

Inhibition of Antigen-Induced Mediator Release from IgE-Sensitized Cells by a Monoclonal Anti-FcεRI α-Chain Receptor Antibody: Implications for the Involvement of the Membrane-Proximal α-Chain Region in FcεRI-Mediated Cell Activation¹

Andreas Nechansky,* Michael W. Robertson,^{2†} Bettina A. Albrecht,[†] John R. Apgar,[†] and Franz Kricek^{2*}

The interaction between human IgE and its high affinity receptor, FcεRI, is a critical event in mediating the allergic response. Aggregation of the α-chain of FcεRI (FcεRIα) occurs via cross-linking of receptor-bound IgE by Ag, resulting in cell activation and the release of mediators of hypersensitivity. Recently, we mapped the epitopes of two anti-FcεRIα mAbs, 15/1 and 5H5F8. In contrast to 15/1, mAb 5H5F8 does not inhibit IgE binding to FcεRIα. Here we demonstrate both 5H5F8 binding to FcεRI⁺ cells as well as a high level of IgE binding to 5H5F8-saturated cells. At the same time 5H5F8 strongly inhibits hexosaminidase release and Ca²⁺ flux after Ag triggering from human IgE-sensitized RBL-2H3 cells stably transfected with human FcεRIα. Further, 5H5F8 and its Fab inhibit sulfidoleukotriene and histamine release from primary human peripheral blood leukocytes, including cells bearing endogenous IgE. Furthermore, we confirm that 5H5F8 maps to a linear peptide sequence in close proximity to the cell membrane. Two chemically synthesized peptides containing the 5H5F8 epitope sequence PREKY were selected for detailed analysis of 5H5F8 and 5H5F8 Fab binding and were found to produce K_d values of similar magnitude to that observed for binding to recombinant FcεRIα. These peptides may prove useful as targets for the identification of antagonists of FcεRIα-mediated biological activity. Moreover, our data indicate that FcεRIα-mediated activation may involve a novel α-chain epitope in an early step of the cell-triggering pathway leading to cellular activation. *The Journal of Immunology*, 2001, 166: 5979–5990.

It has been estimated that at least 20% of the population worldwide is susceptible to atopic disease (1). The allergic response involves the interaction of IgE with the high affinity receptor for IgE (FcεRI)³ expressed on the surface of mast cells and basophils. Allergen cross-linking of FcεRI-bound IgE initiates receptor aggregation and subsequent cellular activation, culminating in the release of both preformed (e.g., histamine) and newly generated (e.g., leukotrienes, PGs) vasoactive and bronchoconstrictive substances that trigger the clinical symptoms of type I immediate hypersensitivity. The molecular basis of the IgE-FcεRI interaction has been the subject of intense scrutiny, and several

strategies aimed at inhibiting this interaction have emerged, including the use of anti-IgE (2) and monoclonal anti-FcεRIα Abs such as 15A5 (3) or 15/1 (4), synthetic peptides (5), and oligonucleotides (6).

FcεRI is a member of the Ig superfamily and binds human IgE with high affinity ($K_d = 10^{-10}$ M) (for review, see Ref. 7). FcεRI is expressed either as a tetrameric ($\alpha\beta\gamma_2$) multisubunit complex in mast cells and basophils or as a trimeric ($\alpha\gamma_2$) complex on monocytes, eosinophils, Langerhans, and dendritic cells (for review, see Ref. 8). The FcεRIα subunit is an integral membrane protein with an extracellular region (ecFcεRIα) comprised of two Ig-like domains (α1, membrane distal; and α2, membrane proximal) that contain the complete high affinity IgE binding site (9). In humans, formation of the IgE-FcεRIα complex involves residues within the α2 domain (10), although high affinity binding is only achieved in receptor molecules containing the contiguous α1 domain (11). Although defined contact residues within ecFcεRIα have been proposed to be involved in IgE binding (12), no therapeutic target has emerged as yet. In contrast, several studies have mapped specific residues in the IgE heavy chain domain Cε3 as crucial in receptor binding (for review, see Ref. 13). The x-ray structure of ecFcεRIα was recently solved (14), and a model was proposed for the IgE binding site, including participation of a prominent loop (designated the FG loop) that projects from the receptor surface. Indeed, this loop has been further implicated in the recently solved structure of the Fcε-ecFcεRIα complex (15). We have shown that the epitope of the inhibitory anti-FcεRIα mAb 15/1 maps to this FG loop, whereby a single amino acid substitution results in loss of binding (16). Additionally, the epitope of another anti-FcεRIα mAb, 5H5F8, which does not compete for the IgE binding site,

*Novartis Forschungsinstitut GmbH, Vienna, Austria; and [†]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037

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² Address correspondence and reprint requests to Dr. Franz Kricek, Novartis Forschungsinstitut, Brunnerstrasse 59, A-1230 Vienna, Austria. E-mail address: franz.kricek@pharma.novartis.com; or Dr. M. W. Robertson, Department of Molecular and Experimental Medicine, MEM-131, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail address: mwr@scripps.edu

³ Abbreviations used in this paper: FcεRI, high affinity receptor for IgE; FcγRIIB, low affinity IgG Fc receptor; RBL-2H3-huα, rat basophilic leukemia cell line stably transfected with the human FcεRI α subunit; pc, polyclonal; DFP, double-fusion protein; ec, extracellular; HSA, human serum albumin; NIP, 4-hydroxy-3-nitrophenylacetyl; SPR, surface plasmon resonance; RU, response units; hPBLs, human PBL; sLT, sulfidoleukotriene; CAST, cellular allergen stimulation test; AP, alkaline phosphatase; MedFI, median fluorescent intensity.

was mapped to a linear peptide stretch residing within the $\alpha 2$ domain in close proximity to the α -chain transmembrane-spanning sequence.

In this study we demonstrate that mAb 5H5F8 inhibits Ag-specific IgE-mediated cellular activation by a mechanism that does not involve inhibition of IgE binding to Fc ϵ RI α . Our results indicate that receptor-mediated cell triggering may involve a discrete and to date unrecognized peptide stretch on the Fc ϵ RI α -chain that spans the 5H5F8 epitope. We further show that 5H5F8 binds with high affinity to a chemically synthesized α -chain peptide corresponding to the epitope. The synthetic receptor peptide may be useful as a screening target for low molecular mass ligands that selectively block mast cell activation.

Materials and Methods

Abs, cell lines, and reagents

Fc ϵ RI α -specific mAb 15/1 (4) was obtained from J.-P. Kinet (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA). The generation and characterization of mouse anti-human Fc ϵ RI α mAbs 6F9G9 and 5H5F8 have been described previously (17). IgG1/ κ isotype control anti-IL8 mAb and the 5H5F8 Fab were obtained from F. Effenberger (Novartis, Vienna, Austria). Human B11 IgE and anti-human IgE mAb Le27 (C ϵ 4 specific) were obtained from B. Stadler (University of Bern, Bern, Switzerland). A Chinese hamster ovary (CHO) cell line stably transfected with the human Fc ϵ RI α -, β -, and γ -chains (CHO $\alpha\beta\gamma$) was provided by J.-P. Kinet and cultured as previously described (18). RBL-2H3/H2/2/C cells were provided by D. Sustarsic (Diagnostic Products, Los Angeles, CA). These cells were subjected to a single round of cell sorting (FACStar^{Plus}; Becton Dickinson, San Jose, CA) to enrich for cells expressing the highest level (brightest 5%) of surface Fc ϵ RI α detected with mAb 15/1. The brightest Fc ϵ RI α cells were expanded in MEM supplemented with Earle's salts, glutamine, penicillin/streptomycin, and 10% heat-inactivated FBS. Polyclonal (pc) anti-Fc ϵ RI α serum raised in rabbits against insect cell-derived Fc ϵ RI α was produced at Novartis. Pc anti-human IgE was purchased from Nordic Immunology (Tilburg, The Netherlands), and pc anti-human IgE antiserum conjugated to HRP was obtained from Sigma (St. Louis, MO). Recombinant Fc ϵ RI α -human serum albumin (HSA)-Fc ϵ RI α (designated DFP for double-fusion protein), consisting of two molecules of the extracellular portion of Fc ϵ RI α (Val¹-Leu¹⁷⁹, numbering scheme according to Ref. 19) fused to the carboxyl and amino terminals, respectively, of HSA was prepared at Novartis (Basel, Switzerland) from culture supernatants of transfected CHO cells and provided by M. Zurini and H. Kocher.

Production and purification of JW8 IgE

Human chimeric IgE (designated JW8, transfectoma JW8/5/13, European Cell Culture Collection (Porton Down, U.K.) ref. no. 87080706) consisting of the mouse variable region (V_H) and a human C_ε constant region (20) and specific for the hapten 4-hydroxy-3-nitrophenylacetyl (NIP), was cultured at high density in a CL 1000 flask (Integra Biosciences, Ijamsville, MD). The secreted Ab was harvested every 3–4 days, following the manufacturer's protocol, and then purified over a NIP-BSA affinity matrix prepared by conjugation of NIP-BSA (synthesized from BSA (fraction V; Sigma) and NIP-succinimide ester (Solid Phase Sciences, San Rafael, CA)) with cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ). Bound IgE was eluted with 0.1 M glycine (pH 2.5)/0.15 M NaCl and immediately neutralized with 2 M Tris (pH 8.0). JW8 IgE produced and purified in this manner showed high affinity binding to immobilized DFP that was nearly identical with that observed for B11 IgE (data not shown).

ELISA

Costar Strip Plate-8 microtiter plates (Costar, Cambridge, MA) were used to immobilize DFP in the amounts indicated for each experiment in Results, in 100 μ l of coating buffer (0.1 M NaHCO₃, pH 9.6) in a humidified chamber at 4°C overnight. The remaining reactive sites were blocked by incubation for 2 h at 25°C with 200 μ l/well of 5% BSA dissolved in coating buffer. JW8 IgE (100 ng/ml) was preincubated for 2 h at 25°C with the indicated amounts of DFP, mAb 15/1, mAb 5H5F8, and anti-IL8 mAb, respectively, in 100 μ l of FCS buffer (PBS (pH 7.5), 2% FCS, and 0.05% Tween 20 (Serva, Heidelberg, Germany)). Plates were washed twice (PBS (pH 7.5) and 0.05% Tween 20), and the preincubation reactions were dispensed and incubated for 2 h at 25°C. Bound IgE was detected by subse-

quent incubation with goat anti-human IgE-HRP (1/1000 dilution in FCS buffer, 90 min, 25°C). After removal of the incubation mixture and washing, color reaction was induced using the substrate 2,2'-azino-di-[3-ethyl-benzthiazoline-6-sulfonic-acid] (Bio-Rad, Hercules, CA). OD values were then measured at 405 nm (Easy Reader; LabInstruments, Vienna, Austria) and corrected for background binding to microtiter plate wells coated with BSA only. The values shown are the mean of duplicate measurements.

Binding of 5H5F8 to B11 IgE-saturated DFP surface

Surface plasmon resonance (SPR) experiments were performed with a Biacore 1000 apparatus (Biacore, Uppsala, Sweden). Using the amine coupling method, 1.25 pmol of DFP diluted in 10 mM sodium acetate buffer (pH 3.7) was immobilized onto a channel of a CM5 certified sensor chip (Biacore AB) according to the previously described procedure (17). This surface was fully saturated with an excess of B11 IgE (slope of response curve, <0.05) followed by injection of the indicated amount of mAb. Re-binding to free Fc ϵ RI α sites upon dissociation of IgE was monitored by control experiments including mAb 15/1, anti-IL8 mAb, and B11-IgE.

Affinity constant determination for binding of 5H5F8, 5H5F8 Fab, B11 IgE, and mAb 15/1 to immobilized DFP by SPR

Using the same DFP-coupled channel as those described above, 5H5F8, 5H5F8 Fab, B11 IgE, and mAb 15/1 were injected separately over a range from 0.833–0.026 μ M. Binding was monitored in real time as the change in response units (RU). Using the Bialogue software (Biacore AB), k_{ass} and k_{diss} were determined, and K_d values were subsequently calculated.

Flow cytometric analysis of 5H5F8/IgE binding to CHO $\alpha\beta\gamma$ cells and a rat basophilic leukemia cell line stably transfected with the α -subunit of Fc ϵ RI (RBL-2H3-hu α)

Staining of 1×10^6 CHO $\alpha\beta\gamma$ cells was performed with 2 μ g of mAb for 120 min at 4°C using 5H5F8, murine IgG1 isotype control (MOPC-21, Sigma), human myeloma IgE (PS, λ light chain, a gift from H. Spiegelberg, University of California, San Diego, CA), or buffer control. RBL-2H3-hu α cells were incubated with 10 μ g of 5H5F8 or MOPC21 for 2 h at 37°C or for 1 h at 4°C. Thereafter all cells were maintained on ice and treated with 1 μ g of biotinylated goat anti-mouse IgG (Sigma) for 1 h. Cells were washed with PBS/0.5% BSA at 4°C and then treated with PE-conjugated streptavidin (0.5 μ g/10⁶ cells; BD PharMingen, San Diego, CA) for 30 min at 4°C. After additional washing, cells were analyzed on a FACSCalibur (Becton Dickinson) using CellQuest software for both data acquisition and analysis. Live cells were gated on forward and side scatter. PE emission was detected in the FL2 channel.

Sulfolipidoleukotriene (sLT) release from human PBLs (hPBLs)

Isolation of hPBLs and sLT quantitation was performed with the cellular allergen stimulation test (CAST)-ELISA (Bühlmann Laboratories, Allschwil, Switzerland) according to the manufacturer's protocol with mAbs and pc sera used at the concentrations indicated in the text for each experiment. Release of sLT from JW8 IgE-sensitized hPBLs was measured in cell supernatants after a NIP-BSA trigger. Within the heterogeneous leukocyte population only the basophils contribute to histamine and sLT release, and it is generally accepted that the amount of basophils per milliliter of blood from a healthy individual is $\sim 4 \times 10^4$ /ml. Briefly, hPBLs were isolated from whole blood (3 ml) after addition of dextran solution (750 μ l) and incubation at 25°C for 90 min. Fourteen hundred microliters of the upper phase was sedimented, and the cell pellet was resuspended in 850 μ l of stimulation buffer. Cell suspension (200 μ l) was added to reaction tubes containing 1.25 μ g of JW8 IgE in 50 μ l of stimulation buffer alone or together with the indicated mAbs, ranging from 0.5–50 μ g/ml, and incubated for 15 h at 4°C with gentle mixing. The cells were then triggered with NIP-BSA (100 ng/ml) for 40 min at 37°C. Secreted sLT was quantitated by competition ELISA using a constant amount of alkaline phosphatase-labeled sLT (sLT-AP) to compete with cellular sLT. Samples were then briefly centrifuged (1000 $\times$ g), and 200- μ l supernatant aliquots were mixed with 200 μ l of a 1/1 mixture of sLT-AP solution and ELISA buffer. Anti-mouse IgG-precoated microtiter plates were incubated with 50 μ l of the mouse anti-sLT mAb (kit component) at 25°C for 2 h. Wells were washed twice with PBS, and 195 μ l of the sLT competition mixture was added and allowed to incubate for 90 min at 25°C. Wells were washed twice with 300 μ l of PBS and developed with substrate for 40 min at 25°C. The OD at 405 nm was recorded, and the amount of secreted sLT was determined according to the CAST-ELISA evaluation protocol.

Inhibition of Ag-specific triggering of JW8 IgE-sensitized hPBLs by 5H5F8

Histamine quantitation was performed with the histamine enzyme immunoassay kit (Immunotech, Marseilles, France) following the manufacturer's protocol. Briefly, human blood was collected and diluted 1/7 with buffer, and 100- μ l aliquots were added to 50 μ l of solution containing either diluted mAbs or buffer-only control and allowed to incubate at 37°C for 30 min. Abs were diluted in histamine release buffer at the concentration indicated in the text. JW8 IgE was then added (1 μ g/ml), and after incubation, cells were triggered by incubation with NIP-BSA (100 ng/ml). One hundred microliters of each cell supernatant was acetylated and subsequently used for histamine determination in a competition assay where secreted histamine competes with a constant and defined amount of a histamine-alkaline phosphatase conjugate for binding to an immobilized anti-histamine mAb. Bound histamine-AP was measured, and quantitation of secreted histamine was performed according to the manufacturer's protocol.

Hexosaminidase release from RBL-2H3-hu α cells

The release of hexosaminidase from sorted RBL-2H3-hu α cells (21) was performed in a manner similar to the published procedure (22). Briefly, adherent cells were treated with dexamethasone (10 μ M final concentration) for 16–24 h to induce human Fc ϵ RI α expression and then were collected by trypsinization. Cells (1×10^6 /ml) were treated with mAb 5H5F8, the isotype-matched control (MOPC-21), or the buffer control for 90 min at 37°C. JW8 IgE was added to a final concentration of 0.2 μ g/ml. Aliquots (350 μ l) were distributed to 24-well tissue culture plates and cultured overnight in a 5% CO₂ incubator. The cells were washed and triggered with NIP-BSA (8–24 ng/ml) for 45 min at 37°C. Secreted and intracellular hexosaminidase levels were measured after incubation with the substrate *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma) for 90 min at 37°C. Upon quenching (pH 10) the OD was read at 405 nm on a Thermomax plate reader (Molecular Devices, Sunnyvale, CA). The percentage of secreted hexosaminidase was calculated as the ratio of secreted to total available hexosaminidase $\times$ 100.

Coincubation of 5H5F8 with JW8 IgE or native IgE (B11) and subsequent trigger by various cross-linking agents

RBL-2H3-hu α cells (2.4×10^4) were seeded in 300 μ l of medium/well of a 96-well plate (Nunc) surface; Nunc, Naperville, IL) and grown at 37°C in 5% CO₂. After 18 h the medium was removed, and 200 μ l of stimulation buffer was added containing 2 μ g/ml IgE (JW8 or native B11) and 20 μ g/ml 5H5F8 or IgE alone and incubated for 50 min at 37°C in 5% CO₂. The medium was then removed, and the bound IgE was cross-linked by incubation with 200 μ l of stimulation buffer containing NIP-BSA at 50 ng/ml, pc anti-human λ -chain (Southern Biotechnology Associates, Birmingham) at 1 μ g/ml, or anti-human IgE mAb Le27 at 2 μ g/ml at 37°C in 5% CO₂ for 40 min. Cell supernatant aliquots (150 μ l) were removed, and the secreted sLT was measured using the components of the CAST-ELISA kit as described above.

Measurement of intracellular Ca²⁺

RBL-2H3-hu α cells were pretreated with either buffer or 5H5F8 for 60 min at 37°C before the addition of human myeloma IgE (PS) or JW8 IgE. After an additional 60-min incubation, medium was added to achieve a cell density of 1×10^6 cells/ml. Fura-2/AM (Molecular Probes, Eugene, OR; 2 μ M final concentration) and sulfinpyrazone (Sigma; 0.25 mM final concentration) were then added, and the cells were incubated for 60 min at 37°C. After three washes in PBS, the cells were resuspended in medium containing 0.25 mM sulfinpyrazone and rotated at 25°C until needed for the experiment. Aliquots were removed and washed, and the cells (1×10^6 cells/ml) were resuspended in HBSS containing 0.25 mM sulfinpyrazone and 0.2% BSA. The cells were placed in a stirred cuvette holder at 37°C, and intracellular Ca²⁺ was monitored using an Aminco-Bowman AB2 spectrofluorometer (SLM Aminco, Rochester, NY) using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Software supplied with the spectrofluorometer was used to calculate the intracellular Ca²⁺ concentration.

Expression, purification, and immunoblot analysis of human Fc ϵ RI α ectodomain fragments

Truncated human Fc ϵ RI α (Ala¹-Ala¹⁷²; numbering according to Ref. 19) was cloned and expressed as previously described (11). The full-length ectodomain was similarly cloned by PCR amplification of the coding sequence (Ala¹-Gln¹⁸⁰) into the same *Escherichia coli* expression construct

encoding an N-terminal leader peptide and both a C-terminal hexahistidine tag adjacent to a c-Myc tag recognized by the mAb 9E10 (CRL-1729, American Type Culture Collection, Manassas, VA). Expression from *E. coli* strain TG1 was accomplished by direct recovery of soluble recombinant Fc ϵ RI α (rFc ϵ RI α) product from the cell periplasm. Briefly, cultures were inoculated from an overnight starter culture in 2YT containing carbenicillin (100 μ g/ml) and glucose (0.1%) and, upon reaching mid-log growth, were induced with isopropyl- β -galactoside (0.25 mM final concentration) and then cultured with shaking for an additional 12–16 h at 20°C. The bacteria were harvested and resuspended in 50 mM Tris, pH 7.5, containing a mixture of protease inhibitors (Complete, Roche, Indianapolis, IN), treated with EDTA and lysozyme (5 mM and 0.5 mg/ml final concentrations, respectively, for 1 h at 4°C) and then centrifuged (44,000 $\times$ g) at 4°C. The supernatant was dialyzed extensively against PBS and rFc ϵ RI α purified over a Ni²⁺-nitrilotriacetic acid resin matrix (Qiagen, Valencia, CA). Purified rFc ϵ RI α was separated by 15% SDS-PAGE and subsequently transferred (SemiPhor, Hoefer, San Francisco, CA) to Immobilon P membranes (Millipore, Bedford, MA). The blot was probed with the anti-Myc tag mAb 9E10 and pc goat anti-mouse IgG-HRP (Sigma) as previously described (11).

Peptide ELISA

Linear Fc ϵ RI α -derived synthetic peptides ESEPLNITVIKAPRE, NITVIKAPREKY, KAPREKYWL, PREKY, and NITVI (negative control peptide) were purchased from piCHEM Research and Development (Graz, Austria). All peptides were > 80% pure and were characterized by mass spectrometry. Peptides were dissolved at 1 mg/ml in coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl (pH 8.5), and NaOH) and stored at –20°C. For coupling, solutions were diluted 1/50 with coupling buffer, and 100 μ l was dispensed to each well of a Labcoat Amine Binding Plate (Costar) and incubated for 12–14 h at 4°C in a humidified chamber. Wells were then washed three times (PBS and 0.05% Tween 20, pH 7.4) and treated with 200 μ l of locking buffer (0.2 M glycine, 1% BSA, and 0.05% Tween 20 in PBS, pH 8.5) for 3 h at 25°C. Plates were washed, and the specified amounts of 5H5F8 or 5H5F8 Fab were added and allowed to incubate for 2 h at 25°C. After washing, 100 μ l/well of a 1/1000 diluted goat anti-mouse IgG-HRP conjugate (Bio-Rad) was dispensed and incubated for 90 min at 25°C. Wells were washed and treated with 100 μ l/well of 2,2'-azino-di-[3-ethyl-benzthiazoline-6-sulfonic-acid] substrate solution as described above. Nonspecific binding was assessed by 5H5F8 and 5H5F8 Fab binding to mock-coupled (peptide-free) wells. OD values measured at 405 nm were corrected for nonspecific binding and represent the mean of duplicate samples.

Affinity measurements for binding of 5H5F8 and 5H5F8 Fab to immobilized peptides by SPR

Peptides KAPREKYWL, PREKY, and NITVI, dissolved in 10 mM sodium acetate buffer (pH 5.5), were immobilized onto a CM5 sensor chip using the amine-coupling procedure cited above. Binding of 5H5F8 and the corresponding Fab was measured within a range of 1.667–0.012 μ M. Regeneration was performed with a pulse of 5 μ l of 100 mM aqueous HCl. Nonspecific binding to the derivatized chip surface was assayed in a reference channel treated with coupling reagents alone.

Inhibition of 5H5F8 and 5H5F8 Fab binding to DFP by synthetic peptides

Twenty-five picomoles of 5H5F8 or 5H5F8 Fab was preincubated in HBS buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.05% BIAcore surfactant P₂₀) with 2500, 250, 25, 2.5, or 0.25 pmol of peptide (KAPREKYWL, PREKY, or NITVI) for 2 h at 25°C. The mixture was subsequently injected over the DFP surface. Maximum binding in the absence of peptide was set at 100%. Inhibition of binding was expressed as a percentage of maximal binding.

Results

Properties of anti-Fc ϵ RI α mAb 5H5F8

We have previously shown that the Fc ϵ RI α -chain epitope recognized by the 5H5F8 mAb is distinct from the IgE binding site and is situated proximal to the transmembrane region, thus lying close to the cell surface (16). In the present study we evaluated the potential of 5H5F8 compared with other Abs to inhibit IgE binding

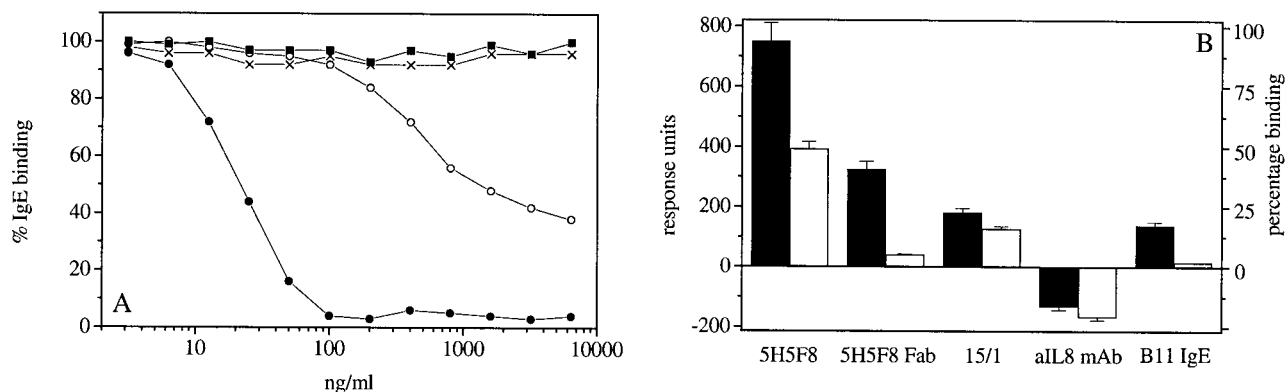


FIGURE 1. A, 5H5F8 does not inhibit IgE binding to FcεRIα (ELISA). JW8 IgE (100 ng/ml) was preincubated with the indicated amounts of DFP (●), mAb 15/1 (○), mAb 5H5F8 (X), and anti-IL8 mAb (■), respectively, for 2 h at 25°C. Mixtures were transferred to DFP-coated wells (50 ng/well), and binding of IgE was detected with pc goat anti-human IgE-HRP. OD values, representing the mean of duplicate assays differing <4%, were converted into percent binding, with 100% defined as the binding of 100 ng IgE. B, Binding of Abs to B11 IgE-saturated DFP surface (SPR). DFP was covalently coupled to the sensor chip and subsequently saturated with B11 IgE (44 pmol in 100 μl), followed by injection of the specified Ab (52 pmol in 30 μl) at a flow rate of 5 μl/min. Maximum binding was recorded in RUs (■). Dissociation of bound Abs was recorded (□) 3 min after initiating buffer only flow of 5 μl/min. The percentage of binding is shown on the right ordinate.

to a recombinant FcεRI α-chain construct (designated DFP) consisting of two FcεRIα molecules (Ala¹-Leu¹⁷⁹) fused to the carboxyl- and amino-terminal ends of HSA, respectively. As summarized in Fig. 1A, 5H5F8 does not interfere with the IgE/FcεRIα interaction, in contrast to the inhibitory mAb 15/1. Preincubation of IgE with DFP as homologous competitor resulted in 100% IgE binding inhibition at a concentration of 100 ng/ml. The anti-IL8 mAb, included as an IgG1/κ isotype control, had no effect on the interaction of IgE with FcεRIα. In a separate ELISA we confirmed that 5H5F8 does not interact with IgE in a nonspecific manner (data not shown).

5H5F8 and IgE bind simultaneously to rFcεRIα

We then investigated whether prebinding of IgE to recombinant FcεRIα (DFP) could modulate binding of 5H5F8 (and the corresponding Fab) to the same receptor molecule. As summarized in Fig. 1B, real-time binding (■) of 5H5F8 to DFP, saturated with B11 IgE, could be readily detected by SPR. As expected, the isotype control mAb (anti-IL8) showed no detectable binding. The marginal binding of inhibitory mAb 15/1 to immobilized DFP saturated with IgE lies within the same range as the rebinding of IgE (B11) to the same surface, indicating that the low amount of rebinding is caused by binding after IgE dissociation from the DFP-coated chip. The 5H5F8 Fab also bound to the receptor/IgE complex, but with an ~50% reduction in RUs compared with that for the whole 5H5F8 Ab. Dissociation of bound protein was also measured (□), with the 5H5F8 Fab displaying a faster dissociation profile than the intact 5H5F8 mAb, suggesting a higher k_{diss} . Using a reverse setup we measured IgE binding to a 5H5F8-saturated DFP surface and again observed significant binding (data not shown). Taken together these results demonstrate that 5H5F8 and IgE can bind simultaneously to immobilized recombinant FcεRIα.

We then assessed whether the mAb 5H5F8 could bind to cell surface FcεRI using CHO cells stably transfected with human FcεRI α-, β-, and γ-chains (18). As shown in Fig. 2A, monophasic 5H5F8 staining was detected on the surface of >99% of the gated cells compared with isotype control, thereby establishing that 5H5F8 can bind to native, membrane-bound FcεRI α-chain. Indeed, a similar level of high density staining was found for both 5H5F8 (median fluorescent intensity (MedFI) = 487) and human IgE (MedFI = 583) staining, as summarized in Fig. 2C. We then investigated the effect of IgE binding to FcεRI⁺ CHO presaturated

with 5H5F8. As shown in Fig. 2B, secondary treatment with IgE had only a nominal effect on the density of bound 5H5F8 (56 MedFI of ~0.2× between A and B). At the same time high density human IgE staining was evident (Fig. 2D), which was ~2-fold reduced from cells stained with IgE only (Fig. 2C). Therefore, we conclude that 5H5F8 and IgE can simultaneously bind to FcεRI⁺ expressed on the cell surface. Additional experiments that used the reverse order of sequential Ab staining also afforded a monophasic

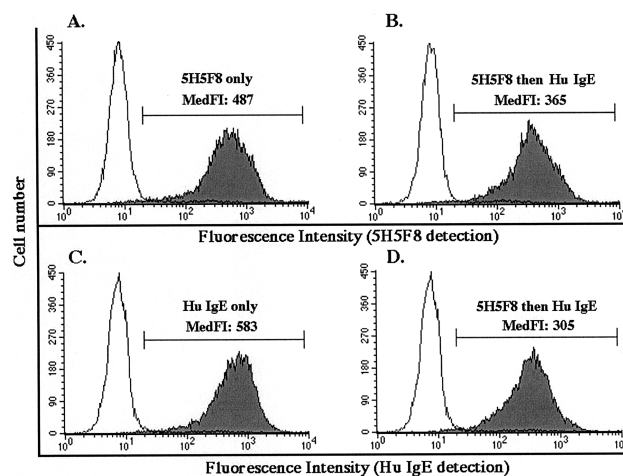


FIGURE 2. Flow cytometric analysis of 5H5F8 and IgE binding to FcεRI⁺-CHO. A, 5H5F8 binding to FcεRI⁺-CHO. Cells were stained with either mAb 5H5F8 (IgG1,κ) or an isotype control mAb (MOPC 21) followed sequentially by goat anti-murine IgG-biotin and then PE-conjugated streptavidin. A marker was set for positive expression based on the isotype control, and the MedFI of the positive-stained cells was calculated using CellQuest software. B, Sequential 5H5F8 and human (Hu) IgE binding to FcεRI⁺-CHO. Cells were first stained with 5H5F8 (or MOPC21) as described above and then with myeloma Hu IgE. Bound 5H5F8 (or MOPC 21) was detected as described in A. C, Hu IgE binding to FcεRI⁺-CHO. Cells were stained with human IgE (myeloma IgE, λ light chain) or buffer control, followed by sequential treatment with biotinylated goat anti-human λ-chain and streptavidin-PE. D, Sequential 5H5F8 and Hu IgE binding to FcεRI⁺-CHO. Cells were first stained with 5H5F8 and then with Hu IgE or buffer control, followed by sequential treatment with biotinylated goat anti-Hu λ chain and streptavidin-PE as described in C. PE fluorescence was acquired on the FL2 channel of a FACSCalibur analyzer.

shift in the doubly labeled cells (90% of gated cells; data not shown), further supporting the conclusion that 5H5F8 and IgE can bind simultaneously to FcεRIα.

K_d determination

We measured the 5H5F8 and 5H5F8 Fab interaction with immobilized DFP (Table I) and confirmed that the Fab binds to the receptor ($K_d = \sim 1 \times 10^{-7}$ M) with ~ 4 -fold lower affinity than intact 5H5F8 mAb ($K_d = 24$ nM). The higher off-rate ($\sim 25\%$) for Fab is consistent with the results shown in Fig. 1B. The measured K_d of 6.5×10^{-9} M for B11 IgE binding to DFP is in good agreement with the well-established high affinity K_d for the IgE-FcεRI interaction (7) and to other SPR measurements between recombinant FcεRIα and IgE constructs (23). The highest affinity for DFP was obtained with mAb 15/1, which was comparable to that with B11 IgE, although the latter showed a significantly faster off rate, consistent with our relative dissociation data summarized in Fig. 1B. Analysis of 5H5F8 binding kinetics to IgE-saturated, immobilized DFP revealed a K_d of $\sim 3.6 \times 10^{-8}$ M, indicating a high affinity interaction between 5H5F8 and IgE-saturated DFP. In addition 5H5F8 dissociation from DFP ($k_{diss} = 1.98 \times 10^{-3}$ s⁻¹; Table I) was comparable to the k_{diss} found for 5H5F8 dissociation from IgE-saturated DFP (2.2×10^{-3} s⁻¹).

Inhibition of IgE-mediated sLT release from hPBLs

As shown in Fig. 3A, coincubation of JW8 IgE with the maximal amount of mAb 5H5F8 tested resulted in a significant reduction ($\sim 50\%$) of sLT release after NIP-BSA trigger. No inhibition was observed using an isotype-matched control anti-IL8 mAb. We also determined that 5H5F8 treatment alone (50 μg/ml) had no direct effect on sLT release. For comparison we tested the inhibitory effect of mAb 15/1, which blocks the binding site for IgE on FcεRIα, and found a concentration-dependent inhibition of sLT release, with maximal inhibition found using ~ 5 μg/ml. Finally, we confirmed in a separate ELISA that NIP-BSA does not bind to 5H5F8, thereby ruling out the possibility that the observed 5H5F8-mediated inhibition is derived from nonspecific inhibition of NIP-BSA binding to the JW8 IgE (data not shown).

Because of the possibility that whole 5H5F8 might simultaneously bind and cross-link FcεRIα (via the Fab portion) and the low affinity IgG Fc receptor (FcγRIIB; via the mAb Fc portion), leading to down-regulation of cellular activation (24), we tested the monovalent 5H5F8 Fab for the capacity to inhibit sLT release. Treatment with the highest concentration of Fab produced approximately a 40% reduction in sLT release, confirming the specificity of this mAb for FcεRIα and arguing against the possibility of 5H5F8-mediated activity derived from FcεRI-FcγRIIB ligation.

Inhibition of IgE-mediated histamine release from hPBLs

We also tested the capacity of 5H5F8 to inhibit histamine release from primary hPBLs. We incubated hPBLs first with 5H5F8 and then added JW8 IgE and subsequently cross-linked the bound anti-NIP IgE with NIP-BSA to induce histamine release (Fig. 3B). 5H5F8 inhibited Ag triggering at 10- and 100-fold molar excesses

(mAb:IgE) compared with the IgG1/k anti-IL8 control mAb as well as the 5H5F8 Fab. The hPBLs used in this assay were also shown to be at least partially loaded with endogenous IgE as seen by the anti-IgE (Le27)-induced histamine release response. It can be further seen that the inhibitory mAb 15/1, with an affinity for FcεRIα comparable to that of IgE, blocks histamine release when used in a 1/1 molar ratio. It should be noted that the relatively larger quantity of 5H5F8 required to inhibit histamine release compared with mAb 15/1 parallels the measured difference in affinities of 5H5F8 and IgE for FcεRIα (see Table I).

Inhibition of IgE-mediated hexosaminidase and sLT release from human FcεRIα-transfected RBL-2H3 cells

To extend the foregoing observations, we tested whether 5H5F8 could inhibit mediator secretion from a rat mast cell line expressing human FcεRIα. To monitor cellular activation, we measured the extent of degranulation of the preformed mediator hexosaminidase from RBL-2H3-huα cells. The cells used in this study were first enriched for a population expressing the highest density of human FcεRIα on the cell surface, as determined by flow cytometry, and exhibited 50–60% net hexosaminidase release upon NIP-BSA triggering of cells sensitized with a saturating quantity (2 μg/ml) of JW8 IgE (data not shown). Interestingly, the process of sorting for a cell population expressing the highest level of FcεRIα produced cells with a markedly higher maximum release phenotype than the original cells (21).

We then tested the capacity of 5H5F8 to modulate degranulation of RBL-2H3-huα cells preincubated for 2 h at 37°C with 5H5F8 before addition of subsaturating JW8 IgE (0.2 μg/ml) and continued (12–14 h) culture. The adherent cells were tested for mediator release upon challenge with an optimal amount of NIP-BSA. Under these conditions the maximum release of hexosaminidase (Fig. 4A) measured in the absence of 5H5F8 was in the range of 25% of the total cellular hexosaminidase, with $< 3\%$ spontaneous secretion. Addition of 5H5F8 produced a marked inhibition ($> 90\%$) in release compared with an isotype-matched control, which had no effect. We further appraised the inhibitory effect of 5H5F8 by titrating the mAb over a 20-fold concentration range. As shown in Fig. 4B, nearly complete inhibition was reached at the highest concentration tested (10 μg/ml), with a steady decline in inhibitory activity at lower 5H5F8 concentrations. Another anti-FcεRIα mAb that does not compete for the IgE binding site, 6F9/G9 (17), was also tested and was found to be completely ineffective in inhibiting hexosaminidase release, although it was capable of binding to CHOαβγ cells (data not shown). To rule out the possibility that 5H5F8-dependent reduction in mediator release was not simply a result of direct 5H5F8 stimulation of RBL-2H3-huα cells leading to depletion of hexosaminidase stores, we tested the capacity of 5H5F8 to induce mediator secretion over a wide range of mAb concentrations. In different assays we could not detect hexosaminidase or sLT release, indicating that 5H5F8 does not have an anaphylactogenic effect on these cells (data not shown).

We further analyzed the capacity of 5H5F8 to inhibit sLT release from these cells. As summarized in Fig. 4C, cells coincubated with 2 μg/ml JW8 IgE and 5H5F8 (20 μg/ml) for 50 min at 37°C and subsequently triggered with either NIP-BSA or anti-IgE mAb Le27 produced essentially complete inhibition of sLT release compared with controls. In a similar experiment cells were treated with native human IgE (B11) and 5H5F8 as before and cross-linked with either a pc anti-human λ-chain Ab or Le27. Complete inhibition of anti-human λ-chain Ab-triggered sLT release was found, whereas anti-IgE-triggered release was $\sim 80\%$ inhibited using the same quantity of 5H5F8. Similar results have been

Table I. Antibody binding constants for immobilized α-HSA-α (DFP)

	$k_{ass} (\times 10^4)$ (M ⁻¹ s ⁻¹)	$k_{diss} (\times 10^{-4})$ (s ⁻¹)	K_D (nM)
5H5F8	8.3	19.77	23.8
Fab 5H5F8	2.6	25.28	97
15/1	4.7	1.84	3.9
B11 IgE	8.8	5.70	6.5

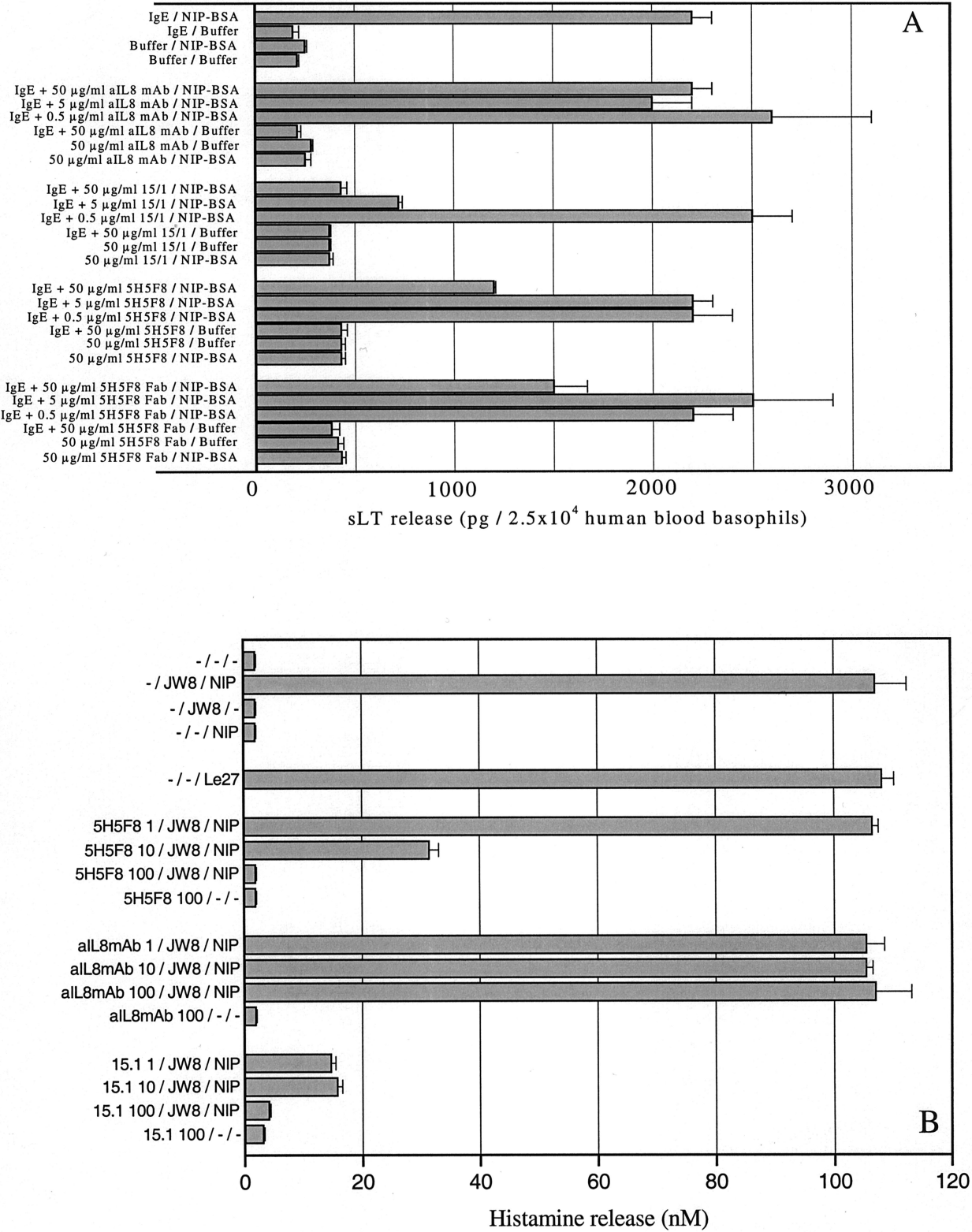


FIGURE 3. A, Inhibition of an NIP-BSA-specific trigger of JW8 IgE sensitized hPBLs. Cells were incubated with JW8 IgE (5 µg/ml) alone, with IgE plus the indicated amount of mAbs, or with only the highest mAb concentration (50 µg/ml) and then triggered with NIP-BSA (100 ng/ml) and assayed for secreted sLT by CAST-ELISA as described in *Materials and Methods*. Pg sLT release represents the mean of duplicate assays of sLT release from 100-µl cell aliquots. B, Sequential incubation of hPBLs with 5H5F8 followed by JW8 IgE and subsequent NIP-BSA treatment results in trigger inhibition. Histamine ELISA was performed as described in *Materials and Methods* with mAbs used at the indicated molar ratio compared with a constant amount of JW8 IgE (1 µg/ml). As a stimulation control a mouse anti-FcεRIα mAb was used to cross-link surface FcεRIα. NIP-BSA (abbreviated NIP)-specific trigger (100 ng/ml) of JW8 IgE (1 µg/ml) sensitized cells is shown. As a negative control the amount of nonspecific histamine release using release buffer only was measured. Nonspecific cell activation was determined by incubation of the cells with each Ab alone at its highest concentration. As an IgG1/κ isotype control an anti-IL8 mAb was used, while the positive control mAb used to establish maximal inhibition was mAb 15/1. Histamine release is shown in nanomolar concentrations and represents the mean of duplicate assays.

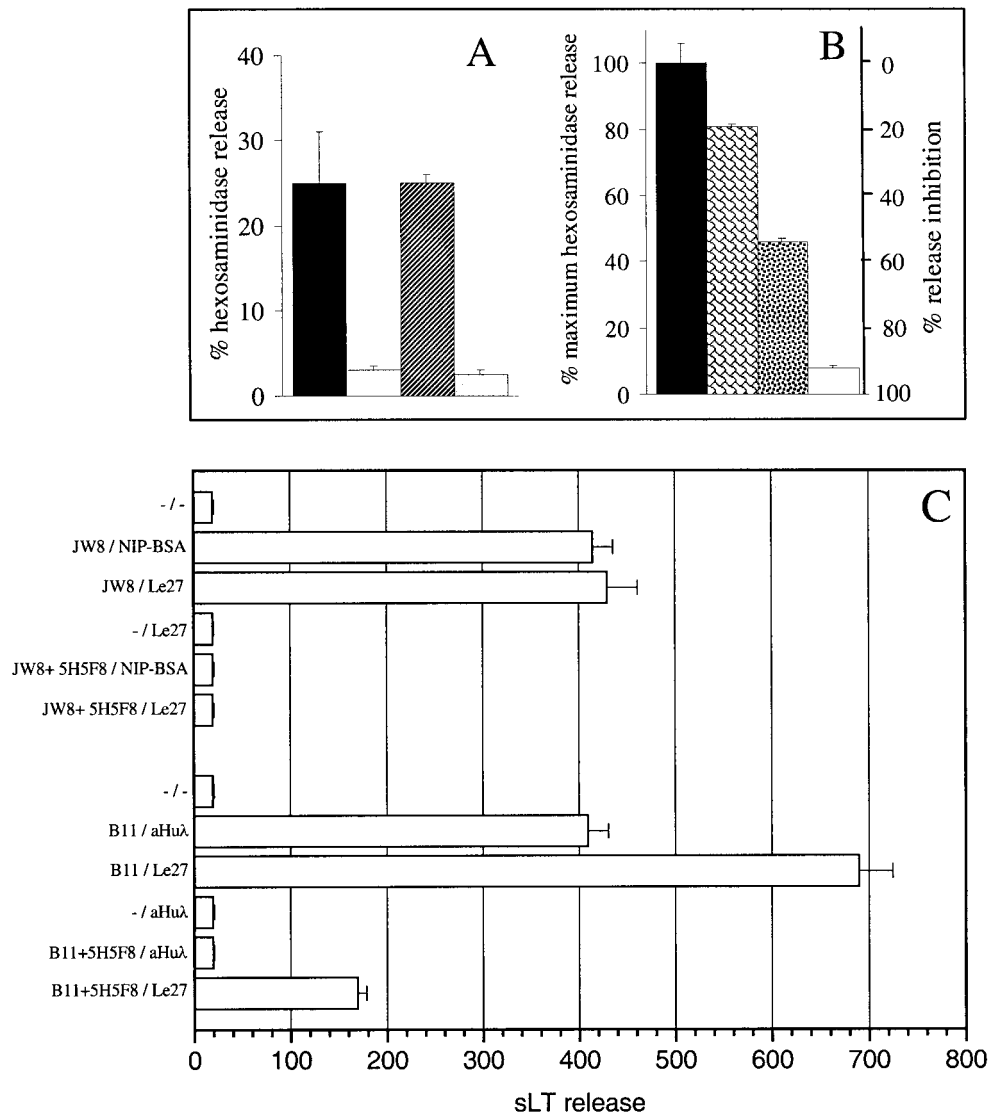


FIGURE 4. A, Inhibition of mediator release from RBL-2H3-hu α cells with mAb 5H5F8. RBL-2H3 cells, stably transfected with a human Fc ϵ RI α expression plasmid, were cocultured with 0.2 μ g/ml JW8 IgE alone (■), with with IgE plus 10 μ g/ml 5H5F8 (□), or with IgE plus 10 μ g/ml of murine monoclonal IgG1 (MOPC-21, ▨) and subsequently triggered to release hexosaminidase with NIP-BSA (8 ng/ml). The amount of released enzyme was measured as described in *Materials and Methods*. The results shown are the average of triplicate assays. Spontaneous release (▤) was measured using a buffer (0.5% BSA/HBSS) control. B, Inhibition of hexosaminidase release by 5H5F8 from RBL-2H3hu α cells cocultured with JW8 IgE (0.2 μ g/ml) and triggered with NIP-BSA (8 ng/ml): titration of 5H5F8 concentration. The maximum release of hexosaminidase from cells cultured in the absence of 5H5F8 is shown (■) and, after subtraction of spontaneous (background) release, is normalized to 100%. The magnitude of mediator release, minus spontaneous release, over a range of 5H5F8 concentrations ((10 μ g/ml (□); 2 μ g/ml (▨); 0.5 μ g/ml (▤)) was expressed as either a percentage of the maximum release (left ordinate) or a percentage of maximal release inhibition (right ordinate). C, Coincubation of 5H5F8 with JW8 IgE or native IgE (B11) with RBL-2H3-hu α cells and subsequent trigger with the indicated cross-linking agents. RBL-2H3-hu α cells were incubated together with IgE (2 μ g/ml) and 5H5F8 (20 μ g/ml) for 50 min at 37°C, and bound IgE were then cross-linked through addition of NIP-BSA, anti-human IgE mAb Le27, or anti-human λ -chain Ab (abbreviated aHu λ). The amount of released sLT was determined with the CAST-ELISA as described above.

obtained with hPBLs and pc anti-human IgE as cross-linking agent (data not shown).

Measurement of intracellular Ca^{2+}

As a first step in determining whether 5H5F8 can inhibit signaling leading to degranulation, RBL-2H3-hu α cells were activated in the presence or the absence of 5H5F8, and changes in intracellular Ca^{2+} were monitored. Cross-linking of Fc ϵ RI is well known to cause an increase in intracellular Ca^{2+} , which is required for degranulation (reviewed in Ref. 25), including RBL cells stably transfected with the human Fc ϵ RI α -chain (26). Cells were sensi-

tized with either myeloma IgE or NIP-specific IgE. When activated with either anti-IgE (Fig. 5A) or NIP-BSA (Fig. 5B), respectively, RBL-2H3-hu α cells had a typical response, characterized by a short delay, an initial rise due to the release of Ca^{2+} from intracellular stores, and a sustained plateau phase due to Ca^{2+} influx across the plasma membrane (27), although the maximal Ca^{2+} response varied by about 50% between the two assays. A significant reduction in the Ca^{2+} response was seen if the cells were preincubated with 5H5F8 before sensitization with human IgE. In both cases (Fig. 5, A and B) preincubation with 5H5F8 lead to an inhibition of $\sim 60\%$ of the Ca^{2+} flux. These data indicate that

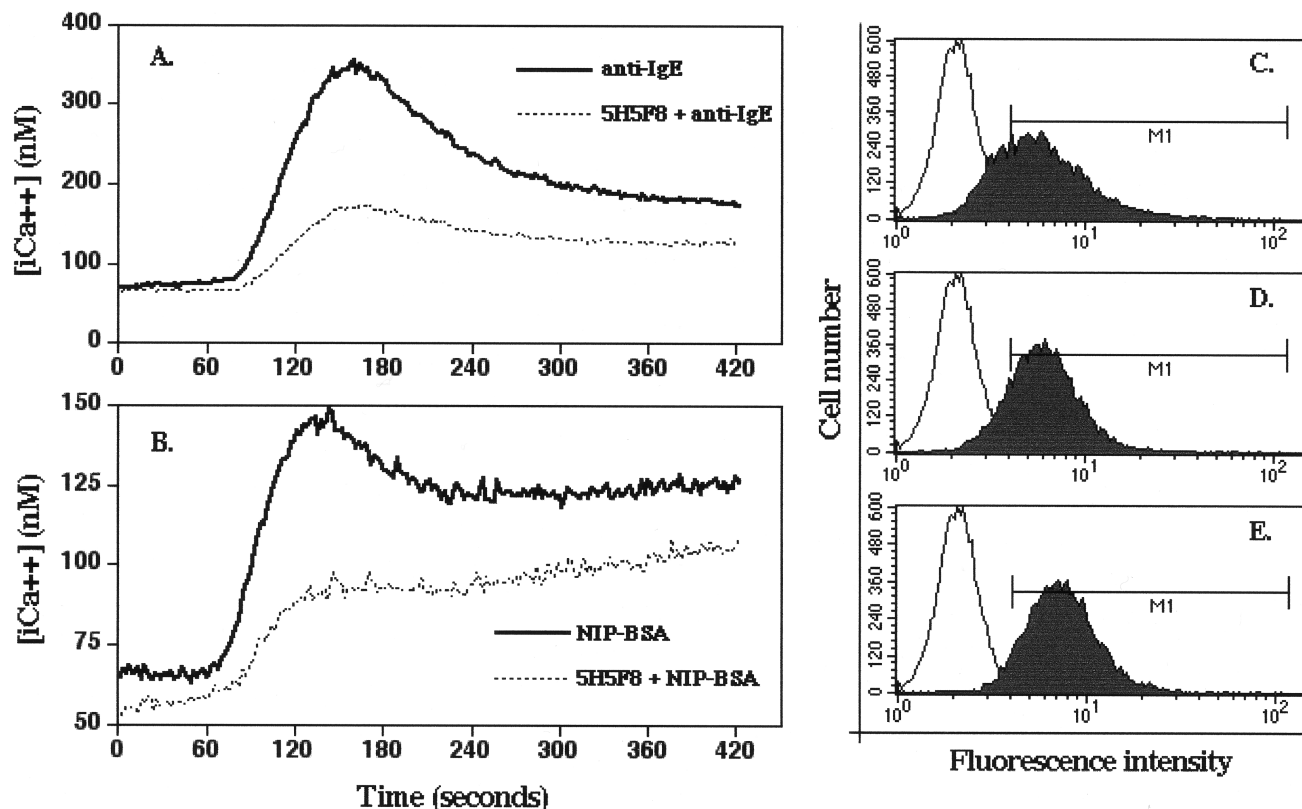


FIGURE 5. Ca^{2+} mobilization assay and flow cytometric analysis of RBL-2H3-hu α cells treated with and without mAb 5H5F8. **A**, Measurement of intracellular Ca^{2+} in human myeloma IgE (PS)-sensitized cells treated with 5H5F8 (dashed line) or without 5H5F8 (dark line) and subsequently triggered with a pc anti-IgE at the 60 s point. Intracellular fura-2 fluorescence was recorded over time. **B**, Measurement of intracellular Ca^{2+} in JW8 IgE-sensitized cells treated with 5H5F8 (dashed line) or without 5H5F8 (dark line) and subsequently triggered with NIP-BSA at 60 s. Intracellular fura-2 fluorescence was recorded over time. **C**, RBL-2H3-hu α cells were incubated for 2 h at 37°C in complete medium without mAb and then stained for constitutive surface FcεRI α expression with mAb 15/1 (1 h 4°C) followed by goat anti-murine IgG-biotin and PE-conjugated streptavidin (shaded histogram). Subsequent analysis proceeded as described in Fig. 2 with the positive staining population (M1) set against negative control cell staining using secondary reagents only (open histogram). **D**, RBL-2H3-hu α were incubated for 2 h at 37°C in complete medium without mAb and then stained for constitutive surface FcεRI α expression with mAb 5H5F8 (1 h, 4°C). Subsequent staining steps and analysis proceeded as described in C. **E**, RBL-2H3-hu α cells were incubated in complete medium containing 5H5F8 (10 μ g/ml) for 2 h at 37°C and then immediately chilled on ice and washed. The amount of cell-bound 5H5F8 was determined by sequential staining using the same secondary reagents and analysis as described above.

5H5F8 inhibits degranulation by significantly reducing the transmembrane signaling involved in the degranulation response.

We then considered the possibility that the 5H5F8-mediated inhibition described above might be derived from intrinsic 5H5F8-mediated receptor internalization leading to a pronounced reduction in FcεRI α surface expression by flow cytometry in RBL-2H3-hu α cells treated with or without 5H5F8 (10 μ g/ml) for 2 h at 37°C. For cells treated without 5H5F8, the level of constitutive human FcεRI α cell surface expression was assayed by standard FACS staining using the anti-FcεRI α mAb 15/1 followed by an anti-mIgG1 mAb as a secondary reagent (Fig. 5C, shaded histogram) compared with negative control staining (open histogram). The level of constitutive FcεRI α expression was also assessed by staining at 4°C with mAb 5H5F8 and the same secondary reagents used in Fig. 5C and found to be very similar (Fig. 5D; M1 = 88%) to the level of expression shown in Fig. 5C (M1 = 77%). For cells treated with 5H5F8 at 37°C the amount of receptor-bound mAb was detected by FACS staining using the same secondary reagents used in Fig. 5, C and D. A comparable pattern of FcεRI α surface expression was seen between the 5H5F8-treated cells (Fig. 5E, M1 = 95%) and the 5H5F8-untreated cells (Fig. 5, C or D), indicating that 5H5F8 did not induce internalization. In separate FACS experiments we confirmed that 5H5F8 does not induce FcεRI α internal-

ization in the presence or the absence of IgE (data not shown) indicating that under these conditions the level of FcεRI α detected on the cell surface is also not affected by 5H5F8.

Epitope characterization

Additional characterization of the membrane-proximal FcεRI α -chain epitope was performed. Two α -chain ectodomain constructs were expressed as previously described (11) in secreted form from *E. coli*: 1) the full-length ectodomain consisting of residues Ala¹-Gln¹⁸⁰ (numbering according to Ref. 19), and 2) a truncation variant (Ala¹-Glu¹⁷²) lacking the eight C-terminal residues P¹⁷³REKYWLQ¹⁸⁰. Both fragments bound IgE equally well as determined by ELISA (data not shown). Immunoblot analysis (Fig. 6A) of each recombinant product showed binding of the anti-Myc tag mAb 9E10 (lanes 1 and 2). As expected, the full-length product migrated with a discernibly higher apparent molecular mass. Probing the same blot with the 5H5F8 mAb showed strong binding to the full-length product (lane 4) and no detectable signal for the truncated product (lane 3), supporting the assignment of the 5H5F8 epitope to the membrane-proximal region of the α -chain ectodomain (16).

To extend the 5H5F8 epitope mapping, various linear peptides were chemically synthesized and tested for 5H5F8 (Fig. 6B) and 5H5F8 Fab (Fig. 6C) binding in an ELISA. The highest 5H5F8

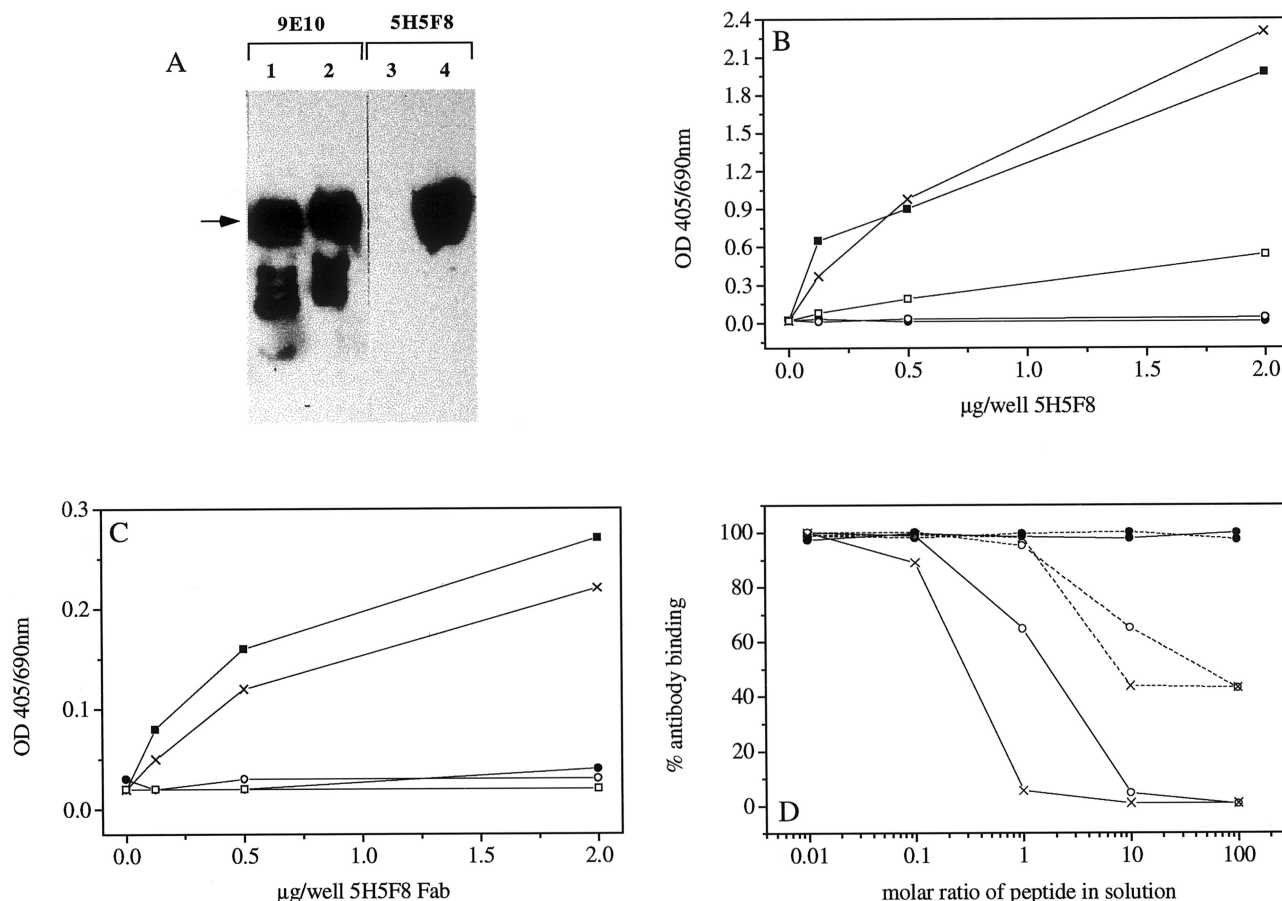


FIGURE 6. Characterization of the 5H5F8 epitope. *A*, Two Myc epitope-tagged rFcεRI α-chains consisting of either a full-length ectodomain sequence (lanes 2 and 4) or a slightly shorter form lacking eight residues from the ectodomain C terminus (lanes 1 and 3) were expressed in *E. coli*, fractionated by SDS-PAGE, and immunoblotted using either the Myc tag mAb 9E10 (lanes 1 and 2) or the 5H5F8 mAb (lanes 3 and 4). The arrow indicates the position of the recombinant ectodomain products. *B*, Binding of 5H5F8 to synthetic peptides. Peptides NITVI (●), ESEPLNITVIKAPRE (○), NITVIKAPREKY (X), KAPREKYWL (■), and PREKY (□) were immobilized to Amine Bind plates at 2 μg/well. After treatment with the indicated amounts of Abs the second step reagent (goat anti-mouse IgG-HRP) was added. OD values were corrected by subtraction of background binding to wells without peptide and represent the mean of duplicate determinations. *C*, Binding of 5H5F8 Fab to synthetic peptides. Peptides NITVI (●), ESEPLNITVIKAPRE (○), NITVIKAPREKY (X), KAPREKYWL (■), and PREKY (□) were immobilized to Amine Bind plates and treated as described in *B*. *D*, Inhibition of 5H5F8 (dashed line) and 5H5F8 Fab (solid line) binding to a DFP-coated surface by the indicated molar excess of synthetic peptides. Peptides NITVI (●), PREKY (○), and KAPREKYWL (X) were used at the indicated molar excess. Twenty-five picomoles of 5H5F8 or 5H5F8 Fab was preincubated for 2 h with various amounts of the indicated peptides and then assayed for 5H5F8 or 5H5F8 Fab real-time binding to a DFP-coupled sensor chip using SPR. The measured relative RUs were converted to percentage of Ab binding, with 100% binding set for samples containing 5H5F8 or corresponding Fab without added peptide.

binding was found for the immobilized peptides NITVIKAPREKY and KAPREKYWL. The pentapeptide PREKY, with sequence common to each of the former peptides, bound to a 6- to 7-fold lower level than the longer peptides, but 5- to 6-fold higher than the background. At the same time no binding was observed to two other peptides (NITVI and ESEPLNITVIKAPRE). Binding of the Fab to the peptides gave a similar pattern, although no binding to the PREKY peptide could be detected in this assay. In general, the ELISA readout with the Fab was ~8-fold lower than that for the intact 5H5F8 mAb. Enhanced binding to the KAPREKYWL and NITVIKAPREKY peptides relative to PREKY suggests that 5H5F8 binds to the PREKY peptide with reduced affinity, and that the full 5H5F8 epitope may include an additional residue(s) at the C- or N-terminal of this sequence. SPR analysis was used to demonstrate that peptides PREKY and KAPREKYWL could inhibit 5H5F8 and 5H5F8 Fab binding to immobilized FcεRIα (DFP). Both peptides inhibited (~60%) 5H5F8 binding to DFP using a 100-fold molar excess of peptide, whereas no inhibition was observed with the NITVI peptide (Fig. 6D). The KAPREKYWL pep-

tide inhibited binding to the same extent at a 10-fold lower molar excess, suggesting that this degree of inhibition represents the maximum possible for this peptide. 5H5F8 Fab binding to DFP was totally inhibited by both peptides when used in a 10-fold molar excess, while the KAPREKYWL peptide exerted complete inhibition at a 1/1 molar ratio. The control peptide NITVI did not inhibit binding over the entire concentration range assayed.

Affinity determination of 5H5F8 for epitope-containing peptides

The kinetic parameters for binding of the synthetic peptides KAPREKYWL and PREKY, immobilized on a biosensor chip, to either 5H5F8 or the 5H5F8 Fab are summarized in Table II. Strikingly, affinities for binding of 5H5F8 and 5H5F8 Fab to KAPREKYWL (Table II) were almost the same as 5H5F8 and 5H5F8 Fab binding to DFP (Table I). This result demonstrates that the immobilized KAPREKYWL peptide is as functional in mAb binding as rFcεRIα, providing additional evidence that 5H5F8 recognizes a linear epitope. Binding to immobilized PREKY peptide could only be demonstrated for the whole 5H5F8 with a measured

Table II. Binding to immobilized KAPREKYWL peptide

	$k_{\text{ass}} (\times 10^4)$ ($\text{M}^{-1}\text{s}^{-1}$)	$k_{\text{diss}} (\times 10^{-4})$ (s^{-1})	K_D (nM)
5H5F8	19	20.57	10.8
Fab 5H5F8	1.36	12.58	93.0

K_d value of about 71 nM (Table III), again in good agreement with the measured values of Fab binding to DFP (Table I). Together the ELISA and SPR experiments define a minimal 5H5F8 epitope (PREKY), but suggest that a slightly extended epitope is probably required to achieve a structure with maximal receptor binding affinity.

Discussion

In the present study we demonstrate that mAb 5H5F8, which does not block the interaction between IgE and FcεRIα, is able to inhibit Ag-induced mediator release from IgE-sensitized cells. Having previously shown that the 5H5F8 epitope lies in close proximity to the cell membrane (16), we first investigated the association of FcεRIα with 5H5F8 and the effect of IgE on this association. Initial experiments showed that 5H5F8 does not inhibit IgE binding to immobilized, recombinant FcεRI α-chain using an ELISA competition assay. By analysis of real-time binding of 5H5F8 to recombinant FcεRIα presaturated with human IgE we showed distinct receptor binding sites for 5H5F8 and IgE. Simultaneous binding of IgE and 5H5F8 to FcεRIα indicates that the 5H5F8 epitope is sterically separated from the IgE binding site within FcεRIα. To assess whether 5H5F8 is able to bind to the native epitope expressed on FcεRI⁺ cells we used CHO cells constitutively expressing human FcεRI. These cells were considered an ideal choice for FACS binding analysis because they express much higher amounts of surface FcεRI compared with other cells (RBL-2H3-huα and hPBLs) used in this study. The finding (Fig. 2) that 5H5F8 stains these cells to about the same extent as IgE (judged by similar MedFIs) was at first surprising in view of the spatial proximity of the 5H5F8 epitope to the plasma membrane. Further, our results indicate that cells sequentially labeled with saturating 5H5F8 followed by IgE produce a staining pattern similar to that of cells labeled with the individual Abs. This indicates that the two-Ab labeling proceeds efficiently and that subsequent IgE binding does not significantly displace prebound 5H5F8. An additional two-color FACS analysis was performed that revealed a monophasic population of double-positive cells (data not shown), supporting our conclusion that these cells simultaneously stain with 5H5F8 and human IgE. Thus the native 5H5F8 and IgE binding epitopes are distinctly accessible for binding by both Abs. Our FACS data are also in good agreement with SPR K_d determinations that showed high affinity 5H5F8 binding (3.6×10^{-8} M) to an IgE-saturated recombinant FcεRIα molecule.

The most surprising finding of this study was that 5H5F8 attenuates FcεRI-mediated cellular activation. We observed 5H5F8-dependent inhibition of mediator release after multivalent hapten challenge of anti-NIP (JW8) IgE-sensitized cells in two different

experimental setups using different cell types. The biological relevance and therapeutic potential of the inhibitory activity of 5H5F8 was tested in a human ex vivo model experiment of the basophil response in atopic disorders. The setup includes the use of an Ag-specific IgE to mimic allergen-specific endogenous IgE. Our results with 5H5F8 show that addition of a mixture of exogenous IgE (JW8) plus 5H5F8 to hPBLs significantly inhibits de novo mediator synthesis (Fig. 3A). Both whole 5H5F8 and the corresponding Fab inhibited sLT release when used in a 10-fold molar excess, while mAb 15/1, which blocks IgE binding to receptor, inhibited at a 1/1 molar ratio. The higher quantities of 5H5F8 and Fab required to inhibit cellular activation are consistent with the lower receptor affinities measured for 5H5F8 binding to a recombinant FcεRIα molecule (Table I) compared with a nearly 10-fold enhanced affinity of mAb 15/1 for the same receptor construct.

We have also shown that 5H5F8 inhibits preformed mediator (histamine) release from primary hPBLs that are at least partially loaded with endogenous IgE (Fig. 3B). Partial loading with endogenous IgE was shown by direct anti-IgE (Le27) activation of these cells. In addition these cells could be equally activated by multivalent Ag (NIP-BSA) following sensitization with exogenous JW8 anti-NIP IgE. Consistent with 5H5F8-mediated inhibition of sLT release, an ~10-fold molar excess of 5H5F8 was required for complete inhibition of Ag-triggered histamine release from these cells. The main significance of these results is that 5H5F8 can inhibit cellular activation even when added to cells preloaded with endogenous IgE.

A similar pattern of 5H5F8 inhibition was found using the RBL-2H3-huα cell line, which has been previously shown to elicit the same series of human FcεRI-mediated biological responses (21). In our experiments incubation of RBL-2H3-huα cells with 5H5F8 and JW8 IgE strongly inhibited hexosaminidase (Fig. 4, A and B) and sLT release (Fig. 4C) after NIP-BSA triggering. In addition, sLT release was inhibited in cells cross-linked with anti-IgE mAb (Le27). In a parallel experiment using native IgE (B11), sLT release triggered by a pc anti-human light chain Ab was completely inhibited by coincubation with 5H5F8 and was ~80% inhibited when triggered with Le27. Thus, inhibition of the activation of these cells by 5H5F8 was not restricted to either the source of IgE or, of greater significance, to whether receptor-bound IgE was cross-linked by polyvalent hapten, anti-IgE mAb, or pc anti-light chain Ab. Regarding the observed differences (Figs. 3 and 4C) in the degree of inhibition, we argue that they are system inherent for the following reasons: 1) the histamine assay measures the release of this mediator from cellular stores, whereas the sLT assay measures the extent of newly synthesized released upon cellular activation; and 2) the experimental setup of the two assays is generally similar, but with the notable difference that the anti-sLT mAb (used for sLT capture) is presented via pc anti-mouse IgG, whereas the anti-histamine mAb is free in solution. Additionally, the released histamine has to be acetylated to be suitable for the assay. Besides these technical differences, the human FcεRI density on the RBL-2H3-huα cells is lower compared with that of the human basophils.

Because both degranulation and eicosanoid production require an increase in intracellular calcium (25), we investigated the effect of 5H5F8 on Ca^{2+} mobilization in RBL-2H3-huα cells. Significant reduction of the Ca^{2+} response was observed after incubation of 5H5F8-sensitized cells with IgE and subsequent cross-linking. These data indicate that downstream signaling is significantly affected by binding of 5H5F8 to FcεRI. We are presently analyzing the effect of 5H5F8 on other components of the FcεRI signaling

Table III. Binding to immobilized PREKY peptide

	$k_{\text{ass}} (\times 10^4)$ ($\text{M}^{-1}\text{s}^{-1}$)	$k_{\text{diss}} (\times 10^{-4})$ (s^{-1})	K_D (nM)
5H5F8	1.58	11.3	71
Fab 5H5F8	ND	ND	ND

^a ND, Not detected.

cascade, including FcεRI subunit tyrosine phosphorylation, to further elucidate the mechanism of 5H5F8 action.

Although the underlying mechanism of 5H5F8 activity is still unresolved and is the subject of continuing investigation, several mechanistic possibilities have been considered for 5H5F8-mediated inhibition of cell activation. They include involvement of inhibitory receptors (28), modulation of receptor mobility (29), FcεRI internalization, and a possible role of the FcεRIα membrane-proximal region in the early stages of FcεRI clustering. Inhibition of Ag-induced cellular activation of hPBLs by 5H5F8 could be caused by cross-linking of FcεRI and FcγRIIB (24) via binding of the Fc portion of FcεRIα-bound 5H5F8 to FcγRIIB. FcγRIIB is known to be expressed on primary human basophils and mast cells (30) and contains an immunoreceptor tyrosine-based inhibition motif that acts to down-regulate cellular activation (24). Our data suggest that inhibition by 5H5F8 is not mediated through FcγRIIB, because we also observed inhibition with the monovalent 5H5F8 Fab, albeit to a lesser extent. Furthermore, human FcγRII can only bind oligomeric or complexed IgG and binds murine isotypes such as 5H5F8 (IgG1,κ) with lower affinity than IgG2a and IgG2b (31). Finally, 5H5F8 was also demonstrated to act on RBL-2H3-huα cells, which are not known to express FcγRIIB.

An alternative mechanistic possibility is that the binding of 5H5F8 to FcεRIα might cause an intrinsic decrease in receptor mobility, resulting in reduced receptor cross-linking/clustering. However, in preliminary experiments we have found that incubation of cells loaded with 5H5F8 with an anti-mouse κ-chain Ab resulted in cellular activation measured by sLT release (data not shown). An additional mechanistic possibility is that 5H5F8 binding, with or without simultaneous IgE binding, might lead to intrinsic loss of receptor cell surface expression. Here we show that FcεRIα surface expression in RBL-2H3-huα cells treated with 5H5F8 for 2 h at 37°C was comparable to constitutive FcεRIα expression detected in these cells in the absence of 5H5F8 treatment (Fig. 5E compared with Fig. 5, C or D). We also determined by FACS analysis that the amount of receptor-bound 5H5F8 in cells incubated with or without IgE at 37°C was unchanged (data not shown), and thus we conclude that the FcεRI-5H5F8 complex does not appear to be intrinsically susceptible to mechanisms, such as internalization, that lead to reduced receptor density on the cell surface.

5H5F8 could also act by inhibiting the interaction between FcεRIα and a hypothetical component of the plasma membrane required to initiate or propagate the signaling pathway. It is known that receptor aggregation results in an increased association of FcεRI with membrane skeletal proteins (32), and it has been suggested that lipid-mediated interactions between FcεRI and the plasma membrane occur before tyrosine phosphorylation (33). Evidence is accumulating that initiation of FcεRI signaling depends on how FcεRI and tyrosine kinase Lyn interact with cholesterol- and sphingolipid-rich regions within the plasma membrane (34). This interaction is not mediated by the FcεRI β-subunit or the cytoplasmic tail of the γ-subunit (35), but does appear to be regulated by the actin cytoskeleton (36). It is intriguing to speculate that the 5H5F8 membrane-proximal epitope functions as a determinant for interaction with other cell surface molecules and/or membrane components and that inhibition of this association by 5H5F8 leads to attenuation of downstream cellular activation. This hypothesis closely parallels the recent finding that the membrane-proximal region of the α₃β₁ integrin complex α-chain contains an epitope necessary for integrin-mediated activities, such as cell motility, via binding to the transmembrane 4 superfamily protein member CD151 (37, 38). In this context we speculate that

the 5H5F8 epitope may interact with specific membrane components, and efforts are now underway to identify such a putative molecule with particular emphasis on the transmembrane 4 superfamily member CD81, which has been recently shown to participate in down-regulation of mast cell activation when treated with the anti-CD81 mAb 5D1 (39). The possible involvement of the 5H5F8 epitope in molecular recognition prompted us to search the Swissprot database for the PREKY sequence using the fasta utility of the GCG program. Only two matches were found (a hypothetical yeast transmembrane protein and the constant region 3 of human and gorilla IgA1), suggesting a sequence-specific function for this epitope. At the same time it remains an open possibility that the PREKY sequence may exist in novel cell surface proteins and possibly in a form that may be involved in cellular activation.

The sequence of the 5H5F8 epitope is not encoded by the two exons that encode the FcεRIα extracellular Ig-like domains (40), which together comprise the complete ligand recognition site. Instead, the 5H5F8 epitope is encoded within the exon that encodes the FcεRIα transmembrane-spanning domain. This information may provide a further clue to the importance of the membrane environment for FcεRIα-mediated signaling. Aspects of the biological importance of the membrane-proximal region of FcεRIα have been recently described. Kershaw et al. (41) expressed various single-chain chimeric FcεRIα on the surface of COS-7 cells and assayed for IgE binding. Their results suggest that the membrane-proximal region, encompassing the 5H5F8 epitope, plays a crucial role in the presentation of the IgE binding residues, but did not appear to be directly involved in IgE binding. Suzuki et al. (42) have suggested that the seven membrane-proximal residues of FcεRIα (PREKYWL) are important to allow expression of FcεRIα on the cell surface with the capacity to bind IgE, although only after coexpression of the γ-chain. They speculate that this region may be required for association with the FcRγ dimer or as a functional spacer required to maintain the native structure of the α-chain extracellular domain on the cell surface. Recently, we presented evidence for the existence of a structural element within the membrane-proximal region that separates the 5H5F8 and 15/1 epitopes (16). The membrane-proximal region might function as a linear stalk (38) to lift the α-chain ectodomain above the membrane environment, making it accessible for IgE binding.

In conclusion, the present study provides evidence for an important and novel role of the membrane-proximal region of the extracellular portion of FcεRIα (Ala¹-Gln¹⁸⁰) in FcεRI-mediated biological activity. The finding that the anti-FcεRIα mAb 5H5F8, which maps to the minimal sequence P¹⁷³REKY¹⁷⁷, blocks Ag-specific cellular activation by a mechanism that does not involve inhibition of IgE binding or 5H5F8-induced receptor internalization implicates a potentially important function of this epitope in FcεRI-mediated signaling. Experiments designed to probe the mechanism of 5H5F8-mediated activity are currently underway, with particular emphasis on whether 5H5F8 binding can modulate FcεRI γ- and/or β-chain immunoreceptor tyrosine-based inhibition motif phosphorylation. Because synthetic peptides containing the PREKY sequence have been shown to bind 5H5F8 (Fig. 6B) with high affinity (Table II), we suggest their use as targets for the identification of ligands that may inhibit cellular activation. The pharmacological potential of such ligands would provide novel options for the rational therapeutic intervention in IgE-mediated allergic responses.

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