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Extraction of IgE-Binding Components of *Helicobacter pylori* by Immunoblotting Analysis in Chronic Urticaria Patients

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Key Words

Helicobacter pylori · Chronic urticaria · IgE · Immunoblotting · RAST

Abstract

Background: An association between *Helicobacter pylori* and chronic urticaria has been suspected previously. An IgE-mediated pathway might be a possible link between *H. pylori* infection and chronic urticaria, and therefore we wanted to prepare an optimal *H. pylori* antigen to detect *H. pylori*-specific IgE antibodies in chronic urticaria patients. ***Methods:*** *H. pylori* antigen extracts were prepared in different ways to find the optimal antigen extract to be used in the assays. Immunoblotting was used to detect IgE-binding bands. The results were applied in an *H. pylori* RAST assay for specific *H. pylori* IgE antibodies in patient sera. ***Results:*** In immunoblotting, the largest number of IgE-stained bands were visualized in the washing fluids and sonicated extracts, while strong heating and denaturing treatments destroyed the epitopes for IgE binding, suggesting that they belonged to the flagellar structures of *H. pylori*. However, in *H. pylori*-specific RAST analysis, specific IgE was found only in 1 of 25 *H. pylori*-infected patients. ***Conclusions:*** Our findings suggest that although IgE-binding epitopes were found in *H. pylori*, *H. pylori*-specific IgE antibodies are not common in chronic urticaria, and the clinical significance of the IgE response is unclear.

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Introduction

Specific immune reactions are important contributing factors in *Helicobacter pylori* gastritis. Methodologically different techniques and antigen extracts have been used to demonstrate IgG and IgA antibodies against *H. pylori* by immunoblotting and ELISA assays. These studies have revealed several important IgG- and IgA-binding antigenic structures of *H. pylori*, including CagA (128 kD) and VacA (87 kD) [1–3]. The presence of *H. pylori*-specific IgG in serum and IgA in gastric juice does not eliminate *H. pylori* infection and it has remained unclear whether an immune response against *H. pylori* plays some role in the pathogenesis of *H. pylori*-associated intestinal diseases. At present, *H. pylori* is considered as one of the infectious agents which have been connected to several idiopathic diseases. Several authors have studied the association of *H. pylori* with chronic urticaria [4, 5].

In addition to IgG and IgA, the IgE-mediated immune response to *H. pylori* has been studied [6–8], but little is known about the antigenic structures of *H. pylori* that are responsible for the binding of IgE antibodies. In previous reports, we found elevated total IgE levels in patients with chronic urticaria, especially in patients with *H. pylori* gastritis [9]. An IgE-mediated pathway might be a possible link between *H. pylori* and chronic urticaria. We therefore carried out an immunoblotting study to characterize the IgE-binding antigens of *H. pylori* in order to establish an *H. pylori* IgE-specific assay for clinical studies.

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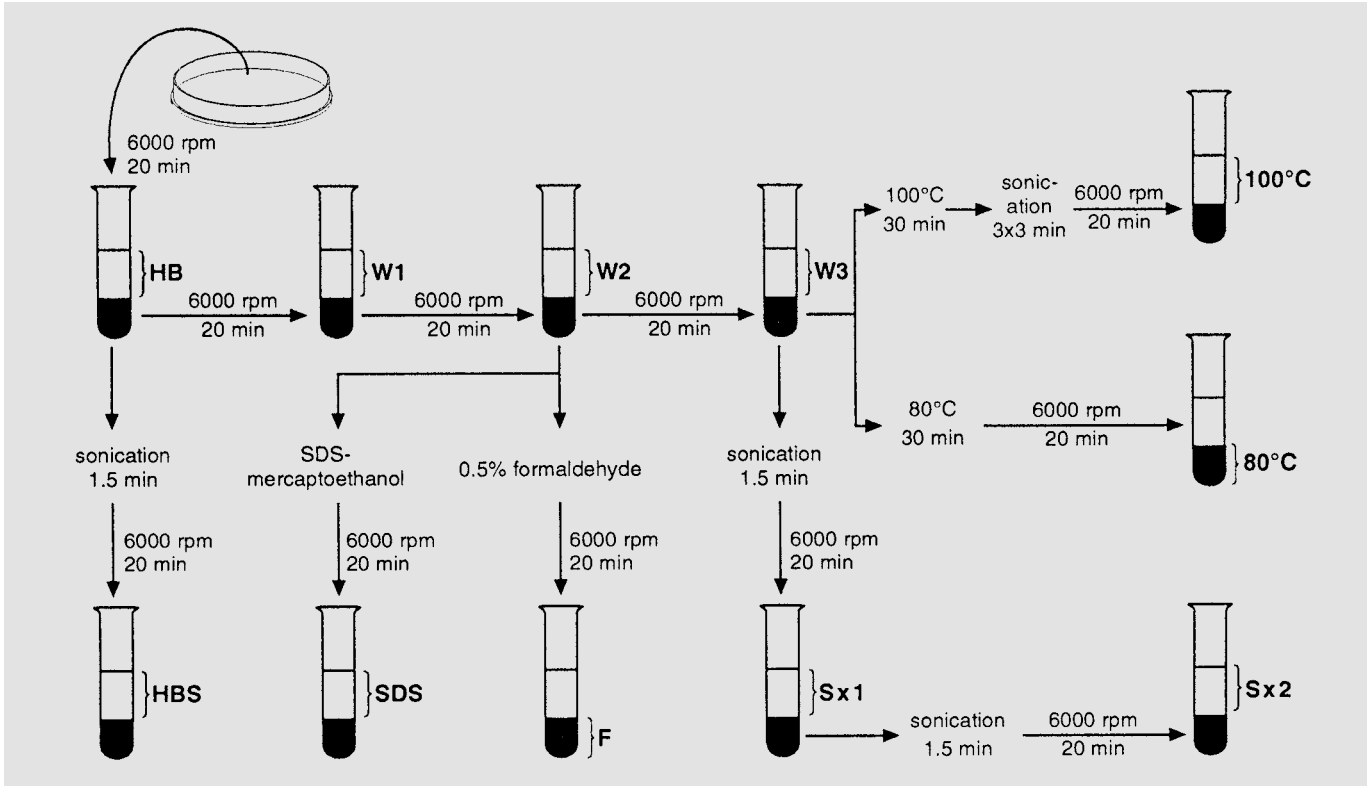


Fig. 1. Purification of different *H. pylori* antigens used in IgE immunoblotting, including HB, W1, W2, W3, HBS, Sx1, Sx2, heating treatments (100°C, 80°C) and SDS and F treatment.

Materials and Methods

Bacterial Strains

Two different *H. pylori* strains were used in this study. The Turku strain was a clinical isolate from a patient investigated for gastritis in Turku University Central Hospital. It was typed by the Department of Medical Microbiology, Turku University, Finland. The other *H. pylori* strain was supplied by the American Type Culture Collection (ATCC; strain type 12301).

Culture Conditions and Preparation of H. pylori *Extracts*

The bacteria were cultured on chocolate agar plates under anaerobic conditions at 42°C for 3 days. Bacterial cells were harvested with 0.9% NaCl from cultured plates and suspended in 0.9% NaCl to be processed further.

Different *H. pylori* antigen preparations of both *H. pylori* strains were produced to be compared in immunoblotting experiments (fig. 1). The antigens consisted of harvesting buffer (HB) and 1st (W1), 2nd (W2) and 3rd (W3) washing fluids after collection and centrifugation (at 6,000 rpm for 20 min) of the harvested cells. In addition, pellets after the 3rd wash were sonicated for 1.5 min (Sx1) and for another 1.5 min (Sx2), and after harvesting for 1.5 min (HBS). The sonicated pellets were centrifuged as earlier described and the supernatants were saved, displaying separate antigens. The effect of denaturating conditions on preparations was analyzed as follows: a pellet after the 2nd wash was suspended in 10 ml of 0.5% formaldehyde in 0.05 mol/l PBS. After incubation at 20°C, the cells were washed once and centrifuged and the pellet was stored at –70°C (F). A pellet obtained after the 2nd wash was suspended in SDS-mercaptoethanol (1 µl of PBS and 5 µl of mercaptoethanol), boiled for 5 min and centrifuged and the supernatants was saved (SDS). A pellet obtained after the 3rd wash was heated for 30 min at 100°C and sonicated for 3 min three times, after which it was centrifuged and the supernatant was saved. A pellet obtained after the 3rd wash was heated for 30 min at 80°C and centrifuged and the pellet was collected and stored at –70°C to be used in the assays. The supernatants were pooled, dialyzed at 5°C against deionized distilled water (molecular weight cutoff of the dialysis membrane being 3,000 D) and freeze dried.

The *H. pylori* antigen used in routine IgG and IgA antibody assays in the Turku University Hospital was prepared as follows: the bacteria were grown anaerobically at +42°C for 48 h, collected in 0.9% NaCl, washed twice with NaCl and heated for 60 min at 100°C. Then, the preparation was suspended in 0.2 *M* glycine-hydrochloride buffer, pH 2.2, sonicated, dialyzed at 5°C against deionized distilled water, frozen at –70°C and freeze dried.

Patients

For the characterization of IgE binding to *H. pylori* antigens with immunoblotting, a serum pool from three patients having *H. pylori*-

specific IgE antibodies with routine antigen was used. Five patients with no detectable *H. pylori* IgE according to RAST results served as controls.

For comparative RAST assays, the sera from 25 *H. pylori* IgG-seropositive chronic urticaria patients (*H. pylori* diagnosis confirmed by biopsy specimens in all of them) and 9 *H. pylori*-seronegative chronic urticaria patients (*H. pylori* infection excluded also by biopsy specimens in 2) were examined. The patients (20 male and 14 female, with an age range from 24 to 70 years, mean age 47 years) had suffered from urticaria symptoms for between 8 months and several years. For the treatment of chronic urticaria, all of the patients used H1 antagonists. In addition, 15 of these patients (12 *H. pylori* positive, 3 *H. pylori* negative) occasionally needed oral prednisone and 6 patients (5 *H. pylori* positive, 1 *H. pylori* negative) occasionally used bronchospasmolytes.

SDS-PAGE and Western Blot

For SDS-PAGE, HB, washing fluids (W1, W2, W3), sonicated extracts (Sx1, Sx2, HBS) and antigen preparations obtained after denaturating treatments (F, SDS, 100°C, 80°C) (fig. 1) were dissolved in sample buffer, applied to a 5–17.5% gradient gel with a molecular weight marker (LMW Kit, Pharmacia LKB Biotechnology, Uppsala, Sweden) and separated according to the method of Laemmli [10]. The gels were stained with Coomassie brilliant blue to visualize separation of the proteins. The separated proteins of non-stained gels were transferred onto nitrocellulose sheets (Millipore 0.22 μm; Millipore, Bedford, Mass., USA) according to the method of Towbin et al. [11].

The specific IgE binding to *H. pylori* on the nitrocellulose membrane was detected according to the method of Bengtsson et al. [12]. The nitrocellulose sheets were incubated with a serum pool from three patients. The allergen-bound IgE was detected using ^{125}I-labeled anti-IgE (Phadebas RAST, Pharmacia) diluted 1/10 with PBS and 0.5% Tween (Phadebas RAST, Pharmacia). The IgE-stained protein bands were detected by autoradiography using X-ray film (Kodak XRP-5, USA) with an intensifying screen at –70°C.

The H. pylori *RAST*

Based on immunoblotting experiments, two *H. pylori* extracts were used in the RAST assays: (1) the one routinely used in *H. pylori* IgG and IgA assays and (2) a mixture comprising W2, W3 and Sx1 (pooled in the ratio 1:1:2).

A modification of the previously described method of Walsh et al. [13] was used. Discs with diameter of 5.5 mm were cut from a nitrocellulose sheet with an office punch (Millipore, pore size 0.22 μm, Millipore). The optimum concentration of *H. pylori* extract (in PBS, pH 7.4) for coupling the discs was determined as 0.5 mg/ml (range 0.01–2.5 mg/ml). The nitrocellulose discs were incubated in *H. pylori* extracts in a shaker for 2 h at 37°C. Then, the discs were washed three times with PBS and were blocked by incubation in 0.5% Tween-20 for 1 h at 37°C. For the assays, 50 μl of undiluted serum was applied onto the discs and incubated at +4°C overnight. Thereafter, the discs were washed three times with NaCl-Tween (0.5%), and 50 μl of diluted (1/2 in PBS-Tween) ^{125}I-labeled anti-IgE (Pharmacia) was added and the discs were incubated at 37°C for 2 h. The discs were washed three times and the bound radioactivity was detected by a gamma counter (1282 Compugamma; Wallac, Turku, Finland). Maximum total activity was determined in two test tubes containing 50 μl of diluted ^{125}I-labeled anti-IgE, and in addition, the radioactivity in two buffer blanks was counted. Duplicated determinations were made for each serum. The results were expressed as an *H. pylori* RAST index value:

$$\frac{\text{sample (cpm)} - \text{blank (cpm)}}{\text{total activity (cpm)} - \text{blank (cpm)}} \times 10^2.$$

Results

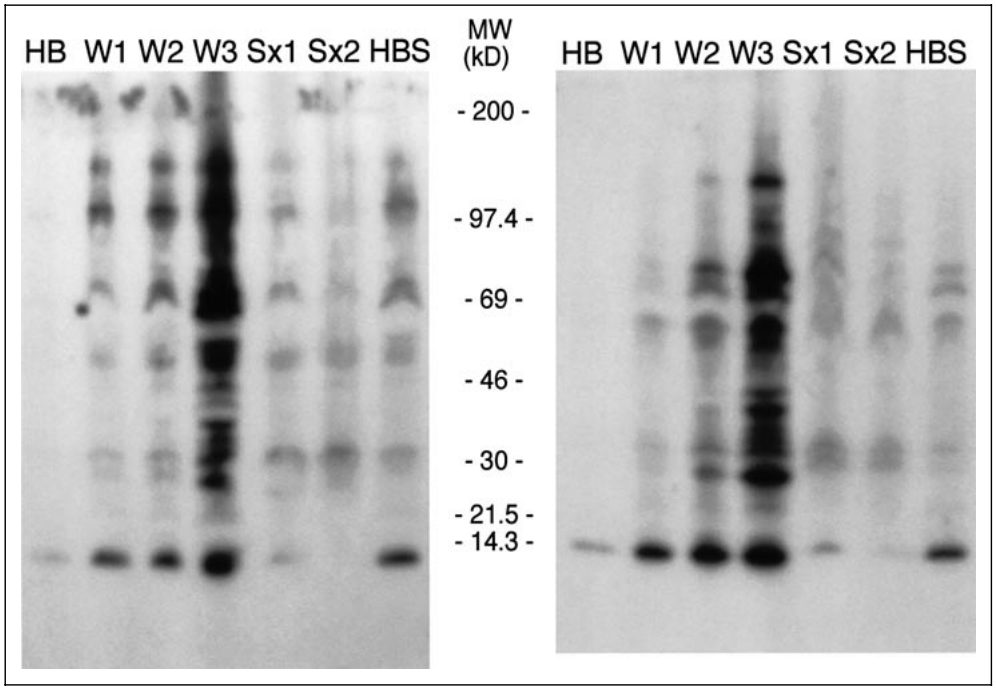
The number of Coomassie brilliant blue-stained bands was equal in both *H. pylori* (Turku, ATCC) strains, with about 35–40 bands visualized in washing fluids and sonicated extracts, while the antigens obtained after heating, SDS-mercaptoethanol and formaldehyde treatment displayed 15–20 bands.

In immunoblotting, the comparison of the IgE staining of different *H. pylori* antigen preparations are shown in figures 2 and 3. The IgE-stained bands were found between 7 and 170 kD, depending on the antigen preparation. The most intensively stained protein bands were found in the washing fluids and sonicated extracts. The greatest number of IgE-stained bands (n = 19) were visualized in W3. Formaldehyde-, SDS-mercaptoethanol- and heat-treated extracts gave only from 0 to 5 weakly IgE-stained bands between 7 and 80 kD (fig. 3). Formaldehyde treatment destroyed all the IgE-binding epitopes of the antigen. The IgE-stained immunoblotting patterns were similar with both *H. pylori* strains (fig. 2). In immunoblotting, no IgE-stained bands were detected with individual control sera by an antigen pool of the extracts W2, W3 and Sx1 (1:1:2).

The results of the *H. pylori*-specific RAST assay are presented in figure 4 as *H. pylori* RAST index values. The routine antigen is the one used in routine *H. pylori* IgG and IgA assays in the Turku University Hospital, and the novel antigen is a pool of the extracts W2, W3 and Sx1 (1:1:2). Of 34 chronic urticaria patients with *H. pylori* gastritis, none was positive using the routine antigen, while the novel antigen detected 1 *H. pylori* IgE-positive patient.

Discussion

To optimize the method of studying *H. pylori* IgE antibodies, a number of antigenic extracts were prepared in several ways and IgE staining was analyzed with a sensitive immunoblotting technique. The majority of IgE-binding bands were found with antigen extracts which were prepared by a 'gentle method' from *H. pylori*. In this method, washing fluids of intact or briefly sonicated bac-



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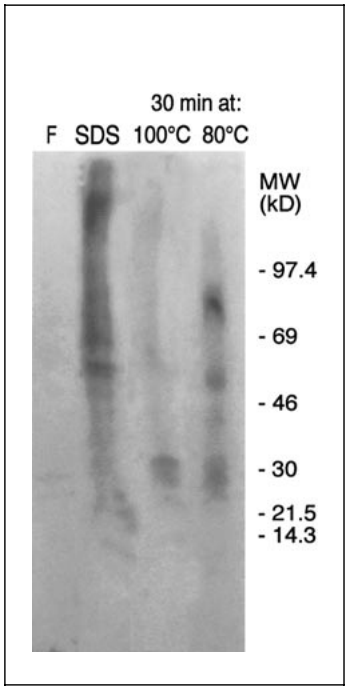

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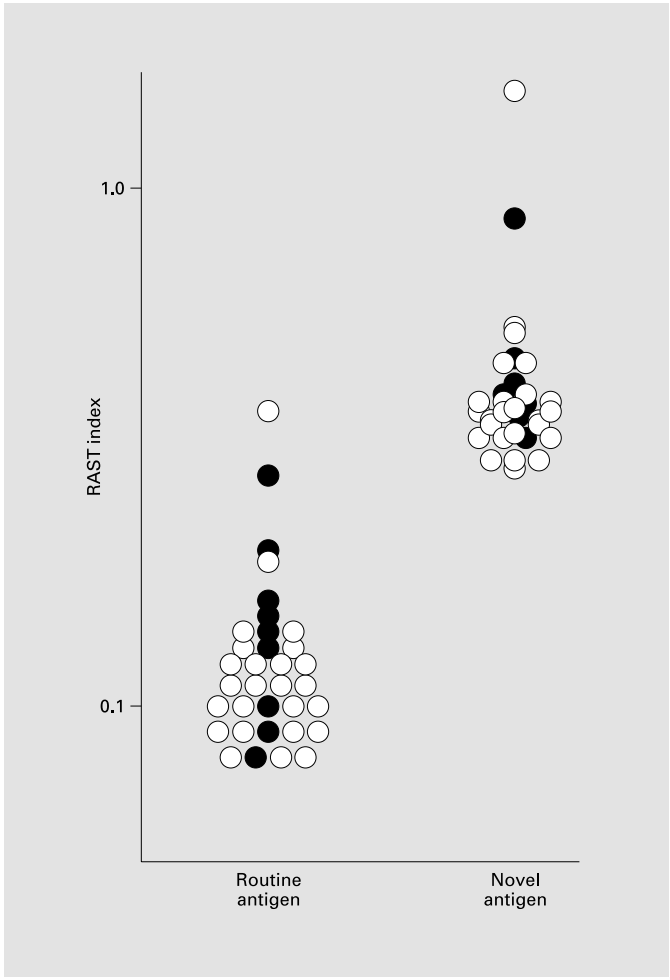

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teria were harvested for antigen preparation. The findings suggest that the epitopes inducing an IgE response would mainly be found in the cell surface structures of *H. pylori*, possibly in the outer membrane proteins of the glycocalyx layer. Further treatment of bacteria with denaturating agents, such as SDS, formaldehyde or heat, gave unacceptable results; the number and intensity of IgE-binding bands was clearly lower than with the milder treatment. Using the optimal antigen preparation for IgE immunoblotting, bands in the 87- and 128-kD region, probably representing known immunodominant VacA and CagA proteins, were visualized. In addition to these, several other bands were also detected, indicating that the lower molecular weight bands also harbor IgE-binding epitopes.

Infection with *H. pylori* results in chronic inflammation of the gastric mucosa. Usually, the infection is

Fig. 2. IgE staining of the *H. pylori* antigens extracted under physiologic conditions using two different *H. pylori* strains (Turku strain on the left, ATCC strain on the right).

Fig. 3. IgE staining of the *H. pylori* extracts treated in denaturating conditions using the Turku strain.

Fig. 4. *H. pylori* RAST assays with an antigen used in routine *H. pylori* IgG and IgA assays and a novel antigen (a pool of W2, W3 and Sx1; 1:1:2). ○ = Chronic urticaria patients with current *H. pylori* infection; ● = chronic urticaria patients with no evidence of *H. pylori* infection.

acquired in early childhood and persists, unless treated with antibiotics, in spite of IgG and IgA responses. However, the role of these antibodies in the pathogenesis of *H. pylori*-induced gastritis and ulceration is not known. More recently, studies of IgE responses to *H. pylori* have been conducted, and some of these indicate that specific IgE might also be associated with the pathophysiology of *H. pylori* infection. Even before the discovery of *H. pylori* by Marshall and Warren [14], Brown et al. [15] proposed a theory of an allergic pathogenesis of gastric erosions. Andre et al. [16] found a marked increase in IgE-producing cells at the edges of duodenal and gastric ulcers. As bacteria-specific IgE antibodies can also function as histamine-releasing factors, symptoms associated with food intake in some patients could be explained by *H. pylori*-specific IgE antibodies at the site of *H. pylori* infection. Such a chronic infection might evoke an allergic reaction locally in the gastrointestinal tract against a variety of allergens.

In order to study the possibility that *H. pylori* might induce a prominent IgE response at least in some patients, which in turn could elicit and maintain symptoms like urticaria, we decided to conduct a study where the detection of serum IgE was first optimized using an immunoblot technique and then the antigen was applied to a more flexible RAST system. The requirements of antigen extracts for IgE assays differ from those for IgG and IgA. IgE-binding components, also in microbes, are usually easily soluble in physiologic conditions [17]. Of a total of 25 chronic urticaria patients with current *H. pylori* infection, only 1 patient appeared positive with *H. pylori*-specific RAST. This indicates that IgE-mediated responses against *H. pylori* are not common in this group of patients and may not be involved in histamine release, for example, contrary to earlier observations by Aceti et al. [6], who found basophil-bound specific IgE in 84% and serum IgE, determined by the histamine release test, in 69% of patients with *H. pylori* infection.

The pathophysiology of *H. pylori* infection remains enigmatic. Although our results do not support the role of IgE-mediated responses to *H. pylori* in chronic urticaria, it has not been excluded that IgE still might play a role in *H. pylori*-associated diseases. Recent findings of Figura et al. [18] and Corrada et al. [19] suggest that *H. pylori* infection may facilitate penetration by allergens and the development of an IgE response to common alimentary antigens and thereby affect the development of food allergy. However, based on the current results, IgE antibodies against *H. pylori* are not common among patients with chronic urticaria.

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