

Membrane IgE Binds and Activates FcεRI in an Antigen-Independent Manner¹

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Interaction of secretory IgE with FcεRI is the prerequisite for allergen-driven cellular responses, fundamental events in immediate and chronic allergic manifestations. Previous studies reported the binding of soluble FcεRIα to membrane IgE exposed on B cells. In this study, the functional interaction between human membrane IgE and human FcεRI is presented. Four different IgE versions were expressed in mouse B cell lines, namely: a truncation at the Cε2-Cε3 junction of membrane IgE isoform long, membrane IgE isoform long (without Igα/Igβ BCR accessory proteins), and both εBCRs (containing membrane IgE isoforms short and long). All membrane IgE versions activated a rat basophilic leukemia cell line transfected with human FcεRI, as detected by measuring the release of both preformed and newly synthesized mediators. The interaction led also to Ca²⁺ responses in the basophil cell line, while membrane IgE-FcεRI complexes were detected by immunoprecipitation. FcεRI activation by membrane IgE occurs in an Ag-independent manner. Noteworthy, human peripheral blood basophils and monocytes also were activated upon contact with cells bearing membrane IgE. In humans, the presence of FcεRI in several cellular entities suggests a possible membrane IgE-FcεRI-driven cell-cell dialogue, with likely implications for IgE homeostasis in physiology and pathology. *The Journal of Immunology*, 2005, 174: 5602–5611.

Interaction of secretory IgE (sIgE)³ with mast cells and basophils, through binding to high affinity receptors (FcεRI), is the prerequisite for allergen-driven cell degranulation (1–3). FcεRI is a multichain (αβγ₂ or αγ₂) membrane glycoprotein, detected solely on the surface of mast cells and basophils in rodents, with a remarkably wider cellular distribution in humans (1, 4). Indeed, the presence of FcεRI on granulocytes, platelets, monocytes, and dendritic cells suggested a multipurpose role for this receptor (1, 4). FcεRI α-chain (FcεRIα) contains the IgE binding site (1–3), while the β-chain functions as a signal amplifier (1, 5). The γ-chain acts in signal transduction, and it is essential for the transport of human α-chain to the cell surface (6). In APCs, FcεRI is present in the αγ₂ form, and it has been reported to enhance MHC peptide presentation, a mechanism most likely involved in the development and potentiation of chronic allergic diseases (7).

IgE, as all other Ig isotypes, is produced as secretory or membrane isoforms (8–10). The main function of sIgE is the recognition of foreign Ags, a role shared by all Abs. Nonetheless, sIgE action is magnified and maintained in an exclusive manner upon its high affinity binding to FcεRI, providing the lever to trigger Ag-dependent degranulation and the specificity to confer a noncanonical immune memory to mast cells (11). Monomeric sIgE binding to FcεRI increases receptor number by stabilizing cell surface sIgE-FcεRI complexes and improves survival of FcεRI⁺ cells by an antiapoptotic effect (12). Recently, monomeric sIgE has also been shown to enhance the sensitization phase of contact sensitivity, in a concerted action with mast cells (13). Similarities and differences in the cellular effects induced by aggregated or monomeric sIgE appear to be dependent on the quantity and duration of FcR γ-chain signaling (14).

Membrane IgE (mIgE) isoforms assemble with Igα and Igβ accessory proteins to constitute εBCRs (10). As all BCRs, the ε isotype is involved in Ag recognition and B cell differentiation. Clearly, mIgE and sIgE wield different tasks, yet both forms retain Ag and Fc receptor binding sites. Ag binding is fundamental for the function of sIgE and mIgE. Equally important, FcεRI and CD23 (the IgE low affinity receptor) binding by sIgE has been extensively characterized (1–3). Conversely, absence of data on the interaction between FcεRI (or CD23) and the Fc domain of mIgE holds unexplored intriguing aspects of mIgE affinity and mechanism of action toward cellular Fc receptors. Indeed, binding by recombinant soluble FcεRIα to mIgE⁺ B cells suggested a possible new mechanism in the regulation of these cells (15, 16). However, soluble FcεRIα moieties were not clearly detected in vivo, while human cellular FcεRI is exposed and accessible in a variety of cell types.

We report in this work the characterization of a direct interaction between human mIgE and human FcεRI and the investigation of the functional effects on FcεRI⁺ cells. Cell-cell contact led to increase of intracellular Ca²⁺ concentration ([Ca²⁺]_i), significant

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Received for publication August 12, 2004. Accepted for publication February 16, 2005.

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¹ This work was supported by Associazione Italiana per la Ricerca sul Cancro Grant (to G.P.), Consiglio Nazionale delle Ricerche Progetto Finalizzato Biotecnologie (to A.G.S.), Ministero dell'Istruzione dell'Università e della Ricerca CoFin 2001 and 2002 (to A.G.S.), and Istituto Superiore di Sanità Progetto AIDS (to A.G.S.). M.C.-G. is a recipient of a predoctoral fellowship from Scuola Internazionale Superiore di Studi Avanzati.

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³ Abbreviations used in this paper: sIg, secretory Ig; [Ca²⁺]_i, intracellular Ca²⁺ concentration; CHO, Chinese hamster ovary; ε_LBCR, εBCR isoform long; ε_SBCR, εBCR isoform short; ER, endoplasmic reticulum; KRH, Krebs-Ringer solution buffered with HEPES; mIg, membrane Ig; m_LIgE, mIgE isoform long; NIP, 4-hydroxy-3-iodo-5-nitrophenyl-acetyl; sα, soluble dimeric FcεRIα; sLT, sulfido-leukotriene; tm_LIgE, truncated m_LIgE.

β -hexosaminidase and sulfido-leukotriene (sLT) release, and biochemical detection of mIgE-Fc ϵ RI complexes. Strikingly, mIgE activated Fc ϵ RI in absence of Ag.

This new mechanism is likely to affect IgE homeostasis, and may have relevant implications in the development of atopic diseases. The confirmation of an *in vivo* role for the mIgE-Fc ϵ RI interaction would set the conceptual stage for the development of innovative antiallergic drugs.

Materials and Methods

Cells and Abs

Chinese hamster ovary (CHO) cells were grown in α -MEM (Invitrogen Life Technologies) containing 40 μ M deoxy-ribonucleosides and 40 μ M ribonucleosides. Mouse plasmacytoma sp2/0 cells were grown in RPMI 1640 medium (Invitrogen Life Technologies). Mouse plasmacytoma J558L cells, mouse myeloma A20 cells, and rat basophilic leukemia RBL-2H3 cells were grown in DMEM medium (Invitrogen Life Technologies). Cells were supplemented with 10% (v/v) FCS. Soluble dimeric Fc ϵ RI α (sd α)-expressing CHO cells (16), truncated mIgE isoform long (tm₁IgE)-expressing sp2/0 cells (sp2/0-tm₁IgE), J558L cells expressing either human anti-4-hydroxy-3-iodo-5-nitrophenyl-acetyl (NIP) sIgE_{S2} (8, 9) or anti-NIP m₁IgE (J558L-m₁IgE) (9), and A20 cells expressing ϵ BCR isoform long (ϵ_L BCR) and ϵ BCR isoform short (ϵ_S BCR), obtained, as described for a mutated m₁IgE (17), by transfection of pCIG-C ϵ CH4-M1'-M2 and pCIG-C ϵ CH4-M1-M2, respectively (9, 10), were all supplemented with 400 μ g/ml Geneticin (G-418 sulfate; Calbiochem). RBL-SX38 cells expressing human Fc ϵ RI α -, β -, and γ -chains (18) were kindly provided by M. Jouvin (Harvard Medical School, Boston, MA) and were supplemented with 800 μ g/ml Geneticin, as previously described (16).

Supernatant from J558L cells expressing the second sIgE isoform (sIgE_{S2}), presenting a C-terminal interchain disulfide bond (8), was used throughout all experiments. Because sIgE_{S2} and sIgE_{S1} have comparable Fc ϵ RI affinity (19), sIgE_{S2} was indicated as sIgE for simplicity. Cell supernatant IgE levels were measured by ELISA. Microtiter plates (Maxisorb; Nunc) were coated with anti-human IgE Abs (Star 96; Serotec). A chimeric human anti-NIP IgE (JW8/1; Serotec) was used as standard. Bound Abs were revealed by HRP-conjugated anti-human IgE Abs (DakoCytomation). *O*-phenylenediamine (Sigma-Aldrich) was used as substrate, and the absorbance was read at 492 nm. The sensitivity of the assay was 5 ng/ml. The mouse anti-human Fc ϵ RI α mAb 9E1 and the mouse anti-SV5 mAb have already been described (16, 20, 21), and the mouse anti-(anti-NIP) Id mAb AC38 (22) was kindly provided by S. Burastero (San Raffaele Scientific Institute, Milan, Italy).

Human basophil activation

Basophils were enriched from fresh peripheral blood by erythrocyte sedimentation. Whole blood was obtained by venipuncture and drawn into Vacutainer tubes containing EDTA anticoagulant (BD Biosciences). The erythrocytes were then allowed to settle for 45 min. The leukocyte-containing supernatant was carefully aspirated to avoid contamination with the erythrocyte-containing sediment. Collected cells were used at a concentration of 10^6 cells/ml in RPMI 1640 medium containing 10% FCS. Assays were performed in duplicate using 100 μ l of cell suspension. Incubation time for anti-human IgE Abs was 15 min and 1 h for J558L cells. Basophils were detected by direct immunofluorescence staining with PE-labeled anti-human CD203c (Beckman Coulter). Analysis was performed on a Cytomics FC 500 flow cytometer (Beckman Coulter). PE fluorescence was measured through a 575-nm band pass filter. To exclude J558L cells, a FITC-conjugated anti-mouse CD45R mAb (BD Pharmingen) was used.

Human monocyte isolation

PBMC were separated by Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation from individual 50-ml heparinized blood donations from allergic and nonallergic donors. Mononuclear cells were resuspended in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, antibiotics, and antimycotics, and cultured at a concentration of 10^6 cells/cm² in 24-well plates (Nunc) at 37°C, 5% CO₂. Monocytes were obtained after 1 h by washing wells with PBS. Preparations contained >80% CD14⁺ cells, as assayed by flow cytometry, and absence of basophils was assessed by toluidine blue staining (data not shown).

Plasmid construction, cell transfection, and selection

A 623-bp *HindIII/Bsu36I* fragment, coding for the Ig secretion peptide described in Li et al. (23), followed by the SV5 tag (21), the human C ϵ 3

domain (including the N-terminal C328), and part of the C ϵ 4 domain, was excised from pcDNA3-SV5-Cys-C ϵ 3C ϵ 4_{S1}, a plasmid coding for a soluble N-terminally tagged C ϵ 3C ϵ 4 with the C terminus of sIgE_{S1} (8) (M. Cesco-Gaspere, L. Vangelista, and O. Burrone, manuscript in preparation). The fragment was inserted into the *HindIII/Bsu36* sites of pcDNA3- ϵ_L -mSIP (17) to obtain pcDNA3-SV5-Cys-C ϵ 3C ϵ 4_{mL}, the construct encoding for tm₁IgE. The *PvuII*-linearized plasmid was then transfected into sp2/0 cells by electroporation (by a single pulse; 950 μ F, 250 V). Geneticin-resistant clones were analyzed by flow cytometry on a FACSCalibur (BD Biosciences) using FITC-conjugated anti-human IgE Abs (Pierce) or with the anti-SV5 mAb, followed by FITC-conjugated anti-mouse IgG Abs (Pierce).

Mediator release

Plastic-adherent human Fc ϵ RI⁺ cells (typically $5\text{--}7 \times 10^4$ cells/well) were incubated in the stimulation buffer provided with the CAST-2000 kit (Buhlmann Laboratories) with 100 ng of human anti-NIP sIgE for 2 h at 37°C. Cells were then washed and incubated in stimulation buffer with 100 ng of NIP-BSA (Biosearch Technologies). Alternatively, suspensions of control or mIgE⁺ cells were added to Fc ϵ RI⁺ adherent cells (typically 1:1 or 2:1 ratio), and plates were centrifuged 5 min at $300 \times g$ and incubated at 37°C. B cells were also added to adherent cells omitting the centrifugation, and no relevant difference in mediator release was observed between sedimented and centrifuged cells (data not shown); hence, centrifugation was used as a means to synchronize cell-cell contact. The release of β -hexosaminidase was monitored on RBL-SX38 cell supernatant added with *p*-nitrophenyl-*N*-acetyl- β -D-glucosamine (Sigma-Aldrich) in 0.1 M citrate buffer (pH 6.2) and incubated at 37°C for 120 min. The reaction was terminated using 0.1 M carbonate buffer (pH 10), and the absorbance was read at 405 nm. Negative control (NS) was the supernatant of nonstimulated cells. Stimulation control (SC) was the maximal stimulation of IgE-sensitized RBL-SX38 cells with 100 ng of NIP-BSA and presented 25–35% of the total β -hexosaminidase content, obtained from a Triton X-100 lysate of the cell monolayer. The results are calculated as percentage of β -hexosaminidase release ($100 \times (A_{405 \text{ nm}} \text{ sample} - A_{405 \text{ nm}} \text{ NS}) / (A_{405 \text{ nm}} \text{ SC} - A_{405 \text{ nm}} \text{ NS})$). Release of sLT was measured by the CAST-2000 kit, a competition ELISA using a constant amount of alkaline phosphatase-conjugated sLT to compete with cellular sLT released in the supernatant. The procedure was conducted following the manufacturer instructions, except for the SC control, which was the same used in the β -hexosaminidase release assay. An sLT standard curve was run with all experiments. SC varied between 0.8 and 1.2 ng/assay, and NS was ≤ 0.1 ng/assay. The results are calculated as percentage of sLT release ($100 - (100 \times (A_{405 \text{ nm}} \text{ sample} - A_{405 \text{ nm}} \text{ SC}) / (A_{405 \text{ nm}} \text{ NS} - A_{405 \text{ nm}} \text{ SC}))$).

Flow cytometry

mIgE was detected by direct immunofluorescence, as previously reported (17). Sp2/0-tm₁IgE, J558L-m₁IgE, A20- ϵ_S BCR, and A20- ϵ_L BCR cells in staining buffer (PBS, 5% BSA, 0.01% NaN₃) were incubated with FITC-labeled anti-human IgE Abs (Pierce) 40 min at 4°C. Cells were then washed and fixed in 0.4% paraformaldehyde. mIgEs were also detected by indirect immunofluorescence, using the supernatant from sd α -expressing CHO cells (and supernatant from nontransfected CHO cells, as control) and FITC-labeled anti-human IgG Abs (Pierce), as previously reported (16). Membrane expression of human Fc ϵ RI α was detected by indirect immunofluorescence, as previously reported (16). RBL-SX38 cells were incubated with the supernatant from human sIgE-expressing J558L cells (or supernatant from J558L cells, as control) 40 min at 4°C. Cells were then washed and incubated with FITC-labeled anti-human IgE Abs 30 min at 4°C, washed, and fixed in 0.4% paraformaldehyde. Human Fc ϵ RI α on RBL-SX38 cells was detected also using 9E1 and FITC-conjugated anti-mouse IgG Abs (Pierce).

Ca²⁺ imaging

RBL-SX38 cells (8×10^5) were plated on glass coverslips. At the beginning of each experiment, cells were washed with Krebs-Ringer solution buffered with HEPES (KRH) (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2 mM CaCl₂, 6 mM glucose, 25 mM HEPES-NaOH, pH 7.4). Cell monolayers were incubated 30 min at 37°C with 4 μ M fura 2-AM dissolved in KRH supplemented with 0.02% pluronic acid F-127 (Calbiochem). Cells were then rinsed in KRH and examined by fura 2 videomicroscopy, according to Grohovaz et al. (24). Before NIP-BSA stimulation (50 ng/ml), RBL-SX38 cells were treated 2 h at 37°C with sIgE. To study cell-cell interactions, RBL-SX38 cell supernatant was removed, and 0.5 ml of KRH containing 1×10^6 (or 5×10^5) sp2/0-tm₁IgE (or sp2/0) cells was added.

The digital fluorescence imaging system is built on an inverted Axiovert 135TV microscope (Zeiss). Cells were excited at 340 and 380 nm by a

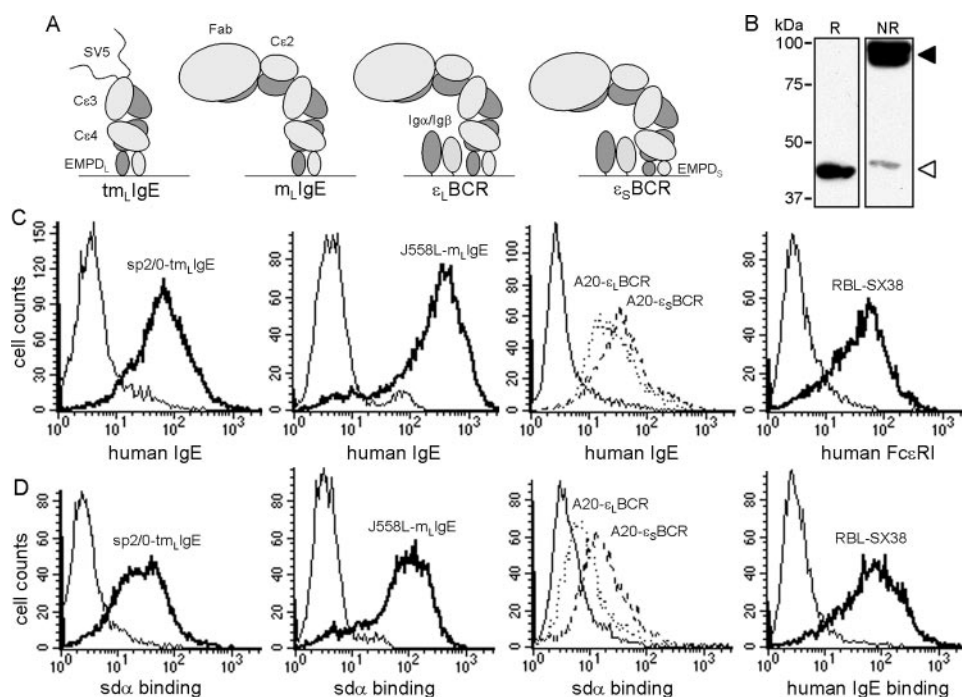


FIGURE 1. Schematic representation of cell surface-exposed human mIgE variants and characterization of their expression. *A*, The four mIgE variants. Cell type determines the presence and association with the Igα/Igβ heterodimer. A20 cells allow BCR formation, while sp2/0 and J558L cells do not. EMPD, extracellular membrane-proximal domain (17). *B*, Western blot analysis of tmIgE using HRP-conjugated anti-human IgE Abs. R, reducing and NR, nonreducing conditions. Δ and $\blacktriangle$, Indicate the monomer and dimer, respectively. *C*, Surface exposure of mIgE variants and FcεRI detected by flow cytometry. Bold lines represent sp2/0 cells expressing tmIgE, J558L cells expressing mLIgE, and RBL cells expressing human FcεRI. Dotted and dashed lines correspond to A20 cells expressing complete BCRs of mLIgE and membrane IgE isoform short (m_SIgE), respectively. Thin lines represent non-transfected cell lines. Mouse B cells were incubated with FITC-conjugated anti-human IgE Abs. RBL cells were incubated with 9E1 and FITC-conjugated anti-mouse IgG Abs. *D*, Binding activity of human FcεRI and mIgE variants investigated by flow cytometry. Bold, dotted, dashed, and thin lines are as in *C*, respectively. Mouse B cells were incubated with sIgA and FITC-conjugated anti-human IgG Abs. RBL cells were incubated with human sIgE and FITC-conjugated anti-human IgE Abs. Data were obtained from one of several experiments yielding similar results.

modified CAM-230 dual wavelength fluorometer (Jasco), and fluorescence images were captured by a low-light level charge-coupled device camera (ISIS; Photonic Science). The Openlab software (Improvision) was used to control acquisition protocol and to perform data analysis. Fluorescence images were converted to $[Ca^{2+}]_i$ maps, using the 340/380-nm excitation wavelength ratio method. Image sequences are available as supplemental videos.⁴ The mean values in regions of interest, corresponding to single cells, were calculated from sequences of ratio images.

Immunoprecipitation, surface biotinylation, and Western blot analysis

Cells were lysed in TNN buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40), containing protease inhibitors (200 μ g/ml phenylmethanesulfonyl fluoride; Sigma-Aldrich; and a protease inhibitor mixture, Complete; Roche) and 20 mM alkylating agent *N*-ethylmaleimide (Sigma-Aldrich). Cell lysates were centrifuged at 4°C for 10 min at $10,000 \times g$, and anti-human IgE Abs (DakoCytomation) or the anti-SV5 mAb were added to supernatants and incubated 1 h at 4°C. Protein complexes were then precipitated with protein A-agarose (RepliGen) 1 h at 4°C. Protein A beads were then washed twice each with TNN, TNN + 1% BSA, RIPA buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 5 mM MgCl₂, 1% deoxycholate, 0.1% SDS), and PBS (all solutions containing 20 mM *N*-ethylmaleimide). Bound proteins were then eluted 5 min at 95°C in nonreducing SDS sample buffer (30 mM Tris-HCl, pH 6.8, 1.5% SDS, 10% glycerol, 0.1 mg/ml bromophenol blue).

For the cell surface biotinylation, 10^6 RBL-SX38 (or RBL-2H3) cells were incubated with 1 mM normal human serum-Sulfobiotin (Pierce) for 1 h at 25°C. Cells were washed in PBS, and the reaction was blocked using Tris-NaCl (50 mM Tris, 150 mM NaCl, pH 8.0) 15 min at 25°C. Cells were then washed in PBS and lysed in TNN, and proteins were immunoprecipitated using 9E1 and protein A-agarose, as described above.

For the Western blot analysis, protein samples were separated under reducing or nonreducing 10% SDS-PAGE and transferred to nitrocellulose (Hybond ECL; Amersham Biosciences). FcεRIα was detected using 9E1 and revealed by HRP-conjugated anti-mouse IgG Abs (Pierce). IgE was detected with HRP-conjugated anti-human IgE Abs (DakoCytomation), and tmIgE was detected using also the anti-SV5 mAb, followed by HRP-conjugated anti-mouse IgG Abs (Pierce). Biotinylated FcεRIα was revealed using HRP-conjugated streptavidin (Amersham Biosciences). Western blots were developed by ECL (Amersham Biosciences) autoradiography.

Purification of sIgE_{S2}

NIP-BSA beads were prepared by cross-linking NIP-BSA to CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences), according to manufacturer instructions. Supernatant from J558L cells expressing human anti-NIP sIgE_{S2} was incubated with NIP-BSA beads 1 h at 25°C and then washed with PBS. Bound sIgE_{S2} was eluted using 0.1 M glycine, pH 2.7, and immediately equilibrated using Tris-HCl, pH 8.0. Purity of sIgE_{S2} was assessed by Coomassie blue staining in reducing SDS-PAGE and by Western blot analysis using HRP-conjugated anti-human IgE Abs (DakoCytomation) (data not shown).

Statistical analysis

ANOVA on mediator release data was evaluated by Student's *t* test (Excel TTEST function; Microsoft). Differences associated with *p* values <0.05 were considered significant.

Results

Expression and soluble FcεRIα binding of mIgE variants

Four mIgE⁺ B cell variants were used to investigate the mIgE-FcεRI interaction (Fig. 1A). Sp2/0 cells were transfected with a plasmid encoding for a truncated version (tmLIgE) of human mIgE isoform long (mLIgE), in which the IgE moiety N-terminal to Cε3

⁴ The online version of this article contains supplemental material.

was replaced by the SV5 tag (21). In tm_LIgE , C328 has been retained to provide N-terminal covalent dimerization (as reported for soluble $\text{C}\epsilon_3\text{C}\epsilon_4$) (25, 26), in addition to the membrane-proximal disulfide stabilization (17). Sp2/0 cells do not produce Ig chains, and $\text{Ig}\alpha$ is silenced by DNA methylation (27). Therefore, the observed cell exposure of tm_LIgE (Fig. 1C) indicates that the protein reaches the cell surface as an isolated moiety (i.e., without $\text{Ig}\alpha/\text{Ig}\beta$ heterodimer). In nonreducing conditions, Western blot analysis (using anti-IgE Abs) on sp2/0- tm_LIgE cell extracts presented a major band at ~ 80 kDa, corresponding to the expected dimer (Fig. 1B). The small amount of monomer (band at ~ 40 kDa) could derive from the endoplasmic reticulum, probably representing an intermediate of tm_LIgE assembly. As expected, only the monomer was detected in reducing conditions (Fig. 1B). The same monomer/dimer pattern was revealed when using the anti-SV5 mAb (data not shown). To compare tm_LIgE and m_LIgE binding to $\text{Fc}\epsilon\text{RI}$, we used J558L- m_LIgE cells, in which m_LIgE is expressed on the cell surface (Fig. 1C) (9). J558L cells do not express $\text{Ig}\alpha$ (28) and Ig H chains, but produce NIP-specific L chains (8, 10). Because our IgE H chain is a chimeric mouse anti-NIP V_H /human $\text{C}\epsilon_1$ - $\text{C}\epsilon_4\text{m}_L$ (9), the resulting mIgE has anti-NIP activity, as shown for sIgE isoforms expressed by the same cell line (8, 19). m_LIgE and membrane IgE isoform short (m_SIgE) were expressed as cell surface BCRs in A20 cells (Fig. 1C) (17). A20 cells express $\text{Ig}\alpha$ and $\text{Ig}\beta$, crucial for the signaling function of BCRs (29). Moreover, human mIgE isoforms can associate with mouse $\text{Ig}\alpha/\text{Ig}\beta$ heterodimers to assemble into functional BCRs (10). In a parallel experiment, tm_LIgE , m_LIgE , $\epsilon_L\text{BCR}$, and $\epsilon_S\text{BCR}$ bound to $\text{sd}\alpha$, a soluble dimeric $\text{Fc}\epsilon\text{RI}\alpha$ (16), although to different extents (Fig. 1D). The lower $\text{sd}\alpha$ -binding activity shown by $\epsilon_L\text{BCR}$ and $\epsilon_S\text{BCR}$, as compared with m_LIgE , parallels their lower expression levels (Fig. 1, C and D).

Human mIgE^+ B cells induce mediator release from human $\text{Fc}\epsilon\text{RI}^+$ RBL cells

Expression of human $\text{Fc}\epsilon\text{RI}$ and sIgE binding by RBL-SX38 cells is shown in Fig. 1, C and D. The functional interaction between the four human mIgE versions (tm_LIgE , m_LIgE , $\epsilon_L\text{BCR}$, and $\epsilon_S\text{BCR}$) and human $\text{Fc}\epsilon\text{RI}$ was assessed by monitoring mediator release by RBL-SX38 cells (19). A competitive ELISA served to analyze the release of newly formed sLT, while enzymatic activity of β -hexosaminidase was assayed to monitor the release of preformed mediators by degranulation. Surprisingly, upon cell-cell contact, all mIgE^+ B cell types used were capable of promoting sLT and β -hexosaminidase release (Fig. 2). Nontransfected B cells triggered only basal mediator release (p values all < 0.0001), comparable to nonstimulated cells. The release induced by tm_LIgE and m_LIgE is not significantly different (p value 0.21), while it is lower for $\epsilon_L\text{BCR}$ and $\epsilon_S\text{BCR}$, as compared with m_LIgE (p values of 0.0005 and 0.0032, respectively). These results parallel the lower $\text{sd}\alpha$ -binding capacity of ϵBCRs , probably due to the lower expression levels of ϵBCRs compared with those of tm_LIgE and m_LIgE (Fig. 1, C and D), as already discussed. The powerful activation of RBL-SX38 cells by mIgE is not reproduced by RBL-2H3 cells (constitutively expressing only rat $\text{Fc}\epsilon\text{RI}$) in identical experimental conditions (data not shown). The selective release obtained by human $\text{Fc}\epsilon\text{RI}$ -expressing RBL cells supports the known incapability of rodent $\text{Fc}\epsilon\text{RI}$ to interact with human IgE (1), further attesting the specificity of human mIgE- $\text{Fc}\epsilon\text{RI}$ interaction. Moreover, the release of mediators (sLT and β -hexosaminidase) was significantly inhibited ($p < 0.01$) by preincubation of sp2/0- tm_LIgE cells with a supernatant from CHO cells transfected with $\text{sd}\alpha$ (Fig. 2), but not with a supernatant from nontransfected CHO cells (data not shown).

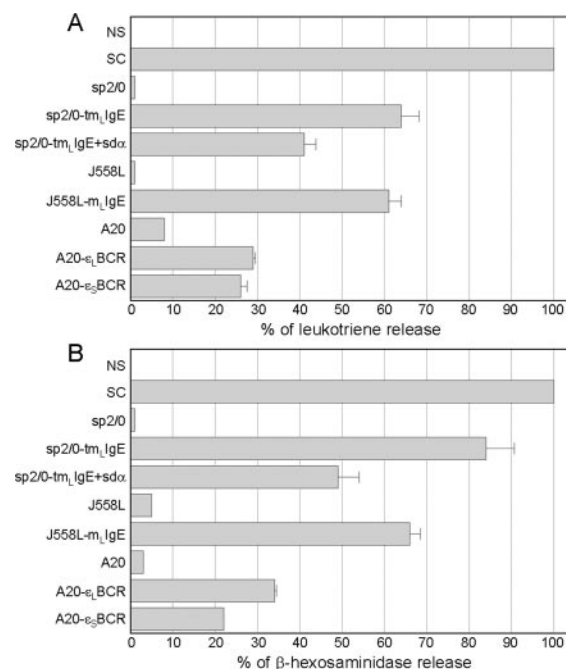


FIGURE 2. RBL-SX38 cells release sLT (A) and β -hexosaminidase (B) upon contact with mIgE^+ B cells. NS, nonstimulated RBL-SX38 cells; SC, stimulation control obtained incubating RBL-SX38 cells with anti-NIP human sIgE and NIP-BSA. Nontransfected sp2/0, J558L, and A20 cells were used as controls. The inhibition of mediator release ($p < 0.01$) after preincubation of sp2/0- tm_LIgE cells with supernatant of $\text{sd}\alpha$ -expressing CHO cells is also shown. Values are mean $\pm$ SD of three determinations from two independent experiments.

To exclude possible artifactual results (e.g., due to mIgE-containing cell membrane fragments activating RBL-SX38 cells), mIgE^+ cells were placed on transwells on top of RBL-SX38 cells. Supernatants from transfected cells (or nontransfected cells, as controls) presented basal sLT release, comparable to nonstimulated cells (data not shown).

mIgE activation of $\text{Fc}\epsilon\text{RI}$ is inhibited by sIgE

Direct mIgE binding to $\text{Fc}\epsilon\text{RI}$ was further demonstrated by sIgE inhibition (Fig. 3). The effect on mIgE-driven sLT release was

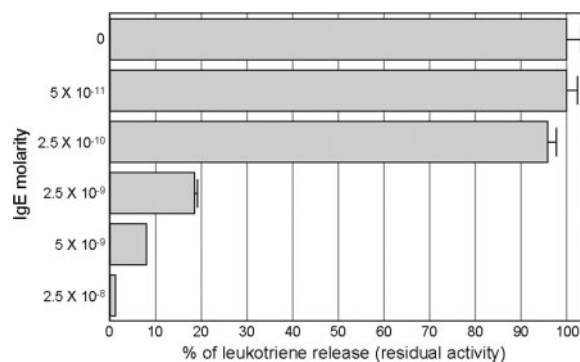


FIGURE 3. $\text{Fc}\epsilon\text{RI}$ activation by mIgE is completely inhibited by sIgE. Plastic-adherent RBL-SX38 cells (7×10^4 cells/well) were incubated with sIgE at different concentrations (IgE molarity). J558L- m_LIgE cells (1×10^5 cells/well) were then added with no further treatment, and supernatants were analyzed for their sLT content. The results are normalized with respect to untreated controls. Values are mean $\pm$ SD of three determinations from two independent experiments.

evaluated by incubating RBL-SX38 cells with sIgE (at concentrations ranging from 5×10^{-11} M to 2.5×10^{-8} M) 2 h before J558L- m_L IgE cell contact. The response was totally inhibited by 2.5×10^{-8} M sIgE, demonstrating that the sites involved in mIgE and sIgE binding to FcεRI are equivalent. A 50% sLT release inhibition was obtained at $\sim 10^{-9}$ M sIgE, a concentration within the range of dissociation constants reported for sIgE-FcεRI interaction (30).

mIgE⁺ cells induce $[Ca^{2+}]_i$ increase in FcεRI⁺ cells

Coverslip-plated RBL-SX38 cells were loaded with fura 2 and analyzed by a videomicroscopy system. Before each experiment, series of 340/380 images were acquired for 5 min, to monitor basal $[Ca^{2+}]_i$ and to exclude spontaneous activity (data not shown). Preincubation of RBL-SX38 cells with sIgE, followed by the stimulation with NIP-BSA, promoted strong elevation of $[Ca^{2+}]_i$ in most cells ($\sim 90\%$ of 100 cells analyzed, in two separate experiments). The delay of Ca^{2+} responses, after administration of NIP-BSA, ranged between 1 and 3 min, and their shape (despite the high variability among cells) was mainly represented by a rapid increase of the 340/380 ratio that was maintained for the entire duration of the experiment (Fig. 4, A and B).

In different experiments, sp2/0- t_m IgE cells were gently added on the top of the chamber. The cell suspension (1×10^6 cells/ml) quickly reached the RBL-SX38 cells, and the contact between the two cell types was characterized by a rapid and transient $[Ca^{2+}]_i$ increase, occurring also when adding the same number of sp2/0 cells (data not shown). Approximately 3 min later, a small proportion of RBL-SX38 cells exhibited $[Ca^{2+}]_i$ elevation. The amount of responding cells increased during time, reaching the maximal percentage ($\sim 80\%$ of 150 cells analyzed, in two separate experiments) ~ 10 min after the initial contact (Fig. 4C). Ca^{2+} responses exhibited different shapes and amplitudes (various os-

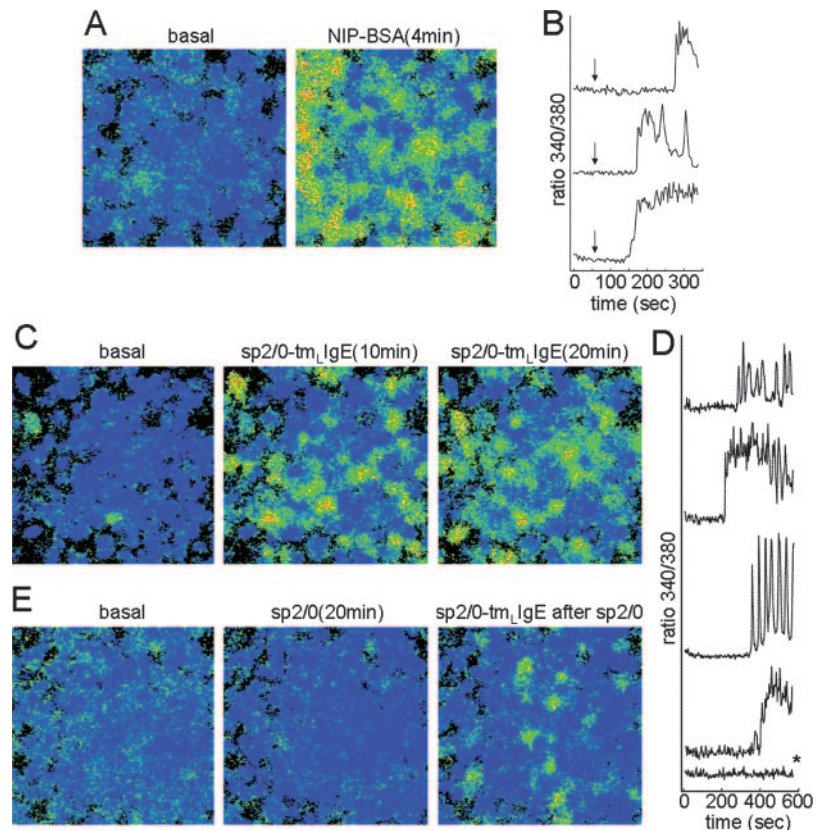
cillatory patterns; slow, but persistent elevation or transient increase) (Fig. 4D), and most of them were maintained during the entire period of analysis (~ 20 min; data not shown). When adding sp2/0 cells, no increase of $[Ca^{2+}]_i$ was observed over the same incubation time (Fig. 4E). After 20 min, sp2/0 cells were partially removed from the RBL-SX38 cell monolayer by a gentle wash, and sp2/0- t_m IgE cells were added. Again, after 3–5 min, a $[Ca^{2+}]_i$ increase was detected in a fraction of the RBL-SX38 cells (Fig. 4E). When using sp2/0- t_m IgE cells at lower density (5×10^5 cells/ml), the amount of responding cells (within the 20-min analysis) decreased considerably ($\sim 12\%$), probably due to a longer sedimentation time required to encounter a significant number of RBL-SX38 cells (data not shown).

The different Ca^{2+} response kinetics, observed in RBL-SX38 cells when adding NIP-BSA (to sIgE-loaded cells) or sp2/0- t_m IgE cells, may reflect two distinct FcεRI activation processes (see supplemental videos 1–3).⁴ In sIgE-loaded cells, NIP-BSA should lead to immediate aggregation of FcεRI, while the cell-cell approach may require a physiological time to allow lateral diffusion, eventually leading to the formation of frontal FcεRI-mIgE clusters.

mIgE-FcεRI complexes form upon cell-cell contact

Western blot analysis of RBL-SX38 cell extracts, using the anti-FcεRIα mAb 9E1, presented a major band migrating at 50–60 kDa and a second FcεRIα form migrating at ~ 30 kDa (Fig. 5, lane 2). Surface biotinylation of RBL-SX38 cells, followed by immunoprecipitation with 9E1 and detection in Western blot using HRP-conjugated streptavidin, revealed only the 50- to 60-kDa band, confirming that it corresponds to the mature cell surface-exposed FcεRIα (Fig. 5, lane 4). To investigate the identity of the ~ 30 -kDa FcεRIα band, RBL-SX38 cells were preincubated with sIgE, washed, lysed in presence or absence of sIgE, and immunoprecipitated using anti-human IgE Abs. Western blot analysis using

FIGURE 4. Increase of $[Ca^{2+}]_i$ in RBL-SX38 cells upon mIgE stimulation. **A**, sIgE-loaded RBL-SX38 cells before and 4 min after addition of NIP-BSA. **B**, Traces represent three examples of single cell Ca^{2+} response; arrows indicate addition of NIP-BSA. **C**, RBL-SX38 cells at time 0, 10, and 20 min after addition of sp2/0- t_m IgE cells. **D**, Traces represent four examples of single cell Ca^{2+} response after addition of sp2/0- t_m IgE cells. The trace marked with the asterisk corresponds to a nonresponsive cell; similar traces were recorded for cells after addition of sp2/0 cells (**E**) (data not shown). **E**, RBL-SX38 cells at time 0, 20 min after addition of sp2/0 cells, and ~ 5 min after subsequent addition of sp2/0- t_m IgE cells. **C–E**, Time 0 is ~ 10 s after contact between the two cell types (see Results). Data were obtained from one of two experiments yielding similar results. Complete image sequences are available as supplemental videos 1–3.⁴



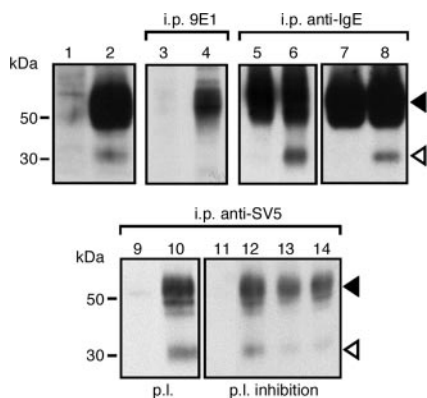


FIGURE 5. Analysis of mIgE-FcεRI complexes. Nonreducing SDS-PAGE was followed by Western blot analysis using the anti-FcεRIα mAb 9E1 and HRP-conjugated anti-mouse IgG Abs (except for lanes 3 and 4, in which HRP-conjugated streptavidin was used). Lanes 1 and 2, Correspond to cell extracts of RBL-2H3 and RBL-SX38 cells, respectively. Lanes 3 and 4, Correspond to cell extracts of RBL-2H3 and RBL-SX38 cells, respectively, after surface biotinylation and immunoprecipitation (i.p.) with 9E1. Lanes 5 and 6, RBL-SX38 cells were preincubated with sIgE, lysed in absence (lane 5) or presence (lane 6) of sIgE, and immunoprecipitated using anti-human IgE Abs. Lanes 7 and 8, RBL-SX38 cells were preincubated with sIgE, challenged with sp2/0 (lane 7) and sp2/0-tm₁IgE (lane 8) cells, and immunoprecipitated using anti-human IgE Abs. Postlysis (p.l.) mIgE-FcεRI interactions are shown in lanes 9 and 10, in which lysed RBL-SX38 cells were mixed with lysed sp2/0 (lane 9) or sp2/0-tm₁IgE (lane 10) cells and immunoprecipitated with the anti-SV5 mAb. To highlight the inhibition of postlysis interactions, sp2/0 (lane 11) or sp2/0-tm₁IgE (lanes 12–14) cells were added to RBL-SX38 cells. Cell lysis and immunoprecipitation (using the anti-SV5 mAb) were conducted in presence (lanes 11, 13, and 14) or absence (lane 12) of sIgE. Purified sIgE_{S2} (lanes 11 and 13) and purified sIgE_{S1} (lane 14) were used. ▲ and △, Represent mature cell-exposed and ER-resident FcεRIα, respectively. Data were obtained from one of three experiments yielding similar results.

9E1 revealed the 50- to 60-kDa FcεRIα band in both conditions (Fig. 5, lanes 5 and 6), while the ~30-kDa band was detectable only in the sample containing sIgE during cell lysis and immunoprecipitation (Fig. 5, lane 6). This result strongly suggests a postlysis interaction between sIgE and the ~30-kDa FcεRIα moiety, thus identified as the endoplasmic reticulum (ER)-resident pool. Conversely, assuming its cell surface exposure, the ~30-kDa FcεRIα form should have been recognized by sIgE also before cell lysis, and it should have been detected in the cell surface biotinylation. After deglycosylation, only a ~25-kDa FcεRIα form is present, which corresponds to the nonglycosylated protein moiety and further confirms the differential glycosylation of the 50- to 60-kDa and ~30-kDa FcεRIα forms (data not shown). The pool of ER-resident FcεRIα has already been characterized and found to be capable of efficient IgE binding (31, 32). Those studies indicated that FcεRIα intracellular folding is achieved before glycosylation and highlighted the role of glycosylation for the efficient export of FcεRIα (31, 32). In a separate experiment, RBL-SX38 cells were preincubated with sIgE, washed, and challenged with sp2/0-tm₁IgE or sp2/0 cells. After cell-cell contact, cells were lysed, and the formation of IgE-FcεRI complexes was investigated by IgE immunoprecipitation (using anti-human IgE Abs) and detection of FcεRIα (using 9E1). Again, cell surface-exposed FcεRIα was revealed in both conditions (Fig. 5, lanes 7 and 8), while the ER-resident FcεRIα was detected only in presence of sp2/0-tm₁IgE cells (Fig. 5, lane 8). Hence, postlysis tm₁IgE-FcεRIα interaction should occur. Indeed, mature and intracellular FcεRIα bound to mIgE after cell lysis, as confirmed by adding

lysed sp2/0-tm₁IgE cells to lysed RBL-SX38 cells (Fig. 5, lane 10). In this case, the anti-SV5 mAb was used to immunoprecipitate the SV5-tagged tm₁IgE.

We then devised an experiment to dissect prelysis from postlysis interaction and finally visualize the mIgE-FcεRI recognition inducing RBL-SX38 cell activation. To achieve this, we used the ~30-kDa FcεRIα form as a probe, because it would only be available for binding once cells are lysed and during immunoprecipitation. sp2/0-tm₁IgE cells were added to RBL-SX38 cells; cell lysis was conducted in presence of sIgE; and immunoprecipitation was performed using the anti-SV5 mAb, to detect only tm₁IgE-FcεRIα complexes (avoiding interference by sIgE-FcεRIα complexes). In this way, a strong inhibition of the intracellular FcεRIα band was observed, while the mature FcεRIα band was only slightly inhibited (Fig. 5, compare lane 12 with lanes 13 and 14), demonstrating that the corresponding mIgE-FcεRIα complexes were formed during live cell-cell contact. Finally, the presence of tm₁IgE after immunoprecipitation was revealed using either anti-human IgE Abs or the anti-SV5 mAb. In nonreducing conditions, the expected disulfide-bonded tm₁IgE dimer, migrating at ~80 kDa (identical with that shown in Fig. 1B), was detected with comparable intensity for all samples (data not shown).

Release of sLT upon mIgE-FcεRI binding is time and cell number dependent

Formation and release of sLT (upon challenging RBL-SX38 cells with J558L-m₁IgE cells) were analyzed in terms of time and cell number dependence. Induction of sLT release was already detectable 10 min after cell-cell contact (Fig. 6A). Synthesis and release of sLT continued until a maximum detected at ~60 min. At 180 min, the lower sLT content suggested termination of the synthesis and partial sLT degradation. Supernatants from RBL-SX38 cells reacted with J558L cells presented basal level release (Fig. 6A). This release kinetics was compared with that of sIgE-loaded RBL-SX38 cells by collecting supernatants after NIP-BSA stimulation. Maximum release was reached in 10–15 min (data not shown).

Release of sLT is also cell number dependent and detectable challenging 5×10^4 RBL-SX38 cells with 5×10^3 J558L-m₁IgE cells (i.e., a 10:1 RBL:J558L ratio) (Fig. 6B). The release increased until the RBL-SX38 monolayer was completely covered by J558L-m₁IgE cells, a situation corresponding to a 1:4 RBL:J558L ratio. When using J558L cells, only basal release was detected (Fig. 6B).

Ag independence of FcεRI activation by mIgE

Figs. 2, 3, and 6 show mediator release by FcεRI⁺ cells upon mIgE interaction in absence of Ag. To further characterize this feature, three cross-linkers recognizing IgE in distinct ways (NIP-BSA, the anti-Id mAb AC38, and anti-human IgE Fc Abs) were tested on mIgE activation of RBL-SX38 cells. J558L-m₁IgE cells were incubated with the cross-linkers and washed before contact with RBL-SX38 cells. Cell supernatants were then analyzed for their sLT and β-hexosaminidase content (Fig. 7). None of the conditions tested revealed a release above that of noncross-linked mIgE, indicating that neither the type of IgE cross-linker nor its concentration increased the activation power of mIgE. Conversely, all cross-linkers inhibited (*p* values ranging from 0.017 to 0.0001) sLT synthesis and release (Fig. 7A), while only two conditions (anti-Id mAb, 1 μg/ml, *p* value of 0.027; NIP-BSA, 1 μg/ml, *p* value of 0.049) caused a significant decrease of β-hexosaminidase release (Fig. 7B). The observed decrease might indicate a degree of hindrance caused by the aggregated mIgE-cross-linker complexes when encountering FcεRI on the surface of RBL cells, or, most likely, the internalization of a fraction of cross-linked mIgE. The lower concentration of anti-Id mAb and NIP-BSA inhibited more

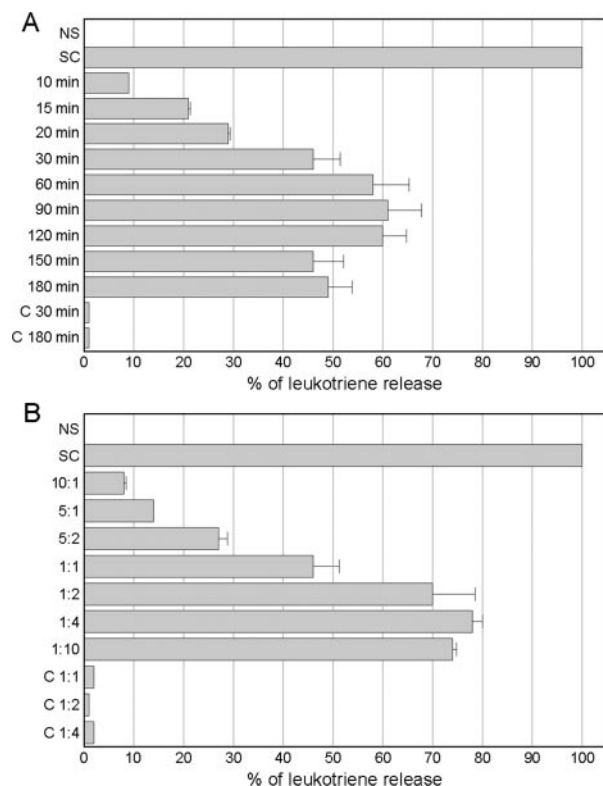


FIGURE 6. Time (A) and cellular ratio (B) dependence of sLT release. RBL-SX38 cells were challenged with J558L-mIgE cells. NS and SC are as in Fig. 2. C, Nontransfected J558L cells used as controls. A, Incubation times after cell-cell contact (5×10^4 cells each type) are indicated. B, Cellular ratios (RBL-SX38:J558L) are indicated (RBL-SX38 always 5×10^4 cells). Incubation time was 30 min for all ratios. Values are mean $\pm$ SD of three determinations from two independent experiments.

than the higher concentration, suggesting that mIg aggregation should be more efficient at an optimal molar ratio than at saturating ligand concentration.

Incubation with NIP-BSA immediately after cell-cell contact provided similar results, i.e., no difference was observed compared with noncross-linked mIgE (data not shown). Moreover, after 2 h of mIgE-FcεRI interaction, cells were washed, and fresh stimulation buffer containing anti-human IgE Abs was added. After additional 2 h, release of mediators was negligible (data not shown). The latter result might indicate that, after 2 h of cell-cell contact, RBL-SX38 cells were desensitized. Alternatively, mIgE-FcεRI cell-cell contact terminates within 2 h. Both possibilities are in agreement with the results shown in Fig. 6A. In conclusion, mIgE cross-linkers (added before, after, or at FcεRI encountering) do not influence FcεRI activation, demonstrating the Ag independence of mIgE effector potency.

Human peripheral blood basophils from nonallergic individuals are activated by mIgE

The reactivity of peripheral blood basophils toward mIgE⁺ cells was tested to verify that results obtained with RBL-SX38 cells could also be obtained with FcεRI⁺ cells from human blood. Experiments were performed monitoring the expression of CD203c, a specific marker of basophils and mast cells (33). Cell surface expression of CD203c increases upon cell stimulation, a feature successfully used to diagnose basophil activation (33). Human peripheral blood basophils were reacted either with anti-human IgE Abs or J558L-mIgE cells. Cells were stained with PE-conjugated

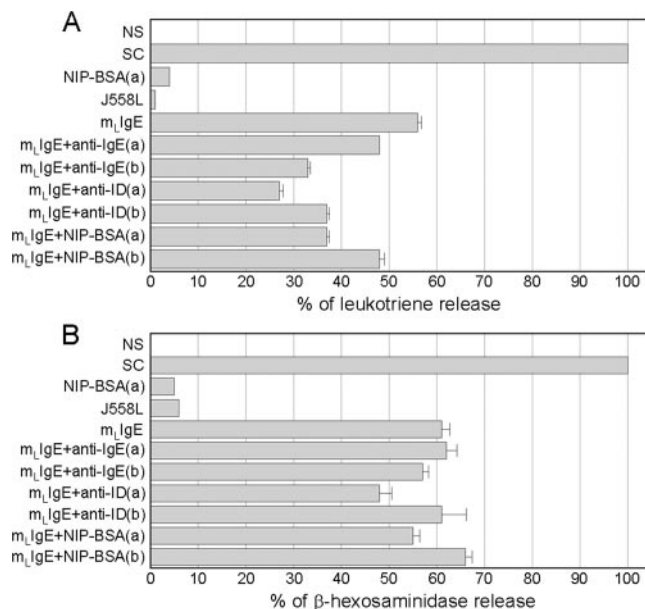


FIGURE 7. Ag independence of FcεRI activation by mIgE. sLTs (A) and β-hexosaminidase (B) release was monitored using various mIgE cross-linkers. NS and SC are as in previous figures. NIP-BSA, polyclonal anti-human IgE Abs, and a mAb recognizing the anti-NIP Id (ID) were used as cross-linkers at two different concentrations: a, 1 μg/ml and b, 10 μg/ml. NIP-BSA (1 μg/ml) and nontransfected J558L cells were used as controls. Values are mean $\pm$ SD of three determinations from two independent experiments.

anti-CD203c Abs and FITC-conjugated anti-mouse CD45R Abs. Stained cells were then analyzed on a flow cytometer using a gating strategy that excluded CD45R-labeled J558L cells and included only peripheral blood leukocytes and CD203c-labeled basophils. Anti-IgE-stimulated basophils differed clearly from nontreated basophils (Fig. 8). There was an even higher increase in the mean fluorescence intensity of basophils stimulated with J558L-mIgE cells, compared with basophils stimulated with nontransfected J558L cells. However, only a fraction of total basophils was activated. This is expected because activation requires direct contact with an mIgE⁺ cell, and, because the frequency of basophils was 1.5% or less of all leukocytes, the likelihood of basophil-mIgE⁺ cell contacts was low. Compared with nontreated basophils, a portion of the basophils incubated with nontransfected J558L cells showed activation, independent of the IgE system. Hence, CD203c expression on basophils activated by J558L-mIgE cells should be compared with that of basophils treated with nontransfected J558L cells. Therefore, the threshold of basophil activation by mIgE was set accordingly, excluding 98% of basophils treated with J558L cells (Fig. 8, gates R and A). Similar results were obtained from nonallergic individuals when testing sp2/0-tm₁LgE vs nontransfected sp2/0 cells (data not shown).

When treated with anti-IgE Abs, basophils from allergic individuals were activated. However, they did not show specific activation when incubated with mIgE⁺ cells, most likely reflecting a full FcεRI occupancy by serum IgE that prevents mIgE recognition (data not shown). These results confirm the data reported in Fig. 3, further suggesting a role for the mIgE-FcεRI interaction in physiological conditions or in the early developmental phase of allergic conditions.

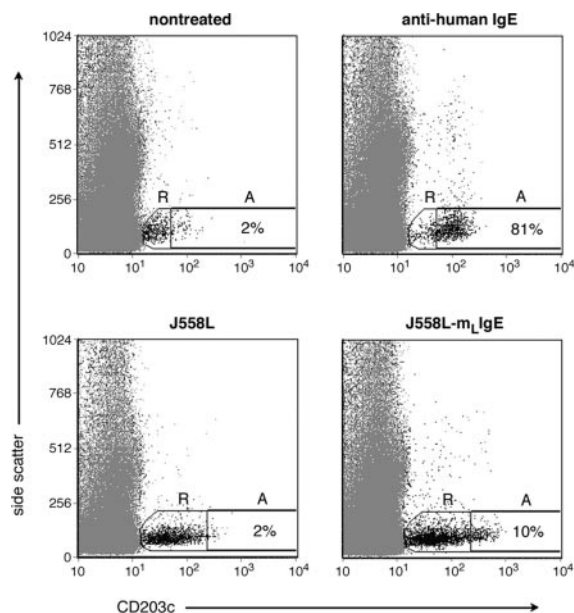


FIGURE 8. Human basophils from nonallergic individuals are activated by mIgE⁺ cells. Flow cytometric dot-plot analysis of whole blood leukocytes from a nonallergic individual. Basophil activation was monitored by the expression level of cell surface CD203c, detected by fluorescence of the PE-labeled anti-CD203c mAb. Nontreated samples and samples treated with anti-human IgE, J558L, and J558L-mL IgE cells are shown. Percentage of total basophils is reported in IgE-mediated basophil activation gates (A). Placement of A was chosen to exclude 98% (R) of the control populations (nontreated basophils and basophils treated with nontransfected J558L cells). Respective mean fluorescence intensity for R and A gates is: nontreated, 33 and 87; anti-human IgE, 83 and 104; J558L, 48 and 301; and J558L-mL IgE, 56 and 359. Identical experiments using whole blood from three other nonallergic individuals yielded similar results.

Human monocytes from allergic individuals release sLT upon contact with mIgE

The reactivity of freshly isolated peripheral blood monocytes toward mIgE⁺ cells was also investigated. Monocytes were obtained from three allergic (Fig. 9, lanes 1–3) and three nonallergic individuals (data not shown). Only monocytes from allergic individuals responded to IgE stimulation. After sensitization with NIP-specific sIgE and subsequent NIP-BSA administration, release of sLT was detected. When incubating monocytes only with sIgE or NIP-BSA, sLT release was basal, comparable to that obtained

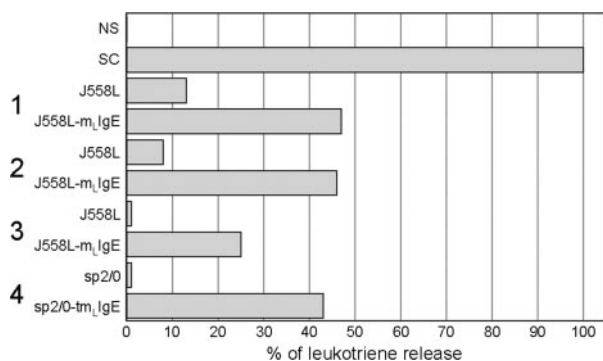


FIGURE 9. Human monocytes from allergic individuals release sLT upon contact with mIgE⁺ cells. NS, nonstimulated monocytes; SC, stimulation control obtained incubating monocytes with human anti-NIP sIgE and NIP-BSA. Nontransfected J558L and sp2/0 cells were used as controls. Lanes 1–4, Represent monocytes from four different allergic individuals.

from nonstimulated cells (data not shown). Challenging monocytes with J558L-mL IgE cells yielded substantial release of sLT, in the order of 25–45% of that obtained with NIP-BSA-aggregated sIgE. The contact with nontransfected J558L cells released significantly less sLT (p values <0.002), 1–13% of that induced by NIP-BSA-aggregated sIgE (Fig. 9). Monocytes from a fourth allergic individual were challenged with sp2/0-tmL IgE cells, providing results at all similar to the J558L-mL IgE-induced release (Fig. 9, lane 4).

Studies on a larger number of individuals are required to investigate the observed variability and the effect of serum IgE levels. Interestingly, our data cannot exclude the possibility that the observed sLT release would be induced by an interaction between mIgE and CD23. However, these preliminary data demonstrate that human monocytes from allergic individuals are capable of responding to mIgE⁺ cells, strengthening the potential *in vivo* relevance for mIgE interaction with its receptors.

Discussion

FcR binding by sIgs drives Ab-mediated cellular functions (34). mIgs present Fc receptor binding sites identical with those of sIgs, suggesting a functional role mediated by the BCR Fc region in the context of the B cell. Indeed, a direct mIgM-C1q interaction has been recently reported (35). However, mIgM-C1q binding occurs between a soluble (C1q) and a cellular (mIgM) ligand, while FcR binding to mIgs would take place among cell membranes. Structural resolution of IgE and IgG Fc moieties complexed with FcR soluble fragments (FcεRI and FcγRIII, respectively) (25, 36) did not reveal any obvious steric hindrance that could hamper receptor interaction with mIgs, an assumption partly proved by the reported mIgE accessibility to soluble FcεRIα (15, 16).

As a model to investigate structural and functional characteristics of this interaction, we used mouse B cell lines (transfected with different human mIgE constructs) and a rat basophilic leukemia cell line transfected with human FcεRI αβγ-chains (18). tmL IgE, an mL IgE truncated at the Cε2-Cε3 junction, was first analyzed. As expected, tmL IgE dimerized covalently, reached the cell surface, and bound to sdα, a soluble dimeric FcεRIα (16). Dimerization of tmL IgE should involve C328 (N-terminal to Cε3) (26) and two of the four εL extracellular membrane-proximal domain cysteines (17). Binding of tmL IgE to FcεRI excluded any possible hindrance caused by the IgE Fab regions in the membrane context, as they were absent. Inhibition due to the BCR accessory proteins Igα and Igβ could also be ruled out, because sp2/0 cells do not express the Igα/Igβ dimer (27). Next, the complete mL IgE, expressed in Igα/Igβ-negative J558L cells (28), was transported to the cell surface (9) and interacted with sdα. Finally, εBCRs (isoforms long and short) were found to bind soluble FcεRIα in the context of human B lymphocytes (15) or the B cell lines used in this work.

Strikingly, all mIgE variants were capable of inducing FcεRI-mediated β-hexosaminidase and sLT release by RBL-SX38 cells. The interaction with mIgE⁺ cells also caused a significant increase of $[Ca^{2+}]_i$ by RBL-SX38 cells. Moreover, specific coimmunoprecipitation of FcεRIα with mIgE provided compelling evidence for the formation of mIgE-FcεRIα complexes. These results indicated clearly that mIgE can bind to FcεRI during a specific cell-cell contact. mIgE cross-linking (using NIP-BSA, an anti-Id mAb, and anti-IgE Abs) activated FcεRI to a level comparable to that obtained in absence of cross-linking agents (monitored by mediator release), indicating that the mIgE-driven FcεRI activation is Ag independent. sIgE and sdα inhibited the mIgE-induced RBL-SX38 mediator release, designating mIgE as the sole determinant for FcεRI activation. Furthermore, human peripheral blood basophils and monocytes were used as an *in vitro* model for primary cells of

human origin. Our results demonstrate that these cells possess functional Fc receptors, recognized by mIgE. Conceivably, FcεRI ubiquity in the human immune system heralds a multifaceted role, including the recognition of mIgE.

To understand the membrane topology established during mIgE-FcεRI cell-cell contacts, data from similar systems have been considered. The seminal work by McConnell and colleagues (37, 38) exploited hapten-containing membrane targets to induce cell degranulation. However, those targets were recognized by the Ag binding sites of FcεRI-bound sIgE, whereas the present work discusses the Ag-independent cell-cell contact driven by the mIgE Fc portion. In a different approach, Yanagihara et al. (15) detected a down-regulation of ϵ transcripts in human mIgE⁺ B cells, subsequent to soluble FcεRIα challenge. This, together with the demonstration of mIgE binding by sdα (16), indicated a possible role played by the FcεRI interaction with mIgE. FcεRI-mIgE complexes may not necessarily exert a similar down-regulatory effect on IgE⁺ B cells. In fact, sIgE binding to cellular FcεRI and soluble FcεRIα binding to mIgE imply monomeric recognition. sIgE-FcεRI complexes require multivalent Ags to induce mast cell degranulation, whereas mIgE-FcεRI interaction is sufficient to cause similar effects. Seemingly, the membrane context is capable of promoting aggregation of the interacting proteins. Resting BCRs appear to be present on the cell membrane in the form of oligomers (39); hence, the existence of preclustered BCR homo-multimers could explain mIgE Ag independence for FcεRI activation. Alternatively, the cell-cell recognition environment could be sufficient to cluster mIgE and FcεRI into large aggregates. It is also possible that both conditions (oligomers and large aggregates) concur for the observed cell activation. Several membrane proteins associate with raft microdomains in physiology and disease (40). FcRs and BCRs locate into membrane rafts upon activation, yet in separate physiologic aggregation events (i.e., FcR upon challenge by soluble Ig-Ag complexes and BCR upon Ag encountering) (41, 42). Cell-cell contacts involving raft-associating immune receptors can lead to the formation of immune synapses (43). Dendritic cells and B cells form immune synapses via soluble immune complexes (Ig-Ag) bridging Fc receptors to BCRs, respectively (44, 45). Overall, the adaptive immune system regulation could occur through the formation of a set of different immune synapses, depending on the cell differentiation route (46). According to the aspects discussed above, it is plausible to hypothesize the formation of an immune synapse upon a direct ϵ BCR-FcεRI recognition.

Analyzing the results obtained preincubating RBL-SX38 cells with sIgE, it appears that mIgE-FcεRI-mediated cell-cell contacts would be allowed in normal individuals (e.g., IgE serum levels ~50 ng/ml, 2.5×10^{-10} M). On the contrary, in atopic patients with IgE serum levels of up to 1 μ g/ml (5×10^{-9} M), the interaction should be severely inhibited. Interestingly, allergic individuals presenting serum IgE levels well below 1 μ g/ml would allow mIgE-FcεRI recognition. Following, the demonstration of the mIgE-FcεRI functional relevance in vivo is now needed. Although circulating mIgE⁺ B cells are rare and difficult to visualize, we are currently characterizing the effects elicited by FcεRI binding to mIgE on human B cells. The wide FcεRI cell expression in humans could present specific mIgE-FcεRI recognition microenvironments, such as those occurring between dendritic and B cells at germinal centers or others that might be present in mucosal-associated lymphoid tissues. Previously unexplored scenarios can be pictured considering the functional effects triggered in mIgE⁺ B cells. It is tempting to speculate that a powerful signaling generated inside the B cell by the mIgE-FcεRI interaction could be capable of inducing apoptosis, similar to the effect produced by anti-IgE mAbs (47). Death of mIgE⁺ B cells induced by FcεRI

could concur to establish the low IgE levels, compared with all other Ig isotypes. Equally intriguing, a cell-cell mIgE-FcεRI-driven contact between mIgE⁺ B cells and mast cells or basophils could play a role in T cell-independent B cell regulation. Indeed, mast cells and basophils can induce B cell class switch and IgE production by expressing IL-4 and CD40L (48), a feature induced by the allergen cross-linking of FcεRI-bound sIgE (49). This could be relevant in peripheral districts, in which local IgE production has been detected (50, 51). The situations outlined above are not mutually exclusive and may be part of a previously unknown cross talk involved in IgE homeostasis.

Recalling the low affinity receptor for IgE (CD23) and its well-known importance in IgE regulation (2), it is clear that mIgE molecules possess intact CD23 binding sites, potentially as efficient as those for FcεRI, a consideration to bear in mind for future research in the field.

In conclusion, the mIgE-FcεRI interaction and its functional implications provide a new feature in the complex regulation of the immune response to parasites and allergens. Furthermore, the extension of our findings to a general BCR-FcR interaction would represent a new paradigm in immunology. In the case of mIgG, a whole array of unexplored cell-cell interactions involving FcγRs could be envisaged, together with a role played by a lateral interaction between FcγRIIb and mIgG in B lymphocyte physiology.

Acknowledgments

We thank Alberto Albanese and Daniele Zacchetti for help with calcium imaging data analysis.

Disclosures

The authors have no financial conflict of interest.

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