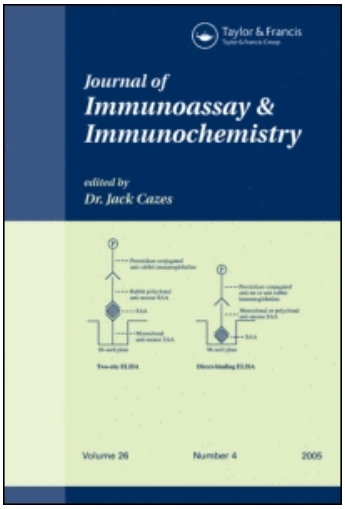

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Abstract: Meningitis is a common life threatening disease which may be caused by a bacterium, fungus, or virus. Here, the presence of a *Helicobacter pylori* antigen was investigated in serum and CSF samples from 173 individuals with meningitis. The influence of *H. pylori* infection on CSF levels of Th1/Th2 cytokines was also evaluated. *H. pylori* antigen was detected using ELISA and Western blot based on specific anti-*H. pylori* antibody. Th1/Th2 cytokines (IFN-γ & IL-10, respectively) were also determined. A target epitope of 58-kDa was detected in selected CSF and serum samples using Western blot. *H. pylori* antigen was detected in the CSF samples of 75% of meningitis patients showing *H. pylori* antigen in their sera. A significant correlation ($p < 0.001$, $r = 0.21$) was shown between serum and CSF levels of 58-kDa *H. pylori* antigen. Only the levels of Th1 cytokine (IFN-γ) were significantly higher ($p < 0.05$) in CSF of meningitis patients positive for *H. pylori* antigen than negative patients with meningitis. In conclusion, the 58-kDa *H. pylori* antigen crossed the blood brain barrier and entered the CSF of patients with meningitis. A significant upregulation of Th1 response may be associated with *H. pylori* infection in patients with meningitis.

Keywords: *Helicobacter pylori*, Antigen, Cerebrospinal fluid, Th1/Th2 cytokines, Meningitis

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a gram-negative microaerophilic organism, is a common infection found primarily in the stomach. Infection with *H. pylori* leads to gastritis and is associated with the development of peptic ulcer disease, gastric carcinoma, and lymphoma.[1–3] During the last decade, *Helicobacter* species have been isolated from human fecal samples, liver, and gallbladder.[4] *H. fennelliae, H. cinaedi, H. westmeadii*, and *H. rappini* have been isolated from patients with septicemia.[5] Orlicek et al.[6] reported a case of septicemia and meningitis by *H. cinaedi* in a neonate whose mother cared for pet hamsters during the first two trimesters of her pregnancy. Chiba et al.[7] have reported the presence of several IgG antibodies against crude *H. pylori* antigens in the cerebrospinal fluid (CSF) of patients with Guillain-Barre syndrome (GBS). Several reports have addressed the roles of T helper (Th) subsets in *Helicobacter* immunity and immunopathology.[8–10] The immunoregulatory and proinflammatory cytokines induced by *H. pylori* may influence the nature of the local T-cell response. The Th1 subset promotes cell-mediated immunity by producing mainly IL-2 and gamma interferon (IFN-γ), and the Th2 subset, which is important for antibody responses and also for down-regulation of chronic inflammatory reactions, produces IL-4, IL-5, IL-6, and IL-13.[11] However, the regulatory roles of Th1 and Th2 cells in immune protection against *H. pylori* infection are not clearly understood. Recently, Attallah et al.[12] identified a *H. pylori*-circulating antigen in sera of *H. pylori* infected individuals using ELISA with a high degree of sensitivity (92%) and specificity (91%). Here, the presence of the *H. pylori* antigen was investigated in CSF of patients with meningitis and the influence of *H. pylori* infection on CSF levels of Th1/Th2 cytokines (IFN-γ & IL-10) was evaluated.

EXPERIMENTAL

Samples

CSF and serum samples of 173 Egyptian individuals (90 males and 83 females, aged 3 mo to 80 yr, mean age 24 $\pm$ 18.14), kindly provided by the staff of Abbassia Fever Hospital, Cairo, Egypt, were included in the present study before antibiotic therapy and after approval from the hospital ethics committee. They were considered likely to have meningitis on the basis of laboratory and clinical criteria, patient history, physical signs, and symptoms. Sera of 16 individuals (10 males and 6 females, age range 16–66 yr) with confirmed *H. pylori* infection and of 16 age matched non-infected individuals (9 males and 7 females) were used as positive and negative controls, respectively. An informed consent was obtained from all individuals who participated in the present study and they were fully informed concerning the nature of the disease and the diagnostic procedures involved.

Preparation of *H. pylori* Whole Cell Lysate Antigen

H. pylori was grown on selective blood agar plates for 48 hr at 37°C under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) and suspended in PBS. Cells were then washed three times in phosphate buffered saline (PBS) by centrifugation at 6,000 × *g* for 10 min at 4°C before being disrupted by freeze-pressing with X-press.[13] The preparation was then filtered through a 0.2 μm membrane filter (Schleicher & Schuell, Dassel, Germany). The protein content was determined by the Bio-Rad protein assay (Hercules, CA), and aliquots were frozen at - 20°C until used.

SDS-PAGE and Western Blotting

Selected CSF and serum samples, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), were electrotransferred onto the nitrocellulose filters (0.45 μm pore size, Sigma) in protein transfer (BioRad Laboratories) according to the method of Towbin et al.[14] The nitrocellulose filter was blocked using a blocking buffer composed of 5% (w/v) non fat milk dissolved in 0.05 M Tris-buffered saline (TBS) containing 0.15 M NaCl, pH 7.4, rinsed in TBS, and incubated with specific anti-*H. pylori* antibody[12] diluted in the blocking buffer with constant shaking overnight. The blots were washed three times (30 min/wash) in TBS, followed by 2 hr incubation with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) dilution in TBS. The blots were then washed three times with TBS (15 min/wash). The target antigen of THE specific anti- *H. pylori* antibody was visualized by incubating the NC filter and soaked in premixed BCIP/NBT alkaline phosphatase substrate (ABC Diagnostics, New Damietta City, Egypt). The reaction was stopped by distilled water after color was observed within 10 min.

ELISA for *H. pylori* Antigen Detection in CSF

Diluted CSF samples (1 : 2) or serum samples (1 : 800) in coating buffer (pH 9.6) were tested (50 μL per well) for *H. pylori* antigen according to Attallah et al.[12] In brief, sample coated ELISA plate was sealed with an acetate plate sealer and incubated overnight at 2–8°C. After blocking of free binding sites, the specific anti- *H. pylori* antibody diluted in PBS-T20 was added (50 μL per well) and incubated at 37°C for 2 h. After washing, anti-rabbit IgG alkaline phosphatase conjugate (Sigma) was added and incubated at 37°C for 1 h. The amount of coupled conjugate was determined by incubation with p-nitrophenyl phosphate substrate for 30 min at 37°C. The reaction was stopped and absorbance was read at 405 nm using an ELISA reader (Σ960; Axiom, Burstadt, Germany). The cutoff level of ELISA, above or below, for which the tested CSF is considered positive or negative

was calculated as the mean ELISA optical densities of 32 CSF samples from *H. pylori* non-infected individuals ± 3 standard deviation (i.e., $0.155 \pm [3 \times 0.034]) = 0.257$. The cutoff level of ELISA, above or below which the tested serum is considered positive or negative, was set at 0.398.[12]

Cytokine Measurements

IFN-γ Th1 cytokine was identified in serum and CSF samples by SDS-PAGE and Western blot, based on specific monoclonal antibody according to the method of Towbin et al.[14] and quantified by a commercially available ELISA kit (ABC Diagnostics, New Damietta, Egypt) according to the manufacturer's instructions. In brief, after 30 min incubation of standard and sample, the coated ELISA plate with monoclonal antibody to IFN-γ was washed 5 times, then 50 μL of polyclonal antibody to IFN-γ was added to each well. Following a wash to remove any unbound antibody, 50 μL per well of anti-rabbit alkaline phosphatase conjugate were added to each well. Enzyme reagent, a substrate solution, is added to the wells and color develops in proportion to the amount of IFN-γ. The IL-10 Th2 cytokine was evaluated with a commercially available ELISA kit (Quantikine Kit, R & D Systems, Inc., 614 McKinley Place N.E., Minneapolis, USA), according to the manufacturer's instructions. After 2 h of sample incubation, plates were washed 3 times and, then, 200 μL of an enzyme linked polyclonal antibody was added to each well. Following a wash to remove any unbound enzyme reagents, the substrate solution is added to the wells and color develops in proportion to the amount of cytokines. The color development is stopped and the intensity of the color is measured.

Statistical Analyses

Data were expressed as mean $\pm$ SD and were analyzed by using the statistical program SPSS for windows, version 12.0 (SPSS Inc., San Diego, CA). *P* values <0.05 were considered significant.

RESULTS

Identification of 58-kDa *H. pylori* Target Antigen in Serum and CSF Samples

The target *H. pylori* antigen was identified by the Western blot at 58-kDa in *H. pylori* whole cell lysate, and in serum and CSF samples of meningitis patients. In addition, a degradation product of 42-kDa was identified in some serum samples. No bands were identified in sera and CSF samples from non-infected controls; see Figure 1.

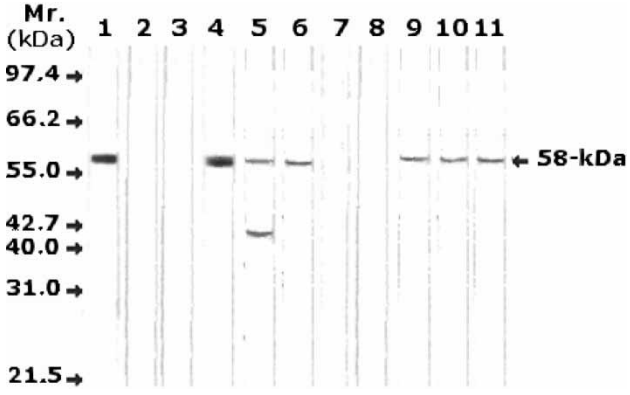

Figure 1. Western blot based on specific anti-*H. pylori* antibody of serum and CSF samples from meningitis patients. Lane 1: whole cell lysate of *H. pylori* as positive control. Lanes 2–3: sera of 2 healthy individuals, Lanes 4–6: sera of 3 meningitis patients, Lanes 7–8: CSF of 2 *H. pylori* non-infected individuals, and Lanes 9–11: CSF of the same 3 meningitis patients. All infected patients showed target 58-kDa antigen. No bands were identified in CSF and sera from non-infected controls. Molecular weight markers (not shown but indicated by arrows) include Phosphorylase B (97.4-kDa), Bovine serum albumin (66.2-kDa), Glutamate dehydrogenase (55.0-kDa), Ovalbumin (42.7-kDa), Aldolase (40-kDa), Carbonic anhydrase (31-kDa), Soybean trypsin inhibitor (21.5-kDa).

Detection of *H. pylori* Antigen in CSF and Serum Using ELISA

Of 173 sera, 84 individuals (49%) were positive for *H. pylori* antigen using ELISA. The target antigen was detected in CSF samples of 75% of 84 meningitis patients showing the target antigen in their sera. A significant correlation ($p = 0.007$, $r = 0.21$) was shown between serum and CSF levels of 58 kDa antigen; see Figure 2.

Detection of Th1/Th2 Cytokines in CSF and Serum Using ELISA

IFN-γ was identified at 26 kDa in serum and CSF of meningitis patients positive for *H. pylori* antigen than in patients negative for *H. pylori* antigen; see Figure 3. The levels of IFN-γ (51.9 ± 57.8 pg/mL) evaluated using ELISA, were significantly higher ($P < 0.05$) in sera of meningitis patients positive for *H. pylori* antigen than in sera of patients negative for *H. pylori* antigen (37.6 ± 37.4 pg/mL). Meningitis patients positive for *H. pylori* antigen in serum had higher levels of IL-10, compared with those patients who were negative for *H. pylori* antigen in serum. However, this increase did not reach a statistically significant level ($p > 0.05$). All *H. pylori* negative controls showed very low serum levels of investigated Th1/Th2

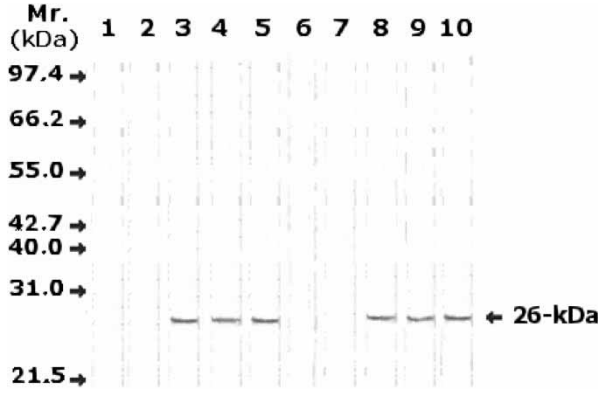

Figure 2. Western blot based on specific anti-interferon gamma monoclonal antibody of serum and CSF samples from meningitis patients. Lanes 1,2: sera of 2 healthy individuals, Lanes 3–5: sera of 3 meningitis patients, Lanes 6,7: CSF of 2 *H. pylori* non-infected individuals, and Lanes 8–10: CSF of the same 3 meningitis patients. All infected patients showed target 26-kDa Interferon gamma. No bands were identified in CSF and sera from non-infected controls. Molecular weight markers (not shown but indicated by arrows).

cytokines (IFN-$\gamma < 30.0$ pg/mL & IL-10 < 15.6 pg/mL), whereas *H. pylori* positive controls showed significantly ($p < 0.05$) high serum levels of IFN-γ (55.4 ± 45.9) and IL-10 (110.7 ± 16.2). Meningitis patients positive for *H. pylori* antigen in CSF had higher levels IFN-γ and IL-10 than the patients negative for *H. pylori* antigen in CSF. However, this increase was only statistically significant in IFN-γ cytokine ($p < 0.05$).

DISCUSSION

Meningitis is a common life threatening disease, which may be caused by a bacterium, fungus, or virus. Orlicek et al.[6] reported a case of septicemia and meningitis by *H. cinaedi* in a neonate whose mother cared for pet hamsters during the first two trimesters of her pregnancy. Chiba et al.[7] found that four of seven CSF samples had several IgG antibodies against *H. pylori* proteins; these antibodies may be involved in the immune responses of patients with Guillain-Barre syndrome. Furthermore, a highly significant association between *H. pylori* ureC and cagA genes was shown in the stomach, trachea, and lung of cases of sudden infant death syndrome.[15] Recently, a 58 kDa *H. pylori* antigen was detected in the serum of *H. pylori* culture positive patients with high degrees of sensitivity and specificity (>90%).[12] Here, we have identified the target *H. pylori* antigen in CSF, as well as in sera of patients with a background of meningitis at the same molecular weight, i.e., 58 kDa. A high incidence of 58 kDa

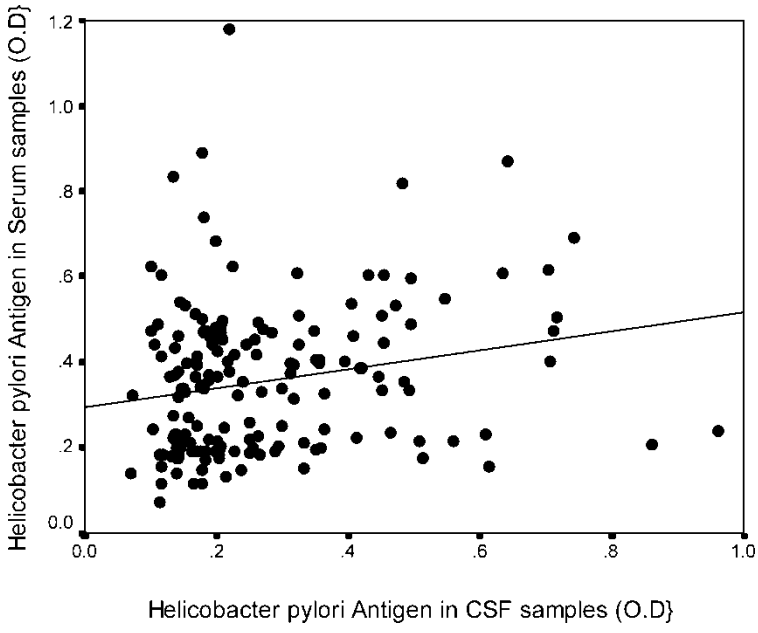

Figure 3. Correlation between HpCA levels in serum and CSF. A significant correlation ($p = 0.007$, $r = 0.21$) was shown between serum and CSF levels of the 58-kDa antigen.

H. pylori antigen was demonstrated in 75% CSF of meningitis patients showing *H. pylori* antigen in their sera. These results suggest the serum 58 kDa antigen originated from *H. pylori* gastric colonization may cross the blood brain barrier and enter the CSF. An experimental model demonstrated the feasibility of parenteral immunization against *H. pylori* and suggest an appropriate balance between Th1 and Th2 type responses is required to achieve complete protection.[16] Recent studies in both humans and animal models strongly suggest the contribution of a host immune response to *H. pylori*-related disease. Bontems et al.[17] found that stomach concentrations of IFN-γ increased in infected children and adults, compared with controls. IL-2, IL-4, IL-10, and TNF-α concentrations were similar in infected and uninfected children and adults. Here, patients positive for *H. pylori* antigen had higher levels of IFN-γ, compared with those patients who were negative for *H. pylori* antigen. Levels of IFN-γ marker were significantly higher in the CSF of patients positive for *H. pylori* antigen than those who were negative. These results suggest that *H. pylori* infection in humans induces a Th1 immune response characterized by increased production of IFN-γ. The gastric IL-4 takes part in the local immune response to *H. pylori*. Treatment of *H. pylori* infected mice with an algal cell extract containing the antioxidant, astaxanthin, reduces the bacterial load and

gastric inflammation.[18–20] These changes are associated with a shift of the T-lymphocyte response from a predominant Th1-response dominated by IFN-γ to a Th1/Th2-response with IFN-γ and IL-4. The results of Borody et al.[21] support the hypothesis that impaired mucosal immunity, particularly involving the secretion of IL-4, may contribute to *H. pylori* eradication failure and measurement of whole blood secretion of IL-4 may predict which patients are more likely to fail standard antibiotic therapy. Acute infection was characterized by a predominantly Th1 (IL-2 and IFN-γ) and proinflammatory (TNF-α and MIP-1β) type of cytokine response and the absence of a Th2 type of response[22] Fan et al.[23] concluded that suppressed Th1 and enhanced Th2 responses in *H. pylori* infection may be involved in the immunopathogenesis of chronic *H. pylori* infection. In conclusion, the 58 kDa *H. pylori* antigen cross the blood brain barrier and enter the CSF of patients with meningitis. A significant upregulation of Th1 response may be associated with *H. pylori* infection in meningitis patients.

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