

A High-Affinity Natural Autoantibody from Human Cord Blood Defines a Physiologically Relevant Epitope on the FcεRIα¹

Tomasz Bobrzynski,* Michaela Fux,* Monique Vogel,* Michael B. Stadler,[†] Beda M. Stadler,* and Sylvia M. Miescher^{2,*‡}

Natural Abs represent the indigenous immune repertoire and are thus present at birth and persist throughout life. Previously, human autoantibodies to the α domain of the high-affinity IgE receptor (FcεRIα) have been isolated from Ab libraries derived from normal donors and patients with chronic urticaria. To investigate whether these anti-FcεRIα Abs are present in the germline repertoire, we constructed a phage Fab display library from human cord blood, which represents the naive immune repertoire before exposure to exogenous Ags. All isolated clones specific to the FcεRIα had the same sequence. This single IgM Ab, named CBMα8, was strictly in germline configuration and had high affinity and functional in vitro anaphylactogenic activity. Inhibition experiments indicated an overlapping epitope on the FcεRIα recognized by both CBMα8 and the previously isolated anti-FcεRIα Abs from autoimmune and healthy donors. This common epitope on FcεRIα coincides with the binding site for IgE. Affinity measurements demonstrated the presence of Abs showing CBMα8-like specificity, but with a significantly lower affinity in i.v. Ig, a therapeutic multidonor IgG preparation. We propose a hypothesis of escape mutants, whereby the resulting lower affinity IgG anti-FcεRIα Abs are rendered less likely to compete with IgE for binding to FcεRIα. *The Journal of Immunology*, 2005, 175: 6589–6596.

The origin and function of natural Abs in the human repertoire are enigmatic (1). They appear spontaneously throughout life, without antigenic stimulation, have none or only a few somatic mutations in the V regions (2), and are usually polyreactive to phylogenetically conserved structures (3). They can be of the IgM, IgG, or IgA isotype (4); may be involved in innate immunity to viruses (5, 6) or elimination of cancer cells (7); and probably function as precursors for hypermutated Abs. Serum natural Abs are frequently identified as autoantibodies (8) and for a long time were considered indicative of ongoing pathological autoimmune disease. Many of their functions were extrapolated from murine model systems, and there is a paucity of human natural mAbs available for research. Nevertheless, extensive studies using affinity-purified autoreactive Ig fractions have illustrated the increasing importance of natural autoantibodies. Such autoreactive Abs are now thought to play an important role in regulation of the normal B cell repertoire and represent the major fraction of serum Abs (9). Recent literature suggests that natural autoantibodies react throughout life with a restricted set of self-Ags (10).

Using phage display technology, we have recently isolated autoantibodies to the high-affinity IgE receptor (FcεRIα) from tonsils

of healthy children and from blood of chronic urticaria (CU)³ patients (11). These widespread Abs are particularly interesting because they cross-link the FcεRIα on basophils leading to cell degranulation and release of inflammatory mediators in vitro and may thus have a role in the pathogenesis of CU (12). This disease is diagnosed by regular appearance of short-lived wheals on the skin for at least 6 wk (13). We suggested that autoimmune CU may be caused by an imbalance of natural Abs, which bind to an epitope exposed after removal of IgE from FcεRIα. Moreover, we have previously detected IgM anti-FcεRIα Abs in human cord blood serum (14). These Abs most likely originated from the fetus itself, because it is believed that only maternal IgG Abs cross the placenta (15).

To verify the presence of anti-FcεRIα Abs in the natural Ab repertoire, we constructed a phage display library from IgM transcripts from pooled human umbilical cord blood samples. The library represents a naive immune repertoire before exposure to exogenous Ags and clonal expansion of hypermutated Abs. In contrast, the libraries from which we previously isolated anti-FcεRIα Abs were limited to donors exposed to environmental Ags. In this study we report the isolation and molecular characterization of a human cord blood natural Ab strictly in germline configuration, which reacted with an epitope common for the previously isolated Abs and overlapping with the IgE binding site on the FcεRIα. The interaction between IgE and FcεRIα is of high affinity; consequently, the autoepitope on the receptor is physiologically masked by IgE. Occasional dissociation of IgE from the receptor has been suggested (11) and could lead to an immune response and selection of specifically reacting B lymphocytes. According to current knowledge, Abs of predetermined specificities

*Institute of Immunology, University of Bern, Bern, Switzerland; [†]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; and [‡]ZLB Behring, Bern, Switzerland

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² Address correspondence and reprint requests to Dr. Sylvia Miescher, Institute of Immunology, Inselspital, Sahli-Haus 2, UG11, Bern CH-3010, Switzerland. E-mail addresses: sylvia.miescher@iib.unibe.ch or sylvia.miescher@zlbbehring.com

³ Abbreviations used in this paper: CU, chronic urticaria; IVIg, i.v. Ig; HSA, human serum albumin; IAsys, interaction analysis system.

mutate and are selected for increased affinity, but against self-recognition. We found an autoantibody in germline configuration that has higher affinity than the overall affinity of the corresponding autoantibodies found in a therapeutic i.v. Ig (IVIg) preparation. This may suggest a previously unrecognized phenomenon, whereby autoantibodies undergo a maturation process resulting in reduced affinity, thus avoiding an autoimmune reaction. We have termed such potentially mutated autoantibodies escape mutants.

Materials and Methods

Phage display library

In brief, members of the V_H , κ , and λ gene families (16–20) from the IgM class were amplified independently from cDNA of cord blood lymphocytes. The downstream primers used for this PCR were tested in silico in a BLAST (21) search and were found to match exclusively the first constant domain of human IgM Fc fragment. The PCR products were cloned into an expression phagemid, yielding a total of 4×10^7 clones.

The cord bloods (20 ml each) were provided from deliveries of 17 male and eight female newborns (Lindenhofspital). Great care was taken not to contaminate the cord blood samples with maternal blood. The lymphocyte fraction was isolated by Ficoll (Amersham Biosciences) density gradient centrifugation. Total RNA was prepared as described previously (22) and pooled. The SMART cDNA library construction system (23) (BD Clontech) was used to ensure complete RT. A first long-distance PCR was performed according to BD Clontech manual PT3000-1. The conditions were as follows: 50 μ l of a mixture containing Pfx Amplification Buffer, Pfx Enhancer Buffer, 1 mM $MgSO_4$, 400 μ M each of the standard dNTPs, 2.5 U of Platinum Pfx polymerase (all from Invitrogen Life Technologies), 10 μ l of first-strand cDNA, 0.4 μ M each of the primers 5' PCR and CDS III/3' PCR; 95°C for 1 min, followed by 20 cycles of 95°C for 15 s and 68°C for 5 min. The resulting double-stranded cDNA was used as a template for amplification of individual H and L chain gene families (or constant domains for control). All primer sequences are written 5' to 3' end.

Set 1

The primers for H chain families V_{H1a} , V_{H1f} , V_{H3a} , V_{H3f} , V_{H4f} , and V_{H6a} were previously described (24). Additionally designed primers were V_{H2} (cag gtc acc ttg ctc gag tct ggt), V_{H4g} (cag gtg cag cta ctc gag tgg gg), V_{H5} (gag gtg cag ctc gag cag tct gg), and V_{H7} (cag gtg cag ctc gag caa tct gc). The following downstream primer for the first constant domain of human IgM was used: gct cac act agt cta ggc aat cac tgg aag agg.

Set 2

The primers for κ families: $V_{\kappa1a}$, $V_{\kappa2a}$, $V_{\kappa3a}$, and $V_{\kappa3b}$ were previously described (24). Additionally designed primers were $V_{\kappa4}$ (identical with the $V_{\kappa1a}$ primer), $V_{\kappa5}$ (gaa acg gag ctc acg cag tct cca), and $V_{\kappa6}$ (gaa att gag ctc act cag ctc cca). The downstream primer for the 3' end of human κ constant domain was tcc ttc tag aac act ctc ccc tgt tga agc tct ttg tga cgg gcg aac t.

Set 3

The designed primers for λ families were $V_{\lambda1}$ (cag tct gag ctc acg gag cc(g/a) ccc tc), $V_{\lambda2}$ (cag tct gag ctc act cag cct gcc tc), $V_{\lambda3}$ (gcc tcc tat gag ctc act cag cca), $V_{\lambda4a}$ (cag cct gag ctc act caa tca tcc tc), $V_{\lambda4b}$ (cag cct gag ctc act cag ccc cgc tc), $V_{\lambda5}$ (cag cct gag ctc act cag cgc (g/t)ct tcc), $V_{\lambda6}$ (aat ttg gag ctc act cag ccc cag), $V_{\lambda7}$ (cag act gag ctc act cag gag ccc), $V_{\lambda8}$ (cag act gag ctc acc cag gag cca tgc ttc), $V_{\lambda9}$ (cag cct gag ctc act cag cca cct tc), and $V_{\lambda10}$ (cag gca gag ctc act cag cca ccc tgc). The downstream primer for the 3' end of the human λ constant region was gca ttc tag atg aac att ctg tag ggg cca c.

The H and L chains were separately pooled. DNA was purified by extraction from bands in 2% agarose using a kit (Qiagen). The H chains were cut with *XhoI* and *SpeI*, and L chains were cut with *XbaI* and *SacI* (Roche). The resulting fragments were separately pooled and sequentially cloned into pMVS (12), a vector based on pComb3H that allows the expression of Fab on the surface of the filamentous phage M13 (25). Ligation, transformation of *Escherichia coli* XL-1 Blue strain, and production of phage particles were conducted as described previously (26–28) (the phagemid DNA was directly amplified in bacteria without performing affinity maturation in vitro). Total phagemid DNA was purified (Plasmid Maxi kit; Qiagen) from infected bacteria.

Biopanning on immobilized Ag

Fc ϵ RI α -HSA-Fc ϵ RI α (provided by Novartis) is a double-fusion construct of the extracellular part of Fc ϵ RI α and human serum albumin (HSA), produced in Chinese hamster ovary cells (14). One of the Fc ϵ RI α is fused by its N terminus, and the other by the C terminus. Binding of human IgE and recombinant human anti-Fc ϵ RI α mAbs was confirmed by ELISA (data not shown). For panning, four wells of microtiter plates (Corning; product 3690) were coated with a total of 100 μ l (panning rounds one to four) or 400 μ l (rounds five to eight) of Fc ϵ RI α -HSA-Fc ϵ RI α at 50 μ g/ml (in 0.05 M $NaHCO_3$ (pH 9.6) at 4°C overnight). HSA (ZLB Behring) was coated at the same concentration and used for two preabsorptions in each panning round. Blocking, washing, and elution were performed essentially as previously described (27), except for preabsorptions on HSA. Wells coated with HSA were each filled with 180 μ l of phages ($\sim 1 \times 10^{13}$ phages/ml in blocking buffer) at room temperature; after 1 h, phages were transferred to the wells with Fc ϵ RI α -HSA-Fc ϵ RI α for 2 h. Eluted phages were amplified in bacteria (26, 27). After each round of panning, the enrichment was monitored by titrating the eluted phage CFU, and the presence of both H and L chain genes was confirmed by restriction analysis of DNA (data not shown).

Sequencing

Infected bacteria were cloned on selective media as previously described (29), and phagemid DNA was purified (Wizard Plus SV Minipreps; Promega). Clones were sequenced at Microsynth. The following primers were used: 5'-gga gga att taa aat gaa ata c (for variable H chain) and 5'-gtg gaa ttg tga gcg gat aac (for variable L chains). The most similar germ lines were found using the University of Cambridge School of Biological Sciences computing facilities and the V BASE index (30).

Phage ELISA and phage inhibition assay

Phages were titrated and diluted ($\sim 10^{12}$ CFU/ml in PBS with 2% FCS and 0.05% Tween 20). To test the specificity of the phages, ELISA plates (same as for biopanning) were coated with Fc ϵ RI α -HSA-Fc ϵ RI α (5 μ g/ml in 0.05 M $NaHCO_3$ (pH 9.6), 50 μ l/well, 4°C, overnight) or, for controls, with equimolar HSA or 10 μ g/ml anti-human Fab (The Binding Site; product PC005). The plates were washed three times with 0.1% Tween 20 in PBS (pH 7.4), blocked with 5% BSA in PBS (2 h, 37°C), washed six times, incubated with the phages (100 μ l/well, 2 h, $\sim 21^\circ$ C), washed eight times, incubated with rabbit anti-phage Abs labeled with HRP (100 μ l/well, 90 min, $\sim 21^\circ$ C), washed eight times, and developed with 3,3',5,5'-tetramethylbenzidine (Fluka)/hydrogen peroxide solution (100 μ l/well, 5 min). The reaction was quenched with 1 M H_2SO_4 (100 μ l/well). OD was measured in an ELISA plate reader (450 nm). The experiments shown in Fig. 2 were performed in essentially the same way, except that for the experiment shown in Fig. 2A Fc ϵ RI α -HSA-Fc ϵ RI α was coated at 1 μ g/ml and CBM α 8 phages were at a concentration of 5×10^{10} CFU/ml after addition. The Abs tested for the ability to inhibit CBM α 8 were incubated in the coated wells for 2 h at room temperature (50 μ l/well at the concentrations indicated in Fig. 2), followed by CBM α 8 phages (50 μ l/well) for 2 h. Mean OD values from duplicate measurements are shown. Bars indicate the values obtained. The anti-phage Abs (raised in our laboratory) were conjugated with HRP (Sigma-Aldrich; P-8375) as previously described (31). LTM α 15 and LTM α 35 were previously produced in our laboratory as IgG Abs (11). Human monoclonal IgE-SUS11 was purified (29) from hybridomas generated in our laboratory (32). Immunoaffinity-purified murine monoclonal anti-human Fc ϵ RI α (5H5F8) was provided by F. Krick (Novartis, Vienna, Austria). Sheep anti-mouse IgG-HRP was purchased from ICN (product 55558); anti-human serum albumin-HRP and anti-human IgE-HRP were obtained from The Binding Site (products PP032 and AP014).

Generation of full-length CBM α 8

Phages were not optimal for IAsys (Affinity Sensors) measurements due to possible steric effects caused by the phage capsids. To generate CBM α 8 as an Ig, the H and L chain genes were fused to the IgG Fc by cloning to modified (33) vectors from the V_H and VKExpress system (34), and the constructs were verified by sequencing. Human embryonic kidney cells (HEK 293; American Type Culture Collection; CRL-1573; adjusted to suspension culture) were cotransfected with the H and L chain constructs (Swiss Federal Institute of Technology) and cultured in EX-CELL 293 serum-free medium (JRH Biosciences) for 5–6 days. The Ab was purified on a protein G-Sepharose 4 Fast Flow (Amersham Biosciences) column, and the concentration was measured by UV absorption and with the DC Protein Assay (Bio-Rad). The Ab reacted with recombinant Fc ϵ RI α , and both chains were detected with anti-human Fd or anti-human λ Abs in an ELISA and visualized by SDS-PAGE (data not shown). The Abs used in

the ELISA were purified in our laboratory from hybridoma culture supernatants (American Type Culture Collection; HP6045 and HP6054, respectively).

Purification of anti-Fc ϵ RI α from IVIg

IVIg (ZLB Behring) was reconstituted with ultrapure water (Millipore) and dialyzed against PBS. Abs reacting with Fc ϵ RI α were purified using an affinity column of Sepharose coupled to Fc ϵ RI α -HSA-Fc ϵ RI α (4.5 mg of protein was taken for coupling). IVIg (1.4 g) was applied to the column, and the eluate was absorbed on a Sepharose-HSA column (10 mg of HSA was taken for coupling) before concentration on a protein G-Sepharose column.

IAsys measurements

Affinities were measured with the IAsys surface plasmon resonance instrument (Affinity Sensors) using previously described methods (14). For the linear analysis of association kinetics of CBM α 8 to Fc ϵ RI α , a monophasic association model was assumed. The previously determined affinity of LTM α 35 was reconfirmed. Inhibition assays were performed on a carboxylate IAsys cuvette coated with Fc ϵ RI α -HSA-Fc ϵ RI α (10 μ g was used for the immobilization) in the first channel and with equimolar HSA in the second channel as a control. Both channels were treated in the same way, and the HSA background was subtracted. Acid elution of bound Abs was performed with 10 mM HCl (three washes, followed by three washes with PBS). The concentrations of Abs in the experiment shown in Fig. 3 were as follows: 7.5 μ g/ml (50 nM IgG) for 5H5F8, LTM α 35, LTM α 15, and CBM α 8; and 19 μ g/ml (100 nM IgE) for the mAb SUS11. For testing the inhibition of enriched anti-Fc ϵ RI α from IVIg, CBM α 8 was incubated in the cuvette at 30 μ g/ml for 2 h and replaced with fresh CBM α 8 solution for an additional 20 min (to ensure saturation). The enriched anti-Fc ϵ RI α Abs were added together with CBM α 8 at 7.5 μ g/ml (to maintain the saturation).

Hexosaminidase release from RBL-2H3 cells expressing human Fc ϵ RI α

The mast cell line RBL-2H3E5.D12.8 (RBL-2H3; American Type Culture Collection; CRL-2256; transfected with human Fc ϵ RI α) was a gift (S. Mécheri, Institut Pasteur, Paris, France) and was confirmed to express human Fc ϵ RI α by FACS analysis. Release of β -hexosaminidase from the cells was performed similarly to previously described procedures (35, 36). Briefly, the cells were distributed to 24-well tissue culture plates (5×10^4 cells in 0.5 ml of antibiotic-free complete DMEM/well). The medium was supplemented with 0.1 mM hydroxyurea to enhance the expression of Fc ϵ RI α (27). After 2 days, the medium was replaced with prewarmed medium either with or without 50 μ g/ml IgE SUS-11 for 90 min. Cells were washed twice with Tyrode's salts (Sigma-Aldrich) supplemented with 10 mM HEPES buffer and treated with Abs (concentrations shown in Fig. 5) in 100 μ l of Tyrode's buffer for 45 min at 37°C. Secreted and intracellular β -hexosaminidase relative levels were measured by means of a chromogenic reaction as previously described (37). The release was expressed as a percentage of the activity of total available hexosaminidase activity of unstimulated cells. Spontaneous release was measured using a buffer control without added Abs. Omalizumab (human anti-IgE Ab used for control) was a gift from Novartis. Triggering with the CBM α 8 Ab was analyzed statistically using the Student's *t* test.

Calculation of solvent-accessible surface of Fc ϵ RI α

We used the MSMS program (M. F. Sanner, The Scripps Research Institute, La Jolla, CA) and crystallography model 1F6A from PDB. Residues not belonging to Fc ϵ RI α (the cocrystallized Fc ϵ) were removed from the model. The probe radius was 1.4 Å.

Results

Analysis of human cord blood Fab library

To examine the complexity of the library, 14 random clones were sequenced. The H and L chains of clones consistently revealed different germline sequences with only occasional scattered mutations and with differing hypervariable region 3 (CDR3). Thus, the library can be considered to represent the natural Ab repertoire. The most frequently occurring H chain germline families were V_H4 and V_H1 (four clones each), then V_H3 and V_H6 (two clones each), and V_H5 and V_H7 (one clone each); no V_H2 clones were found. These frequencies are similar to the reported V_H use: V_H3 > V_H4 > V_H1 > V_H5 > V_H2/V_H6/V_H7 (38, 39). The fol-

lowing L chain (V_L) gene families were found: V_L3 (three clones), V_L4 (one clone), V_L7 (two clones), V_L2 (three clones), V_L3 (two clones), and V_L5 (three clones).

Cord blood-derived natural Ab recognizes Fc ϵ RI α

The library phages were expressed in bacteria and selected by biopanning on recombinant Fc ϵ RI α fused to HSA. Phages were preabsorbed on HSA. To take account of possible low affinity clones present in this cord blood library, washing was less stringent for the first round of panning.

Analysis by ELISA confirmed the enrichment scores, in that the strongest reaction to Fc ϵ RI α was observed after the eighth panning round. Interestingly, we also found a strong reactivity of the cord blood library phages to CRM, a *Diphtheria* toxin mutant (data not shown), which points at a possible role of natural Abs in innate immunity to bacteria. This initial reactivity was diluted out by the rounds of panning on Fc ϵ RI α . Nine clones specific to Fc ϵ RI α were randomly selected from the enriched library and sequenced. All these clones had identical V_H and V_L, indicating that only one Ab, named CBM α 8, was isolated. As expected, the V_H and V_L sequences of CBM α 8 were entirely in germline configuration. The sequences were aligned to the previously isolated anti-Fc ϵ RI α Abs for comparison (Fig. 1). UG α 8, and an Ab identical with LTM α 15, previously described as UM α 16, were isolated from libraries generated from blood of CU patients (11). The most similar Ab to CBM α 8 is LTM α 35. The LTM α 15 and LTM α 35 Abs were previously isolated from a library constructed from tonsils of healthy children (11). Among the human anti-Fc ϵ RI α Abs isolated to date, CBM α 8 had a unique CDR3 of the H chain. The CDR3 of the L chain of CBM α 8 was similar to that of LTM α 35 (one amino acid difference). The V_L sequences of CBM α 8 and LTM α 35 differed by several amino acid residues. The V_H and V_L sequences of both CBM α 8 and LTM α 35 belonged to the V_H5 and V_L1 families (17, 40), respectively (Table I). These Abs had the same canonical classes of CDR loops 1 and 2 of V_H and V_L. The V_H of CBM α 8 belonged to the DP-73 germ line, and that of LTM α 35 belonged to the V_HVCW germ line. These germ lines differ only at the DNA level by one nucleotide (silent mutation) (16).

Human anti-Fc ϵ RI α Abs and IgE inhibit binding of the natural Ab CBM α 8 to Fc ϵ RI α

Using an inhibition ELISA, we evaluated whether the described sequence differences between CBM α 8 and the recombinant human anti-Fc ϵ RI α Abs affect the epitope specificity. Binding of phages expressing the Fab of CBM α 8 to human Fc ϵ RI α was inhibited by increasing concentrations of the human anti-Fc ϵ RI α Ab LTM α 35 or human IgE, but not by a control murine mAb 5H5F8 (37, 44) (Fig. 2A), which has a membrane-proximal epitope on Fc ϵ RI α . The data indicate that the epitope of the germline-encoded Ab CBM α 8 can be masked with IgE, as previously shown for the human anti-Fc ϵ RI α Abs LTM α 15 and LTM α 35, which have somatic mutations. Moreover, binding of the phage-displayed CBM α 8 Fab to Fc ϵ RI α was equally well inhibited by LTM α 15 and LTM α 35 individually and when added together (Fig. 2B). The result suggests that the three epitopes are overlapping, and binding of one of the Abs renders the epitope inaccessible for the remaining Abs.

To allow affinity measurements, a full-length CBM α 8 Ab was constructed by DNA engineering and produced in eukaryotic cells. Although the Fab of this Ab was isolated from an IgM library, an IgG Fc was added. The IgG isotype was chosen because our interest was Ag binding characteristics, not Fc-mediated interactions, and because the previously isolated Abs LTM α 15 and LTM α 35 were also produced as IgG. The specificity of the purified

Heavy chains								
	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	
CBM α 8	EVQLLEQSGAEVKKPGESLKISCKGSGYSFT	SYWIG	WVRQMPGKGLEWMG	IIPGSDSTRYSFSFGG	QVTISADKSISTAYLQWSSSLKASDTAMYYCAR	LLELGGYWFYDL	WGRGTLTVTS	
LTM α 35	-----	-----	-----	-----	-----	-GYDFWGSDDYYMDV	--K--T-----	
LTM α 15	-----GGLVQ--G--RL--AA--FT-S	--AMS	---A-----VS	A-SGSGGS-Y-AD-VK-	RF---R-N-KN-L---MN--R-E--V---K	GERWLPSY-M-V	--K--T-----	
UG α 8	-----GGLVQ--G--RL--AA--FT-S	--AMS	---A-----VS	A-SGSGGS-Y-AD-VK-	RF---R-N-KN-L---N--R-E--V---K	GERWLPSY-M-V	--Q---S---	
Light chains								
	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	
CBM α 8	AAELTQPPSVSGAPGQRTVISC	TGSSSNIGAGYDVH	WYQQLPGTAPKLLIY	GNSNRPS	GVPDFRSGSGKSGTSASLAITGLQA	EDEADYYC	QSYDSSLSVVV	FGGGTKLTVLGQP
LTM α 35	QSV-----	-----N	-----	--N---	-----I---	-----A--	-----	-----
LTM α 15	ELVV---H---ES--KT-----	-C--GS- -SIY-Q	---R--SS-TTV--	-DNQ---	-----IDSS-N-ASL-ISGLKT-----	-----NNH--	-----	-----
UG α 8	-----H---ES--KT-----	-R--DS- -SNY-Q	---RR--S--TTV-F	ADDR---	-----A-IDYS-N-ASL-ISGLRT-----	-----NNLW-	-----	-----

FIGURE 1. Comparison of CBM α 8 to previously isolated human anti-Fc ϵ RI α Abs. Amino acid sequences of H and L chain variable regions are aligned (hyphens represent identical residues). The CDR3 of the H chains are more diverse than those of the L chains. The most similar sequences are found in the CBM α 8 and LTM α 35 Abs. The entire L chains of CBM α 8 and LTM α 35 differ by only a few amino acids. The H chain of CBM α 8 has a unique CDR3. Amino acids at the beginning of framework 1 (FR1) of the H chains (leucine, followed by glutamic acid) and of the L chains (two alanine residues, followed by glutamic acid) are changed due to introduction of restriction sites for cloning the V_H and V_L chains into the pMVS vector. The sequences of CBM α 8 are available from the EMBL Nucleotide Sequence Database (accession no. AJ580927 and AJ580928).

Ab was verified by a sandwich ELISA in which the full-length CBM α 8 Ab was shown to contain the L and H chains with the Fc and to react with recombinant Fc ϵ RI α . The affinity of CBM α 8 was assessed by on-line monitoring of binding kinetics using the IAsys cuvette system. The K_d was 3×10^{-9} M and was similar to the values obtained for the previously isolated anti-Fc ϵ RI α Abs LTM α 15 (7×10^{-9} M) and LTM α 35 (1×10^{-8} M) (11). In comparison, our previous unpublished observations showed that the UG α 8 Ab, which was isolated from an IgG library, did not bind Fc ϵ RI α as strongly as did the LTM α 15 Ab (UG α 8 used at 100 μ g/ml gave an OD of 0.5, whereas LTM α 15 at 5 μ g/ml gave an OD of >1 in the same ELISA experiment).

The ELISA results were also validated using full-length CBM α 8 in the IAsys. The interaction profiles of anti-Fc ϵ RI α Abs, human monoclonal IgE, and Fc ϵ RI α are shown in Fig. 3. In these experiments Fc ϵ RI α was covalently bound to the solid phase. The first curve (Fig. 3A) reflects binding of CBM α 8 Ab to Fc ϵ RI α . Upon further addition of the Ab, there was no additional binding, which indicates saturation with CBM α 8. Addition of LTM α 35 or LTM α 15 at the same concentrations as CBM α 8 also showed no significant binding. However, a control murine mAb 5H5F8 bound to Fc ϵ RI α . The epitope of the 5H5F8 Ab is known to be separate from the IgE binding site (37, 44). The results show that CBM α 8 interferes with binding of LTM α 35 and LTM α 15 and suggest that these Abs have an overlapping epitope different from that of the murine control Ab 5H5F8. After removal of the bound Abs with an acid wash, the LTM α 15 and LTM α 35 Abs could bind the receptor

(~60 arc s response; data not shown). In another experiment, Fc ϵ RI α was saturated with human monoclonal IgE (Fig. 3B). Under these conditions, CBM α 8 did not bind, indicating that its epitope overlaps with the IgE binding site on the Fc ϵ RI α receptor. As expected, binding of the control 5H5F8 Ab in the presence of saturating amounts of IgE was not affected.

Evidence for CBM α 8-like specificity in normal human serum

To investigate whether the epitope specificity of CBM α 8 can be found in human serum, we used IVIg (45), which represents IgG Abs from a large pool of healthy donors. Abs specific to recombinant Fc ϵ RI α were enriched by affinity chromatography and were shown to react with Fc ϵ RI α by ELISA. Using the IAsys system, we have demonstrated binding of the serum-derived anti-Fc ϵ RI α Abs to the Fc ϵ RI α in the absence of CBM α 8 (Fig. 4). The serum-derived anti-Fc ϵ RI α Abs were partially inhibited (~45%) by the natural mAb CBM α 8, suggesting that IVIg contains CBM α 8-like Abs. These anti-Fc ϵ RI α Abs purified from IVIg may be oligo- or polyclonal. The average affinity of these Abs demonstrated a K_d value of 1×10^{-6} M, as determined by IAsys.

Natural Ab CBM α 8 induces degranulation in vitro

The potential of CBM α 8 to trigger mast cells was evaluated by monitoring the release of a marker of secretory granules, β -hexosaminidase (Fig. 5), from RBL-2H3 cells expressing human Fc ϵ RI α (RBL-2H3E5.D12.8 cells). To up-regulate Fc ϵ RI α and increase the sensitivity of the assay, we used hydroxyurea (33), not

Table I. Number of mutated amino acids present and classification of human anti-Fc ϵ RI α Abs^a

	H Chain Mutations	Germline	Family	Canonical Loop Structure			L Chain Mutations	Germline	Family	Canonical Loop Structure		
				H1	H2	H3				L1	L2	L3
CBM α 8	0	DP-73	V _H 5	1	2	k	0	DPL8	V _L 1	10	1	nc
LTM α 35	0	V _H VCW	V _H 5	1	2	k	3	DPL8	V _L 1	10	1	nc
LTM α 15	0	DP-47	V _H 3	1	3	k	3	V1-22	V _L 6	nc	1	nc
UG α 8	0	DP-47	V _H 3	1	3	k	10	V1-22	V _L 6	nc	1	nc

^a The total numbers of mutated amino acid residues in the frameworks and in the CDR1 and CDR2 regions of the Abs were determined by comparison to the corresponding germline sequences. Canonical structures of Ag-binding loops were identified using Chothia definitions of canonical rules (41, 42). All of these Abs have the C-terminal structure of the H chain loop 3 classified as "kinked" (k) and none as "extended" according to published rules (43). Noncanonical (nc) loops are indicated. The germlines DP-73 and V_HVCW differ only by a silent mutation (16).

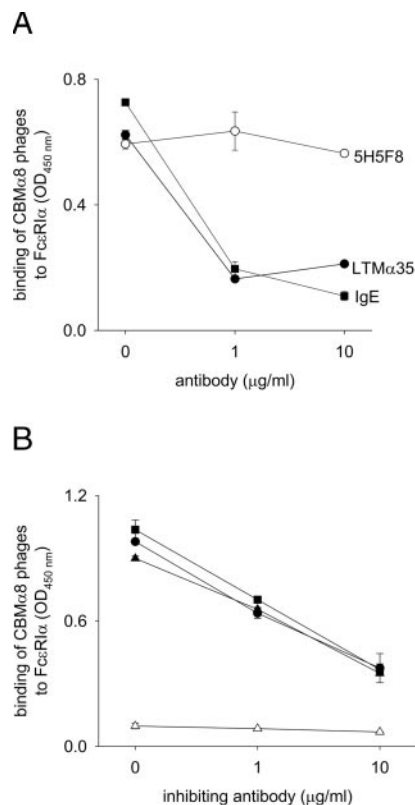


FIGURE 2. Human IgE and the previously isolated anti-FcεRIα Abs inhibit binding of CBMα8 phages to FcεRIα (ELISA). *A*, Recombinant human FcεRIα was immobilized (at 5 μg/ml) and incubated with increasing concentrations of human IgE (■), human anti-FcεRIα Ab LTMα35 (●), or murine anti-FcεRIα Ab 5H5F8 (○), followed by a constant concentration (~10¹² CFU/ml) of phages displaying anti-FcεRIα Fab (derived from a cord blood library and named CBMα8). The bound phages were detected using rabbit anti-phage peroxidase-labeled Abs. Binding was assessed by measuring the OD at 450 nm. CBMα8 phages were inhibited by the mAb LTMα35. Inhibition with IgE maps the epitope of CBMα8 to the IgE binding site on FcεRIα. The murine mAb 5H5F8 (control) did not inhibit the phages. This is in agreement with the known location of the 5H5F8 epitope, which is distant from the IgE binding site on FcεRIα. Background (binding of wild-type phages) has been subtracted. *B*, Combining LTMα35 and LTMα15 Abs has no additive effect on inhibition of CBMα8 phages. Recombinant FcεRIα was coated at 1 μg/ml. Wells were blocked with BSA and incubated with 0, 1, or 10 μg/ml solutions of LTMα35 or LTMα15 Abs or with an equal volume of a 1:1 mixture of the two solutions. The Abs were replaced with 5 × 10¹⁰ CFU of CBMα8. Bound phages were detected with peroxidase-labeled Abs (OD was measured at 450 nm). Binding of CBMα8 phages to FcεRIα was inhibited by LTMα35 (●) and LTMα15 (■) to the same extent as by the two Abs combined (▲), suggesting that the Abs have an overlapping epitope on FcεRIα. Additional wells, representing background binding (△), were pre-incubated with the mixture of Abs followed by wild-type phages in place of CBMα8.

IgE, because IgE interferes with binding of the natural Ab CBMα8. For a positive degranulation control, the cells were triggered with a mouse anti-human FcεRIα Ab 22E7 or with human IgE followed by a cross-linking anti-IgE Ab. Triggering using IgE is a suitable control, because previous experiments have shown that it strongly stimulates degranulation of purified human basophils (46). In the cell culture system, the release levels of β-hexosaminidase were maximally 17%, which is higher than published results obtained by cytotoxin-induced degranulation of RBL-2H3 cells (47). We stimulated the transfected cells with different concentrations of CBMα8. Hexosaminidase release was relatively

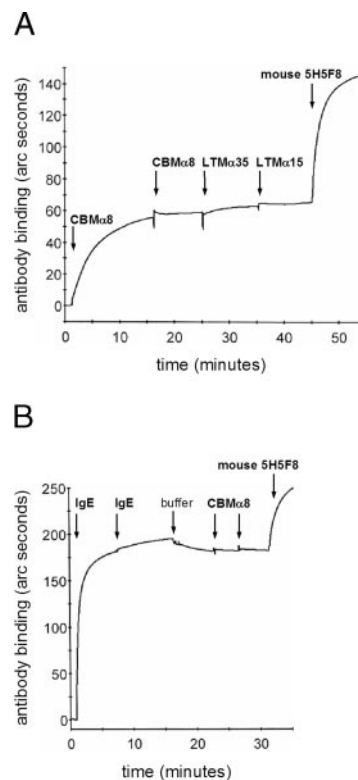


FIGURE 3. CBMα8 and other human anti-FcεRIα Abs selected by phage display share an epitope overlapping with the IgE binding site on FcεRIα. The molecular interactions with immobilized recombinant FcεRIα were monitored online with an IAsys resonant mirror biosensor. *A*, FcεRIα was saturated with the full-length CBMα8 Ab (50 nM, applied twice). Under these conditions, the human Abs LTMα35 and LTMα15 were inhibited, but the murine mAb 5H5F8 was not (all the Abs were adjusted to 50 nM). The results show that CBMα8 interferes with the binding of LTMα35 and LTMα15, but not that of 5H5F8. The epitope of the 5H5F8 control Ab is on the membrane-proximal part of FcεRIα, separate from the IgE binding site. *B*, FcεRIα was saturated with human monoclonal IgE (100 nM, applied twice and washed with buffer to remove excess IgE). Inhibition of CBMα8 (50 nM) binding to FcεRIα showed that the epitope of CBMα8 overlaps with the site for binding IgE.

strong and was dose dependent up to 16% of the total intracellular content with 4% spontaneous release. A negative control isotype-matched nonanaphylactogenic Ab, omalizumab (48, 49), showed 6% secretion.

We have confirmed these results using isolated human basophils stripped of IgE with lactic acid as described previously (11). The degranulation response to stimulation with CBMα8 was positive: 0.2 μg/ml CBMα8 induced 10% release, 2 μg/ml induced 17% release, and 20 μg/ml induced 18% release (data not shown). The buffer control was only 3%, indicating that degranulation resulting from any possibly remaining IgE-allergen complexes was negligible.

Discussion

Human autoantibodies specific to either FcεRIα or IgE have been ascribed a central role in up to 30% of CU cases (50). Our former data suggested that cross-linking anti-FcεRIα Abs are also present in healthy individuals (11). We have now cloned and characterized an IgM Ab from a representative human natural Ab repertoire. This anti-FcεRIα Ab, CBMα8, is remarkable, because it originates from human umbilical cord blood and is entirely in germline configuration. The human anti-FcεRIα Abs previously isolated by

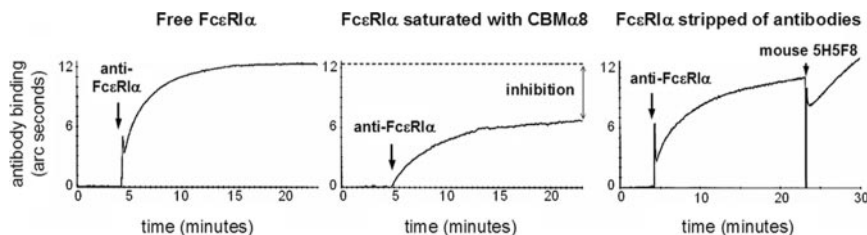


FIGURE 4. CBMα8-like specificity is present in IVIg. A putative CBMα8-like epitope specificity in IVIg was confirmed by IAsys. For simplicity, washing steps are not shown, and time measurement was reset at the beginning of each part of the experiment. Binding of anti-FcεRIα Abs (500 nM; i.e., 75 μg/ml) affinity purified from IVIg was monitored online (left panel). The bound Abs were eluted with acid, and FcεRIα was saturated with CBMα8 (data not shown). Under these conditions, binding of the Abs from IVIg was weaker (center panel). The initial binding to free FcεRIα was reproduced (right panel) after another elution of the bound Abs using acid. The results indicate that the mAb CBMα8 partially inhibits natural serum anti-FcεRIα Abs purified from IVIg. The murine 5H5F8 mAb (1.7 nM; i.e., 0.25 μg/ml) was used as a positive binding control.

phage display (from tonsils of children and peripheral blood of CU patients) differ from CBMα8 by several mutations in the L chains and have totally different CDR3s of the H chains. Nevertheless, the germline Ab CBMα8, like the previous Abs, interfered with the binding of IgE to FcεRIα. By means of an IAsys inhibition assay, we have also shown that IVIg, the IgG fraction from plasma of multiple healthy donors (51), contains the epitope specificity of the natural mAb CBMα8. Remarkably, this single Ab inhibited by ~45% the anti-FcεRIα Abs affinity purified from IVIg. This suggests that the epitope specificity of CBMα8 persists throughout life. The observed inhibition of Abs from IVIg by the mAb CBMα8 may be partially accounted for by steric hindrance. We calculated the solvent-accessible surface of an FcεRIα crystallographic model to be $\sim 1.1 \times 10^4 \text{ Å}^2$, of which even less is available to Abs due to the bent shape of the receptor. The solvent-accessible surface of a typical Ab binding site is known to be $\sim 1.7 \times 10^3 \text{ Å}^2$ (52). Therefore, a large part of the available surface on FcεRIα is already buried under a single binding site of an anti-FcεRIα Ab. However, CBMα8 did not block the membrane-proximal epitope on FcεRIα, as seen by binding of the control mAb 5H5F8. In CU cases, the presence of anti-FcεRIα Abs in serum has been frequently reported (53–55), but the epitopes of these Abs have not been elucidated.

We have confirmed that CBMα8 induces degranulation *in vitro* by measuring the release of the preformed granular enzyme β-hexosaminidase from RBL-2H3 cells expressing functional human

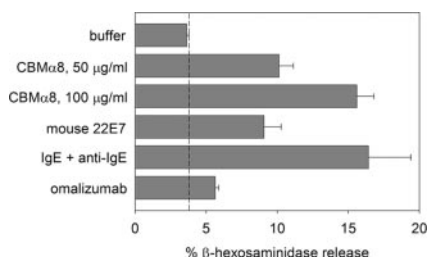


FIGURE 5. The natural Ab CBMα8 triggers degranulation of RBL-2H3 cells transfected with human FcεRIα. The cells were stimulated with the human anti-FcεRIα Ab CBMα8 at the given concentrations. Anaphylactogenic anti-FcεRIα mouse mAb 22E7 (50 μg/ml) and human IgE (clone SUS-11, 50 μg/ml), followed by anti-IgE (clone Le27; 10 μg/ml), were used as positive triggering controls. Omalizumab (100 μg/ml) was used as the isotype-matched control. Values are the mean percentage of total hexosaminidase release $\pm$ SD of determinations from three culture wells and are representative of three experiments. CBMα8 triggered degranulation ($p < 0.01$ for both concentrations). High concentrations of CBMα8 had to be used, possibly reflecting the relatively low density of the human FcεRIα on the surface of the transfected rat cells (CBMα8 at 1 and 10 μg/ml did not show a response).

FcεRIα and by measuring histamine release from purified human basophils. Thus, an Ab in strict germline configuration can be isolated from human cord blood by phage display and exhibits biological activity *in vitro*. This is in line with previous results describing biologically active germline Abs cloned from human cord blood B cells (56).

The affinity of CBMα8 to FcεRIα is high (3×10^{-9} M). Other reported natural Abs have affinities from 10^{-5} to 10^{-8} M (57), and mature Abs typically have affinities from 10^{-7} M upward. We hypothesized that mutations in autoantibodies to FcεRIα may facilitate escaping from tolerance mechanisms. Rather than undergoing a separate process, the escape mutants may occur just like other mutated Abs. Random somatic mutations in the V genes are likely to lower the affinity of Abs that have a relatively high affinity already in germline configuration. In addition, because FcεRIα appears saturated with strongly bound IgE (58), human autoantibodies to FcεRIα presumably do not physiologically exhibit a strong autoreactivity because their epitope is masked by the IgE. Thus, random mutations can occur to lower the affinities of the autoantibodies below a threshold level that would be needed for deletion of the autoreactive clone. To test the hypothesis of escape mutations, we measured the overall affinity of CBMα8-like natural autoantibodies found in normal human serum. Indeed it was relatively low (1×10^{-6} M), which is in agreement with previous observations (14). Because IVIg contains a pool of IgG Abs from human serum, these anti-FcεRIα may have undergone a class switch and may result from an immune response that lowers their affinity. This is also consistent with our previous observations that an anti-FcεRIα IgG Ab isolated by phage display from CU patients (UGα8) appeared to have a lower affinity than the IgM Ab LTMα15, as judged by functional assays (25). The recognition of FcεRIα was not as strong as that by the LTMα Abs (UGα8 used at 100 μg/ml gave an OD of 0.5, whereas LTMα15 at 5 μg/ml gave an OD of >1 in the same ELISA experiment; our unpublished observations).

Physiologically these Abs may not be able to bind FcεRIα expressed on mast cells unless the cryptic epitope on FcεRIα is unmasked by dissociation of IgE. This may be reflected by the occasional induction of mild urticaria, for example, by administration of the therapeutic anti-IgE Ab omalizumab, which complexes IgE and prevents its binding to FcεRIα (49). Additional mechanisms protecting from autoreactivity to FcεRIα may include anti-idiotypic Abs to the anti-FcεRIα Abs. Indeed, the symptoms of CU could be alleviated in clinical trials (50) with IVIg, which is hypothesized to contain natural Abs (45, 51) and is believed to restore an unbalanced anti-idiotypic network (59). In addition, the exposed receptor is likely to be protected from CBMα8-like Abs

by its turnover and thus by its limited availability for the autoantibodies. Binding of IgE to FcεRIα reduces the receptor turnover (60–62), so conversely, dissociation of IgE is likely to increase the turnover. Despite having no apparent physiological function, anti-FcεRIα Abs may give rise to beneficial mature Abs to viruses or bacterial Ags, not necessarily cross-reactive with the FcεRIα.

To date, human Abs to other epitopes on FcεRIα have not been isolated, although this would be of potential therapeutic interest. Indeed, the anti-FcεRIα Ab (5H5F8) raised in immunized mice has been shown to down-regulate signaling by the human receptor in vitro (37). Additionally, pathological autoantibodies in CU may target other epitopes on the FcεRIα. Although the existence of such Abs cannot be excluded, only Abs specific to one site on FcεRIα were isolated both from the natural Ab repertoire and previously from donors exposed to environmental Ags, including CU patients.

In summary, we have shown that the cord blood-derived IgM Ab CBMα8 is entirely in germline configuration, but, nevertheless, has a high affinity, competes with IgE in vitro, and is biologically active in functional assays. All the human anti-FcεRIα Abs isolated to date are mutually inhibited. A similar specificity is found in IVIg. However, the overall affinity of the IVIg-derived IgG Abs was low, suggesting a mechanism leading to a decrease in the affinity of autoantibodies. We hypothesized that the anti-FcεRIα autoantibodies in IVIg may have escape mutations, reducing their affinity. It remains an open question whether these Abs have a role in immune regulation of FcεRIα-expressing cells or evasion of pathogens.

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Disclosures

The authors have no financial conflict of interest.

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