

Lack of a Role for Cross-Reacting Anti-Thyroid Antibodies in Chronic Idiopathic Urticaria

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The etiology of chronic idiopathic urticaria (CIU) is attributed to autoantibodies directed against the α -chain of the high-affinity IgE receptor (Fc ϵ RI α) or IgE on mast cells in 30–60% of patients. Approximately 30% of CIU patients have Hashimoto's thyroiditis (HT). We investigated the pathophysiologic relationship of anti-thyroid and anti-Fc ϵ RI α antibodies. Nine individuals with both CIU and HT underwent autologous serum skin testing (ASST) and sera were assayed for thyroid autoantibodies, thyroid-stimulating hormone, and anti-Fc ϵ RI α antibodies. Serum samples were studied for their ability to activate a human mast cell line (LUVA) as determined by cysteinyl leukotriene (CysLT) production. Experiments were performed to determine whether epitope cross-reactivity could explain the high incidence of HT found in CIU patients. A significant proportion of CIU patients had a positive ASST (nine of six) and anti-Fc ϵ RI α antibodies (six of nine). Incubation of patient sera with Fc ϵ RI α , but not thyroglobulin or thyroid peroxidase, resulted in the decreased ability to detect anti-Fc ϵ RI α antibodies. Incubation with thyroid antigens did not inhibit CysLT production by mast cells. Epitopic cross-reactivity does not explain the increased prevalence of HT found in CIU patients. The frequent concurrence of HT and CIU likely reflects a genetic tendency toward autoimmune diseases.

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INTRODUCTION

Chronic idiopathic urticaria (CIU) is defined as recurrent episodes of hives with erythema and pruritus occurring over a period of 6 weeks or longer (Sabroe *et al.*, 1999). CIU is a common disorder with a prevalence of 0.1% in the general population (Charlesworth, 2002). In the 1980s, Grattan *et al.* (1986) reported the presence of a serum factor that induced a wheal and flare response following intradermal injection of autologous serum, thought to be reflective of the presence of autoantibodies that are capable of inducing mast cell (and basophil) degranulation. The autologous serum skin test (ASST) has since been regarded as a reliable *in vivo* test in predicting autoimmunity in CIU patients (Sabroe *et al.*, 1999).

Studies have demonstrated that 30–60% of CIU patients possess IgG autoantibodies directed against the α -subunit of the high-affinity IgE receptor (Fc ϵ RI α) on mast cells and basophils (Hide *et al.*, 1993; Fiebiger *et al.*, 1995; Ferrer *et al.*, 1998). An additional 5–10% of CIU patients reportedly possess autoantibodies directed against IgE (Gruber *et al.*, 1988). Previous studies have demonstrated that IgG from patients with CIU stimulates

histamine release from donor basophils *in vitro* suggesting a pathogenic role for autoantibodies (Grattan *et al.*, 1991). Basophil activation tests have been considered the “benchmark” diagnostic test to identify autoimmunity as the cause of hives in patients with CIU (Yasnowsky *et al.*, 2006; Greaves and Tan, 2007; Altrich *et al.*, 2009). However, the main limitation to this approach is the inherent variability of basophils, which requires rigorous screening of basophils from numerous donors both to identify “releaser” basophils capable of Fc ϵ RI-dependent activation (Lichtenstein and MacGlashan, 1986) and also to ensure that donor basophils do not spontaneously secrete high basal levels of histamine. In addition, although basophils *in vitro* provide useful diagnostic information, CIU is primarily a disease of cutaneous mast cells. As basophils are not the primary cellular target of CIU, a mast cell model is the more ideal framework to study CIU pathophysiology.

We have developed an immortalized human mast cell line (LUVA) displaying high concentrations of Fc ϵ RI (Steinke *et al.*, 2009). These cells are useful in the study of immune mechanisms of mast cell-dependant disorders such as CIU, insofar as they degranulate and release arachidonic acid-metabolites in an Fc ϵ RI-dependant manner. The LUVA cells are activated when incubated with CIU serum, and this activation correlates with the presence of a positive ASST (Tiñana A, Mozena J, Steinke J Screening for autoimmune mechanisms in chronic idiopathic urticaria (CIU) utilizing an immortalized human mast cell line (LUVA), abstract submitted to the American Academy of Allergy, Asthma and Immunology annual meeting, February 2010).

Thyroid autoantibodies (TA) have a prevalence of ~3–6% in the general population versus ~15–30% in CIU patients (Leznoff and Sussman, 1989; Turktas *et al.*, 1997;

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Abbreviations: ASST, autologous serum skin test; CIU, chronic idiopathic urticaria; CysLT, cysteinyl leukotriene; Fc ϵ RI α , α -subunit of the high-affinity IgE receptor; HT, Hashimoto's thyroiditis; TA, thyroid autoantibodies; TG, thyroglobulin; TPO, thyroid peroxidase

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Verneuil *et al.*, 2004; Palma-Carlos and Palma-Carlos, 2005; Cebeci *et al.*, 2006). Although these studies establish the coincidental occurrence of TA and CIU, no current data exist to determine the basis for this linkage. The confluence of these two diseases could reflect an underlying genetic predisposition for the development of autoimmune illnesses. Alternatively, however, it is plausible that these diseases are pathogenically linked, possibly reflecting a shared epitope targeted by these different autoantibodies. Elucidation of the role that TA have in CIU may ultimately help in understanding the pathologic basis of this condition.

We initiated the current studies to further study the relationship between the presence of anti-FcεRIα antibodies and TA in patients with CIU. Specifically, we investigated whether the high incidence of Hashimoto's thyroiditis (HT), defined as the presence of antibodies to thyroglobulin (TG) and/or thyroid peroxidase (TPO), observed in patients with CIU could be explained by epitope cross-reactivity of anti-FcεRIα antibodies with thyroid antigens.

RESULTS

Study population

Twenty patients with CIU were enrolled in the study. The characteristics of patients enrolled into the study are presented in Table 1 and summarized in Table 2. The majority of patients with CIU and HT were females (17 females/3 males), with an average age of 38 ± 13 years.

ASST and anti-FcεRIα antibodies

Thirteen of 20 subjects had a positive ASST. In addition, 12 of 20 patients had positive anti-FcεRIα antibodies. Nine of 20 patients had both a positive ASST and anti-FcεRIα antibodies (Tables 1 and 2).

Thyroiditis prevalence

Nine of 20 subjects were selected for further study based on screening for the presence of either anti-TG or anti-TPO antibodies. Six of nine of these study patients required thyroid replacement therapy, the remainder had euthyroid HT (Table 1).

Epitope sharing between FcεRIα and thyroid proteins

Initially, a CIU patient who possessed anti-FcεRIα, anti-TPO, and anti-TG antibodies was evaluated for cross-reactivity regarding epitope binding. The ability to detect serum anti-FcεRIα antibodies was inhibited in a dose-dependent manner with preincubation of the serum sample with increasing concentrations of soluble FcεRIα. However, neither recombinant TPO nor purified TG competed with binding to FcεRIα (Figure 1).

Subsequently, four additional subjects with anti-FcεRIα, anti-TPO, and anti-TG antibodies were studied for epitope binding cross-reactivity using high concentrations of FcεRIα, TPO, and TG. The detection of anti-FcεRIα antibodies by ELISA was inhibited by an average of $93 \pm 11\%$ when these patient sera were incubated with human recombinant FcεRIα (Table 3). However, the ability to detect anti-FcεRIα antibodies was not inhibited when sera from the same patients were incubated with TPO or TG (Table 3).

Functional epitope cross-reactivity

As previously reported, serum obtained from CIU patients induced mast cell activation as demonstrated as cysteinyl leukotriene (CysLT) secretion (Tiñana *et al.*, 2009). However, in CIU patients who possessed anti-FcεRIα, anti-TPO, and anti-TG antibodies, CysLT secretion by mast cells was not affected by preincubation with TPO or TG (Figure 2).

Functional antibody-mediated mast cell activation

As expected, incubation with anti-FcεRIα induced significant CysLT production. In contrast to TPO and TG, this effect was inhibited by preincubation with recombinant FcεRIα (>90% inhibition) (data not shown). However, incubation with either anti-TG or anti-TPO induced mast cell activation. In addition, incubation with TPO and TG did not interfere with activation of mast cells with anti-FcεRIα (Figure 2).

DISCUSSION

Consistent with many other autoimmune diseases and similar to what other investigators have reported (O'Donnell *et al.*, 2005; Kulthanan *et al.*, 2006), we found that CIU typically affects females in the third or fourth decade of life (Table 1). In support of an autoimmune etiology to CIU, an increased prevalence of HT in patients with CIU is well recognized with a reported range of ~15–30% (Leznoff and Sussman, 1989; Turkas *et al.*, 1997; Verneuil *et al.*, 2004; Palma-Carlos and Palma-Carlos, 2005; Cebeci *et al.*, 2006). In contrast, the prevalence of TA in healthy control populations is reported to be 3.3–5.5%. In our CIU population (Table 1), a slightly higher prevalence of TA was noted (45%).

We confirmed the use of the ASST in demonstrating the presence of mast cell-activating function in the serum of CIU patients (Hide *et al.*, 1993; Toubi *et al.*, 2004; Yasnowsky *et al.*, 2006). However, a positive ASST was equally prevalent in patients who did not have TA (67 vs 64%). We also confirmed a high prevalence of anti-FcεRIα antibodies in the serum of CIU patients as detected by ELISA (60%). However, similar to the ASST results, an equivalent proportion of CIU patients with and without TA had positive anti-FcεRIα antibodies (67 vs 55%, respectively). Therefore, the presence of TA does not predict the presence of an autoimmune "signature," specifically the presence of a positive ASST or anti-FcεRIα antibodies. The possibility that a larger sample size could detect a CIU/HT autoantibody profile that correlated with the presence of anti-FcεRIα antibodies cannot be discounted.

The mechanism by which HT is linked to CIU has not been elucidated. Concha *et al.* (2004) demonstrated that 2 of 20 patients with CIU and HT possessed measurable IgE anti-thyroid antibodies, although this has not been confirmed (Tedeschi *et al.*, 2001). We addressed the possibility that epitope binding cross-reactivity could explain the concomitant occurrence of these two autoimmune conditions. However, this was comprehensively ruled out. Neither anti-TG nor anti-TPO antibodies are capable of inducing activation of connective tissue mast cells (Figure 2). In our patients with CIU who possessed anti-FcεRIα, TPO, and TG antibodies, the detection of anti-FcεRIα antibodies by ELISA

Table 1. Characteristics of CIU population

Patient	Sex	Age	Thyroid replacement	Anti-TPO antibodies	Anti-TG antibodies	ASST ¹	Anti-FcεRIα antibodies ²
<i>Thyroiditis cohort</i>							
1	F	56	No	No	Yes	Negative	Positive
2	F	28	No	No	Yes	Positive	Positive
3	F	45	Yes	Yes	No	Positive	Positive
4	M	65	Yes	No	Yes	Positive	Positive
5	F	40	Yes	Yes	Yes	Positive	Positive
6	F	29	Yes	Yes	Yes	Negative	Negative
7	F	61	Yes	Yes	Yes	Negative	Negative
8	F	21	Yes	Yes	Yes	Positive	Negative
9	F	24	No	No	Yes	Positive	Positive
<i>Non-thyroiditis cohort</i>							
10	F	32	—	—	—	Positive	Negative
11	F	35	—	—	—	Positive	Positive
12	F	61	—	—	—	Positive	Positive
13	M	29	—	—	—	Negative	Negative
14	F	27	—	—	—	Positive	Positive
15	M	47	—	—	—	Negative	Positive
16	F	46	—	—	—	Positive	Negative
17	F	27	—	—	—	Positive	Positive
18	F	37	—	—	—	Negative	Negative
19	F	33	—	—	—	Negative	Positive
20	F	25	—	—	—	Positive	Negative

Abbreviations: ASST, autologous serum skin testing; CIU, chronic idiopathic urticaria; F, female; FcεRIα, α-chain of the high-affinity IgE receptor; M, male; TG, thyroglobulin; TPO, thyroid peroxidase.

¹Positive ASST defined as wheal >5 mm in diameter more than saline controls.

²Positive anti-FcεRIα titer defined as >3.9 μg ml⁻¹.

Table 2. Summary of characteristics of CIU population

	Sex (# female)	Age (mean years ± SD)	ASST (# positive)	Anti-FcεRIα antibodies (# positive)
Thyroiditis cohort (n=9)	8/9	41 ± 16	6/9	6/9
Non-thyroiditis cohort (n=11)	9/11	36 ± 10	7/11	6/11

Abbreviations: ASST, autologous serum skin testing; CIU, chronic idiopathic urticaria; FcεRIα, α-chain of the high-affinity IgE receptor.

was not inhibited by the addition of TG or TPO (Figure 1 and Table 3). Finally, when we investigated functional antibody cross-reactivity, we demonstrated that preincubation with these thyroid proteins did not decrease the capability of CIU serum to activate mast cells (Figure 2).

It should be noted that our diagnostic assays for the presence of anti-FcεRIα antibodies might exaggerate their frequency. The recombinant FcεRIα we used was produced in a baculovirus cell line that has the machinery to decorate proteins with N-linked glycans thought to have a role in protein folding. Many humans possess pre-existing antibodies to this host cell line-specific glycosylation motif (Hancock

et al., 2007). As such, some of the CIU patients in whom we report anti-FcεRIα antibodies may, in fact, only have antibodies to this glycosylation motif. However, our studies (Figure 1) do demonstrate that this glycosylation motif does not interfere with the ability of soluble recombinant FcεRIα to capture IgE receptor-targeted antibodies. This latter observation validates our reporting the absence of cross-reacting functional antibodies.

In summary, our data confirm the high prevalence of TA and anti-FcεRIα antibodies in CIU. However, this phenomenon cannot be explained by the presence of a shared epitope recognized by these two different antibodies.

An alternative explanation for the increased incidence of HT in CIU is that these conditions reflect a shared genetic predisposition toward the development of autoimmune disease. Along the same lines, autoimmune thyroid disease also has a tendency to occur concurrently with type 1 diabetes. This co-association has frequently been found to occur with patients who express HLA-DR3, suggesting that their etiology may involve common genetic factors (Levin *et al.*, 2004). This possibly reflects shared thymic-programmed mechanisms for developing self-tolerance. In summary, our data confirm the high prevalence of TA and anti-FcεRIα antibodies in CIU and strongly suggests that anti-thyroid and anti-FcεRIα antibodies do not share epitope specificity.

MATERIALS AND METHODS

Patient enrollment

Patients with CIU were recruited from the outpatient adult allergy/immunology clinics at the University of Virginia. Subjects gave written informed consent under a protocol approved by the University of Virginia human institutional review board. The study was carried out in accordance with the Declaration of Helsinki principles. Subjects were diagnosed with CIU based on the presence of urticarial lesions of >6 weeks duration who had hives present at least 3 days per week. All patients had their blood screened for thyroid-stimulating hormone, free T₄ (if thyroid-stimulating hormone abnormal), anti-TPO antibody, and anti-TG antibody. Patients with significant elevation in circulating antibodies to either

TPO (>0.7 IU ml⁻¹) or TG (>0.6 IU ml⁻¹) were defined as having HT and invited to participate in further studies. Patients with a history of autoimmune disease (other than HT), chronic infection, or malignancy were excluded. Antihistamines were discontinued for a minimum of 5 days before skin testing. Patients on immunosuppressive medications (for example cyclosporine, plaquenil) were excluded, as were patients who had used corticosteroids within the previous 2 months.

Anti-FcεRIα antibody assay

Phosphate-buffered saline (PBS) with or without 200 ng l⁻¹ of recombinant human FcεRIα (Heska, Fort Collins, CO) were incubated overnight and adsorbed onto sterile flat-bottomed Immulon 2HB 96-well ELISA plates (Beckman Coulter, Fullerton, CA). The concentration of 200 ng l⁻¹ FcεRIα was determined experimentally

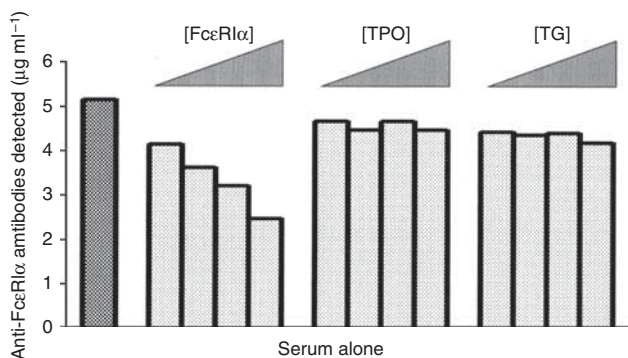


Figure 1. Inhibition assay using recombinant FcεRIα, TPO, and thyroglobulin. Serum from one patient who had anti-FcεRIα and TA was diluted 1:50 in PBS containing 0.05% sodium azide and incubated overnight at 37 °C with increasing concentrations of recombinant human FcεRIα, purified human TG, or recombinant human TPO. Titers of anti-FcεRIα antibodies were measured using the methodology described.

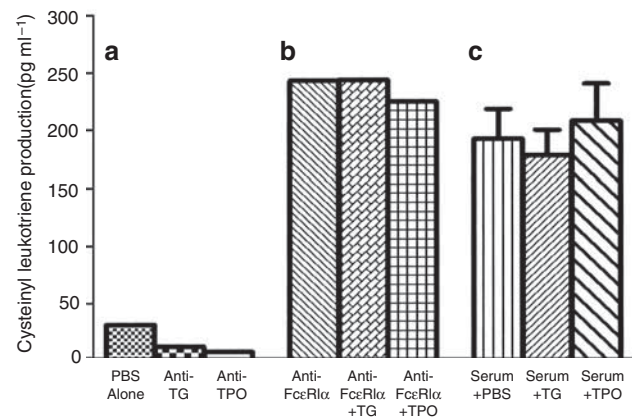


Figure 2. Anti-FcεRIα functional assay. Comparison of CysLT production after: (a) activation with PBS, anti-thyroglobulin, and anti-TPO antibodies; (b) preincubation with thyroid antigens followed by activation with anti-FcεRIα; and (c) preincubation with thyroid antigens followed by activation with patient serum. (a) LUVA mast cells were activated with PBS, anti-TG, or anti-TPO antibodies to evaluate if anti-thyroid antibodies caused mast cell activation. (b) Additional mast cells were preincubated with recombinant TPO or purified TG followed by activation with anti-FcεRIα to determine if thyroid antigens interfere with activation of cells. (c) Sera from patients with positive anti-TG (*n* = 8) or anti-TPO (*n* = 5) antibodies were diluted 1:20 and incubated with thyroid antigens overnight. This was followed by activation of LUVA mast cells to determine if preincubation with the thyroid antigens inhibited CysLT production. For all experiments, activation took place over 30 minutes at 37 °C. Supernatants were collected and CysLT production quantified by ELISA as described. Results presented in panels a and b were from a single experiment with the LUVA cells and thus have no error bars, whereas in panel c the error bars represent the standard error of the mean.

Table 3. Anti-FcεRIα inhibition assay with FcεRIα, thyroid peroxidase, and thyroglobulin

	Serum incubated with recombinant FcεRIα	Serum incubated with purified thyroglobulin	Serum incubated with recombinant thyroid peroxidase
% Inhibition of anti-FcεRIα antibody detection (± SD)	93 ± 11*	17 ± 16	8 ± 12

FcεRIα, α-chain of the high-affinity IgE receptor.

**P* < 0.05.

and found to yield comparable results to higher concentrations. The next day, wells were washed and blocked with 10% goat serum in PBS (pH 7.4; Sigma, St Louis, MO) for 4 hours. After washing, plates were incubated with 100 µl human sera in duplicate diluted 1:100 in PBS containing 5% goat serum at 4 °C for 1 hour. Plates were rinsed before adding biotinylated anti-human IgG 1:10,000 (Rockland Immunochemicals, Gilbertsville, PA) in 5% goat serum and incubated for 1 hour at 4 °C. After washing, 100 µl of horseradish peroxidase-conjugated streptavidin (Sigma) diluted 1:10,000 in PBS was incubated in wells for 20 minutes. The ELISA was developed with o-phenylenediamine dihydrochloride 0.4 mg ml⁻¹ (Sigma) in 0.05% phosphate-citrate buffer with sodium perborate (Sigma) for ~20 minutes before the reaction was terminated with 3 N sulfuric acid (after the optical density of the most concentrated portion of the standard curve was between 1.5 and 2.0 (see below)). Measured values were translated into antibody concentrations by logarithmic regression analysis using standardized human IgG (Sigma) directly adsorbed to the plate (comprising a linear range from 0 to 1,000 ng ml⁻¹). On the basis of preliminary testing performed in a large cohort of CIU patients (data not shown), a positive anti-FcεRIα assay was defined as a concentration of >3.9 µg ml⁻¹.

Autologous serum skin test

ASST was performed using standard methodology (Sabroe *et al.*, 1999). Blood was drawn into a glass tube, allowed to clot, and centrifuged at 5,000 r.p.m. for 15 minutes to separate sera. Patient sera (50 µl) was injected intradermally into the ventral forearm and compared with saline and histamine controls. Skin tests were read after 30 minutes with wheals measuring >5 mm in diameter more than saline defined as positive.

Anti-FcεRIα inhibition binding assay

Inhibition assays were performed to determine whether a shared epitope recognized by both anti-FcεRIα and TA could explain the high incidence of HT associated with CIU. Initially one serum sample from a subject who possessed anti-FcεRIα, anti-TG, and anti-TPO antibodies was diluted 1:50 in PBS and incubated overnight at 37 °C with either PBS, recombinant human FcεRIα, human TG (Advanced Immunochemicals, Long Beach, CA), or recombinant human TPO (Research Diagnostics, Flanders, NJ) at a range of concentrations and then assayed for the presence of anti-FcεRIα antibodies by ELISA as described. An experiment was then performed with four representative patients who also had all three classes of autoantibodies with high concentrations of recombinant human FcεRIα, TG, or TPO (at 65 µg ml⁻¹ each).

Anti-FcεRIα inhibition functional assay

Serum was diluted 1:20 in PBS and incubated with PBS, recombinant TPO, or purified TG (65 µg ml⁻¹ each) overnight as described for the binding assay. The next day, the serum samples were incubated with LUVA mast cells at 37 °C for 30 minutes and supernatants collected. LUVA activation was determined as CysLT secretion as quantified by ELISA according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Anti-FcεRIα functional assay

LUVA mast cells were incubated with PBS, and commercially available anti-TG (Sigma) and anti-TPO (Biodesign International,

Saco, ME) antibodies for 30 minutes at 37 °C and supernatants were collected. Similarly, LUVA mast cells were incubated with recombinant anti-FcεRIα (Upstate, Lake Placid, NY) alone and anti-FcεRIα after preincubating with recombinant soluble FcεRIα (Heska) for 15 minutes. In addition, LUVA mast cells were preincubated with recombinant TPO and purified TG for 15 minutes followed by incubation with anti-FcεRIα for 15 minutes. LUVA activation was determined as CysLT secretion as described above.

CONFLICTS OF INTEREST

The authors state no conflict of interest.

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