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Evaluation of the humoral response against mycobacterial peptides, homologous to $MOG_{35–55}$, in multiple sclerosis patients

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ABSTRACT

Bacillus Calmette-Guérin (BCG) and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) have been associated with multiple sclerosis (MS). Clinical data indicates that BCG vaccination exerts anti-inflammatory effects in MS; conversely, MAP is thought to be one of the possible infectious factors responsible of MS through a molecular mimicry mechanism.

A peptide-based indirect ELISA was used to detect antibodies against the encephalitogenic myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ epitope, and two mycobacterial peptides sharing sequence homology with the latter: $MAP\_2619c_{352–361}$/$BCG\_1224_{355–364}$ and $BCG\_3329c_{64–74}$. Among 40 MS patients and 39 healthy volunteers included in the study, only $MOG_{35–55}$ was capable of inducing a significantly higher humoral response in MS subjects compared to controls. Indeed, 11 out of 40 MS subjects (27.5%) and only 2 out of 39 controls (5%) were antibody-positive for $MOG_{35–55}$ ($p = 0.01$, AUC = 0.65).

These findings strengthen the importance of $MOG_{35–55}$ in MS pathogenesis. The MAP and BCG MOG-homologues epitopes investigated were not recognized in MS patients. Overall, the results allow us concluding that sharing homology of linear epitopes is necessary but not sufficient to induce antibody-mediated cross-reactivity.

1. Introduction

Multiple sclerosis (MS) is an autoimmune-mediated inflammatory disease affecting mainly the white matter but also the grey matter of the central nervous system [1]. T cells, B cells and autoantibody play a key role in MS-related demyelination [2]. MS etiology is generally thought to be the consequence of interactions between genetic and environmental factors, resulting in a dysregulation of the immune system [3]. Concerning the environmental triggers, there is increasing evidence that mycobacteria may play a role in MS pathogenesis [4].

A number of studies proposed the molecular mimicry theory to explain how *Mycobacterium avium* subspecies *paratuberculosis* (MAP) might trigger an autoreactive immune response associated with MS [4–6]. This response could involve the activation of autoreactive T cells due to the cross-reactivity between self and bacterial epitopes during MAP infection. Of note, MAP has been also associated with other human autoimmune diseases such as Crohn's disease, Type 1 diabetes (T1D) and Hashimoto's thyroiditis [7–9].

Concerning T1D, several works hypothesized that molecular mimicry between MAP proteins and human proteins may be at play in triggering the autoimmune response responsible in turn of the destruction of pancreatic β cells [8,10]. Indeed, it was proposed that the high degree of homology found between human glutamic acid decarboxylase 65 and MAP heat shock protein 65 (44% overall identity) [10], and the presence of highly conserved regions (boasting 41.2% aminoacid identity) in human zinc transporter 8 and MAP3865c [8,11] lend support to the hypothesis which ascribe at MAP a role in triggering autoimmunity in T1D subjects. The presence of antibodies (Abs) against these two T1D related autoantigens and against the respective MAP homologous proteins corroborated the aforementioned hypothesis. On the other side, it was demonstrated that systemic infection with *Mycobacterium bovis* bacille Calmette-Guerin (BCG) could suppress autoimmune responses in experimental autoimmune encephalomyelitis (EAE), the animal model of MS [12]. It was showed that BCG induces apoptosis, which deletes $CD4^+$ T cells in the periphery and reduces autoreactive T cells in the CNS [13]. Recently, it has been reported that BCG vaccination is associated with significantly reduced development of lesions in MS compared to placebo [14].

Although these bacteria have been associated with MS, at present it is not known if mycobacterial infections can be beneficial or harmful for MS.

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EAE can be induced by myelin oligodendrocyte glycoprotein (MOG)-specific encephalitogenic T-cells. In this model, mice are immunized with MOG_{35-55} peptide in complete Freund's adjuvant containing killed *Mycobacterium tuberculosis*, to develop inflammation and axon damage [15]. MOG, is one of the major antigens recognized by T cells and a target of demyelinating autoantibodies [16], especially MOG_{35-55} is an encephalitogenic peptide capable of inducing strong T and B cell responses in several variants of EAE [15].

After performing an *in silico* analyses, two mycobacterial epitopes sharing linear homology with MOG_{35-55} have been identified: MAP_2619c$_{352-361}$ identical to BCG_1224$_{355-364}$ and BCG_3329c$_{64-74}$, deriving from MAP and BCG, respectively.

The present study was undertaken to explore the relationship between MS disease and the presence of anti-MOG_{35-55} Abs in MS sera; moreover, we aim to establish whether these mycobacterial homologues epitopes are capable of eliciting a strong humoral response in MS patients and, eventually, if they are able to be cross recognized by anti MOG_{35-55} Abs.

In order to achieve this goal, a peptide-based indirect ELISA was developed aiming at detecting Abs against human MOG_{35-55}, MAP_2619c$_{352-361}$/BCG_1224$_{355-364}$ and BCG_3329c$_{64-74}$ epitopes in the peripheral blood of 40 MS patients and 39 healthy volunteers.

2. Materials and methods

2.1. Subjects

This study included 40 MS patients (F/M = 25/15; mean age ± SD was 37.6 ± 11.0) and 39 healthy controls (HCs) (F/M = 25/14; mean age 37.9 ± 6.5). All subjects fulfilled the revised McDonald diagnostic criteria [17] and were recruited from the Multiple Sclerosis Center, University of Cagliari, Italy. The study was commenced immediately after obtaining the approval from the University ethical committees, and an informed consent was obtained from all participants before their enrollment into the study. Clinical characteristic of the MS cases are reported in Table 1. All sera were kept frozen at −80 °C until assayed.

2.2. Peptides

The following synthetic peptides were included: the 21-mer MOG_{35-55} [MEVGWYRPPFSRVVHLYRNGK] derived from MOG_HUMAN protein (UniProtKB accession number: Q16653); the 10-mer MAP_2619c$_{352-361}$/BCG_1224$_{355-364}$ [WYIPPLSPVV] derived from MAP_2619c protein (UniProtKB accession number: Q73WP1) and from BCG_1224 protein (UniProtKB accession number: W8SWZ3); the 11-mer BCG_3329c$_{64-74}$ [PPGSVVHLYRD] derived from BCG_3329c protein (UniProtKB accession number: A1KNV2). All peptides were synthesized at >90% purity commercially (LifeTein, South Plainfield, NJ 07080 U.S.). Purified peptides were prepared as [10 mM] stock solutions, and were stored in single-use aliquots at −80 °C.

Table 1
Clinical characteristics of the subjects and antibodies distribution.

	MS patients n = 40	Healthy controls N = 39
Age, years	37.6 ± 11.0	37.9 ± 6.5
Female/male ratio	25/15	25/14
Disease Course (RR/SP/PP)	35/4/1	NA
Duration of MS, years	9.5 ± 10.0	NA
Age at MS onset, years	31.0 ± 10.0	NA
Expanded disability status scale	2.0 ± 0.6	NA
MOG_{35-55} IgG positive n (%)	11 (27.5)	2 (5)
MAP_2619c$_{352-361}$ IgG positive n (%)	3 (7.5)	3 (7.7)
BCG_3329c$_{64-74}$ IgG positive n (%)	8 (20)	3 (7.7)

Values are expressed as mean ± SD; RR, relapsing remitting MS; SP, secondary progressive MS; and PP, primary progressive MS.

2.3. ELISA

The optimal dilutions of each coated peptide, serum sample and secondary Ab were determined by checkboard titration in microtiter plates. Wells were coated with 10 μg/ml of peptides dissolved in 50 mM carbonate/bicarbonate buffer and incubated at 4 °C overnight. The day after, 200 μl of blocking solution (PBS-Tween containing 5% milk) was added to each well and the plate was further incubated at room temperature for 1 h. After rinsing with PBS-T, serum samples were added at 1:100 dilutions in PBS-T for 2 h at room temperature. After five washing with PBS-T, alkaline phosphatase-conjugated goat antihuman IgG polyclonal Ab (1:1000) was added for 1 h at room temperature. Finally, after five washing with PBS-T, para nitrophenylphosphate substrate solution was added to each well and the plates were incubated at room temperature in the dark for 5, 10 and 15 min. The optical density (OD) was read at a 405 nm wavelength using VersaTunable MAX microplate reader. Data was normalized to a positive control serum included in all experiments, the reactivity of which was set at 10.000 arbitrary units (AU)/ml.

2.4. Statistical analysis

Data was analyzed using a statistical software package (Prism 6, GraphPad Software, Inc., La Jolla, CA, USA). The frequencies of Abs distribution among MS cases were compared to that of HCs using Fisher's exact test with 95% confidence interval. The cut-off values were determined based on the receiver operating characteristic (ROC) curve, and the specificity was set at 95% and the sensitivity was chosen accordingly. A minimum level of statistical significance was considered at a p level of <0.05.

3. Results

Among the 40 MS patients, MOG_{35-55}, MAP_2619c$_{352-361}$/BCG_1224$_{355-364}$ and BCG_3329c$_{64-74}$. The alignment of peptides' sequences is shown on Table 2. Abs were detected in the sera of 11 (27.5%), 3 (7.5%) and 8 (20%) patients, respectively (Fig. 1). None of the HCs had high-titer Abs for these peptides (ranging from 5% to 8%) as displayed in Fig. 1. Compared to HCs Ab-positive subjects (5%), only the percent fraction of anti-MOG_{35-55}Ab-positive MS subjects was statistically significant higher ($p = 0.01$ by Fisher's exact test). In contrast, the prevalence of MAP_2619c$_{352-361}$/BCG_1224$_{355-364}$ and BCG_3329c$_{64-74}$ Ab-seropositivity was found to be not statistically significant when comparing MS patients with HCs ($p = 1$; $p = 0.2$). Concerning the clinical features, 10 out of 11 MOG_{35-55} Ab-positive sera belonged to relapsing remitting MS patients, while only one positive serum belonged to a secondary progressive MS subject. In addition, the prevalence of MOG_{35-55} Ab-seropositivity was found to be higher during acute phase than during remission periods.

4. Discussion

There is increasing data suggesting that a synergy between autoreactive T and B cells may be at play in immune-mediated demyelination. Different antigens have been proposed as targets of the autoantibody response, and MOG is one of the best candidate target self-antigen [16]. MOG_{35-55} peptide can be used to induce EAE in many species, even if anti-MOG_{35-55} Abs alone cannot induce EAE. In humans, there are conflicting lines of evidences regarding the pathogenic role of anti-MOG Abs [18].

In silico analysis identified two peptides, MAP_2619c$_{352-361}$/BCG_1224$_{355-364}$ and BCG_3329c$_{64-74}$, belonging to MAP and BCG, which share sequence homology with MOG_{35-55} (Table 2). These