HELICOBACTER PYLORI INFECTION IN CONNECTIVE TISSUE DISORDERS IS ASSOCIATED WITH HIGH LEVELS OF ANTIBODIES TO MYCOBACTERIAL HSP65 BUT NOT TO HUMAN HSP60

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Abstract

Background: To investigate whether the Helicobacter pylori (H. pylori) status influences levels of antibodies against mycobacterial heat shock protein (hsp) 65 and human hsp60 in systemic autoimmune diseases and studied the concentration of anti-H. pylori antibodies in autoimmune patients and healthy controls.

Materials and Methods: Antibodies against human heat-shock protein hsp60, mycobacterial heat-shock protein hsp65 were analyzed by ELISA. Anti-Helicobacter antibodies were determined by enzyme immunoassay.

Results: There was markedly higher prevalence of H. pylori infection in undifferentiated connective tissue disease (82%) (n = 33) and systemic sclerosis (78%) (n = 55) but not in systemic lupus erythematosus (n = 49), polymyositis/dermatomyositis (n = 14), rheumatoid arthritis (n = 21) or primary Raynaud's syndrome (n = 26) compared to controls (59%) (n = 349). In autoimmune diseases H. pylori infection was associated with elevated levels of anti-hsp65 ($p = 0.008$) but not of anti-hsp60. Anti-hsp65 levels were significantly higher in H. pylori-infected (n = 129) than in uninfected patients (n = 69) ($p = 0.0007$).

Conclusions: These findings indicate that in autoimmune diseases the infection with the H. pylori bacterium is associated with increased concentration of anti-mycobacterial hsp65.

Introduction

Helicobacter pylori (H. pylori) is a Gram-negative organism that colonizes the acid-secreting portion of the stomach. It always causes inflammatory histological lesions, which predispose to gastro-duodenal peptic disease, gastric cancer and lymphoma [1, 2, 3].

Several studies have been published on the issue of *H. pylori* and autoimmunity, and almost all of them seemed to give credit to *H. pylori* for influencing the occurrence or course of some autoimmune diseases. In idiopathic thrombocytopenic purpura successful eradication of *H. pylori* resulted in a significant decrease of anti-platelet antibody titers and increase of platelet count [4]. Conversely, no significant differences were found in patients who were treated but not cured. Cross-mimicry mechanisms have been suggested to explain this phenomenon [4]. In rheumatoid arthritis the eradication of *H. pylori* infection led to a significant improvement of all indices of disease activity. No significant changes were observed in controls [5]. In one study about 80% of patients with primary Sjögren's syndrome (SjS) had antibodies against *H. pylori* [6]. The prevalence was significantly higher than that found in various autoimmune diseases and healthy persons. Increased seroprevalence of *H. pylori* in systemic sclerosis (SSc) has been reported by several groups [7, 8, 9, 10]. Some studies indicated association between *H. pylori* infection and Raynaud's syndrome, speculating that increased levels of cytokines and acute phase reactants, C-reactive protein, and fibrinogen cause vasospasm and

platelet aggregation [11, 12]. By contrast, no increased titers of anti-*H. pylori* antibodies were found in the sera from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and polymyositis/ dermatomyositis (PM/DM) [6].

H. pylori sheds extracellular products that elicit local and systemic immune responses that could be responsible for tissue damage [2, 13, 14]. Among these, a 60 kDa protein, belonging to the heat-shock proteins (hsp) seems to play an important role. Since microbial hsp have a high level sequence homology with their mammalian counterparts, and some antigenic determinants may be present in both agent and host proteins, it has been postulated that immune response to *H. pylori* hsp could be involved in the pathogenesis of systemic and organ specific autoimmune diseases [2, 15, 16, 17].

Previously we have reported on the measurement of antibodies against human hsp60 and mycobacterial hsp65 in various autoimmune diseases (SLE, undifferentiated connective tissue disease [UCTD], primary Raynaud's syndrome, [PRS], RA, PM/DM), and healthy blood donors. No significant differences in the anti-hsp60 and anti-hsp65 antibody levels were found between any patient group and the controls except significantly higher anti-hsp concentrations in UCTD (anti-hsp60: $p = 0.0094$, anti-hsp65: $p = 0.0108$, respectively) [18]. According to several reports serum levels of antibodies against the hsp60 of *H. pylori* are higher in the *H. pylori*-infected than in the uninfected subjects [2, 19, 20]. In addition, titers of these antibodies were

reduced after *H. pylori* eradication [21]. Therefore we measured anti-*H. pylori* antibodies in the sera tested previously by us for anti-hsp antibodies of patients with autoimmune diseases, and assessed whether the *H. pylori* status influences the levels of antibodies to mycobacterial hsp65 and human anti-hsp60. Another purpose of the present work was to systematically measure of *H. pylori* antibodies in a high number of patients with different systemic autoimmune diseases and healthy controls.

Methods

Patients

All patients of these groups were cared at the 2nd Department of Internal Medicine, University Medical School, Pécs, Hungary. Forty-nine patients with SLE (10 males, 39 females, age: 41.7 ± 15.4 years, mean ± SD), 14 patients with PM/DM (2 males, 12 females, 48.1 ± 12.7 years), 21 patients with RA (6 males, 15 females, 46.0 ± 17.6 years), 55 patients with SSc (6 males, 49 females, 49.9 ± 12.1 years), 33 female patients with UCTD (42.5 ± 11.7 years) and 26 patients with PRS (6 males, 20 females, 39.4 ± 10.6) were included in the study. All cases fulfilled the accepted international criteria (A-E) [22, 23, 24, 25, 26, 27, 28, 29]. The study was approved by the local Ethical Committee of the 2nd Department of Internal Medicine.

Healthy controls

Two groups of healthy blood donors served as controls for the present study. The first group tested for both anti-*H. pylori* and anti-hsp antibodies consisted of 192 healthy blood donors (111 males, 81 females, 47.1 ± 9.8 years old). Serum samples from 157 other blood donors (117 males, 40 females, 44.4 ± 9.7 years old) were tested only for anti-*H. pylori* antibodies.

Analysis of antibodies to heat-shock proteins

The levels of IgG-type antibodies reacting with proteins of the chaperonin 60 family (recombinant human hsp60, StressGen, SPP-740, Victoria, Canada; recombinant *M. bovis* hsp65 [batch MA14, GBF, Braunschweig, Germany, supported by the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases]) was assessed by ELISA. ELISA plates were coated with 0.1 μg/well human hsp60 or *M. bovis* hsp65. After washing and blocking (PBS, 0.5 % gelatin) the wells were incubated with 100 μl of serum samples diluted 1:500 in PBS containing 0.5% gelatin and 0.05% Tween 20. Binding of anti-hsp antibodies was determined using γ-chain specific anti-human IgG peroxidase labelled antibodies (Sigma, St. Louis, USA) and o-phenylene-diamine (Sigma) detection system. The optical density was measured at 490 nm (reference at 620 nm) and the means of duplicate wells were calculated [30]. A serial dilution of a control anti-hsp60 rabbit polyclonal antiserum (StressGen, SPA-804, reacting with all hsp tested) was used as standard. Data obtained as optical density values were calculated to arbitrary unit/ml values related to this standard.

Measurement of the IgG antibodies against *Helicobacter pylori*

Titer of anti-*H. pylori* antibodies was determined by the recomWell Helicobacter IgG test (Mikrogen GmbH, Martinsried, Germany). In this indirect ELISA test recombinant *H. pylori* antigens bound to the solid phase were incubated with 10 μl of serum samples (1:101 dilution) at 37 °C for 1 hour. After washing the plates were then incubated with peroxidase-conjugated anti-human IgG antibodies at 37 °C for 30 minutes followed by washing. The antibody titer was detected by a color reaction using TMB solution at room temperature for 30 minutes, stopped by phosphoric acid. The optical density was measured at 450 nm. The results were expressed as an index value. The cut-off value was based on the study of a large control group. A positive control (= calibrator) in combination with a Factor value was used to determine the OD of cut-off. The value of the Factor was given in the control certificate included with each kit. The OD cut-off in our laboratory was 1.0. Subjects with a *H. pylori* antibody concentration equal to or below this value were considered as not infected whereas subjects with concentration exceeding cut-off were considered to be infected with the bacterium.

Statistical analysis

Data were analyzed with GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego California USA). As the values of antibody to heat-shock proteins and *H. pylori* index values were not normally distributed, non-parametric tests and *post hoc* tests were used for group comparisons.

Categorical variables were compared with the $\chi 2$ and Fisher's exact tests. Multiple regression analysis was performed by the SPSS program, version 10.0 (SPSS Inc., Chicago, Ill, USA).

Results

Antibodies against *H. pylori* in the sera of patients with different autoimmune diseases and healthy persons

Anti-*H. pylori* antibody levels were determined in the sera of both groups of healthy blood donors (altogether 349 subjects) and in 198 patients with different autoimmune diseases (UCTD, SSc, SLE, PRS, RA, and PM/DM). Antibody levels to *H. pylori* were significantly ($p < 0.0001$) higher in the group of patients with autoimmune diseases (median [interquartile range] of the index: 2.4 [0.7-5.4]) as compared to control subjects (1.4 [0.5-3.0]. Since the proportion of females to males was markedly higher in the latter group (174 to 24 as compared to 121 to 227 among the healthy subjects) we compared the antibody levels to *H. pylori* by using multiple regression analysis adjusted to age and gender of the subjects tested. Patients had significantly ($p = 0.014$) higher *H. pylori* indices than the controls even after adjustment to age and gender.

The difference between healthy subjects and patients with autoimmune diseases was due to the significantly higher prevalence of *H. pylori* infection in two diseases: UCTD (82%) and SSc (78%) compared with healthy controls (59%) (Table 1). The median value of the *H. pylori* index was significantly

higher in these two diseases than in the healthy controls as demonstrated by ANOVA completed with a *post hoc* test. By contrast, *H. pylori* infection did not occur significantly more frequently and *H. pylori* index was not significantly higher in SLE, PRS, RA or PM/DM patients than in the healthy subjects (Table 1).

Relationship between anti-*H. pylori* antibodies and anti-60 kD heat shock protein antibodies in the sera of patients with autoimmune diseases

In the whole group of 198 patients with different autoimmune diseases the serum concentration (index) of anti-*H. pylori* antibodies exhibited a highly significant positive correlation with antibodies against *M. bovis* hsp65 ($r = 0.335$, $p < 0.0001$) whereas no correlation with serum levels of antibodies against human hsp60 was found ($r = 0.056$, $p = 0.441$). By contrast, in the sera of healthy controls there was a weak but highly significant positive correlation between the levels of anti-hsp60 and anti-*H. pylori* antibodies (data not shown).

When patients and controls were divided into two groups (those with a *H. pylori* index equal to or exceeding 1.0 (considered infected with the bacterium) and those with a *H. pylori* index <1.0 considered to be not infected) marked differences were found in the levels of antibodies to mycobacterial hsp65 (Table 2). The levels of these antibodies were significantly ($p = 0.0007$) lower in the sera of the 69 not infected patients than in those from the 129 *H. pylori*-infected patients whereas no difference in the anti-hsp65 antibody concentration was found between infected and not infected healthy controls

(Table 2). By contrast, median serum concentration of anti-hsp60 antibodies did not differ between *H. pylori*-infected and not infected patients whereas a slight but significant difference was observed in the group of healthy controls (Table 2).

Significant differences between the patients and controls were found only In the *H. pylori* positive group: anti-hsp65 levels were almost 1.5 times higher in the patients than in the controls ($p = 0.008$) whereas in the *H. pylori* negative group anti-hsp65 antibody concentration was about the same in the healthy subjects and the patients. No differences in the anti-hsp60 antibody levels were found between patients and controls in either group (Table 2).

By using ANOVA, we did not find significant differences among groups with different autoimmune diseases either in the levels of anti-hsp60 or anti-hsp65 antibodies (data not shown).

Discussions

We have studied two questions: i) is there any difference in the frequency of *H. pylori* infection between patients with different autoimmune diseases and healthy persons, and ii) does infection with *H. pylori* influence the occurrence of antibodies against human and mycobacterial hsp in the same patients and healthy subjects?

As for the frequency of *H. pylori* infection, patients with autoimmune diseases as a whole had significantly higher average IgG anti-*H. pylori* levels than healthy controls. This difference, however, could be solely due to an increased serum concentration in UCTD and SSc. More importantly, the frequency of high ($\geq$ 1.0) anti-*H. pylori* indices, (considered as the sign of infection), was higher than those in the healthy control group only in these two diseases. In accordance with previous results the patients with SLE, PRS, RA and PM/DM did not differ from the healthy group in this respect either [9]. Thus our present findings indicate that the frequency of *H. pylori*-infected subjects exceed that in the healthy normal population only in some autoimmune diseases.

Of particular relevance is the preferential occurrence of *H. pylori* infection in UCTD that refers to diseases in evolution toward particular differentiated particular connective tissue disorders including SSc. To our best knowledge, no data on the frequency of anti-*H. pylori* antibodies in this disease have been published yet. Further studies are needed to evaluate the possible diagnostic and prognostic significance of *H. pylori* infection in such cases. In accordance with our results several recent findings indicate increased rate of *H. pylori* infection in patients with SSc [7, 8, 9, 10]. The preferential occurrence of *H. pylori* infection in SSc may be explained in two ways. First, an increased prevalence of *H. pylori* infection might be favored by the disturbed gastrointestinal motility, a clinical phenomenon well known in patients with SSc. The second explanation may be that *H. pylori* infection and the immunological mechanisms operative in the course of SSc may be related.

H. pylori-associated hsp60, human hsp60 and mycobacterial hsp65 are members of the 60-kD family of heat shock proteins, they are believed to share high sequence homology. Thus potential exists for cross-reaction of antibodies directed against these proteins [16, 20, 31, 32, 33, 34]. Several recent observations indicate that the titer of antibodies against *H. pylori* hsp60 is significantly higher in *H. pylori*-infected than in the not infected subjects [2, 19, 20]. Furthermore, the titration of these antibodies can be used for early serological monitoring of *H. pylori* eradication treatment [21]. Only scarce data are, however, available on the influence of *H. pylori* infection on serum concentration of antibodies against other members of the 60 kD hsp family [31, 35]. To our best knowledge no such study has been performed in patients with autoimmune diseases.

By using sensitive ELISA methods that were found to be suitable to measure the serum concentrations and study the properties of antibodies against different members of 60 kD hsp [30, 36, 37] we obtained only weak differences between *H. pylori*-infected and not infected healthy individuals in the levels of anti-human hsp60 and anti-mycobacterial hsp65 IgG antibodies [18]. No influence of *H. pylori* infection on the serum concentration of these antibodies has been found previously whereas IgA type antibodies to hsp60 and hsp65 were elevated in patients with atrophy compared to *H. pylori* negative controls [35]. Sharma et al - in accordance with our present findings - found IgG antibodies recognizing human hsp60 that could be detected by immunoblotting

in *H. pylori*-infected persons but found no difference between *H. pylori*-infected and not infected individuals in antibodies to *M. bovis* hsp65 [31].

The normal level of anti-hsp60 antibodies in patients with autoimmune diseases supports our recent hypothesis on the regulation of the autoantibodies against human hsp60 [36]. According to this hypothesis anti-hsp60 antibodies can be considered as bona fide natural autoantibodies. Our unpublished observations indicate that the level of these antibodies is constant for years and even decades as that of other natural autoantibodies [38, 39]. If this assumption is true, polyclonal B cell activation, characteristic for some autoimmune disease, such as SLE is expected to have no influence on their serum concentration. Our present findings are compatible with this assumption as is our previous observation on the normal anti-hsp60 antibody levels in HIV-infection, a disease that is also associated with polyclonal B cell activation [40].

In contrast to anti-hsp60, serum concentration of antibodies against mycobacterial hsp65 correlated to that of anti-*H. pylori* antibodies in patients with autoimmune diseases. Moreover, in these diseases anti-hsp65 antibodies were found in significantly higher titers in *H. pylori*-infected than in not infected patients. Human hsp60 and mycobacterial hsp65 share several common epitopes therefore anti-hsp60 and anti-hsp65 antibodies cross-react with each other. However, the overlap between the two proteins is only partial, they have different epitope specificities and complement activating abilities [30]. Since anti-mycobacterial hsp65 represents antibody against bacterial hsp combined

infection with *H. pylori* and some other bacteria might contribute to the observed results.

There are marked differences in the epitope specificity between the *H. pylori* associated hsp60, other bacterial 60 kD hsps and human hsp60 as well. The amino acid sequences of two regions (residues 181 to 204 and 204 to 229) on *H. pylori* hsp60 were different from the amino acid sequences of other bacterial hsp60s, whereas the amino acid region of residues 189 to 203 on *H. pylori* hsp60 is conserved among several bacterial hsp60s and human hsp60 [21]. Our present findings indicate that a part of the antibodies reacting with mycobacterial hsp65 represent antibodies against cross-reactive epitopes between mycobacterial hsp65 and *H.pylori*-associated hsp60 and their elevated titer in *H. pylori*-infected patients reflects high serum concentration of antibodies against *H. pylori* hsp60 [2, 19, 20]. Increased formation of these antibodies in *H. pylori*-infected patients with some autoimmune diseases may be a part of the immune dysregulation characteristic for the given disease. More studies are needed for a better understanding of the development and the possible pathological significance of anti-hsp antibodies with a large array of specificity in autoimmune diseases.

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Table 1

Association of different autoimmune diseases and infection with *Helicobacter pylori*

Patients with	**Number of subjects tested/infected (H. pylori prevalence %)[+]**	***H. pylori* index, median (interquartile range)**
Healthy controls	349/206 (59%)	1.50 (0.50-3.95)
Undifferentiated connective tissue disease (UCTD)	33/27 (82%)*	3.40 (1.10-6.00)*
Systemic sclerosis (SSc)	55/43 (78%)**	3.50 (1.40-5.65)**
Systemic lupus erythematosus (SLE)	49/28 (57%)	1.50 (0.60-4.40)
Primary Raynaud's syndrome (PRS)	26/12 (46%)	2.25 (0.60-5.35)
Rheumatoid arthritis (RA)	21/11 (52%)	1.65 (0.60-4.75)
Polymyositis/dermatomyositis (PM/DM)	14/11 (79%)	2.10 (0.50-4.35)
p value	0.0049 (χ2 test)	0.0029 (Kruskal-Wallis test)

[+]*H. pylori* index > 1, *p<0.05, **p<0.01, Fisher's exact test and Dunn post hoc test for the column 1 and 2, respectively

Table 2

Comparison of the serum concentration of the antibodies to mycobacterial hsp65 and human hsp60 in patients with autoimmune diseases and healthy subjects infected or not infected with *Helicobacter pylori*

	Anti-hsp60, median (interquartile range) (number of subjects)			Anti-hsp65, median (interquartile range) (number of subjects)		
	Healthy subjects	Patients with autoimmune diseases	P value*	Healthy subjects	Patients with autoimmune diseases	P value*
H pylori index ≤ 1.0	87.7 (50.2-140.5) (79)	93.1 (64.7-164.9) (69)	0.340	9.8 (4.6-21.5) (79)	10.4 (4.7-17.0) (69)	0.949
H pylori index > 1.0	116.3 (71.1-179.4) (113)	119.2 (72.3-202.5) (129)	0.533	11.3 (4.9-24.2) (113)	15.8 (8.4-31.3) (129) (129)	0.008
P value	0.024	0.182	-	0.473	0.0007	-
Total	100.8 (64.4-170.9) (192)	110.6 (66.3-199.6) (198)	0.187	10.7 (4.9-21.7) (192)	12.7 (7.2-25.5) (198)	0.023