samples to make normalized volumes. Supernatants were collected at the end of incubation and frozen at −80 °C until assay. Cell pellets were also kept at −80 °C until analysis. Cell viability in each culture was assessed by trypan blue dye exclusion. All reagents used were tested before use for endotoxin (<10 pg/ml; Associates of Cape Cod, Inc., Woods Hole, MA, USA) and mycoplasma contamination (General-probe II; General-probe Inc., San Diego, CA, USA) and found negative. The same batch of serum and medium were used in all experiments. All media and reagents were purchased from Sigma (Mi, Italy).

Table 1
Characteristics of the subjects involved in this study.

Variable	Control group (n = 15)	RR-MS group (n = 15)	p-value
Gender, n (%)			0.908[a]
Male	2 (13.3)	3 (20.0)	
Female	13 (86.7)	12 (80.0)	
Age (years), median (range)	40 (19–76)	38 (18–59)	0.680[b]
BBB impairment, median (range)	–	5.5 (2.8–15.2)	
Duration of disease (years), median (range)	–	5 (3–6)	
EDSS, median (range)	–	2.5 (0.0–6.0)	

BBB: Blood–brain Barrier; EDSS: Expanded Disability Status Scale.
[a] Fisher's exact test.
[b] Mann–Whitney U test.

2.3. IL-1β and IL-17 ELISA assay

Supernatants from the PBMC cultures were collected and frozen at −80 °C until the ELISA assay was performed at a single time, so as to avoid a "batching" effect, and preliminary analyses have showed that storage of supernatants for ≈ 8 months had no effect on cytokine levels. IL-1β and IL-17 release were evaluated by commercial ELISA kits (Thermo Fisher, Rockford, IL, USA) following the manufacturer's instructions. A standard curve was generated using known amounts of recombinant cytokine and IL-1β and IL-17 levels were then calculated plotting the optical density (O.D.) of each sample against the standard curve. All samples were analyzed in duplicate. The detection limit of the assay was ≤5 pg/ml for IL-17 and ≤1 pg/ml for IL-1β. The range of analysis was between 31.25 to 2000 pg/mL for IL-17 and 10.2 to 400 pg/mL for IL-1β. The intra- and inter-assay reproducibility was >90%. Duplicate values that differed from the mean by greater than 10% were not considered.

2.4. RNA extraction and mRNA expression analysis

Total RNA was extracted from PBMC using TRIzol reagent (Invitrogen, Life Technologies, Paisley, U.K.) and then digested with DNAse I (Ambion-Life Technologies Italia, Monza, Italy). The RNA concentration was estimated by measuring the absorbance at 260 nm (λ) using a Bio-Photometer (Eppendorf AG, Hamburg, Germany). RNA samples were kept frozen at −80 °C until use. Purified RNA was electrophoresed on a 1% agarose gel to assess the integrity of the purified RNA. One microgram of RNA was reverse transcribed into cDNA using a High Fidelity Superscript reverse transcriptase commercially available kit (Applied Biosystems, Foster City, CA, USA), in accord with the manufacturer's instructions. PCR was performed using specific primer pairs, following reported:

IL1 β: forward, 5′ TGAGGATGACTTGTTCTTTGAAG-3′;
reverse, 5′-GTGGTGGTCGGAGATTCG-3′
IL17 α: forward, 5′-CAACGATGACTCCTGGGAAG-3′;
reverse, 5′-GGGATTGGTATTGGTATTCCGG-3′
18S: forward, 5′ CTTTGCCATCACTGCCATTAAG-3′;
reverse, 5′-TCCATCCTTTACATCCTTCTGTC-3′

All polymerase chain reactions (PCRs) were performed in PCR-express cyclers (Hybaid, Heidelberg, Germany). To be within the