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IgE/Anti-IgE Immune Complexes in Sera from Patients with Crohn's Disease Do Not Contain Food- Specific IgE

Abstract

Background: An association of Crohn's disease (CD) with food allergy has been discussed, but the role of food-specific IgE has not been clarified yet. Since CD is combined with immune complex formation, we examined in the present study whether anti-IgE autoantibodies in such complexes might hinder the determination of specific IgE. **Methods:** In order to elucidate the role of food-specific IgE in CD, we tested sera from CD patients (n = 107), healthy controls (n = 65) and allergic subjects (n = 7) for their IgE binding to food antigens (yeast, corn, celeriac, wheat) by an immunodot assay. After determining levels of IgE/IgG anti-IgE immune complexes, we purified them from serum pools of patients with CD, allergic subjects and healthy controls by affinity absorption using a monoclonal anti-IgE antibody. These purified immune complexes were treated by low pH (pH = 4) in order to dissolve them and to increase the detectability of food-specific IgE by RAST and CAP assay. **Results:** In CD sera no food-specific IgE could be detected, but levels of immune complexes of IgE and IgG anti-IgE autoantibodies were statistically significantly increased compared to healthy controls. pH treatment of purified IgE/IgG anti-IgE immune complexes resulted in a significant increase in specific IgE to yeast, corn, wheat and celeriac detected by RAST, however, only in the serum sample purified from allergic subjects. After pH treatment of CD immune complexes, specific IgE levels remained still very low. **Conclusion:** Thus, even if IgE seems to represent an autoantigen in CD, it is unlikely to specifically participate in the pathophysiology of the putative food adverse reactions.

Key Words

Anti-IgE autoantibodies
Crohn's disease
Immune complexes
Food-specific IgE

Introduction

Crohn's disease (CD) is a chronic inflammatory disease for which an immunologic etiology has been proposed. Patients suffer from diarrhea, abdominal pain, malabsorption, but also from food intolerance. Whether food hypersensitivity might contribute to the pathogenesis of CD is not clar-

ified and is discussed controversially. Among patients with inflammatory bowel disease, those with CD had the highest levels of total IgE [1], but normal levels have also been found [2]. No significant difference was seen between CD, ulcerative colitis and healthy controls regarding total IgE levels, in contrast to specific IgE, which was significantly higher in patients with CD and ulcerative colitis [3]. How-

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ever, specific IgE levels were reinvestigated later by ELISA experiments and found to be normal [4], in accordance with elevated IFN- γ secretion by mucosal lamina propria CD4+ T cells [5]. However, CD is associated with elevated levels of immune complexes occasionally leading to arthritis for example [6]. IgE might be an autoantigen in CD participating in immune complex formation.

Since the first description of anti-IgE autoantibodies [7] substantial evidence has accumulated that IgG anti-IgE autoantibodies play a role in different atopic diseases [8, 9]. It has been shown that a large proportion of serum IgE occurs in complexes with IgG anti-IgE autoantibodies [10]. Moreover, these IgG anti-IgE autoantibodies hide specific IgE and preclude its precise determination in immunoassays, such as immunodot and RAST [11]. Furthermore, in the same immunoassays, the IgG anti-IgE autoantibodies bound to specific IgE were misinterpreted as 'specific IgG', if detection was performed by an anti-IgG antibody. This might be a pitfall in determining specific IgE also in other diseases, e.g. CD. Here, food-specific IgE might still be involved in food intolerances and local inflammation.

The aim of our study was, therefore, to unmask specific IgE in CD patients from residing anti-IgE autoantibodies. We purified immune complexes by affinity absorption on a monoclonal anti-IgE antibody and treated them by low pH in order to detect 'real' levels of specific IgE in RAST immunoassay.

Materials and Methods

Patients and Sera

Sera from 107 CD patients (47 males, 60 females; mean age 34.6 years) were collected at the Department of CD, Clinic of Internal Medicine IV, AKH Vienna [12]. Diagnosis was based on standard clinical, radiological and histopathological criteria [13]. None of the patients had resective surgery, more than 10 mg prednisolone equivalents or parenteral nutrition within 2 months before the study. Patients with stoma or short bowel syndrome were excluded. The mean duration of the disease was 83 months. 62 patients underwent bowel resections, 42 were treated with 5-aminosalicylic acid, 13 with 10 mg prednisolone equivalents at most daily. The activity of the disease was evaluated according to the CD activity index established by Best et al. [14]. The mean CD activity index was 100. 65 healthy controls (33 males, 32 females; mean age 33.8 years) formed the control group. Food adverse reactions of patients were recorded [12].

The allergic group consisted of 14 patients (9 males, 5 females, age range: 9–68 years) with clinical symptoms of pollen allergy during summer and high specific IgE to wheat (evaluated by RAST, Pharmacia, Uppsala, Sweden).

Serum Pools. Equal parts of 10 sera from CD patients were pooled. This CD serum pool was analyzed by immunodot, RAST, ELISA and affinity chromatography. The normal human serum (NHS) pool consisted, in equal parts, of 10 sera of non-allergic healthy controls with-

out detectable specific IgE. The serum pool of allergic subjects was composed of equal parts of sera from 8 wheat-allergic subjects.

Protein Extraction

Yeast was bought from Oetker (Villach, Austria), wheat, corn and celeriac were purchased from a local retail shop. 5 g of each raw food were homogenized and extracted in 10 mM K_2HPO_4 , 10 mM sodium-diethyldithiocarbamate, 2% polyvinylpyrrolidone, 2 mM EDTA, 0.1% PMSF, 0.01% pepstatin, 3 mM NaN_3 , at 4°C overnight with constant stirring. After centrifugation (325 g, 30 min, 10°C), supernatants were collected and again centrifuged (40,000 g, 60 min, 4°C). Then supernatants were dialyzed against 10 mM K_2HPO_4 , 3 mM NaN_3 at 4°C (Dialysis membranes: Spectra/Por, Huston, Tex., USA, cutoff 6,000–8,000). Dialysates were frozen at –70°C and lyophilized. The extracts were stored in aliquots at –20°C. Extract quality was controlled by SDS-PAGE analysis (not shown). The protein concentrations were determined by the method of Bradford [15].

Immunoblot

Detection of Specific IgE 'Total' IgE. Food antigens (yeast, wheat, corn and celeriac) or monoclonal anti-IgE antibodies (4F4, kindly provided by Dr. Klimovich, Hybridoma Technology Laboratories, St. Petersburg, Russia [16]; BSW 17, LE 27 [17, 18], kindly provided by Prof. Dr. Beda Stadler, Bern, Switzerland) were diluted in PBS and dotted in duplicates onto nitrocellulose (Millipore, Bedford, Tex., USA, 0.45 μ m). Extracts of yeast, wheat, corn and celeriac were dotted at 4 μ g/dot (1 μ l/dot), monoclonal anti-IgE antibodies BSW 17 and 4F4 at 0.25 μ g/dot and LE 27 at 1 μ g/dot. Dots were air dried and blocked with buffer (50 mM Na_2HPO_4 , 0.5% BSA, 0.05% NaN_3 , 0.5% Tween 20, pH = 7.4) containing 1% dry milk. Then, sera were diluted 1:4 for the detection of IgE and applied onto strips overnight at 4°C with constant shaking. Control strips were incubated with buffer and washed. Specific IgE and total IgE were detected by ^{125}I -labeled rabbit anti-human IgE (Phadebas RAST kit; Pharmacia, 100,000 cpm/ml buffer) overnight at room temperature (RT). For quantification, standard IgE (SU-11; from an immortalized B cell line, kindly provided by Prof. Dr. B.M. Stadler) was dotted in serial dilution and developed with ^{125}I -labeled rabbit anti-human IgE (Pharmacia).

Autoradiography was performed at –70°C for 1, 7 or 21 days, using hyperfilm MP (RPN 6, Amersham, Sweden) in exposure cassettes (X-omatic, Kodak) containing intensifying screens (X-omatic regular, Kodak). Dot density was measured using a hand-held reflection densitometer (Vipdens 130, Viptronic GmbH, Brixen, Italy).

ELISA

For the detection of IgE/anti-IgE immune complexes, sera were tested by ELISA. Plates (Dynatek M129A, Centax) were coated with anti-IgE antibodies (BSW 17 0.05 μ g, 4F4 0.05 μ g, LE 27 0.05 μ g/ml) in carbonate buffer at 4°C overnight. For quantification, wells were coated with a dilution series of human myeloma IgG (40 ng to 0.1 ng/ml). After washing with PBS, 0.05% Tween 20 (30 min, three changes), remaining binding sites were blocked by incubation with PBS containing 1% BSA at 37°C for 30 min. Sera (diluted 1:20 in PBS/Tween/BSA) were incubated at 37°C for 1 h. After washing, bound immune complexes were detected by peroxidase-conjugated mouse anti-human IgG (Zymed Laboratories, San Francisco, Calif., USA) 1:1,000 in PBS/Tween/BSA, at 37°C, 1 h. The reaction was developed with benzoe acid (pH = 6), 0.05% H_2O_2 (9:1) as substrate at RT for at least 30 min. Optical density was measured at 450 nm with a Dynatech microplate reader (MR 600, Germany).

Affinity Chromatography

Preparation of Reversed Immunosorbent. 1 mg of mouse monoclonal anti-human IgE antibody (LE 27) [17] was bound to 1 g CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Affinity Purification of Immune Complexes using LE 27. CD-, NHS- and allergic subjects serum pools were diluted 1:2 in 0.9% (w/v) NaCl and dialyzed against 0.9% (w/v) NaCl at 4°C using dialysis membranes (Spectra/Por, USA, cutoff 100 kD) before application to the respective column. Sera (5 ml per separation run) were loaded and recirculated for 5 h at 4°C. Then effluents were collected. After extensive washing with 0.9% (w/v) NaCl, bound material was eluted with 3 M KSCN/5 mM K₂HPO₄ (pH = 6.5). 1-ml fractions were collected, neutralized with 0.1 M NaOH8 (pH = 7.5) and immediately dialyzed against 0.9% (w/v) NaCl at 4°C. Eluted samples were concentrated using ultrafiltration membranes (Centriprep-100; Amicon, Beverly, Mass., USA) with a cutoff value of 100 kD. The protein concentration of these samples was adjusted to 1 mg/ml PBS by the method of Bradford [15]. The composition of the eluted fractions was controlled by an immunodot assay for the presence of IgE complexed with anti-IgE autoantibodies (data not shown).

Modified RAST

Squares (6 × 6 mm) cut from nitrocellulose (Schleicher & Schüll, Dassel, Germany) were coupled with antigens by incubation in a solution of 1 mg yeast, corn, wheat or celeriac extract/ml PBS at 4°C overnight. For IgE quantification, squares were prepared by dotting a serial dilution of standard IgE (SUI-11) onto nitrocellulose (0.4–25 ng/μl). Dried discs were washed with 0.9% (w/v) NaCl containing 0.05% (v/v) Tween 20 and washing solution (Pharmacia RAST kit). Protein concentrations in eluent samples from affinity chromatography were determined by the method of Bradford [15], and 2 ng/RAST disk in 100 μl PBS were applied and incubated at RT for 3 h. Alternately, eluent samples were incubated in PBS, pH = 4, for 30 min at RT before application. Buffer control discs were incubated with PBS or PBS pH = 4. After repeated washing, discs were incubated with ¹²⁵I-labeled rabbit anti-human IgE (Pharmacia) at RT overnight. Discs were washed again, and bound radioactivity was measured in a γ-counter (Pharmacia, Wallac Oy, Finland).

Results

Detection of Specific and Total IgE by Immunodot Assay

We selected four food antigens according to their antigenic potential in food adverse reactions of CD patients (wheat, corn and yeast) [19] and in food allergy (wheat and celeriac) [20–23]. Specific IgE directed against food antigens was analyzed by immunodot. Figure 1 shows the levels of specific IgE antibodies (ng/ml serum) in sera from CD patients, healthy controls and allergic subjects. Median levels of specific IgE to all four antigens were similar in CD patients (n = 107) and healthy controls (n = 65). Elevated median levels of specific IgE to wheat and celeriac could be detected in the allergic group (n = 7; composed of wheat-

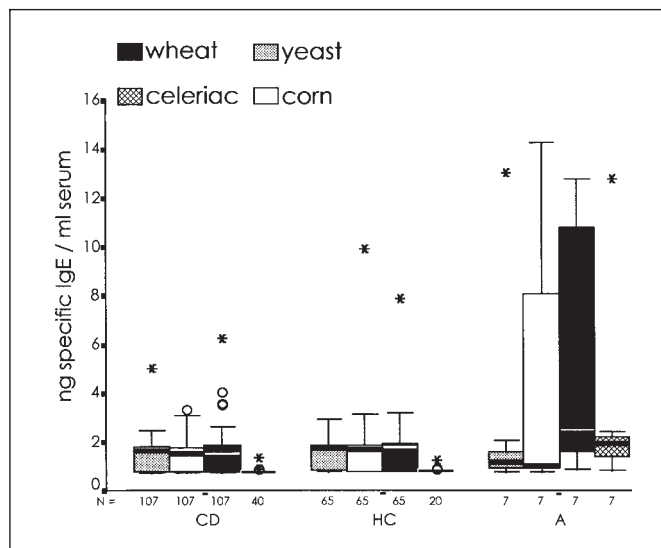


Fig. 1. Box plot graph showing specific IgE antibody levels (ng/ml serum) in sera from CD patients, healthy controls (HC) and allergic subjects (A) determined by immunodot. Antigens (yeast, corn, wheat and celeriac) were dotted onto nitrocellulose (4 μg per dot), incubated with sera (diluted 1:4) and developed with ¹²⁵I-labeled rabbit anti-human IgE. Autoradiographic signals were densitometrically analyzed and quantified in comparison with dotted standard IgE. The boxes represent the range of the inner quartiles of the sample, the 'whiskers' the outer 25% of sera. The box is divided by the median. Sera with titers showing more than 1.5-fold deviation from the end of the box were defined as outliers and marked as circles, sera with titers with more than 3-fold deviation were defined as extremes and marked as asterisks.

allergic patients). Thus, by immunodot assay, 'normal' levels of food-specific IgE were detected in CD sera compared to allergics.

As with specific IgE, no difference was found comparing 'total' IgE levels in sera from CD patients (n = 96) and healthy controls (n = 55; fig. 2). The monoclonal anti-IgE antibodies BSW 17 and 4F4 (both anti-Fcε3) detected significantly more IgE than LE 27 (anti-Fcε4) in sera of the allergic group. In all three patient groups, the anti-IgE antibody 4F4 detected about ten times more IgE than LE 27.

Detection of Immune Complexes by ELISA

Immune complexes of IgE and IgG anti-IgE autoantibodies in sera from CD patients, healthy controls and allergics were detected by a sandwich ELISA using the monoclonal anti-IgE antibodies BSW 17, 4F4 or LE 27 as catching antibodies (fig. 3). With all three antibodies, levels of immune complexes appeared increased in CD sera (n = 10) as compared to the healthy control group (n = 10). We found statistically significant increases when catching with antibody

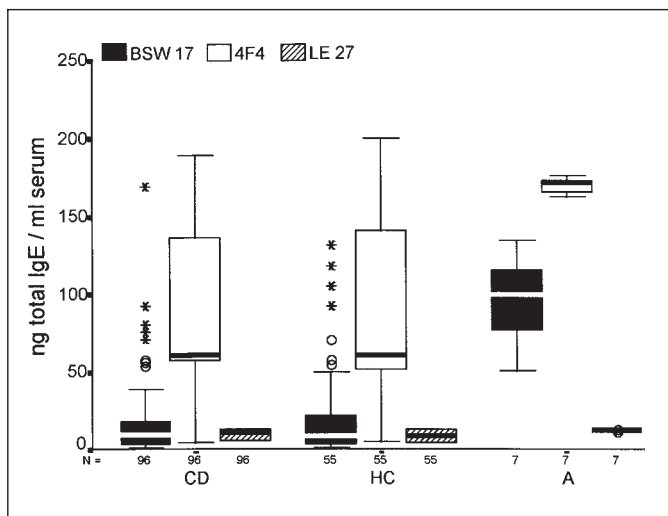


Fig. 2. Box plot graph (described in the legend to fig.1) showing total IgE antibody levels (ng/ml serum) in CD patients, healthy controls (HC) and allergic subjects (A) by immunodot. Monoclonal anti-IgE antibodies (BSW 17, 4F4 and LE 27) were dotted onto nitrocellulose. Strips were incubated with sera (diluted 1:4) and developed with ^{125} I-labeled rabbit anti-human IgE. Autoradiograph signals were densitometrically analyzed and quantified in comparison with dotted standard IgE.

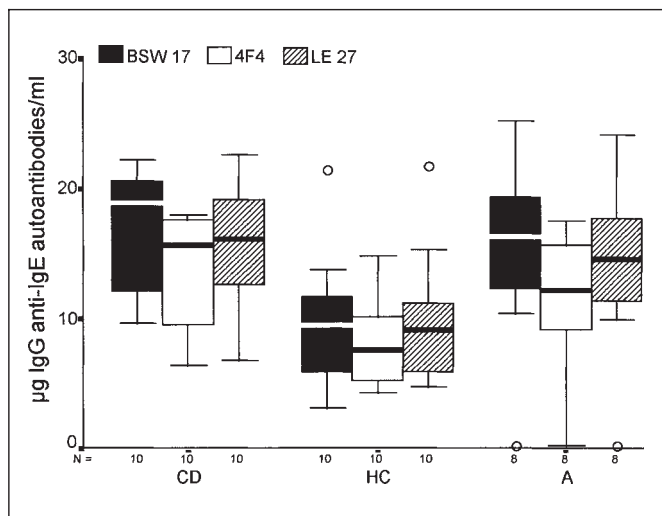
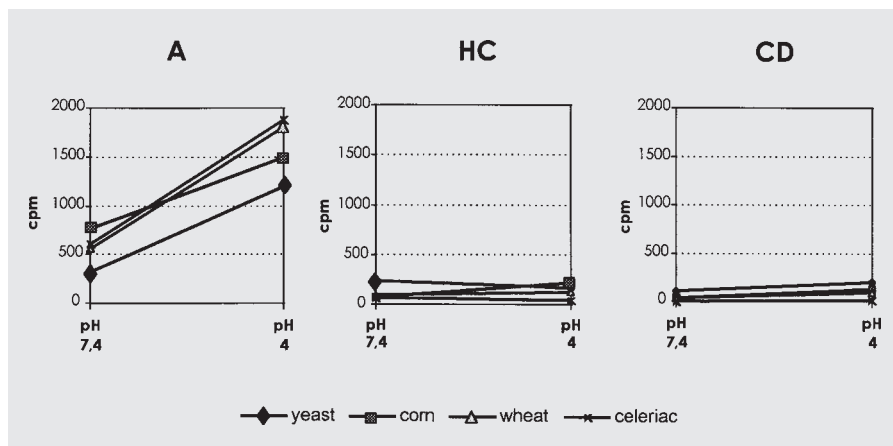


Fig. 3. Box plot showing levels of IgG anti-IgE autoantibodies (μ g/ml serum) complexed with IgE in sera from CD patients, healthy controls (HC) and allergic subjects (A) analyzed by ELISA. Plates were coated with monoclonal anti-IgE antibodies (BSW 17, 4F4 and LE 27). Signals were quantified by comparing with standard human IgG. After incubation with diluted sera, bound immune complexes were detected by peroxidase-conjugated mouse anti-human IgG.

Fig. 4. RAST. Nitrocellulose discs were coupled with antigens (yeast, corn, wheat, or celeriac) and incubated with pH = 4-treated immune complexes, affinity purified from sera of patients with food allergy (A), of healthy controls (HC) or CD patients. Bound IgE was detected by ^{125} I-labeled rabbit anti-human IgE.



4F4 ($p < 0.009$) and BSW 17 ($p < 0.003$), and a trend toward elevated immune complex levels with LE 27 ($p < 0.024$) with the Student's *t* test.

pH = 4 Treated IgE/Anti-IgE Immune Complexes by RAST

We purified IgE/IgG anti-IgE immune complexes from serum pools of allergic subjects, healthy controls and CD patients by affinity absorption using monoclonal anti-IgE

antibody LE 27. The eluents were treated by pH = 4 in order to dissolve immune complexes and make more IgE detectable. Figure 4 shows the reactivity of specific IgE (complexed with anti-IgE autoantibodies in the eluent) after pH = 4 treatment in RAST. Regarding eluents from allergic subjects' sera, pH = 4 treatment made significantly more specific IgE to all antigens detectable. In contrast, pH = 4 treatment had no effect on the detection of specific IgE in samples from CD patients and healthy controls. Thus, sera

from the CD and allergic patients contained equal amounts of IgE/anti-IgE immune complexes, but no IgE specific for the tested food antigens.

In order to increase sensitivity, purified immune complexes were also tested in a commercial CAP assay. However, a similar pattern as in the above-mentioned RAST analysis was seen (data not shown).

Discussion

Ingestion of different types of food, e.g. yeast, wheat products, corn and vegetables, is sometimes attributed to worsening of clinical symptoms in CD patients [19, 24]. Therefore, elimination diets are recommended by some physicians and have been reported to be helpful [19], thus implying a direct association of food intake and the pathophysiology of CD. The role of food-specific antibodies in CD, both IgG [4, 12; Gscheidlinger et al., manuscript submitted] and IgE [3, 4], remained so far controversial. In the present study, we have demonstrated that specific IgE does not contribute to the putative pathophysiology of food adverse reactions in CD, in accordance with the observed predominant TH1 cytokine pattern [5]. With the immunodot sandwich assay, we also determined levels of 'total' IgE ranging between 10 and 170 ng/ml in the tested patient groups (reflecting an underestimation of the 'real' total IgE levels when using monoclonal anti-IgE antibodies for catching, as observed previously [25]). Also, the levels of 'total' IgE did not distinguish the group of CD patients from healthy controls.

Immune complexes of IgE and anti-IgE autoantibodies play a substantial role in food allergy [26]. As CD has been proposed to be associated with food adverse reactions on the one hand, and with immune complex formation on the other hand [6], we determined levels of immune complexes of IgE and IgG anti-IgE autoantibodies. Compared to healthy controls, in CD sera statistically significant increases in the levels of such complexes were also found. Anti-IgE autoantibodies are present in sera from patients with different atopic diseases [8, 9] and harbor distinct biological activities depending on the domain specificity on IgE (Fcε) [27–29]. Moreover, it has been shown that these anti-IgE autoantibodies interfere with the determination of total and specific IgE in immunoassays [10, 11]. In order to elucidate the possibility that such autoantibodies might also hinder the detection of IgE in CD sera, we purified immune complexes of IgE and IgG anti-IgE autoantibodies by affinity absorption using a monoclonal anti-IgE antibody. Interestingly, the composition of the eluted immune complexes var-

ied between the different patients groups: in allergic subjects, the ratio of IgE to IgG in immune complexes was approximately 1:4, whereas in CD immune complexes the ratio was much higher in favor of IgG (data not shown).

For dissolving immune complexes, purified fractions were treated by low pH and then tested by RAST. Dramatically more IgE was detected in low-pH-treated samples from allergic subjects, indicating that in these sera a large proportion of the specific IgE is masked by anti-IgE autoantibodies, in agreement with our previous observations [11]. However, in CD patients' samples, detection of specific IgE was not enhanced after pH = 4 treatment of immune complexes. One possible reason might be a lower avidity of CD IgE as compared to atopic IgE. In that case, the lower pH could hinder binding of CD IgE to the allergens. But, our results might also indicate that the levels of food-specific IgE in sera from CD patients are too low to participate in food adverse reactions.

Nevertheless, the levels of immune complexes of IgE and IgG anti-IgE autoantibodies in CD sera were increased as compared to NHS. This might be seen in context with a general trend towards immune complex formation in CD, with IgE representing one of several autoantigens. Whether these immune complexes contribute to the pathophysiology of CD by direct stimulation of FcεR-positive proinflammatory cells, or by antibody-dependent cell-mediated cytotoxicity remains to be answered.

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