

Natural anti-Fc ϵ RI α autoantibodies isolated from healthy donors and chronic idiopathic urticaria patients reveal a restricted repertoire and autoreactivity on human basophils

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The role of autoantibodies against the α -subunit of the human high-affinity IgE receptor (Fc ϵ RI α) in the pathogenesis of chronic idiopathic urticaria (CIU) is controversial. We have shown that these antibodies are widespread, apparently non-pathogenic and belong to the natural antibody repertoire. To clarify this controversy, we constructed antibody libraries from both healthy donors and CIU patients with active disease.

Here we describe the first three high affinity IgM anti-Fc ϵ RI α autoantibodies isolated from normal and urticaria libraries. Sequence analysis revealed germline VH in both cases paired with a slightly mutated VL, thus supporting their classification as natural antibodies. Strikingly, one major IgM clone was present in both CIU patients and normal donors.

The anti-Fc ϵ RI α autoantibodies recognize Fc ϵ RI α on cells, but are non-anaphylactogenic on blood basophils, except when IgE is removed from the receptor. Based on their functional activities we propose a concept of "conditional autoimmunity" where natural anti-Fc ϵ RI α autoantibodies can become pathogenic dependent on the state of occupancy of the Fc ϵ RI α by its natural ligand IgE.

Abbreviations: Fc ϵ RI α , α -subunit of the human high-affinity IgE receptor; CIU, chronic idiopathic urticaria; CU, chronic urticaria; LTM α , UM α , anti-Fc ϵ RI α clones from a tonsillar and CIU IgM Fab library respectively; IVIg, intravenous Immunoglobulin.

1. Introduction

It is generally accepted that pathogenic autoantibodies are normally only detectable in autoimmune disorders. Thus, autoantibodies directed against the α -subunit of the human high-affinity IgE receptor (Fc ϵ RI α) were claimed to be of pathogenic relevance in a subset of chronic urticaria (CU) patients [2,3]. Chronic urticaria is defined by the occurrence of short-lived wheals, appearing daily or almost daily, over a period of more than 6 weeks. Quality of life can be severely affected and it is a chronic disease [19]. However, we have previously reported the presence of anti-Fc ϵ RI α autoantibodies in the serum of healthy donors as well as in multidonor intravenous IgG (IVIg) preparations [4]. Thus, these anti-Fc ϵ RI α autoantibodies may belong to the natural antibody repertoire reacting with a restricted set of self-antigens [8,10]. Functional studies of anti-Fc ϵ RI α autoantibodies using serum or IVIg preparations are difficult to interpret due to the polyclonal nature of the antibody preparations which probably contain mixtures of different types (specificities) of anti-Fc ϵ RI α autoantibodies. Therefore, in order to characterize these natural anti-Fc ϵ RI α autoantibodies and to investigate their functional activity *in vitro* and *in vivo*, we generated human recombinant anti-Fc ϵ RI α antibodies by repertoire cloning from IgM libraries constructed from a pool of normal donors versus a pool of 2 patients with documented active CIU.

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Here we show that recombinant human natural anti-FcεRIα autoantibodies can be isolated from antibody libraries representing both normal donors and CIU patients. Indeed, the unique IgM anti-FcεRIα autoantibody isolated from the CIU library showed the same sequence as one of the antibodies found in the normal donors. Furthermore, these antibodies recognized the FcεRIα displayed on human blood basophils and triggered histamine release but only in the absence of IgE. Based on these results we propose a new model of conditional autoimmunity.

2. Material and methods

2.1. Antibodies

Human monoclonal hybridoma IgE-SUS11, mouse monoclonal anti-human IgE antibody Le27, and peroxidase conjugated rabbit anti-phage antibody were produced in our laboratory as described previously [7,20,21]. Mouse anti-FcεRIα monoclonal antibody 15-1 was kindly provided by Prof. J.P. Kinet (Boston, MA). Human anti-Tetanus toxoid monoclonal antibody ST-18 was donated by Dr. A. Lang (Swiss Serum and Vaccine Institute, Bern, Switzerland). Human multi-donor IgG, IVIg was donated by ZLB Bioplasma, Bern, Switzerland.

Construction of IgM Fab libraries. An IgM Fab library was constructed from children's tonsil B cells and displayed on pIII of the filamentous bacteriophage M13. Messenger RNA was isolated from CD19-positive B cells from the tonsils of 4 children (Age: 5.5 ± 1.6 years; 3–6.6 years), and cDNA and PCR reactions were performed as described [9]. IgM Fab library UM was constructed from blood cells of two CIU patients. Patient 1, a male donor, aged 21 years, was suffering from CIU since 4 months. Patient 2, a female, aged 75 years, was suffering from CIU since more than 4 years. Patients were asked to stop corticosteroid and antihistaminic therapy 2 weeks prior to blood donation. Without therapy they were suffering from urticaria as diagnosed by a dermatologist. Blood was taken during this active urticarial phase.

For the generation of the Fd fragments six upstream primers were used which hybridize to each of the six VH families as described [20]. The downstream IgM primer was the following: 5'-GCT CAC ACT AGT AGG CAG CTC AGC AAT CAC-3'. The Fd fragments were cloned into the pMVS vector. The pMVS vector (unpublished results, M. Vogel) has been reconstructed based on pComb3H [1] and an oligonucleotide containing the myc and 6His tags has been inserted at the Xba I site.

2.2. Isolation of human recombinant anti-FcεRIα Fab clones

The original library was amplified in *E. coli* XL-1 cells (Stratagene), and phages were produced and precipitated as described [1]. Anti-FcεRIα Fab phages were selected on immobilized human recombinant FcεRIα [4] in polystyrene immunotubes (Becton Dickinson). After six to nine rounds of panning, clones were analyzed by nitrocellulose filter lift technique as described [20] and the variable regions of the heavy and light chains of FcεRIα-positive clones were sequenced at Microsynth GmbH (Balgach, Switzerland) and compared with the V Base Sequence Directory [18].

2.3. Generation of full-length IgG

This was produced using the integrated vector system (kindly provided by Dr. A. Bradbury, Trieste, Italy). The VH and λ light chain regions were re-cloned using the primers as recommended by Persic et al. [12].

HEK-293T cells were grown in DMEM:F12 medium (GIBCO BRL) supplemented with 2% FCS at 37°C in 5% CO₂. Plasmid-DNA (heavy chain:light chain, 7:3, total 10 μg/2 × 10⁶ cells) was transfected with Lipofectamine 2000 reagent (GIBCO BRL) according to the manufacturer's instructions, and the cells were cultured for 5 days. Antibodies in the cell supernatant were purified on protein G sepharose columns (Pharmacia) and purity was controlled on a 9% SDS-Acrylamide-gel (data not shown). The concentration of the purified IgG was determined by sandwich ELISA using two goat anti-human IgG antibodies (TAGO).

2.4. Affinity measurement of full length IgG anti-FcεRIα autoantibodies

Affinity of the full-length IgG for the FcεRIα was determined using the IAsys cuvette system exactly as previously described [4]. Briefly, the cuvette with the immobilized FcεRIα was incubated with 180 μl PBS-T to reach a stable baseline. For the association antibodies were added in different concentrations in 20 μl samples (finally diluted 1/10 in order to minimize buffer effects). The cuvette was washed with 5 × 200 μl PBS-T for monitoring the dissociation of the antibodies. For regeneration, the cuvette was washed 1 × 30 sec. with 20 mM HCl and washed again with 5 × 200 μl PBS-T.

2.5. Competitive inhibition of binding of anti-FcεRIα autoantibodies on immobilized FcεRIα

Simultaneous binding to the FcεRIα experiments was assayed in the IAsys cuvette system by online monitoring of the anti-FcεRIα binding. Antibodies were diluted in PBS-Az and added in 100 μl samples (100 nM) to the cuvette. Prior to secondary addition of antibodies the cuvette was washed with 2 × 100 μl PBS-Az.

2.6. IgE inhibition assay

RIA/EIA plates (Costar, Integra Biosciences) were coated with FcεRIα (5 μg/ml) and blocked with PBS containing 0.15% Casein (PBS-C). A constant concentration of LTMα15 or LTMα35 (11 ng/ml and 6 ng/ml, respectively) was incubated with different amounts of IgE-SUS11 (diluted in 2-fold dilution steps starting at a concentration of 24 μg/ml) on the FcεRIα for 4 hrs at 37°C. IgG binding was detected with peroxidase-conjugated sheep anti-human IgG (The Binding Site) and visualized with TMB (3,3',5,5'-tetramethylbenzidine; Fluka) and the reaction was stopped with 1 volume 1 M H₂SO₄. Plates were read at 450 nm with a ν_{max} kinetic microplate reader (Molecular Devices).

2.7. FACS analysis

CHO cells transfected with the human FcεRIα and FcεRIγ chains (kindly provided by Prof. J.P. Kinet, Boston, MA) were maintained in RPMI containing 10% FCS and 1 mg/ml Geneticin G-418 (GIBCO BRL). FACS staining was performed by incubating 10⁴ cells with 5 μg/ml of the following antibodies LTMα15, LTMα35, or ST-18 in PBS containing 0.5% BSA and 0.02% NaN₃ (PBSA-Az) in 96-well V-bottomed polystyrene plates (Dynatech) for 30 min at 4°C. Subsequently, cells were washed 2x with 150 μl PBSA-azide and antibodies binding to the cells were detected by FITC-conjugated sheep anti-human IgG antibody (The Binding Site), and analyzed by Epics Coulter FACS.

2.8. Histamine release from basophils

Basophil enriched peripheral blood leucocytes from three donors were purified by dextran sedimentation and Percoll gradient (Pharmacia) and each individual sample was divided into treated and non treated samples. The treated samples were incubated with lactic acid buffer (0.13 M NaCl, 0.005 M KCl, 0.01 M lac-

tic acid, pH 3.9) for 10 min on ice. After washing 1x with wash buffer (Hepes buffer containing 0.25 mg/ml BSA), cells were resuspended in cell buffer (wash buffer containing 1 mM MgCl₂ and 1 mM CaCl₂) supplemented with or without 50 μg/ml IgE-SUS11. Both treated and non treated basophils were first stimulated with 10 ng IL-3 (kindly provided by Novartis AG, Basel, Switzerland) for 10 min, followed by the addition of different antibodies for 20 min at 37°C. Triggering of basophils was stopped by incubating the cells on ice for 20 min. Samples were analyzed using an automated fluorimetric method [16] and calculated as a percentage of total histamine.

2.9. Histamine release by plasma of healthy donors and CIU patients

Plasma was diluted 1/10 and heat denatured at 56°C for 3 h to destroy IgE, followed by incubation with basophil enriched human leucocytes as above.

3. Results

3.1. Generation of recombinant antibodies against the FcεRIα

A total of 200 clones were screened after 6 rounds of panning and 6 positive clones were found. Two human monoclonal anti-FcεRIα autoantibodies, LTMα15 (4/6 clones) and LTMα35 (2/6 clones), were isolated from the IgM Fab phage library constructed from children's tonsil B cells. Comparison with the known germline sequences of human V_H and V_L segments (V Base Sequence Directory) [18] indicated that both clones showed 100% homology to the nearest germline V_H sequences, whereas λ light chains were slightly mutated (Table 1). Recent results have shown that using essentially the same panning and screening procedure as above only one anti-FcεRIα autoantibody, UMα16 (7/7 clones) was isolated from the IgM Fab urticaria library. Sequence analysis showed this to be exactly the same antibody as LTMα15 (Table 1).

The anti-FcεRIα autoantibodies isolated first from the tonsil library were produced as full length IgG molecules, as described in materials and methods to allow functional assays e.g. histamine release which requires bivalent recognition and signaling via the FcεRIα expressed on basophils.

Table 1
Restricted repertoire of anti-FcεRIα autoantibodies isolated from healthy donors and chronic idiopathic urticaria patients

Source of antibody library		IgM antibody clone	% Homology to germ line gene			
			Heavy chain		Light chain	
Normal donors	Tonsil	LTMα15	DP 47	100%	V1-22	91%
		LTMα35	VHVCW	100%	7DPL-8	93.9%
Urticaria patients	Blood	UMα16	DP 47	100%	V1-22	91%

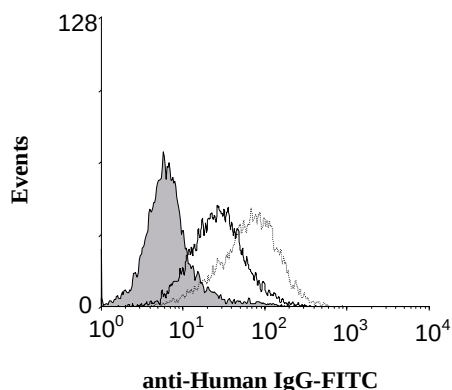


Fig. 1. FACS staining of FcεRIα transfected CHO cells with full-length IgG of LTMα15 (solid line) and LTMα35 (dashed line). Human monoclonal anti-Tetanus toxoid antibody ST-18 was used as an isotype control (gray shaded). Antibody binding to FcεRIα was detected with FITC conjugated sheep anti-human IgG.

3.2. *In vitro* analysis of recombinant full-length IgG₁ anti-FcεRIα autoantibodies: FACS analysis

Anti-FcεRIα autoantibodies may only be functional *in vivo* when they are able to react with the FcεRIα displayed on the cell surface of effector cells. FACS analysis (Fig. 1) demonstrated that both anti-FcεRIα autoantibodies recognized cell surface expressed FcεRIα on transfected CHO cells as compared to the negative control ST18, a Tetanus toxoid specific human IgG monoclonal antibody. The UMα16 clone as a phage preparation and developed with an in house rabbit anti-phage FITC also recognized cell surface FcεRIα (data not shown).

3.3. Affinity measurements, IgE inhibition assay and competitive inhibition of binding of anti-FcεRIα autoantibodies

The affinity of both constructed full length IgG₁ recombinant anti-FcεRIα autoantibodies was assessed by online monitoring of the binding kinetics using the IAsys cuvette system. The affinities were 7.2×10^{-9} M for antibody LTMα15, and 1.4×10^{-8} M for antibody LTMα35.

As IgE, the natural ligand of the FcεRIα, binds with even higher affinity (10^{10} M), we tested whether IgE was capable of inhibiting the binding of LTMα15 and LTMα35 to FcεRIα in an inhibition ELISA (Fig. 2). Binding of both antibodies to FcεRIα was inhibited by human IgE in a dose dependent manner indicating that both antibodies share with IgE an overlapping epitope on the FcεRIα. The binding of LTMα15 was less effectively inhibited by incubating LTMα15 30 min. prior to the addition of IgE (Fig. 2(A)) whereas there was no difference in the inhibition observed for LTMα35 (Fig. 2(B)), indicating that the two human anti-FcεRIα autoantibodies may recognize different epitopes on FcεRIα.

In order to confirm whether these two antibodies recognize different epitopes on FcεRIα, they were tested in an online inhibition assay for their binding capacity to FcεRIα in the simultaneous presence of each antibody (Fig. 3). First, saturating amounts of LTMα15 were added to the FcεRIα cuvette. All LTMα15-specific epitopes on the FcεRIα cuvette were occupied by LTMα15, as secondary addition of LTMα15 resulted in no additional binding of LTMα15. In contrast, addition of the same amount of LTMα35 showed an enhancement of the binding curve, indicating that there were still epitopes available for LTMα35. These data indicated that the two human monoclonal anti-FcεRIα autoantibodies probably recognized different or overlapping epitopes on the FcεRIα.

3.4. Biological activity of the recombinant anti-FcεRIα autoantibodies

Anaphylactogenic potential was assessed in a histamine release assay using freshly isolated peripheral blood lymphocytes enriched for basophils (Fig. 4). Under physiological conditions, represented by the untreated basophils, neither the two anti-FcεRIα autoantibodies LTMα15 and LTMα35 nor a mouse monoclonal anti-FcεRIα antibody, 15-1 triggered the cells for histamine release. In contrast, an anaphylactogenic mouse monoclonal anti-human IgE antibody, Le27 trig-

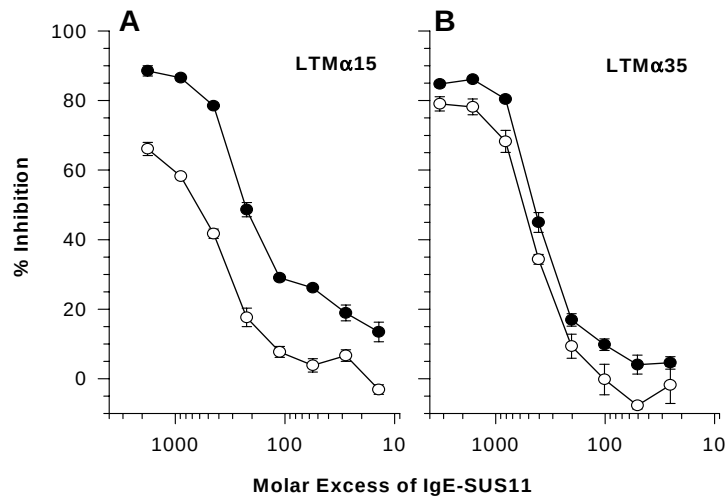


Fig. 2. IgE inhibits the binding of anti-FcεRIα autoantibodies to FcεRIα. EIA/RIA plates were coated with FcεRIα. Different amounts of IgE-SUS11 were titrated 30 min before (closed symbols) or 30 min after (open symbols) the addition of a constant amount of human recombinant anti-FcεRIα antibodies LTMα15 (A) or LTMα35 (B). Antibodies binding to FcεRIα were detected with peroxidase conjugated sheep anti-human IgG.

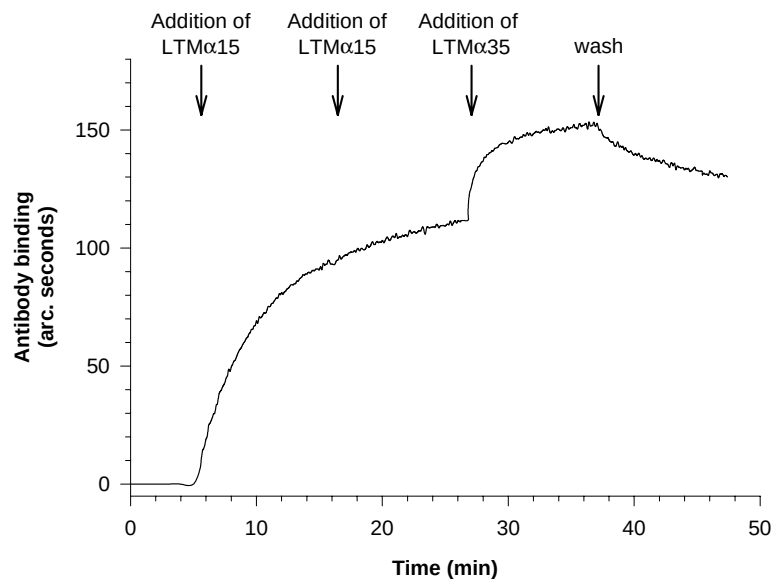


Fig. 3. Online monitoring of anti-FcεRIα antibodies, LTMα15 and LTMα35, binding to FcεRIα. LTMα15 (10 nmoles) was incubated with immobilized FcεRIα for 10 min. The volume was replaced again with 10 nmoles LTMα15. Subsequently, 10 nmoles LTMα35 were added to the cuvette and incubated for 10 min. Finally the cuvette was washed with $5 \times 100 \mu\text{l}$ PBS-Az. Arrows indicate the addition of either antibodies or wash-buffer. Antibody binding is displayed as arc seconds of angle correction for maximal laser reflection.

gered high amounts of histamine release compared to the spontaneous release in the presence of IL3 alone.

However, FcεRIα expressed on freshly isolated basophils is usually occupied by endogenous IgE [13]. Furthermore, in Fig. 2 we showed the inhibition of antibody binding by IgE. Thus, both findings suggest that FcεRIα is normally not accessible for anti-FcεRIα

autoantibodies such as LTMα15 or LTMα35. Upon stripping the cells with lactic acid in order to remove cell bound IgE from the FcεRIα [13], there was a clear increase in histamine release induced by the two human recombinant anti-FcεRIα autoantibodies. The control mouse monoclonal anti-FcεRIα antibody 15-1, whose binding is also inhibited by IgE, showed the same result.

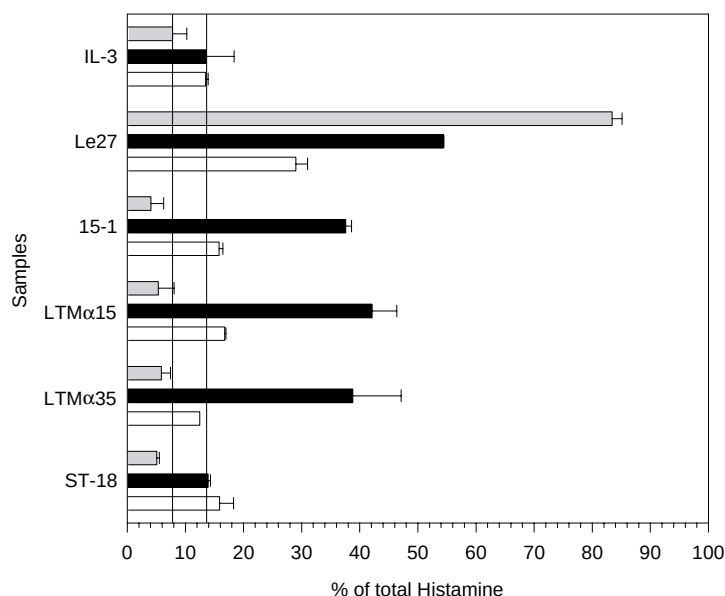


Fig. 4. *In vitro* histamine release induced by human recombinant anti-FcεRIα antibodies LTMα15 and LTMα35. Freshly isolated and enriched basophils isolated from peripheral blood were used either directly (grey bars), desensitized with lactic acid (black bars), or desensitized and re-sensitized with 50 μg/ml IgE (white bars), and primed with IL-3. Mean ± s.d. of duplicate cultures is shown. A: Le27, an anaphylactogenic anti-IgE antibody was used at 1 μg/ml. All other monoclonal antibodies were used at 5 μg/ml. Monoclonal human anti-Tetanus toxoid antibody, ST-18, was used as a negative control.

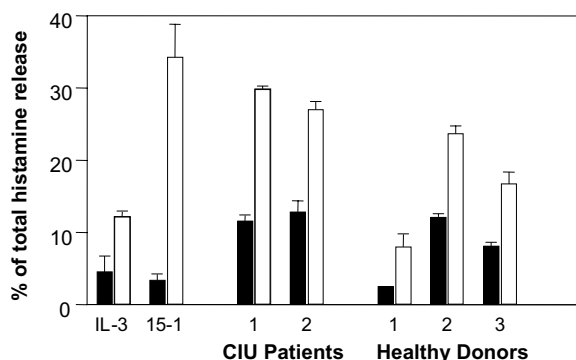


Fig. 5. *In vitro* histamine release induced by plasma of healthy donors and CIU patients. The *in vitro* assay was performed as in Fig. 4 but the plasma was diluted 1/10 and heat denatured at 56°C for 3 h in order to destroy IgE. The plasma-preparations were incubated with basophil-enriched human leukocytes. The cells were used either directly (black bars) or desensitized by lactic acid (white bars) and primed with IL-3. Mouse mAb 15-1 was used as a positive control.

The histamine releasing activity of anti-human IgE antibody Le27 was reduced after removal of IgE but still measurable, indicating that lactic acid treatment only results in a partial removal of surface bound IgE. Re-sensitization of lactic acid treated cells with an excess of IgE (50 μg/ml) resulted in a complete block of the anaphylactogenic activity of both anti-FcεRIα autoantibodies LTMα15, LTMα35, as well as the mouse anti-

FcεRIα antibody 15-1 (Fig. 4). The observed reduction of the triggering activity of anti-human IgE antibody Le27 was most probably due to the excess of IgE in the cell buffer, such that all available receptors were occupied with IgE and the remaining IgE blocked Le27 in solution. The negative control antibody, ST-18, showed no triggering activity on human basophils under any conditions. These results indicated that the anaphylactogenic potential of the recombinant anti-FcεRIα autoantibodies was dependent on the degree of occupancy of the FcεRIα by IgE.

3.5. The same antibody activity found in plasma of healthy donors and CIU patients

The same phenomena was observed using plasma of healthy donors and documented CIU patients. Histamine release was induced only after removal of IgE from the FcεRIα expressed on basophils (Fig. 5). This suggests that similar conditionally anaphylactogenic anti-FcεRIα autoantibodies like LTMα15, UMa16 or LTMα35 may exist *in vivo*.

4. Discussion

Our data demonstrate that human natural anti-FcεRIα autoantibodies can be isolated from healthy

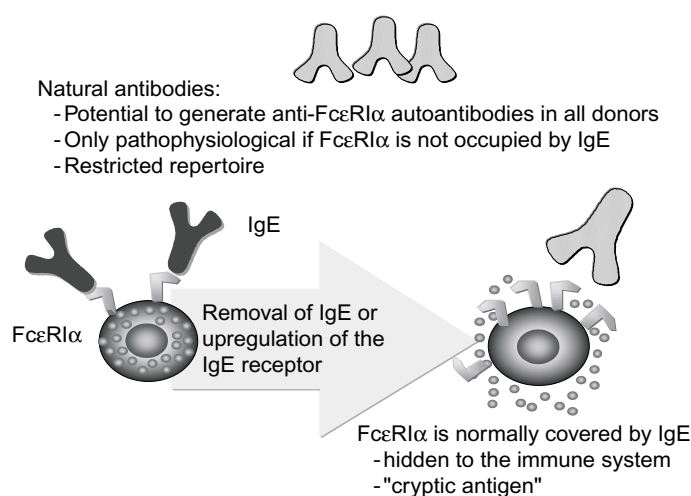


Fig. 6. Scheme portraying a possible patho-physiological mechanism for human natural anti-FcεRIα antibodies. It is envisaged that anti-FcεRIα antibodies are ubiquitous and belong to the natural antibody repertoire. Under normal physiological conditions i.e. when the FcεRIα is occupied by IgE, the anti-FcεRIα antibodies have no access to the receptor and cannot trigger an anaphylactic response on effector cells. Thus, the FcεRIα can be regarded as a "cryptic antigen". Upon dysregulation of the balance between free and receptor bound IgE the FcεRIα may become free and therefore accessible to the conditional anaphylactogenic activity of anti-FcεRIα antibodies.

donors and CIU patients either by phage display or as we previously reported by affinity purification from IVIg preparations [4]. We report the isolation of the first recombinant human natural anti-FcεRIα autoantibodies, LTMα15, LTMα35 and UMα16 which, under physiological conditions are not anaphylactogenic. However, removal of IgE from freshly isolated human basophils resulted in their activation by anti-FcεRIα autoantibodies followed by mediator release.

Originally we had assumed there would be a difference between anti-FcεRIα autoantibodies isolated from normal donors and CIU patients which would account for the proposed anaphylactogenic activity in CIU patients. In fact the same antibody was isolated from both libraries which alters our previous ideas on the presence of pathogenic anti-FcεRIα autoantibodies. Furthermore these antibodies were isolated from antibody libraries not only constructed at totally different time points but also the panning procedures were performed at different times. Therefore the isolation of the same anti-FcεRIα autoantibody, LTMα15 and UMα16 from different sources cannot be due to a contamination.

Additionally, sequence analysis revealed germline VH paired with a slightly mutated VL thus supporting their classification as natural antibodies [11]. There are many reports showing that natural antibodies react with self antigens and can be detected in the serum of normal donors [6]. Our data confirm and extend these previous findings by the isolation of recombinant human natural autoantibodies from the IgM repertoire of both

normal donors and CIU patients. Further the same biological activity shown for the recombinant anti-FcεRIα autoantibodies was also found in the serum of both normal donors and CIU patients indicating that the antibodies isolated by phage display are representative of those present in the serum. Additionally, we have previously reported the presence of IgM anti-FcεRIα autoantibodies in human cord blood [4].

Until now it has been assumed that natural autoantibodies are non pathogenic under physiological conditions for a variety of reasons including low affinity reactions and their role in establishing V-region mediated networks [5]. However, our results indicate that even natural autoantibodies may become pathogenic when the accessibility of their target antigen changes. In this case the FcεRIα is normally not seen by the immune system and can be regarded as a cryptic antigen (as depicted in Fig. 6).

During the recent clinical evaluation of therapeutic anti-IgE antibodies it has been shown that they down-regulated the IgE receptor density on basophils and mast cells [15]. Thus, the IgE level alone may be responsible for the regulation of IgE receptor expression, which might link autoimmune forms of urticaria to the IgE mediated triggerings of allergic urticaria.

Several reports have documented upregulation of the FcεRIα during allergic inflammation which also lend support to its role in the late phase reaction [14,17].

In all these examples the binding of the natural anti-FcεRIα autoantibodies to the naked, newly expressed

FcεRIα would be possible resulting in mediator release. We would like to propose these phenomena be defined as conditional autoimmunity.

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