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Intrathecal chitotriosidase and the outcome of multiple sclerosis

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Activated macrophages are major effectors at all stages of lesion formation in multiple sclerosis (MS) brain. Here, we report that the macrophage enzyme chitotriosidase (Chit) is significantly elevated both in plasma and cerebrospinal fluid (CSF) of patients with MS as compared to healthy controls and other neurological patients ($P < 0.001$). Furthermore, the Chit activity in blood significantly associates with the MS clinical course (higher in secondary progressive relative to relapsing–remitting, $P = 0.01$) and the clinical severity as measured by Kurtkze's Expanded Disability Status Scale ($P < 0.001$). Also, we found that Chit activity is compartmentalized in the central nervous system of early MS patients and that its CSF/plasma quotient, in the presence of a preserved albumin quotient, correlates with the extent of future clinical deterioration ($r = 0.91$; $P < 0.001$). These findings confirm that innate immunity, here represented by Chit, is clinically relevant in MS and allows, if confirmed, reconsidering novel MS therapeutic strategies specifically aimed at this branch of the immune response. *Multiple Sclerosis* 2006; **12**: 551–557. www.sagepub.co.uk

Key words: cerebrospinal fluid; chitotriosidase; macrophage; multiple sclerosis; prognosis

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). Despite adaptive T- and B-cell antigen-specific responses being advocated at its pathophysiological basis [1], studies indicate that the innate immune response predominates in most MS lesion subtypes, involving both resident and infiltrating macrophages [2–4]. Despite this clear pathologic association, there are only a few relevant routine methods available for the examination of the macrophage function within the cerebrospinal fluid (CSF) of MS patients. Macrophage-derived products such as tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, metalloproteinase, nitric oxide (NO) and reactive oxygen species (ROS) show only a modest correlation with MS clinical activity, and they are not utilized in clinical routine [4]. Very recently, TNF-α was reported to differentiate relapsing from progressive MS patients through a complex multivariate analysis on mRNA levels of as many as 25 biomarkers [5].

The human chitotriosidase (Chit) belongs to the glycoside hydrolase family 18 and is highly secreted by fully activated mononuclear cells and, to a lesser extent, by polymorphonuclear leukocytes [6–8]. Little is known about the physiological role of Chit although it has been demonstrated that it hydrolyzes chitin, a structural and functional component of many insects and pathogens [6]. Variable Chit activity has been described in plasma of normal individuals and a mutation in exon 10 of its polymorphic CHIT1 gene causes an asymptomatic Chit activity deficiency [9,10]. Pathophysiological implications of the enzyme deficiency are not known.

Chit activity has been used as a marker of macrophage activation being highly elevated in plasma of chronic conditions with strong

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phagocyte activity such as the lysosomal Gaucher and Niemann–Pick diseases [11], beta-thalassemia [12], atherosclerosis and stroke [13,14], and in acute and chronic parasitic infections such as malaria [15]. Evidence of intrathecal Chit activity has been reported in Gaucher's disease [16] and preliminarily also in some chronic inflammatory neurological diseases, including MS [17]. However, association studies of peripheral and intrathecal Chit activities with clinical features of MS have been never conducted to date.

The aim of our study is to test, on a case–control basis, whether, as seen for other macrophage-derived markers, Chit activity is increased in blood and CSF of MS patients. Also, we aim at studying whether Chit peripheral activity may differentiate between distinct MS clinical courses (relapsing or progressive) and correlate with the extent of CNS damage as scored by the Extended Disability Status Scale (EDSS) [18]. In addition, on a distinct MS patients series, we test whether the intrathecal Chit production is eligible as a prognostic marker for future MS severity, as already demonstrated for immunoglobulins [1,2].

Subjects and materials

Case–control study

Patients with MS and other neurological diseases

Seventy-seven Sardinian patients (54 females and 23 males, ratio 2.2) diagnosed as having MS [19], were recruited from the MS Centre of the Institute of Clinical Neurology, University of Sassari. Relapsing–remitting (RR), secondary progressive (SP) and primary progressive (PP) MS were classified according to established guidelines [20]. Disability was measured according to Kurtzke's EDSS [18]. Mean age was 40.2 ± 10.1 years (range 21–59), average disease duration 8.9 ± 6.3 years (range 1–29) and mean EDSS score 3.7 ± 1.7 (range 1–8). Of the 77 MS patients, 53 have a RR course (mean EDSS 2.9) and 24 a progressive course (2 PP and 22 SP; mean EDSS 5.5). Plasma aliquots were stored at -80°C until Chit activity determination.

In addition, 58 frozen (-80°C) CSF samples were used for determination of Chit activity. Of these, 22 were from MS patients (mean age 38.6 ± 7.8; mean disease duration 10 ± 4.8; mean EDSS 3.8 ± 1.5) and 36 from patients with other neurological diseases (OND), mean age 45.3 ± 8.2, including subarachnoid haemorrhage (5 patients), headache (5), cerebral venous thrombosis (5), paraesthesia of undetermined origin (5), amyotrophic lateral sclerosis (5), alcoholic and other toxic polyneuropathies (5), head trauma (3), acute myelitis of undetermined origin (2) and trigeminal neuralgia (1). With the known increased Chit level in atherosclerotic and stroke patients given in the literature [13,14,17], these two OND categories have been ruled out from the study.

Healthy controls

Three-hundred and nine (309) healthy unrelated subjects (mean age 50.7 ± 12.4, range 20–68) of the general population served as control for the plasma determination of Chit. All participants were interviewed for the absence of chronic disorders, psychiatric and neurological diseases.

All patients and controls gave informed consent to participate in the study according to the Helsinki declaration.

Observational study in early MS patients

Plasma and CSF were simultaneously collected for diagnostic purposes from a new series of consecutive neurological patients with acute CNS episodes highly suspected of demyelinative origin. Of these, 23 patients (18 women and 5 men, mean age 28.6 ± 7.8) eventually developed definite MS [19] and were selected for the study. Selection criteria were either the presence of at least two oligoclonal IgG bands (OCB) in the CSF or an IgG index above 0.7, which indicates an intrathecal IgG production [21]. The presence of blood–brain barrier (BBB) integrity, as calculated by the normal CSF/serum albumin quotient (Table 1) was also an inclusion criterion. Patients were subjected to a comprehensive neurological examination with EDSS score [18] calculation every 6 months.

Standard CSF examination

CSF was obtained by lumbar puncture and standardized CSF cell counting performed at study entry. CSF aliquots were immediately centrifuged at 10 000 rpm and the supernatant frozen at -80°C. IgG index (CSF IgG/serum IgG):(CSF albumin/serum albumin) was calculated and OCB detected to disclose intrathecal IgG production. To this purpose, unconcentrated CSF together with diluted plasma have been examined by agarose isoelectric focusing combined with immunoblotting and peroxidase staining. Oligoclonal bands were considered to be present (OCB+) when demonstrated in CSF only and at a number of at least two [19].

Table 1 Clinical and demographic features of the MS patient series in the observational study

	Chit level		CSF/plasma quotient	
	CSF	Plasma	Chit	Albumin
Median	15	20	0.5	0.0031
25th percentile	5.5	18	0.3	0.003
75th percentile	38.5	35.5	1.5	0.004

	EDSS		Demography	
	Entry	Follow-up	Age	Follow-up duration
Mean	1.5	3.6	28.6	4.9
±SD or range	±0.5	±1.5	(20–46)	(2.5–8)
Median	1.5	3.5	25	5
25th percentile	1	2.5	23	4
75th percentile	2	5	32.5	5.8

Chit level and Chit quotient are expressed as nmol/mL per h; albumin quotient is expressed as mg/dL. Age and follow-up duration are expressed as years.

Chit activity determination

Measurement of enzymatic Chit activity was performed in duplicate as already described in previous papers [12,15]. Briefly, Chit activity was measured in plasma and CSF by incubating 5 μL of undiluted plasma or 30 μL of CSF with 100 μL of a solution containing 22 μmol/L of the fluorogenic substrate 4-methylumbelliferyl-beta-D-N,N′,N″-triacetyl-chitotriose (Sigma Chemical Co.) in 0.5 M citrate–phosphate buffer pH 5.2, for 15 min at 37°C, as originally described by Hollak *et al*. [11] The reaction was stopped by using 2 mL of 0.5 mol/L Na_2CO_3–$NaHCO_3$ buffer, pH 10.7. The fluorescence of formed 4-methylumbelliferone was read on a Hitachi 2500 fluorimeter, on 365 nm excitation and 450 nm emissions. Chit activity was expressed as nanomoles of substrate hydrolyzed per millilitre per hour (nmol/mL per h). This reaction was specific as the effect of lysozyme, which has some catalytic activities, or of another chitinase, acidic mammalian chitinase (AMcase), was excluded with this substrate [11]. Moreover, the activity of each individual person was stable in the same sample and in time after repeated measurement. The large experience of operator and the simultaneity of determinations excluded processing artefacts. Patients with plasma Chit activity below 2.5 nmol/mL per h were considered as Chit deficient and excluded from this study.

Statistical analysis

The management of the data was automatically performed by using the SigmaStat 3.0 software. Chit levels were not normally distributed and for this reason the median and the interquartile range (IQR: 25 percentile or 1st quartile and 75 percentile or 3rd quartile) were used. The Mann–Whitney Rank Sum test for non-parametric variables and *r* Spearman correlation coefficient were used for statistical analysis; significance was conventionally established for P values <0.05. To better analyse the relation between the Chit level and the EDSS score, a multiple linear regression analysis was carried out. A correlation analysis between the main variables (Chit in the CSF, Chit in plasma, EDSS at study entry, EDSS at follow-up, patient's age) was first performed.

Results

Case–control study

Chit activity in plasma

The median Chit plasma level in MS patients was 27 nmol/mL per h (IQR 19.7–36.5) and 6.4 in HC (IQR 3–10), the difference being highly significant (Mann–Whitney test, $P<0.001$; Figure 1a). MS patients were stratified according to their EDSS score: from low to moderate (EDSS 0–3.5; 40 patients) and from moderate to severe disability (EDSS > 4; 37 patients). Chit was significantly higher in the group with higher EDSS (39.6, IQR 22.4–44) as compared to that with lower EDSS (19.7, IQR: 8–23.4; Mann–Whitney test $P<0.001$; Figure 1b).

As the high disability group was mainly composed of progressive patients (both SP and PP, 61%) and the low disability group mainly of RR patients (95%), we evaluated the possible different Chit activity in the two MS courses. The group of 24 progressive MS patients (SP and PP; mean EDSS 5.58) had higher mean Chit activity (36, IQR 15.4–45) as

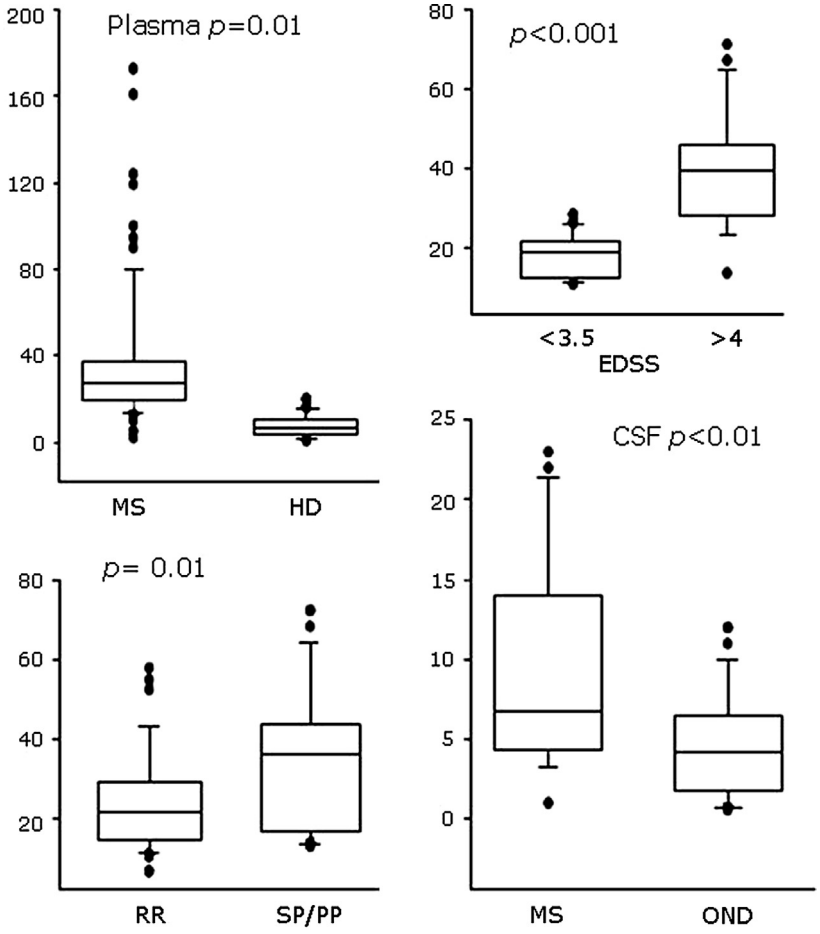

Figure 1 Case–control study: Chit activity differentiates MS from healthy and other neurological disease subjects both in plasma and in the CSF. Chit activity is significantly higher in more disabled and progressive MS patients. (a) Median Chit level expressed as nmol/mL per h on the *y* axis is significantly elevated in plasma of MS patients (77 subjects) as compared to matched healthy controls (HC; 309 subjects). Chit level is also higher in plasma of more disabled (b) and progressive (c) MS patients; (d) the CSF of MS patients (22 subjects) contains higher levels of Chit activity as compared to patients with other neurological diseases (OND; 36 subjects). Mann–Whitney test *P* values are indicated.

compared to the 53 RR patients (23.7, IQR 11–31.5; mean EDSS = 2.98; $P = 0.01$, Figure 1c).

Chit activity in CSF

The average Chit activity in the CSF of 22 MS patients (13.9, IQR 3.9–19 nmol/mL per h) was significantly higher as compared to the 66 OND controls (4.2, IQR 1.9–6.5; $P < 0.001$; Figure 1d).

Furthermore, Chit activity and EDSS were directly correlated both in plasma ($r = 0.46$, $P < 0.01$) and in the CSF, the direct relationship being stronger in the latter ($r = 0.7$, $P < 0.0001$).

Observational study

Data are summarized in Table 1. In the cohort of 23 early MS patients the median Chit plasma level was 20 (IQR 18–35.5). As for the CSF, median Chit activity was 15 (5.5–38.5).

MS patients were followed-up for an average 4.9-year period (range 2.5–8 years). The initial (MS onset) and the final (follow-up) EDSS scores of the individual patients were compared to the CSF and plasma Chit activity level as shown in Table 2. EDSS score and CSF Chit activity were significantly correlated at follow-up ($r = 0.73$, $P < 0.01$) but not at disease onset ($r = 0.39$, $P = 0.07$). EDSS and plasma Chit activity were also significantly correlated at follow-up ($r = 0.54$, $P = 0.01$) but not at disease onset ($r = 0.30$).

Given the normal CSF/serum albumin quotient of our patients, we consider the CSF Chit activity to be of intrathecal and not of peripheral origin (Figure 2). This allowed us to calculate a so-called Chit Quotient (CSF Chit/plasma Chit) and to compare the initial and final EDSS score of the

Table 2 Correlation between clinical and demographic features of the MS patient series as presented on Table 1

Variables		Statistics	
x	*y*	*r*	*P*
Chit quotient	Albumin quotient	−0.12	ns
Chit quotient	EDSS entry	0.24	ns
Chit quotient	EDSS follow-up	0.91	<0.001
CSF Chit	Age	0.32	ns
CSF Chit	EDSS follow-up	0.73	<0.01
Plasma Chit	EDSS follow-up	0.54	0.01
Plasma Chit	Age	0.21	ns

Correlation coefficient *r* and significance *P* values are indicated; ns, not significant.

individual patients with both OCB number (no correlation) and Chit quotient at the time of CSF withdrawal. Given the known influence of age on Chit activity level,[8] we performed a correlation analysis between plasma and CSF Chit activity and age. The main results are indicated in Table 2. Briefly, Chit Quotient was correlated with EDSS at follow-up ($r = 0.91$, $P < 0.001$) but neither with the initial EDSS nor with albumin quotient (Figure 1). The patients' age has an influence on CSF Chit ($r = 0.32$, $P < 0.08$) but not on plasma Chit. Obviously, the age at onset also influences the final EDSS ($r = 0.57$, $P < 0.03$), the latter being also influenced by the disease duration ($r = 0.7$, $P < 0.01$).

Regression analysis

With the aim of better analysing the influence of patients' age, EDSS at entry and at follow-up on the Chit activity level within the CSF, a regression analysis has been carried out. Preliminary correlation analyses are indicated in Table 2. Then, all the variables were entered (EDSS at entry, EDSS at follow-up and patients' age) as independent variables: the regression coefficients for EDSS at follow-up were associated with high significance, those of age and initial EDSS were not significant. In this model, the coefficient of determination was 0.93 (adjusted $R^2 = 0.86$; $P < 0.001$), meaning that 86% of Chit total variability is explained by the variable 'EDSS at follow-up', which is the most statistically significant positive predictor of Chit.

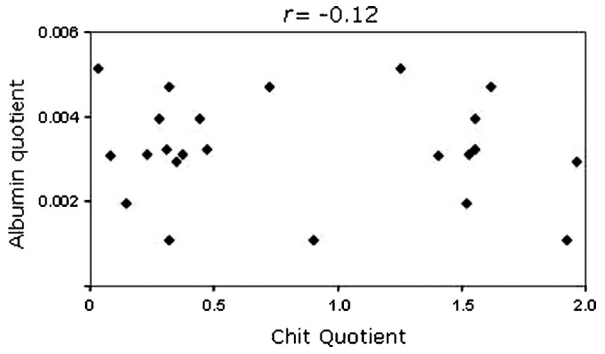

Figure 2 Observational study: the CSF Chit activity in MS patients is of intrathecal and not of peripheral origin. The presence of blood–brain barrier integrity has been calculated by the albumin quotient (mg/dL on the *y* axis: CSF albumin/serum albumin, see also Tables 1 and 2). This allowed us to calculate a so-called Chit quotient (nmol/mL per h on the *x* axis: CSF Chit/plasma Chit) which correlates with MS clinical severity (see Table 2) but not with the albumin quotient ($r = -0.12$, see also Table 2).

Discussion

Understanding of the burden of macrophage-mediated immunity in the pathogenesis and clinical evolution of MS may, in principle, allow the development of new diagnostic and therapeutic strategies. In the present study we report confirmational evidence that the macrophage-derived enzyme Chit is significantly elevated in MS and, for the first time, that its plasma and CSF level parallels the degree of clinical MS deterioration. Also, in agreement with observations on OND [16,17], we found that Chit activity is compartmentalized within the CNS. In addition, we found for the first time that this intrathecal Chit activity better correlates with the extent of CNS damage than the previously proposed macrophage-derived markers [4,5]. These salient points require a discussion in the light of the current knowledge of MS pathology and Chit functions.

In physiological conditions Chit production is influenced by demographic factors including age and race [8,9,13,14]. In pathological conditions Chit elevation is demonstrated in acute and chronic systemic and neurological diseases characterized by high macrophage activity, including MS [9–17].

In this regard, there are several links between macrophages and MS. Depletion of macrophages leads to the suppression of experimental acute encephalomyelitis (the animal model for MS) and infiltrating macrophages within the MS plaques display an activated phenotype with expression of inducible NO and MRP8/14 [4]. The presence of macrophage is a common denominator in at least three out of four patterns of MS lesion [2,3] and macrophages may contribute to myelin and axonal damage through a group of potentially neurotoxic factors [4,22]. Macrophages are major effectors in myelin removal from the axon, even in the early stages of MS lesion formation, and a local gradual transformation of microglia into activated macrophages has been described, perhaps reflecting a continuous antigen stimulation within the MS brain environment [23]. We do not underestimate

the historically important role of an adaptive immune response in MS pathogenesis, recently confirmed by the presence of a compartmentalized B-cell response within the CNS [1]. Furthermore, IgM OCB directed towards myelin antigens are described as an unfavourable prognostic marker in MS, whilst the routine IgG OCB merely constitutes a static hallmark of the disease [24]. These IgM antibodies may exacerbate MS not only by inducing a complement-dependent demyelination but also by enhancing myelin phagocytosis by macrophages and microglia via Fc and complement receptors [1,3,4,24]. In this light, our findings confirm that the role of macrophages in MS phenomenology is still important and allows a proposal of Chit determination in CSF and plasma as a diagnostic and monitoring method in a MS laboratory.

Chit activity is compartmentalized in MS because its CSF production is unrelated to its plasma level. At a cellular level, this enzymatic activity realistically derives from infiltrating macrophages resident microglia. However, cells might also be induced to produce Chit by virtue of their gradual phenotypic transformation into activated macrophages [23]. Chit elevation, besides representing a specific marker of activation of compartmentalized macrophages, has the advantage of being prognostically associated with a future severe MS course.

The increased levels in plasma and the correlation with EDSS are interesting, as plasma markers would be easier in routine patient monitoring. Indeed, EDSS scores reflect cumulative CNS damage over time, perhaps influenced by additional factors other than initial Chit activity; however, dynamic variation of Chit activity could be predictive of EDSS change in the long run. Thus, longitudinal studies are warranted to correlate the plasma Chit level with MS activity as documented by serial gadolinium-enhanced MRI.

Other points remain to be clarified. When and why do infiltrating or perhaps resident macrophages such as microglia activate their gene to produce Chit? Also, does Chit have a direct influence on the CNS damage occurring in MS or does it simply reflect an epiphenomenon? We cannot argue for any such hypotheses yet. Our results indicate that Chit activity, a hallmark of an ancestral innate macrophage response, could be a novel marker of MS clinical deterioration and of the intrathecal macrophage/microglia activation occurring in MS phenomenology. Its plasma and, to a larger extent, CSF level better correlates with the extent of CNS damage as compared to the previously proposed macrophage-derived markers such as TNF-α, IL-6, IL-1, NO and ROS. The innate immunity in MS, here represented by Chit, may be clinically relevant and induces a reconsideration of, if confirmed, novel therapeutic strategies specifically aimed at this branch of the immune response.

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