

Assessment of autoimmunity in patients with chronic urticaria

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Background: The etiology of chronic urticaria is unknown, and an exogenous allergen cannot be identified as the cause in the vast majority of subjects. Thus the concept has evolved that it might be autoimmune.

Objective: We have prospectively assessed sera obtained from 50 consecutive patients with chronic urticaria for the presence of autoantibodies that could be pathogenic.

Methods: We tested sera for their ability to release histamine from human basophils and to activate rat basophil leukemia cells that were transfected with the α subunit of the IgE receptor. We also tested selected sera for anti-IgE antibodies and for IgG anti-Fc ϵ RI α by Western blot.

Results: Sera from 38 of 50 patients with chronic urticaria released β -hexosaminidase from transfected rat basophil leukemia cells, whereas only one of 20 control sera did so ($p < 0.001$); in 30 subjects this could be attributed to IgG anti-Fc ϵ RI α . When human basophils were used, sera from 20 of 50 patients with chronic urticaria released a significant quantity of histamine compared with one of 20 control subjects ($p < 0.01$). Six patients with chronic urticaria and one control subject had IgG anti-IgE. In selected sera we could demonstrate IgG anti-Fc ϵ RI α by Western blot; however, some sera are positive for histamine release but do not demonstrate such binding.

Conclusion: A large fraction of patients with chronic urticaria have antibody directed to Fc ϵ RI α that is functional (60%). A smaller number have IgG anti-IgE (10%). A third group may also have circulating factors capable of activating basophils or mast cells of which the identity is unknown. Thus chronic urticaria may be autoimmune in origin. (*J Allergy Clin Immunol* 1997;99:461-5.)

Key words: Urticaria, histamine release, IgE receptor, autoimmunity

Chronic urticaria has been considered to be a disease of unknown origin ("idiopathic"); and although it resembles allergen-mediated hives, there is no identifiable specific antigen that precipitates episodes of hives, and it

Abbreviations used

Fc ϵ RI α :	α Subunit of the human IgE receptor
HBS-HSA:	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid-buffered saline containing 0.03% human serum albumin
mAb:	Monoclonal antibody
RBL:	Rat basophil leukemia

is just as likely to occur in nonatopic individuals as in those with a personal or family history of allergic rhinitis, asthma, or eczema (atopic dermatitis).¹ Currently, the concept has evolved that the disease might be autoimmune in origin, at least for a subpopulation of patients. Gruber et al.² indicated an increased incidence of anti-IgE antibodies in the sera of patients with chronic urticaria, and Leznoff et al.³ and Leznoff and Sussman⁴ reported a prominent association of chronic urticaria with Hashimoto's thyroiditis in which antimicrosomal antibodies are present.^{3,4} Most recently, Hide et al.⁵ reported an increased incidence of IgG antibodies directed to the Fc ϵ RI α in a subpopulation of patients with chronic urticaria, and this observation has been recently confirmed.⁶ Nevertheless, the incidence of these antibodies is unclear, and their pathogenic significance remains to be determined. In this study we report our data, in which sera from 50 patients with chronic urticaria and 20 normal control subjects were prospectively assessed for their ability to activate either human basophils or rodent basophils transfected with the α subunit of the high-affinity IgE receptor (Fc ϵ RI α), and we present preliminary data regarding the demonstration of such autoantibodies by Western blot.

METHODS

Patients and control subjects

Fifty patients with chronic urticaria, defined as recurrent hives lasting from 4 to 36 hours and occurring at least four times a week for more than 3 months, were recruited. They were selected consecutively and not screened as to severity. Patients with clinical evidence of urticarial vasculitis or physically induced urticaria (e.g., cold urticaria, cholinergic urticaria, dermatographism) were excluded. Antihistamine treatment was stopped at least 48 hours before serum samples were collected, and none of the patients were taking corticosteroids or immunosuppressive drugs at the time of venipuncture. Sera from 20 healthy volunteers served as controls. All sera were separated

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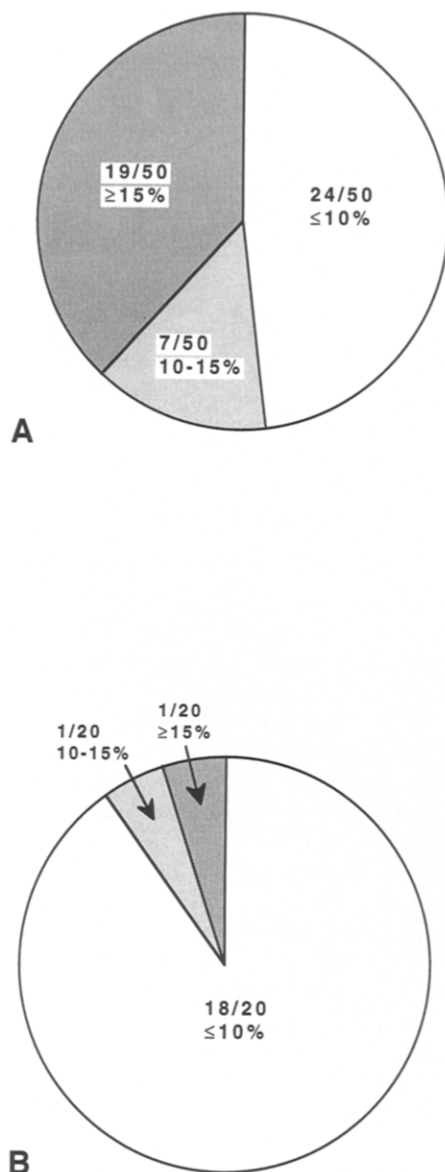


FIG. 1. Activation of human basophils. Human basophils of a healthy donor were challenged with sera from 50 patients with chronic urticaria (**A**) or 20 normal control subjects (**B**), and the percentage of histamine release was determined. The difference between the two groups was significant ($p < 0.01$).

by centrifugation at 300 g for 10 minutes and stored at -20°C . Sera were thawed to room temperature and heated at 56°C for 30 minutes before investigation in order to denature IgE and inactivate complement.

Isolation of IgG fractions

IgG was purified from serum samples with HiTrap 5 ml protein G columns (Pharmacia Biotech, AB, Uppsala, Sweden) according to the manufacturer's instructions. Eluates containing IgG were concentrated with an Omega series membrane 100k (Pharmacia, Piscataway, N.J.) or a microconcentrator (Corning Inc., Corning, N.Y.) and used at a concentration of either 0.1 mg/ml or 1.0 mg/ml. Protein was quantitated by

absorbance at 280 nm and calculated as IgG concentration (mg/ml) = $A_{280}/1.35$.

Histamine release from basophils and histamine assay

Basophils from five normal subjects were initially tested for histamine release with a subgroup of sera from patients with urticaria and for responsiveness to anti-IgE antibody. Considerable variability of response was noted. A single donor whose cells were reactive with anti-IgE antibody and were responsive to many of the sera was chosen for studies reported herein. This donor was not atopic.

Leukocytes were prepared by dextran sedimentation (0.6% dextran, 0.6% glucose, 0.02 mol/L ethylenediaminetetraacetic acid). The basophil-containing layer was washed twice with N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid-buffered saline containing 0.3% human serum albumin (HBS-HSA), and the cells were resuspended in HBS-HSA containing 2 mmol/L MgCl_2 and 2 mmol/L CaCl_2 . Fifty microliters of serum or buffer was incubated with 50 μL of leukocyte suspension (2×10^5 cells/ml) for 40 minutes at 37°C . After incubation, the supernatants were separated by centrifugation at 700 g for 5 minutes at 4°C , and histamine release was determined. Two replicate aliquots of cells were boiled to determine total basophil histamine content.

Histamine release was measured with enzyme immunoassay (Immunotech International, Marseille, France) according to the manufacturer's instructions. All histamine release experiments were done in duplicate, and the results are expressed as a percentage of total histamine content. Spontaneous histamine release from the cells was less than 5% of total histamine.

β -Hexosaminidase release from rat basophil leukemia cells

The rat basophil leukemia (RBL) cell line 4RBL 48, transfected with the human IgE receptor α subunit (Fc ϵ RI α) and anti-Fc ϵ RI α monoclonal antibody (mAb 22E7) were gifts from Dr. Alasdair Gilfillan and Dr. Jarema Kochan.⁷ The cell line was grown in Iscove's modified Dulbecco's media (Gibco Laboratories, Life Technologies, Inc., Grand Island, N.Y.) in tissue culture plates (Corning Inc.). The medium was supplemented with 10% fetal calf serum (Gibco Laboratories) and 1.2 mg/ml Geneticin (Sigma Chemical Co., St. Louis, Mo.). One day before experiments, cells were recultured in 6-well plates (Corning Glass Works) with Geneticin-free medium. In all experiments, adherent monolayers (1.5×10^6 cells per well) were used. Monolayer RBL cells in Tyrode's buffer (Tyrode's buffer with 1 mmol/L MgCl_2 and 1 mmol/L CaCl_2 , pH 7.2) were exposed to 100 μL /ml undiluted sera, 0.1 mg/ml or 1 mg/ml purified human IgG, or Tyrode's buffer at 37°C for 40 minutes. β -Hexosaminidase in supernatants was quantified. Total β -hexosaminidase content was determined by sonicating two replicate wells of cells.

β -Hexosaminidase assay

β -Hexosaminidase in supernatants was quantified by release of p-nitrophenol from the substrate p-nitrophenyl N-acetyl- β -D-glucosaminide (Sigma Chemical Co.). Two hundred microliters of supernatant was collected from each well into glass tubes and mixed with 133 μL of p-nitrophenyl N-acetyl- β -D-glucosaminide⁸ (10 mmol/L in 40 mmol/L citrate buffer, pH 4.5). The reaction was allowed to proceed for 2 hours at 37°C and was terminated with 0.2 mol/L glycine, pH 10.7. The release of

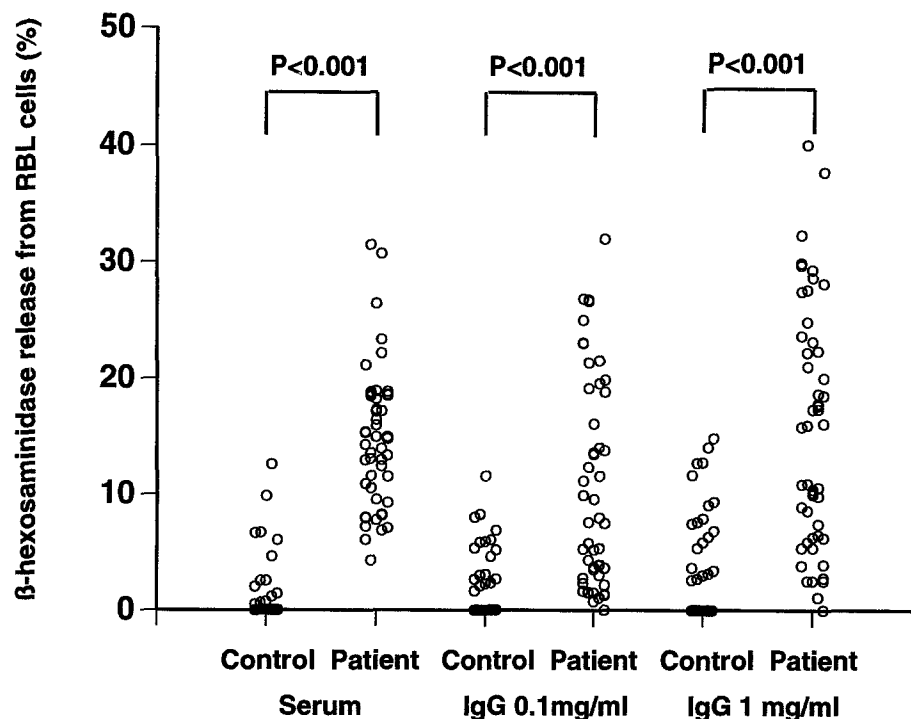


FIG. 2. Activation of transfected RBL cells by sera of patients with chronic urticaria and normal control subjects. Monolayer RBL cells were stimulated with whole serum or purified IgG from 50 patients with chronic urticaria and 20 normal control subjects at 0.1 mg/ml or 1.0 mg/ml. β -Hexosaminidase release was determined. If more than 10% histamine release is chosen as significant, 38 of 50 chronic urticaria sera are positive versus 1 of 20 normal sera. When IgG is tested at 0.1 mg/ml, 20 of 50 chronic urticaria sera are positive versus 1 of 20 normal sera. IgG at 1.0 mg/ml yielded 30 of 50 positives with chronic urticaria sera and 4 of 20 normal sera.

p-nitrophenol was quantitated by absorbance at 410 nm and compared with a p-nitrophenol standard.

Immunoblotting

Purified soluble recombinant Fc ϵ RI α (800 ng/lane), which was prepared as previously described,⁹ was submitted to electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels and blotted onto nitrocellulose membranes (Costar Scientific Corp., Cambridge, Mass.), which were then blocked with 5% dry milk in phosphate-buffered saline for 16 hours. Membranes were reacted with serum (1:5) or monoclonal mouse anti-Fc ϵ RI α antibody (mAb 22E7). Reactivities of anti-Fc ϵ RI α antibodies were detected with a goat anti-human IgG (Fc fragment-specific) alkaline phosphatase conjugate (1:5000; Jackson ImmunoResearch Laboratories Inc.) or a goat anti-mouse IgG alkaline phosphatase conjugate (1:5000; Jackson ImmunoResearch Laboratories Inc.). Antibody binding was visualized by incubation of the membrane with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

Anti-IgE antibodies were similarly sought by Western blot. IgE myeloma at 10 ng/ml was run in 10% sodium dodecylsulfate gels and blotted onto nitrocellulose membranes. A monoclonal anti-IgE antibody served as positive control, and unknown sera were assessed for anti-IgE by the methods described above.

Statistical analysis

Statistical analyses were performed with Student's *t* test by using the computer program Microsoft Excel (Microsoft Co.). Fisher's exact probability test was used to compare patient groups, and the Mann-Whitney U test was used to compare the mean percentage of histamine release between groups.

RESULTS

The ability of sera to stimulate histamine release was assessed as indicated in Fig. 1. Human basophils from a healthy donor were incubated with sera obtained from patients with chronic urticaria or healthy volunteers, and histamine release was determined. Twenty-six of 50 sera from patients elicited histamine release that was greater than 10%. The range of histamine release was 11% to 100%. However, only two of 20 normal sera tested elicited histamine release that was greater than 10%. The difference between the two groups was significant ($p < 0.01$). These results can be explained if the sera of patients with chronic urticaria contained a histamine-releasing agent such as a cytokine capable of degranulating basophils (e.g., monocyte chemotactic protein-1 or RANTES),⁹ anti-IgE antibodies, or antibodies to the IgE receptor.

Anti-IgE antibodies were sought by Western blot. Six

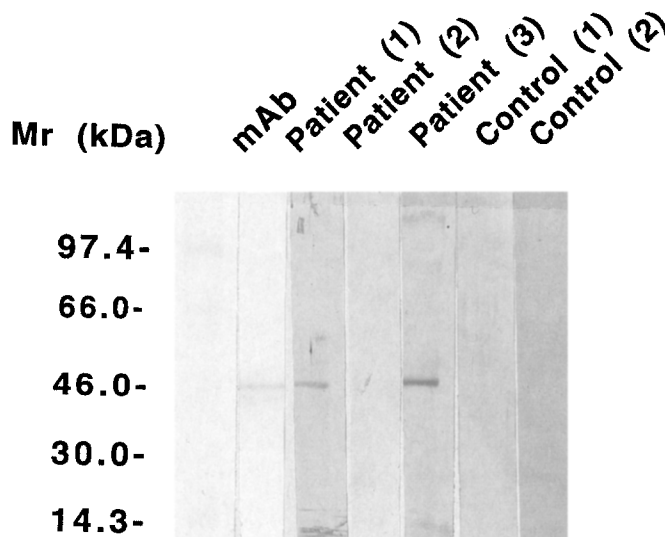


FIG. 3. Immunoblotting to ascertain the presence of IgG anti-FcεRIα. Soluble recombinant FcεRIα was subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis and blotted onto nitrocellulose. Membranes were reacted with monoclonal mouse anti-FcεRIα antibody (*mAb*) or whole sera from patients with chronic urticaria or normal control subjects. Antibody binding was detected with alkaline phosphatase–conjugated goat anti-mouse IgG antibody or goat anti-human IgG Fc fragment–specific antibody, respectively.

of 50 patients with chronic urticaria had such antibodies, and one of 20 normal sera was positive. Because anti-FcεRIα antibodies have been reported to be present in such sera,^{5,6} we incubated sera with RBL cells that had been transfected with FcεRIα and measured release of β-hexosaminidase, which is liberated concomitantly with histamine but is more readily determined by using these cells.

As shown in Fig. 2, heat-treated sera from 38 of 50 patients with chronic urticaria induced β-hexosaminidase release of 10% or more from these cells. Among them, 16 and 22 of the patients' sera induced β-hexosaminidase release of 10% to 15% and 15% to 31%, respectively. Serum from only one of 20 normal control subjects induced β-hexosaminidase release of more than 10%. The levels of β-hexosaminidase released by sera from patients with urticaria were significantly greater than those induced by normal sera at the $p < 0.001$ level.

To determine whether the β-hexosaminidase release activity was detectable in the IgG fraction, serum IgG from each patient and normal control subject was isolated by protein G chromatography and incubated with RBL cells, and β-hexosaminidase release activity was measured. The IgG fraction of 20 of the patients elicited β-hexosaminidase release that was greater than 10% when the IgG concentration was 0.1 mg/ml, whereas 30 of patients' sera were positive if the IgG were tested at 1 mg/ml. RBL cells that were not transfected with FcεRIα produced no β-hexosaminidase release. These data demonstrated that the β-hexosaminidase release activity of the sera was largely attributable to the presence of IgG autoantibodies directed against FcεRIα.

To further define this reactivity, we wished to demon-

strate direct binding to the α subunit of the IgE receptor rather than rely strictly on a functional assay. In addition, a test of this sort might be used as a method to screen unknown sera. Thus we performed sodium dodecyl–polyacrylamide gel electrophoresis of the purified recombinant FcεRIα and sought to detect binding by Western blot. Fig. 3 is representative and shows five sera consisting of serum samples from two normal control subjects and from three patients with chronic urticaria, which were strongly positive for histamine release when incubated with human basophils. A clear band is seen with two of the three sera from patients with chronic urticaria at a molecular weight of 46 kd, corresponding to the size of the FcεRIα. One chronic urticaria serum is negative, as are the two control sera. None of them are positive for anti-IgE.

DISCUSSION

The data presented here suggest that in a subpopulation of patients, chronic urticaria may have an autoimmune etiology, although there is likely to be heterogeneity even within this group. Anti-IgE antibodies are found in 5% to 10% of such patients,¹⁰ and there is an increased incidence of Hashimoto's thyroiditis in patients with chronic urticaria and angioedema. There is also an association with a high titer of antimicrosomal antibodies even if the patients are euthyroid.⁴ The concept of an autoimmune origin for chronic urticaria was then further delineated by the observation that certain sera, when injected into the skin of the donor, caused a wheal and flare reaction (i.e., a positive autologous skin test response.)¹⁰ This implies the presence of a serum substance that causes mast cell degranulation

and also suggests that a late-phase reaction or some variation thereof¹¹ accounts for the cellular infiltrate that characterizes this disorder.¹²

Most subsequent studies have sought the presence of autoantibodies by using blood basophils rather than cultured mast cells because of their ready availability, and the data are consistent with the presence of an antibody that is reactive with the IgE receptor or with IgE itself.^{2, 5, 6, 10} Fiebiger et al.⁶ noted that antibodies directed to the IgE receptor were specific for chronic urticaria, whereas anti-IgE antibodies were also seen in normal subjects, as well as in patients with atopic dermatitis. Their value for anti-IgE antibodies was unusually high (i.e., 26% of their normal control subjects and 69% of patients with chronic urticaria). Our values are 5% and 11%, respectively, and this much lower value is consistent with prior observations.² In this study, we demonstrate that approximately 52% of our patients' sera caused histamine release from human basophils and that 76% of sera were positive when tested with transfected RBL cells bearing the FcεRIα. The data with RBL cells are more specific, from a mechanistic point of view, because these cells lack IgE and are therefore unresponsive to anti-IgE antibody. The RBL cell assay may also be more sensitive than using human basophils because the incidence of positive sera was greater. For example, basophils that are saturated with IgE may not respond to anti-receptor antibody. Regardless, the incidence of these autoantibodies is substantial with either method.

Hide et al.⁵ chose 26 patients who had positive autologous skin test responses for further study, but the denominator (i.e., the total number of patients with chronic urticaria who were skin tested) was not reported. Thus the incidence of a positive autologous skin test response is uncertain. Of these 26, 17 (or about 65%) were positive for histamine release when human basophils were used, but there was also considerable variability on the basis of the basophil donor chosen. Four patients were then studied in detail, and their sera and IgG fractions behaved functionally as if anti-FcεRIα were present. Fiebiger et al.⁶ did neither autologous skin testing nor a survey of histamine-releasing capability of chronic urticaria sera, but reported a 37% incidence of IgG anti-FcεRIα in chronic urticaria by Western blot. Some of these sera were capable of human basophil histamine release, but some were not.

We have also begun to use both ELISA methods (data not shown) and Western blot analysis to assess sera for binding to FcεRIα. Fig. 3 shows some representative sera. None of the normal sera that were tested have IgG anti-FcεRIα antibody as determined by this method, but we have found fewer positive sera in our chronic urticaria population than we have observed with either of the two basophil release approaches (Figs. 1 and 2). Three sera that were strongly positive as determined by basophil histamine release are shown in Fig. 3, but binding to FcεRIα was observed in only two of them.

The nonbinding serum also did not contain anti-IgE antibody. If we assume that the patient has no IgG anti-FcεRIα, we could still have a positive functional assay result, if the antibody to FcεRIα were contained within another class of immunoglobulin, for example, within IgM or IgA. Alternatively, the antibody might be directed to some other subunit of the IgE receptor (β or γ), or the mechanism of release could involve some other factor, such as a chemokine or complement-derived peptide. Thus other mechanisms of basophil or mast cell reactivity may be present in some subjects.

Our data are consistent with an autoimmune initiating mechanism in a substantial percentage of patients with chronic urticaria. Nevertheless, the relationship between the presence of such antibodies and the histology of the lesion characteristic of chronic urticaria remains to be determined. Further, the reason that such antibodies may be clinically significant with regard to the skin, but are seemingly not active in the nose or lungs of such patients, is unknown.

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