Basophil Phenotypes in Chronic Idiopathic Urticaria in Relation to Disease Activity and Autoantibodies

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Potentially pathogenic IgG autoantibodies to IgE or its receptor, FcεRIα, have been detected in ~40% of chronic idiopathic urticaria (CIU) patients. CIU patients' basophils display distinct altered FcεRIα-mediated degranulation. CIU patients with basophil histamine release in response to polyclonal goat anti-human IgE ⩾10% are classified as CIU responders (CIU-R) and <10% are CIU non-responders (CIU-NR). We compared the presence of autoantibodies to basophil degranulation phenotypes and to disease status (active or inactive). Sera were collected from non-CIU subjects and CIU subjects who participated in a longitudinal study of disease severity and had defined basophil degranulation phenotypes. Immunoenzymetric assays (IEMA) quantified IgG anti-FcεRIα and anti-IgE. IgG anti-FcεRIα antibody was detected in 57% of CIU-R ($n=35$), 55% of CIU-NR ($n=29$), and 57% of non-CIU subjects ($n=23$), whereas IgG anti-IgE was present in 43% of CIU-R, 45% of CIU-NR, and 30% of non-CIU subjects. Both the autoantibody levels and the functional basophil phenotype remained stable in subjects with active disease ($n=16$), whereas there was an enhancement in basophil function as subjects evolved into a state of remission ($n=6$), which appears independent of the presence of autoantibody. IEMAs detected a similar frequency of autoantibodies in CIU-R, CIU-NR, and non-CIU subjects. Basophil function may be independent of IEMA-detected autoantibodies.

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INTRODUCTION

Chronic idiopathic urticaria (CIU) is a clinical state that is defined by >6 weeks of hives with no identifiable cause (Greaves, 1995). Its prevalence is approximately 0.1% in the United States (Greaves, 2000). CIU has the greatest impact on the quality of life of any allergic disease (O'Donnell *et al.*, 1997; Grob and Gaudy-Marqueste, 2006) and this impact is similar to that of coronary heart disease and severe atopic dermatitis (O'Donnell *et al.*, 1997; Poon *et al.*, 1999). The economic burden is comparable to that of other skin conditions such as bullous skin diseases and vitiligo, which require multiple medications, medical evaluations, work absences, and emergency department use (Bickers *et al.*, 2006; Delong *et al.*, 2008).

Although the pathogenesis of the majority of cases is unknown, an autoimmune process has been proposed for a subset of CIU subjects (Kaplan, 2004). Approximately 35–40% of patients with CIU are thought to possess IgG autoantibodies to the α-subunit of the high-affinity IgE receptor (FcεRIα) (Hide *et al.*, 1993; Fiebiger *et al.*, 1995; Zweiman *et al.*, 1996; Tong *et al.*, 1997) and approximately 5–10% have naturally occurring IgG anti-IgE autoantibodies (Gruber *et al.*, 1988; Grattan *et al.*, 1991). These autoantibodies are thought to participate in the pathogenesis by directly activating skin mast cells in a complement-dependent manner to generate urticaria.

Functional IgG autoantibodies have been demonstrated *in vitro* using the histamine release activity (HRA) assay (Hide *et al.*, 1993; Fiebiger *et al.*, 1995, 1998; Kikuchi and Kaplan, 2001; Soundararajan *et al.*, 2005). The histamine-releasing activity factor has been localized to the IgG fraction of serum of CIU patients (Hide *et al.*, 1993; Zweiman *et al.*, 1996; Fiebiger *et al.*, 1998; Kikuchi and Kaplan, 2001), and the specificity for the FcεRIα has been demonstrated in a limited number of samples (Hide *et al.*, 1993; Kikuchi and Kaplan, 2001). ELISA and western blot analyses have been developed to measure IgG anti-FcεRIα, and the concordance with HRA has been poor (Fiebiger *et al.*, 1998; Kikuchi and Kaplan, 2001; Soundararajan *et al.*, 2005). Thus, defining the presence of autoantibodies in CIU serum is complicated by the lack of a ''gold standard'' test that confirms the diagnosis of autoimmune urticaria (Sabroe and Greaves, 2006).

Another line of investigation has shown that blood basophils from CIU subjects have altered IgE receptor-mediated degranulation (Greaves *et al.*, 1974; Kern and Lichtenstein, 1976; Sabroe *et al.*, 1998; Luquin *et al.*, 2005; Vonakis *et al.*, 2007) Recently, we reported that the *ex vivo* activation of basophils from CIU subjects with an optimal

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Abbreviations: CIU, chronic idiopathic urticaria; CIU-NR, CIU non-responder; CIU-R, CIU responder; FMLP, N-formyl-met-leu-phe; HRA, histamine release activity; IEMA, immunoenzymetric assay; PBS-BSA, phosphate-buffered saline containing 1% BSA

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dose of polyclonal anti-IgE antibodies segregates them into two groups on the basis of degree of their histamine release: responders (CIU-R) and non-responders (CIU-NR) (Vonakis *et al.*, 2007). CIU subjects with non-responder basophils showed depressed histamine release, whereas basophils of CIU subjects with a responder phenotype displayed histamine release similar to that of healthy control subjects (Vonakis *et al.*, 2007). Basophil functional phenotype is stable on or off medication (Vonakis *et al.*, 2007). Demographics of the groups have been published elsewhere (Baker *et al.*, in press).

Given the existence of basophil functional phenotypes, we examined the relationship of autoantibody presence and the basophil functional phenotype in CIU subjects with active and remissive CIU disease. In this study, we used a panel of analytically sensitive immunoenzymetric assays (IEMA) to measure IgG anti-FcεRIα and naturally occurring IgG anti-IgE autoantibodies in the serum of CIU subjects over time and in relation to their basophil phenotype (CIU-R and CIU-NR).

RESULTS

IgG anti-FcεRIα in the sera from CIU and non-CIU subjects

Sera collected from CIU subjects as part of basophil phenotyping studies ($n=73$) were analyzed for the level of IgG anti-FcεRIα autoantibodies. Of these subjects, 64 had sufficient basophils present in their venous blood sample to allow characterization of their basophil histamine release profile as CIU-R ($n=35$) or CIU-NR ($n=29$). The remaining nine subjects were basopenic and labeled ''CIU-unclassified''. Using the minimal detectable limit of 200 ng ml^{-1}, 57% of CIU-R subjects ($n=35$) and 55% of CIU-NR subjects ($n=29$) had positive IgG anti-FcεRIα antibody levels (two-tailed Fisher's exact test, $P=1.00$), whereas 67% of CIU-unclassified subjects ($n=9$) had detectable antibody (two-tailed Fisher's exact test CIU-R + CIU-NR versus CIU-unclassified subjects, $P=0.72$) (Figure 1a). The latter result was shown to compare the presence of autoantibodies in the sera of CIU subjects with measurable basophil histamine content with that of CIU subjects with extremely low basophil histamine content. The average IgG anti-FcεRIα antibody titers were significantly higher for the CIU-unclassified subjects (CIU-R: 330 ± 58 ng ml^{-1} (SEM), CIU-NR: 322 ± 50 ng ml^{-1}, and CIU-unclassified: 815 ± 262 ng ml^{-1}; two-tailed *t*-test CIU-R + NR versus CIU-unclassified, $P<0.001$).

For non-CIU subjects, 57% ($n=23$) had detectable IgG anti-FcεRIα in their serum, which was not significantly different from CIU subjects (59%, $n=73$) (two-tailed Fisher's exact test, $P=1.00$). The mean concentration of IgG anti-FcεRIα in CIU subjects versus non-CIU subjects was also not significantly different (CIU: 389 ng ml^{-1}, non-CIU: 304 ng ml^{-1}; two-tailed *t*-test, $P=0.38$).

IgG anti-IgE presence in the sera from CIU and non-CIU subjects

The same sera were also analyzed for IgG anti-IgE antibodies. Forty-three percent of CIU-R subjects ($n=35$) and 45% of CIU-NR subjects ($n=29$) had detectable levels (two-tailed Fisher's exact test, $P=1.00$), whereas 67% of unclassified subjects without a clear basophil functional category ($n=9$) had detectable levels (two-tailed Fisher's exact test CIU-R + CIU-NR versus CIU-unclassified, $P=0.29$) (Figure 1b). The average IgG anti-IgE antibody titers were significantly higher for CIU-unclassified subjects (CIU-R: 267 ± 63 ng ml^{-1} (SEM), CIU-NR: 197 ± 22 ng ml^{-1}, and CIU-unclassified: 950 ± 436 ng ml^{-1}; two-tailed *t*-test CIU-R/NR versus CIU-unclassified, $P<0.01$).

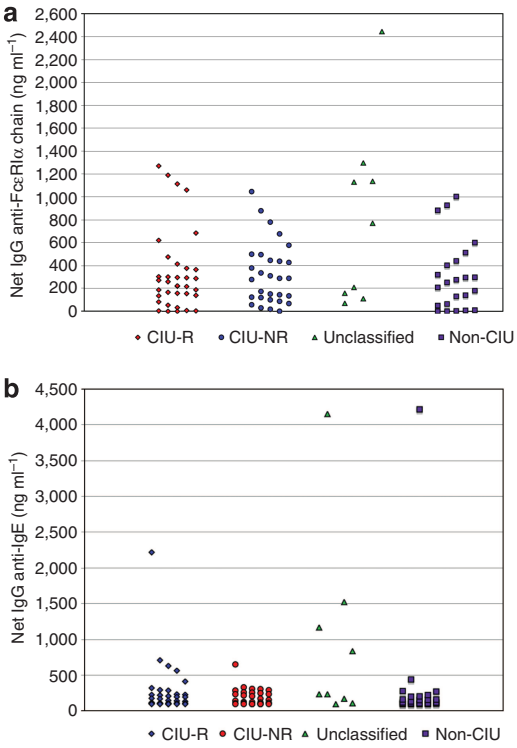

Figure 1. Autoantibody levels in CIU and non-CIU subjects. (**a**) IgG anti-FcεRIα levels in CIU subjects and non-CIU subjects. (**b**) IgG anti-IgE levels in CIU and non-CIU subjects. CIU subjects are categorized based on basophil phenotype.

For non-CIU subjects, 30% ($n=23$) had detectable IgG anti-IgE in their serum, which was not significantly different from CIU subjects ($n=73$) (two-tailed Fisher's exact test, $P=0.24$). The mean IgG anti-IgE concentration in CIU versus non-CIU subjects was not significantly different (CIU: 322 ng ml^{-1}, non-CIU: 339 ng ml^{-1}; two-tailed *t*-test, $P<0.91$).

The distributions of both antibodies in CIU and non-CIU subjects are shown in Tables 1 and 2. Notably, no correlation was seen between the degree of basophil IgE receptor-stimulated response and the IgG anti-FcεRIα or IgG anti-IgE titers (data not shown).

Specificity of the IgG anti-FcεRIα IEMA

Specificity of the anti-FcεRIα autoantibody assay was confirmed with a dose-dependent competitive inhibition using an overnight incubation of soluble FcεRIα with six sera containing the highest levels of IgG anti-FcεRIα detected in the study (Figure 2). The serum of each subject contained >400 ng ml^{-1} of IgG anti-FcεRIα antibody and was shown to

Table 1. Frequency of IgG autoantibodies in CIU (*n*=73)

	IgG anti-IgE-positive 33 (45%)	IgG anti-IgE-negative 40 (55%)
IgG anti-FcεRIα-positive 43 (59%)	23 (32%)	20 (27%)
IgG anti-FcεRIα-negative 30 (41%)	10 (14%)	20 (27%)

CIU, chronic idiopathic urticaria.

Table 2. Frequency of IgG autoantibodies in non-CIU subjects (*n*=23)

	IgG anti-IgE-positive 13 (57%)	IgG anti-IgE-negative 10 (43%)
IgG anti-FcεRIα-positive 7 (30%)	6 (26%)	1 (4%)
IgG anti-FcεRIα-negative 16 (70%)	7 (30%)	9 (39%)

CIU, chronic idiopathic urticaria.

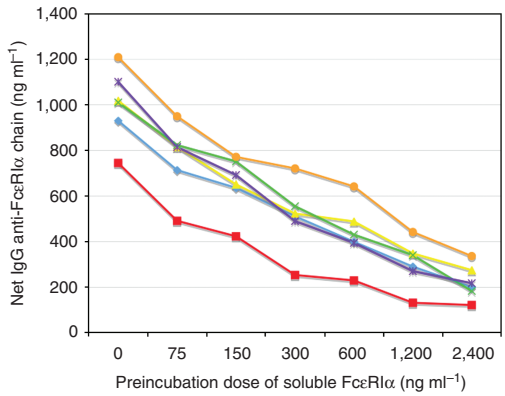

Figure 2. Dose-dependent inhibition of detectable IgG anti-FcεRIα antibodies of six CIU subjects' sera with increasing concentrations of soluble FcεRIα.

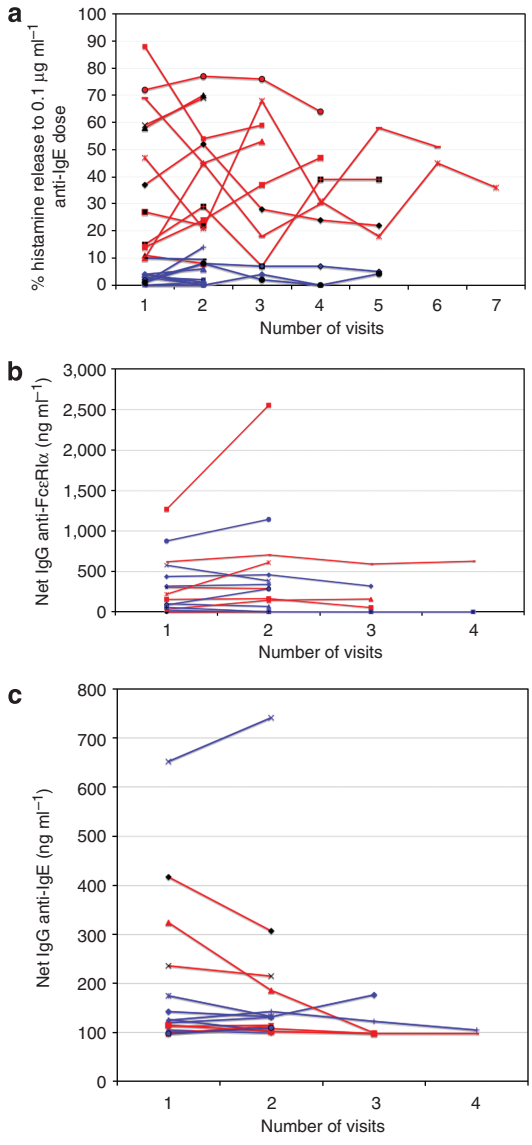

Figure 3. Basophil phenotypes and autoantibody levels during active disease. (**a**) Basophil histamine release to 0.1 μg ml^{-1} anti-IgE over time in CIU subjects with active disease (CIU-R (red circles): $n=12$; CIU-NR (blue triangles): $n=13$). (**b** and **c**) IgG anti-FcεRIα levels (**b**) and IgG anti-IgE levels (**c**) over time in CIU subjects with active disease (CIU-R (red): $n=7$; CIU-NR (blue): $n=9$).

contain FcεRIα-specific IgG antibody by demonstrating inhibition with 2,400 ng ml^{-1} soluble FcεRIα. The mean inhibition for 39 subjects' sera was 79% (SEM = 2%), with a maximum inhibition of 97% (data not shown).

Correlation of basophil functional phenotype and IEMA-measured IgG anti-FcεRIα and IgG anti-IgE antibody levels in subjects with persistent CIU disease

Of the 64 CIU subjects with defined basophil phenotype, basophil functional profiles were measured repeatedly over time in 25 CIU subjects (CIU-R: $n=12$ and CIU-NR: $n=13$) with active disease (Figure 3a). In 12 subjects who were initially CIU-R, 31 of 33 (94%) subsequent basophil functional profile measurements repeated over time during active disease remained within the CIU-R group. In 13 subjects who were initially CIU-NR, 20 of 21 (95%) subsequent basophil functional profile measurements repeated over time during active disease remained in the CIU-NR category. The time between visits is listed in Table 3.

IgG anti-FcεRIα (Figure 3b) and IgG anti-IgE (Figure 3c) levels were determined over time in serum samples from 16 (CIU-R: $n=7$ and CIU-NR: $n=9$) of the 25 CIU subjects with active disease and established basophil functional profiles described above. In 16 subjects with persistent disease, 20 of 23 (87%) repeated measurements showed no change in their IgG anti-FcεRIα antibody status, whereas 22 of 23 (96%) IgG anti-IgE antibody levels did not change statistically over time. Interestingly, the subject with the greatest change, a twofold increase in IgG anti-FcεRIα antibody level, had no detectable

Table 3. Time between visits in CIU subjects with active disease

Visits	1–2	2–3	3–4	4–5	5–6	6–7
CIU-R range in months (mean)	0.5–41.5 (10.5)	1–59 (12.2)	2–18.3 (7.6)	2–6 (3.2)	3.8–13 (8.4)	7.8 (7.8)
CIU-NR range in months (mean)	1–23.8 (8.1)	0.5–6 (2.5)	2.6–8 (4.5)	1.75–2.1 (1.9)	—	—

CIU, chronic idiopathic urticaria; CIU-R, CIU responders; CIU-NR, CIU non-responders.

change in CIU severity as measured by a standardized disease status questionnaire (Baker *et al.*, in press).

Correlation of basophil functional phenotype and IgG anti-FcεRIα and IgG anti-IgE autoantibodies in subjects experiencing disease remission

During the study period, six CIU subjects (CIU-R: $n=1$ and CIU-NR: $n=5$) entered disease remission, defined by no hive symptoms or medication use for 2 months. In disease remission, the CIU-R donors' basophils demonstrated heightened sensitivity to 0.01 μg ml^{-1} anti-IgE. Interestingly, the basophils of the five CIU-NR subjects who experienced disease remission demonstrated an increase in the magnitude of maximal histamine release in response to the optimal concentration of anti-IgE (0.1 μg ml^{-1}) (two-tailed *t*-test, $P=0.03$) (Figure 4a). In contrast to these dramatic shifts in basophil IgE receptor function, IEMA-measured IgG anti-FcεRIα (Figure 4b) and IgG anti-IgE (Figure 4c) antibody levels did not exhibit any significant change in CIU subjects with disease remission. The range of time between active disease and disease remission was 6–54 months (mean = 20 months).

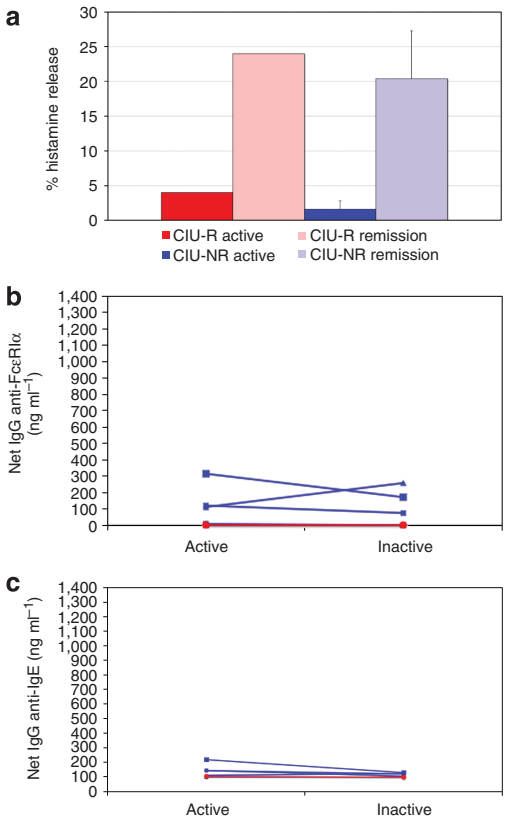

Figure 4. Basophil phenotypes and autoantibody levels in disease remission. (**a**) Basophil histamine release in CIU subjects (CIU-R: $n=1$, to 0.01 μg ml^{-1} anti-IgE; CIU-NR: $n=5$, to 0.1 μg ml^{-1}) with active disease who enter disease remission. (**b** and **c**) IgG anti-FcεRIα levels (**b**) and IgG anti-IgE levels (**c**) over time in CIU subjects who enter disease remission.

DISCUSSION

In this study, we demonstrate that the prevalence and quantitative levels of IgG anti-FcεRIα and anti-IgE in CIU subjects were similar to those measured in the serum of the non-CIU population. The specificity of the FcεRIα-specific autoantibody IEMA was also confirmed with ~80% soluble FcεRIα-specific inhibition for all antibody-positive samples above 400 ng ml^{-1}. Moreover, our data indicate that the pattern of basophil functional phenotypes among CIU patients (CIU-R and CIU-NR) appears to be independent of both the presence and the relative levels of these autoantibodies. Among CIU subjects, both the basophil functional phenotypes and the IgG anti-FcεRIα and anti-IgE autoantibody levels remain stable in subjects with persistent disease. Importantly, however, we noted a remarkable enhancement in basophil function as subjects evolved into a state of remission, which appears to be independent of the presence of an autoantibody.

The observed shift in patterns of basophil histamine release with remission suggests that abnormal basophil function may be a primary feature of the pathogenesis associated with urticaria. These shifts observed in our study are consistent with the observation of Kern and Lichtenstein (1976), who also reported a dramatic increase in basophil responsiveness to anti-IgE in CIU patients who experienced remission. Although the level of autoantibodies was the highest in select subjects with extreme basopenia, the measures of disease severity were not clearly elevated in these subjects. These findings may point to a primary abnormality in the basophil and/or unknown serological factors affecting basophils in CIU patients as more relevant to urticarial disease pathogenesis than the presence or level of either FcεRIα- or IgE-reactive autoantibodies as measured by IEMA.

Historically, IgG anti-FcεRIα and IgG anti-IgE antibodies have been measured by ELISA or western blot analysis and then compared with mediator release results from the HRA assay. Although the HRA assay, in theory, can detect ''functional'' autoantibodies that crosslink the IgE–FcεRIα receptor complex, it depends on the unique characteristics of

the normal basophils that have been selected to perform the assay (Grattan *et al.*, 1991; Hide *et al.*, 1993; Zweiman *et al.*, 1996; Kikuchi and Kaplan, 2001). In addition, this test has been limited by the behavior of the basophils from the various donors (MacGlashan, 1994; Zweiman *et al.*, 1996) and a general lack of universal standardized reagents to permit reproducible and verifiable comparisons across laboratories. Our empirical data indicate extensive variability in the HRA assay (data not shown); therefore, we elected not to use the HRA assay in the present analysis. Previous studies have reported a poor correlation between the presence or relative levels of IgG anti-FcεRIα, as measured by western blot or ELISA, and the magnitude of histamine, as detected in the HRA assay (Fiebiger *et al.*, 1998; Kikuchi and Kaplan, 2001; Soundararajan *et al.*, 2005). We acknowledge that ''non-functional'' autoantibodies may exist. However, the western blot approach used in the largest study to date (Kikuchi and Kaplan, 2001) may not have been sufficiently sensitive to detect the lower concentrations of functional antibodies in the sera of CIU subjects. In the western blot study, the serum was diluted 1:500, in contrast to the 1:100 dilution required for the ELISAs of autoantibodies. This higher-than-desired serum dilution may have prevented the detection of potentially non-functional antibodies with low avidity in non-CIU subjects. In addition, western blot measurements of IgG anti-IgE were not performed in these studies, which could explain some of the discrepancies that exist between previous analyses and our present results. Previous ELISAs reportedly detected autoantibodies regardless of the HRA result (Soundararajan *et al.*, 2005). In another study, an ELISA was unable to detect ''functional'' autoantibodies in some subjects with a positive HRA (Fiebiger *et al.*, 1998). Neither study measured potentially functional, naturally occurring IgG anti-IgE antibodies that we were able to detect at similar frequencies in CIU and non-CIU populations. Although our IEMA does not differentiate between ''functional'' and ''non-functional'' antibodies, we maximized the analytical sensitivity of our assays for both IgG anti-FcεRIα and IgG anti-IgE autoantibodies. However, we have provided the IgE receptor function of blood basophils obtained simultaneously with the serum sample analyzed for autoantibodies to detect evidence of functional autoantibodies on a cell commonly used in the HRA.

Given the importance of precision and accuracy in diagnostic tests, the ability to run many samples in a single assay (in contrast to western blot analysis), and the inherent problems with HRA, we decided to focus in this study on an IEMA for the measurement of IgG anti-FcεRIα and IgG anti-IgE in serum. The design of our IgG anti-FcεRIα assay is similar to the assay used by Fiebiger *et al.* (1998), with several important differences. First, we used a heterologous calibration curve that employed a humanized IgG anti-IgE antibody (omalizumab) to estimate the amount of autoantibody in each serum. This strategy contrasted with the use of the assay response (optical density) by others (Fiebiger *et al.*, 1998), which is not always directly proportional to concentration. Second, we defined a ''positive'' autoantibody presence on the basis of the assay's analytical sensitivity rather than a response level detected in non-CIU subjects. Using a different strategy for defining autoantibody-positive CIU subjects, we detected a higher prevalence of FcεRIα-reactive autoantibodies in normal subjects. Moreover, using Fiebiger's ''positive'' criteria as the mean in non-CIU subjects plus 2.5 times the SEM, IgG anti-FcεRIα would still have remained positive in 26% of our CIU subjects and 22% of our non-CIU subjects. Using these same criteria, IgG anti-IgE would have been positive in 7% of CIU subjects and 4% of non-CIU subjects. Thus, using either strategy for defining autoantibody-positive subjects, both CIU and non-CIU groups had similar frequencies of autoantibody positivity. An explanation for the detection of autoantibodies in non-CIU subjects in our study, in contrast to previously reported studies, may be a consequence of our assay's heightened analytical sensitivity and our method of establishing the detection threshold based on each assay's actual analytical sensitivity rather than simply using the levels of binding detected with sera from a non-CIU population. However, other groups have reported naturally occurring anti-IgE and anti-FcεRIα in non-CIU subjects (Quinti *et al.*, 1986; Fiebiger *et al.*, 1998; Horn *et al.*, 1999). In addition, the autologous serum skin test, considered to reflect the presence of functional anti-IgE and anti-FcεRIα, was positive in 35% of non-CIU subjects in one study (Guttman-Yassky *et al.*, 2007).

The limitations of our IEMA include the lack of a true autoantibody standard for determining the precise amount of antibody present. Nevertheless, a heterologous calibration curve has been successfully used to calibrate other antibody assays (Butler, 1991). Second, we did not measure the subclass distribution of the IgG antibodies. It was originally suggested by Fiebiger *et al.* (1998) that complement-activating IgG1 and IgG3 are present more frequently in CIU subjects than in non-CIU subjects. This observation was later supported by the lack of IgG2 antibodies in the sera of CIU subjects but evidence for IgG4 autoantibodies in sera from CIU subjects (Soundararajan *et al.*, 2005). With no known subclass-specific standard for the antibodies of interest and different binding constants for the different subclass detection antibodies, the accurate detection of these subclasses remains challenging (Hamilton, 1987). Finally, because our assay does not differentiate potentially functional from non-functional autoantibodies, it is possible that there are still differences in the amount of functional antibodies between CIU and non-CIU subjects.

In summary, these studies support the conclusion that altered basophil IgE receptor function is a reproducible finding in active CIU that appears to reflect disease remission. This basophil functional abnormality appears to be independent of autoantibody presence as measured by IEMA and suggests unknown serological factors or primary basophil abnormalities in the pathogenesis of CIU.

MATERIALS AND METHODS

Reagents

Pharmaceutical grade omalizumab was purchased from Genentech (South San Francisco, CA) and reconstituted as recommended in the

package insert. The 147 mg ml^{-1} stock was further diluted with phosphate-buffered saline containing 1% BSA (PBS-BSA). Omalizumab was then coupled to Sepharose at 1 mg ml^{-1} after activation with cyanogen bromide (Schellenberg and Adkinson, 1975) and washed in PBS-BSA. Human serum (diluted 1:10, 0.1 ml) was added to 5% (vol/vol) omalizumab–Sepharose in PBS-BSA and rotated overnight at 23 °C to remove IgE for some assay analyses.

Murine IgM anti-human IgE mAb (clone HP6061; Reimer, 1986; Hybridoma Reagent Laboratory, Baltimore, MD) was added to microtiter plates at 10 μg ml^{-1} in PBS (pH 7.4) and used as a human IgE capture reagent. Streptavidin (Sigma-Aldrich, St Louis, MO) was added to microtiter plates at 20 μg ml^{-1} in PBS (pH 7.4) to capture biotinylated human FcεRIα chain. Horseradish peroxidase-conjugated murine monoclonal anti-human IgG Fc (clone HP6043; Hamilton and Morrison, 1993; Hybridoma Reagent Laboratory) was used at 1 μg ml^{-1} as the human IgG detection protein.

A recombinant truncated form of the extracellular domain (amino acids 1–172) of the α-chain of the high-affinity human IgE receptor (FcεRIα; Heska Corporation, Fort Collins, CO) was produced in a baculovirus expression system and biotinylated as described previously (Stedman *et al.*, 2001).

Polyclonal IgE (JK, Baltimore, MD; diluted to 2 μg ml^{-1} in PBS-BSA) was used as a source of human IgE.

Human specimens

After consent was obtained, whole blood for basophil and serology studies was collected from subjects with a physician-determined diagnosis of CIU ($n=73$) as part of a longitudinal study (Gober *et al.*, 2006; Baker *et al.*, in press) or from non-CIU, healthy control subjects ($n=23$; 10 atopic and 13 non-atopic from history) using protocols approved by the Johns Hopkins Institutional Review Board (IRB) and the Western IRB and in adherence to the Declaration of Helsinki Principles.

Basophil isolation and histamine release

Basophil studies were performed as described previously (Vonakis *et al.*, 2007). Briefly, venous blood was drawn into a syringe containing 5–10 mM EDTA. Mixed leukocytes were obtained using dextran sedimentation with average basophil purity <1%. These cells were stimulated for histamine release with polyclonal goat anti-human IgE (0.01–3 μg ml^{-1}) or *N*-formyl-met-leu-phe (FMLP; 1 μmol l^{-1}) for 45 minutes at 37 °C. Histamine was quantified in cell-free supernatant using an automated fluorometric assay (Siraganian, 1975). Results were computed as the percentage of total histamine release in a total cell lysate of leukocyte aliquots after subtraction of the spontaneous histamine release.

CIU subjects were grouped on the basis of their ability to release histamine after incubation of basophils with an optimal dose of anti-IgE (0.1 μg ml^{-1}) as described previously (Vonakis *et al.*, 2007). Subjects with histamine release <10% were classified as CIU-NR, whereas subjects with histamine release ⩾10% were classified as CIU-R. Subjects with total (complete) histamine release <5 ng ml^{-1} were designated CIU-unclassified.

Human IgG anti-FcεRIα IEMA

Plate preparation. Streptavidin diluted in PBS was adsorbed (0.1 ml per well) onto the bottom six rows of a sterile flat-bottom polystyrene 96-well Bacti plate (Nalge-Nunc International, Rochester, NY). Concurrent with the streptavidin-coating step, a murine IgM anti-human IgE Fc (clone HP6061P diluted in PBS; 0.1 ml per well) was adsorbed onto the top two rows of each plate to establish a calibration curve. The plates were incubated 16–18 hours at 2–8 °C. This was followed by a buffer wash (PBS containing 0.05% Tween 20 and 0.01% thimerosal (PBS-Tween)). Unreacted sites were then blocked with PBS-BSA (0.3 ml per well) for 1 hour at 23°C.

Unknown and calibration serum preparation. IgE-depleted test sera (diluted to 1:100 in PBS-BSA) were incubated for 3 hours at 37 °C with biotinylated FcεRIα (final concentration 60 ng ml^{-1}) or PBS-BSA (sham control). For the calibration portion of the assay, 11 twofold dilutions of omalizumab were prepared with human IgG anti-IgE Fc concentrations from 1 ng ml^{-1} to 1 μg ml^{-1}.

Antibody binding and detection. The blocked plate was washed five times with PBS-Tween and all wells in the top two calibration rows with bound mouse anti-human IgE Fc received polyclonal human IgE (2 μg ml^{-1} final IgE concentration, 0.1 ml per well). The bottom wells containing streptavidin received 0.1 ml PBS-BSA. After 1 h incubation, the whole plate was washed with PBS-Tween and the IgE-depleted sera of test subjects (0.1 ml per well, with or without biotin–FcεRIα) were added to their respective wells in the bottom of the plate coated with streptavidin. The omalizumab (1 ng ml^{-1} to 1 μg ml^{-1}) was pipetted into replicate wells in the top calibration portion of the plate. The entire plate was incubated for 1 h at 23°C.

Detection of bound human IgG. The entire plate was washed five times with PBS-Tween and peroxidase-conjugated mouse anti-human IgG (HP6043-HRP, 1 μg ml^{-1}, 0.1 ml per well) was added to all wells. The plate was incubated for 1 h at 23 °C and then washed five times in PBS-Tween. 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (0.1 ml per well), containing 1 μl of H_2O_2 per milliliter of ABTS, was pipetted into each well. After 60–75 minutes, the plate was read in an ELISA plate reader (MR4000; Dynatech Technologies, McLean, VA) at 405 nm. This calibration curve constructed with omalizumab binding to insolubilized human IgE was used to interpolate the level of IgG anti-FcεRIα in the test sera. Reproducibility was confirmed with interassay coefficients of variation <9% ($n=8$) of reference specimens analyzed on each plate.

Competitive inhibition of anti-FcεRIα assay

To confirm specificity, a competitive inhibition format of the IgG anti-FcεRIα antibody IEMA was performed. Unlabeled soluble FcεRIα (serial dilutions from 75 ng ml^{-1} to 2.4 μg ml^{-1}) was added to IgE-depleted sera containing IgG anti-FcεRIα. After an overnight incubation at 4 °C, the specimens were analyzed in the IgG anti-FcεRIα antibody IEMA at a final concentration of 1:100 as discussed above. Percentage inhibition of binding was computed.

Human IgG anti-IgE immunoenzymetric assay

Naturally occurring IgG anti-IgE in human sera was measured using a solid-phase IEMA as discussed previously (Lichtenstein *et al.*, 1992). In brief, monoclonal murine IgM, anti-human IgE Fc in PBS (clone HP6061P) was absorbed onto sterile flat-bottom polystyrene 96-well Bacti plates (0.1 ml per well; 16–18 hours at 2–8 °C). The

plates were washed five times with PBS-Tween and subsequently blocked with PBS-BSA (0.3 ml per well). Two hours later, the plates were washed five times with PBS-Tween, and then polyclonal IgE (2 μg ml^{-1} final IgE concentration, 0.1 ml per well) was added to each well. After a 1-hour incubation at 23 °C, the plates were washed five times with PBS-Tween and sera of the subjects (non-IgE depleted) were added to the wells (0.1 ml per well, 1:100 final dilution). HP6043-HRP (1 μg ml^{-1}, 0.1 ml per well) was then added, and the plates were incubated for 1 h at 23 °C. After an additional buffer wash, ABTS substrate (0.1 ml per well), containing 1 μl of H_2O_2 per milliliter of ABTS, was added and processed as discussed for the IgG anti-FcεRIα assay. Adsorbance at 280 nm for the unknown specimens was interpolated from the omalizumab heterologous calibration curve into estimates of IgG antibody bound as described above. Reproducibility was demonstrated with interassay coefficients of variation $<20\%$ ($n=8$) of reference curve dilutions analyzed on each plate.

Minimal detectable concentration

The minimal detectable concentration of IgG anti-FcεRIα and IgG anti-IgE antibodies was determined using a *t*-test to determine what human IgG anti-IgE (omalizumab) concentration was significantly different (>95% confidence) from the buffer blank (absence of antibody). The minimum detectable concentration was determined to be 200 ng ml^{-1} of IgG anti-human IgE. Values $\geqslant$200 ng ml^{-1} were considered to be significantly detectable, or ''positive''.

Statistical design

Two-tailed Fisher's exact test was used for nonparametric analysis, whereas two-tailed *t*-test was used for normally distributed data. A *P*-value less than 0.05 was considered significant. All error bars represent SEMs.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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