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Anti-hsp60 antibody responses based on *Helicobacter pylori* in patients with multiple sclerosis: (ir)Relevance to disease pathogenesis

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ABSTRACT

In view of published data suggesting that *Helicobacter pylori* (Hp) is a trigger of multiple sclerosis (MS), we assessed anti-heat shock protein 60 (hsp60)Hp antibody reactivity in 129 MS patients and 48 demographically-matched healthy controls (HCs). Anti-Hp antibodies by ELISA were more elevated in MS than HCs but did not differ between different MS phenotypes. All anti-Hp-positive MS sera, irrespectively of their clinical phenotype, were anti-anti-hsp60 positive. Anti-hsp60 Hp seropositivity correlated with age at disease onset. In conclusion, anti-hsp60 Hp antibodies are present in all anti-Hp positive MS patients, and their relevance to disease pathogenesis is questionable.

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disorder (Katz Sand, 2015). In the majority of patients (85%), the clinical course of the disease is characterized by a bout-onset (relapses) while a progressive-onset is seen in approximately 15% of patients (Katz Sand, 2015; Milo and Miller, 2014). Over time, most of patients with relapsing-remitting MS (RRMS) acquire a secondary progressive MS (SPMS) clinical phenotype (Katz Sand, 2015). Many immunomodulatory or immunosuppressive treatments are currently available only for (RRMS) (Hadjigeorgiou et al., 2013; Ontaneda et al., 2015), while these are uneffective for primary progressive MS or SPMS (Ontaneda and Fox, 2015).

The aetiology of MS remains unknown. Several environmental factors, including microbial agents, have been considered potential inducers of the disease (Grigoriadis and Hadjigeorgiou, 2006). Amongst the microbial agents, *Helicobacter pylori* (Hp) has been considered a possible infectious trigger of the disease (Smyk et al., 2014; Deretzi et al., 2015). At the antigen level, several Hp antigens have been considered important for the loss of immunological tolerance to myelin antigens, particularly heat shock proteins (hsp) (Chiba et al., 2006; Panchapakesan et al., 1992; Salvetti et al., 1992; Scotti et al., 2010; Tishler and Shoenfeld, 1996). Several approaches have been used to assess whether hsps are implicated in the pathogenesis of MS, including studies on immune responses to hsp in MS and the extent of cross-reactivity between hsp and CNS myelin, as well as the expression of hsps in the brain of patients with MS (Salvetti et al., 1992; Tishler and Shoenfeld, 1996; Horvath et al., 2001; Gruber et al., 1996; Chiba et al., 2006). Because of the conserved nature of hsp there is a high degree of amino acid homology amongst hsp60 of different bacteria; thus, immune responses against hsps show a high degree of cross-reactivity (Van Eden et al., 2005).

The most immunogenic bacterial hsps are hsp60 and hsp70, and immune responses against these microbial hsps may initiate cross-reactive humoral and cellular immune responses against their human

Abbreviations: CagA, cytotoxin-associated gene A; CSF, cerebrospinal fluid; Hp, *Helicobacter pylori*; Hsp, heat shock protein; MS, multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; VacA, vacuolating cytotoxin A.

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counterparts. When such molecular mimicry mechanisms are in place, myelin-targeted tissue injury leads to the induction of overt clinical disease in a susceptible individual.

In the past, high levels IgG antibodies against hsp70 have been reported in the cerebrospinal fluid (CSF) of patients with MS (Chiba et al., 2006). However, significant difference in the levels of antibodies against hsp27, hsp60 or hsp90 was not observed (Chiba et al., 2006). The presence of cellular and humoral responses against bacterial and mycobacterial hsp70 in CSF and sera from patients with MS, gave rise to the expectation that immunological cross-reactivity involving infectious/self hsp70 may be involved in the pathogenesis of MS.

Antibody responses to Hp-specific hsp60 (Gonzalez-Lopez et al., 2013) has not been studied in great detail in MS. In the present study, we assessed such antibody reactivities in patients with RRMS and SPMS.

2. Material and methods

2.1. Patients and controls

A total of 133 MS patients were tested (93 females, 69.9%, 40 males, 30.1%) including 102 (76.7%) with RRMS, 27 (20.3%) with SPMS and 4 (3%) patients with primary progressive MS (PPMS). Because of their small number, the PPMS patients were excluded from further analysis. Table 1 shows the major demographic and clinical parameters of MS patients. At the time of serum sample collection, 30 MS patients (23.3%) were not on treatment. Amongst the remaining patients, 33 patients (25.6%) were receiving interferon-β (including 19 on interferon β-1α and 14 on interferon β-1β); 23 (17.8%) natalizumab (a humanized monoclonal antibody against the cell adhesion molecule α4-integrin); 20 (15.5%) fingolimod; 16 (12.4%) glatiramer; 5 (3.9%) teriflunomide; and 2 (1.6%) were receiving mitoxantrone.

Forty eight demographically-matched individuals (including 31 females, 64.6% and 17 males, 35.4%, mean age 46.6 ± 14.3SD years) were included as healthy controls (HCs).

The investigation conformed to the principles outlined in the Declaration of Helsinki. A written informed consent was obtained from patients and controls. The study was carried out following an approval from the Ethical Committee of the University General Hospital of Larissa, Thessaly, Greece.

2.2. Anti-Hp antibody testing by ELISA

Initially, all MS serum samples were tested for IgG anti-Hp antibodies by ELISA (Euroimmun), according to manufacturer's instructions. Positivity for anti-Hp was set at >20 RU/ml.

2.3. Anti-CagA and VacA antibody testing by line immunoassay

CagA is the important virulence factor of Hp (Kusters et al., 2006). Anti-CagA antibodies are frequent in Hp infected patients. VacA is another important virulence factor and anti-VacA Hp antibody responses are also present in Hp infected patients but to a lesser extent compared to VacA (Kusters et al., 2006). To this end, and in order to compare the data obtained against hsp60 Hp, we tested anti-CagA and VacA antibodies by a line immunoassay (EUROLINE-WB), according to the manufacturer's instructions.

2.4. Anti-hsp60 Hp antibody testing by immunoblotting

Antibody responses against hsp60 Hp (Gonzalez-Lopez et al., 2013) were tested by western immunoblotting, as previously described with slight modifications (Bogdanos et al., 2004; Koutsoumpas et al., 2009). Briefly, blot strips with electrophoretically separated Hp extract (Hp strain ATCC 43504) (Euroimmun, Germany) were used as a source of the hsp60 Hp antigen. In order to minimize the risk of background noise of the immunofixed bands, titration experiments were performed to determine the ideal concentration of antigen and serum dilution for serum sample screening tests. The membranes were cut at the corresponding height of hsp60 based on rainbow molecular weight markers, an internal control of the manufacturer (Euroimmun) and a polyclonal anti-GroEL-specific antibody (Enzo Life Sciences) (1/40,000) which cross-reacts with the corresponding Hp protein (Vanet and Labigne, 1998; Gonzalez-Lopez et al., 2013) used as positive control. Subsequently, the membrane pieces were incubated with individual serum samples at a dilution of 1/51 for 30 min. In each anti-hsp60 testing experiment, the positive control (anti-GroEL-specific antibody) and a negative control (rabbit polyclonal anti-VacA-specific antibody, Abcam at 1/2000) were used as internal controls with the respective revealing agents. After three 5-min washes, the serum sample related membranes were incubated with predetermined optimal dilutions of alkaline phosphatase (ALP)-conjugated anti-human IgG antibody (Euroimmun). Ready made NBT/BCIP (Euroimmun) was used as substrate for ALP-conjugated antibodies. Then, membrane strips were evaluated using the EUROLineScan software to obtain densitometric quantitative data. Only the amplitude of the curve describing optical density was used for further analysis. Pretests authenticated that the amplitude compared with the integral of the curve was a valid variable; arbitrary units (AU) of the amplitude were applied. The cut off value for the borderline positives is set at <12 AU. Subanalysis of results were performed in between low positive (21–25 AU), moderate positive (>25–50 AU)

Table 1
Major demographic and clinical characteristics of 129 patients with multiple sclerosis (MS) patients, including 102 with relapsing remitting MS (RRMS) and 27 with secondary progressive MS (SPMS). Patients have been stratified in accordance to their anti-*Helicobacter pylori* (Hp) antibody seroprevalence tested by ELISA. Four additional patients with primary-progressive MS (1 anti-Hp + and 3 anti-Hp-) were not included in the analyses because of their very small number.

Characteristics	Total (n = 129)	Hp+ (n = 55)	Hp-(n = 74)	*p* (Hp + *vs* Hp-)
Sex (M/F)	38 (29.5%)/91(70.5%)	17/38	21/53	ns
Age	42.43 ± 11.7	45.6 ± 12.3	40.1 ± 10.8	0.010[b]
Age on onset	31.22 ± 10.2	33.9 ± 10.5	29.3 ± 9.5	0.012[b]
(>20/<20 years)	128 (99.2%)/1(0.8%)	55/0	73/1	ns
(>30/<30 years)	106 (82.2%)/23(17.8%)	49/6	57/17	ns
(>50/<50 years)	35 (27.1%)/94(72.9%)	20/35	15/59	0.048[a]
MS Type				
RRMS (yes, %)	102 (79%)	41 (74.5%)	61 (82.4%)	ns
SPMS (yes, %)	27 (21%)	14 (25.5%)	13 (17.6%)	
Duration of disease	11.2 ± 7.2	11.7 ± 7.7	10.9 ± 6.9	ns
EDSS	3.3 ± 2.1	3.3 ± 2.2	3.3 ± 2	ns
Number of relapses	5.1 ± 3.6	4.4 ± 3.7	5.6 ± 3.4	ns
Anti-Hp antibody titres (mean titres ± SD) by ELISA in Hp + MS	62.6 ± 82	131 ± 87.1	11.8 ± 4.6	0.000[b]

Data represent mean ± standard deviation.
[a] *p*-Values were calculated using Fisher's Exact Test (2-sided).
[b] *p*-Values were calculated using 2-tailed *t*-test for Equality of Means, equal variances are not assumed. Abbreviations: M, male; F, female; EDSS, expanded disability status scale; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary-progressive multiple sclerosis; NS, not significant; SD, standard deviation.