Regulation of High-affinity IgE Receptor-mediated Mast Cell Activation by Murine Low-affinity IgG Receptors

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Abstract

Allergic symptoms result from the release of granular and lipidic mediators and of cytokines by inflammatory cells. The whole process is initiated by the aggregation of mast cell and basophil high-affinity IgE receptors (FcεRI) by IgE and antigen. We report here that IgE-induced release of mediator and cytokine can be inhibited by cross-linking FcεRI to low-affinity IgG receptors (FcγRII) which are constitutively expressed on mast cells and basophils. Using a model of stable transfectants in RBL-2H3 cells expressing endogeneous rat FcεRI and recombinant murine FcγRII, we showed that inhibition requires that FcεRI be cross-linked to FcγRII by the same multivalent ligand. Inhibition of cross-linked receptors left non–cross-linked FcεRI capable of triggering mediator release and was reversible upon disengagement. Both isoforms of wild-type FcγRII were equally capable of inhibiting FcεRI-mediated mast cell activation provided they had an intact intracytoplasmic domain. Our results demonstrate that mast cell secretory responses triggered by high-affinity receptors for IgE may be controlled by low-affinity receptors for IgG. This regulation of FcεRI-mediated mast cell activation is of potential interest in mast cell physiology and in allergic pathology. (J. Clin. Invest. 1995. 95:577–585.) Key words: Fcγ receptors II • Fcε receptors I • immune complexes • mediators and cytokines • allergy

Introduction

Allergic manifestations are initiated by the aggregation of high-affinity IgE receptors (FcεRI)[1] expressed on mast cells and basophils, when FcεRI-bound IgE antibodies bind multivalent allergens (1). FcεRI are multichain-receptors composed of an IgE-binding α subunit, associated with a four-transmembrane domain β subunit and a homodimeric γ subunit (2). A consensus tyrosine-containing activation motif, in intracytoplasmic (IC) domains of FcεRIβ and γ (3), is thought to account for the cell-activating properties of FcεRI (4).

Mast cells (5, 6) and basophils (7, 8) also express low-affinity IgG receptors (FcγR). Depending on their subtype, mouse mast cells express FcγRII and FcγRIII in variable proportions (6). FcγRIII are multichain-receptors composed of a IgG-binding α subunit (9), associated in mast cells with the same β (10) and γ (11) subunits as FcεRIα. When aggregated by multivalent IgG immune complexes, FcγRIII also trigger the release of inflammatory mediators (12) and cytokines (13). Under the same conditions, FcγRII do not (12, 13). FcγRII are single-chain receptors which exist as two isoforms generated by alternative splicing of sequences of the first IC exon, FcγRIIb1 and FcγRIIb2, which differ by a 47–amino acid insertion in the IC domain of FcγRIIb1 (9). Human basophils express FcγRII (8), but no FcγRIII, and they failed to release histamine when challenged with anti-FcγRII (C. Lawrence and M. Daëron, unpublished data). Ancient experiments aiming at demonstrating the ability of IgG immune complexes to trigger histamine release in human basophils were contradictory (14, 15). Human FcγRII are of two types: FcγRIIA/C and FcγRIIB. FcγRIIA/C are single-chain receptors the IC domain of which possesses a tyrosine-containing activation motif (9) with cell-triggering capability (16). FcγRIIB resemble closely murine FcγRII and they share a common IC aminoacid sequence (9).

Evidence that FcR aggregation is a necessary and sufficient signal for triggering a cellular response (17–20) was based on studies using multivalent ligands specific of a single FcR. Most cells, however, express several types of FcR and antibodies of more than one isotype are usually produced together against a given antigen. When antigen reaches FcεRI-bound IgE antibodies on mast cells, it is therefore probably complexed to IgG antibodies, if IgG antibodies were generated against the same antigen. This is likely to occur during immunotherapy in allergic patients, when high titers of anti-allergen IgG antibodies have been raised (21), but also during a normal immune response. Under these conditions, FcεRI are therefore not only aggregated, they are also cross-linked to adjacent FcγR. In the present work, we examined the consequences of cross-linking FcεRI to FcγRII on IgE-induced mast cell activation.

We found that, when cross-linked to FcεRI, both FcγRII isoforms inhibit mediator and cytokine release triggered by FcεRI aggregation and that inhibition depends on FcγRII intracytoplasmic sequences common to FcγRIIb1 and b2. We showed that inhibition requires FcεRI-FcγRII cross-linking, affects only crosslinked FcεRI and is reversible. Our results provide a new hypothesis for the development of allergic manifestations and offer a possible mechanism for desensitization in

1. *Abbreviations used in this paper:* BCR, B cell receptor; BMMC, bone marrow-derived mast cells; FcεRI. high-affinity receptors for the Fc portion of IgE; FcγRII and III, murine type II and III low-affinity receptors for the Fc portion of IgG; IC, intracytoplasmic; MAR, mouse anti-rat immunoglobulins; RAM, rat anti-mouse immunoglobulins; rIgE, rat IgE.

allergic patients. Because mediator release could be abrogated in mast cells previously sensitized with IgE, they suggest new therapeutic approaches of allergic diseases.

Methods

Antibodies, antibody fragments, and antigens. The rat monoclonal IgE LO-DNP-30 and IR162 were from IMEX (Université de Louvain, Bruxelles, Belgium). The mouse IgE anti-DNP 2682-I mAb was a gift of Dr. Ulrich Blank (Institut Pasteur, Paris). It was purified from culture supernatant of a subclone of DNP-H1-ϵ-26 hybridoma cells (22). The mouse IgE 14.205 A1 mAb (23) was purified by immunoaffinity chromatography from ascitic fluid, generously offered by Dr. Colette Kanellopoulos (Institut Jacques Monod, Paris). IgE-rich anti-ovalbulin serum raised in the Copacabana strain of Swiss mice was kindly provided by Dr. Annie Prouvost-Danon (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil). Rat and mouse IgE bound equally well to rat and mouse mast cell FcϵRI. Mouse IgG1 anti-DNP mAb, purified on Protein G-sepharose, was a gift of Dr. Jacques Couderc (Institut Curie, Paris). Unlabeled and FITC-labeled polyclonal mouse anti–rat Ig (MAR) F(ab')2 fragments, as well as rat anti–mouse Ig (RAM) F(ab')2 fragments were from Jackson Immunoresearch Laboratories (West Grove, PA). MAR F(ab')2 reacted with rat Ig, but not with mouse Ig, and RAM F(ab')2 reacted with mouse Ig, but not with rat Ig. This was checked both by indirect immunofluorescence and by serotonin release on RBL cells sensitized with mouse and rat IgE respectively. The anti-FcγRII/III 2.4G2 mAb (24) was purified by affinity-chromatography on Protein G–sepharose from ascitic fluid of nude mice inoculated with 2.4G2 hybridoma cells intraperitoneally. F(ab')2 fragments were obtained by pepsin digestion for 48h. The purity of 2.4G2 IgG and F(ab')2 fragments was assessed by SDS-PAGE analysis. 2.4G2 reacted with mouse FcγR, but not with rat FcγR.

Heteroconjugates made by chemically crosslinking Fab fragments of 2.4G2 and affinity-purified rabbit polyclonal anti-DNP antibodies (referred to as [2.4G2 × a-DNP] Fab2) were described previously (25). (2.4G2 × a-DNP) Fab2 bind to FcγRII via the 2.4G2 Fab moiety and to dinitrophenyl conjugates via the anti-DNP Fab moiety. They were a generous gift of Dr. David M. Segal (NIH, Bethesda, MD).

Mouse IgE 14.205 A1, BSA (Sigma Chemical Co., St. Louis, MO) and ovalbumin (Sigma Chemical Co.) were dinitrophenylated using 2-,4-dinitrobenzene sulfonic acid (Eastman Kodak Co., Rochester, NY). After passage over Sephadex G25 (Pharmacia, Uppsala, Sweden), the average substitution numbers of dinitrophenylated proteins were 7.5 moles DNP/mole IgE, 13 moles DNP/mole BSA and 4 moles DNP/mole ovalbumin. Rat IgE IR162 was biotinylated using the kit from Sigma Chemical Co. Streptavidin was from Pierce (Rockford, IL), L-lysine and N-ϵ-DNP-L-lysine from Sigma Chemical Co.

Cells. BMMC were prepared from BALB/c bone marrow cells as described (26). They were maintained in culture in RPMI medium (Seromed, Biochrom A. G., Berlin, Germany) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50% conditioned medium from WEHI-3 cells. P815 mouse mastocytoma cells and L929 cells were cultured in RPMI supplemented with 10% FCS, 100 IU/ml penicillin and 100μg/ml streptomycin. RBL-2H3 cells (27) were cultured in DMEM supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Only adherent RBL cells were used. They were recovered with trypsin-EDTA. Except RPMI, all culture reagents were from GIBCO BRL (Paisley, Scotland, UK).

Immunoprecipitation of iodine-labeled mast cells. BMMC or P815 cells were ^{125}I-labeled using lactoperoxydase. Membrane FcγR were precipitated with 2.4G2 and analyzed by SDS-PAGE under reducing conditions, before and after endoglycosidase-F (Bohringer Biochimica, Mannheim, Germany) treatment as previously described (6).

cDNA constructions and transfectants. RBL-2H3 cells stably transfected with cDNAs encoding murine FcγRIIb1 (28) and FcγRIIb2 (29), IC domain-deleted FcγRIIb2(IC1) (30), or chimeric FcγRII-ICIIIα (12) were described previously (12). All transfectants used were cloned as described (12). They were maintained in culture in the presence of 250 μg/ml G418 (Geneticin; GIBCO BRL) and the expression of recombinant receptors on clones remained stable over the duration of experiments.

Immunofluorescence. Nontransfected and transfected RBL-2H3 cells were incubated for 1 h at 0°C with 10 μg/ml rat monoclonal IgE LO-DNP-30 or 2.4G2 mAb in HBSS containing 5% FCS, or with medium alone. Cells were washed and stained by being incubated for 30 min at 0°C with 50 μg/ml FITC-labeled MAR F(ab')2. Fluorescence was analyzed by flow cytometry using a FACScan (Becton-Dickinson, Mountain View, CA).

Serotonin release. Transfected or nontransfected RBL-2H3 cells, resuspended in RPMI medium supplemented with 10% FCS at 1 × 10^6 cells/ml, were incubated at 37°C for 1 h. with 2 μCi/ml [^{3}H]serotonin (Amersham, Les Ulis, France), washed, resuspended in RPMI-FCS, incubated for another hour at 37°C, washed again, resuspended in the same medium and distributed in 96-well microculture plates at 2 × 10^5 cells/well and incubated for 1 h at 37°C with IgE in a final volume of 50 μl. Adherent cells were washed four times with 200 μl HBSS (GIBCO BRL), 25 μl culture medium were added to each well and cells were warmed at 37°C for 15 min before challenge. Cells were challenged for 30 min at 37°C with 25 μl of MAR F(ab')2, RAM F(ab')2, DNP-BSA or streptavidin, previously warmed at 37°C for 15 min. Reactions were stopped by adding 50 μl ice-cold medium and by placing plates on ice. 50 μl of supernatants were mixed with 200 μl Aqualuma-Plus scintillation fluid (Lumac, The Netherlands) and counted in a β-plate counter (Pharmacia, Uppsala, Sweden). The percentage of serotonin release was calculated using as 100% cpm contained in 50 μl harvested from wells containing the same number of cells that were lysed in 100μl of 0.5% SDS and 0.5% NP40.

BMMC were sensitized by an overnight incubation at 37°C with IgE-rich anti-ovalbumin immune serum before being loaded with [^{3}H]-serotonin under the same conditions as RBL cells. After washings and warming, cells were challenged for 10 min at 37°C with DNP-ovalbumin in Eppendorf tubes.

TNF release and assay. Aliquots of 7 × 10^5 RBL cells, previously sensitized by a 1-h incubation at 37°C with indicated concentrations of IgE, were resuspended in 150 μl culture medium and incubated for 3 h at 37°C with 50 μg/ml MAR F(ab')2 fragments. Cell-free supernatants were harvested and assayed for TNFα. TNFα was measured by a cytotoxic assay on the TNFα-sensitive L929 cells as described (13). Briefly, 3 × 10^4 L929 cells were plated in each well of 96-well tissue culture plates. After a 20-h incubation at 37°C, culture medium was replaced by serial twofold dilutions of cell-free supernatants from stimulated cells. Supernatants were diluted in complete medium containing 1.5 μg/ml actinomycin D (Boehringer). After a 20-h incubation at 37°C, medium was removed, adherent cells were stained with 0.2% crystal-violet (Sigma Chemical Co.) in 1% ethanol for 15 min, wells were washed, dried, and the absorbance was measured at 570 nm using a Titerteck spectrophotometer (Labsystems, Helsinki, Finland).

Results

Inhibition of IgE-induced secretory responses by complexation of antigen to IgG antibodies in FcγRII-expressing mast cells. Bone marrow–derived mast cells (BMMC), sensitized with polyclonal IgE anti-ovalbumin (ova), released serotonin upon challenge with DNP-ova (Fig. 1 *a*). Nonsensitized BMMC did not release serotonin when challenged with DNP-ova or with DNP-ova complexed to monoclonal IgG1 anti-DNP (Fig. 1 *a, open circles*). DNP-ova–induced IgE-dependent serotonin release was found to decrease progressively if DNP-ova was complexed to increasing concentrations of IgG1 anti-DNP (Fig. 1 *a, closed symbols*). Inhibition was also observed when DNP-ova was complexed with dilutions of ascitic fluid of the same IgG1 anti-DNP hybridoma (not shown). The same was ob-
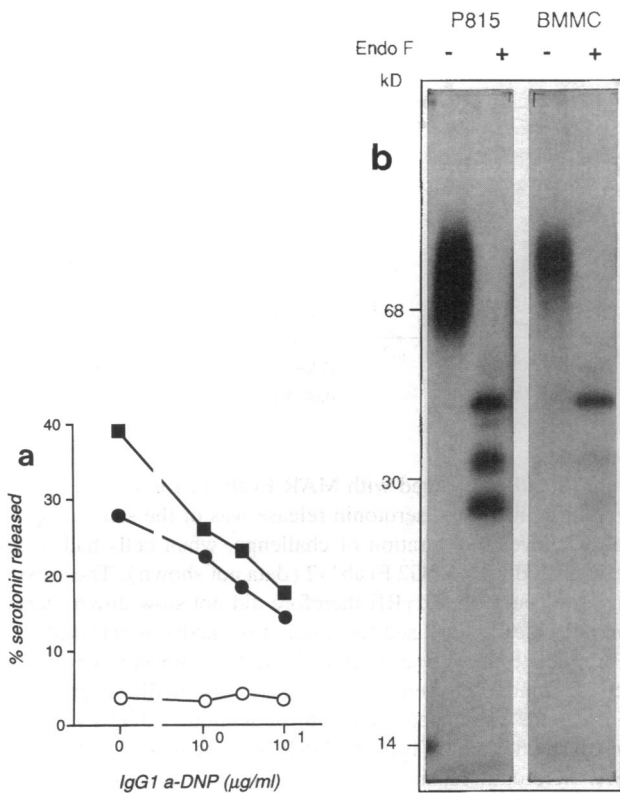
Figure 1. Inhibition of IgE-induced bone marrow-derived mast cell (BMMC) activation by IgG immune complexes and FcγR expressed by BMMC. (a) BMMC were sensitized or not with polyclonal mouse IgE anti-ova by an overnight incubation at 37°C with IgE-rich anti-ovalbumin serum diluted 1/1000 (squares) or 1/3000 (closed circles), or with culture medium only (open circles). Cells were challenged for 10 min at 37°C with 20 μg/ml DNP-ova, not complexed, or complexed with indicated concentrations of purified IgG1 anti-DNP mAb. The figure shows the percentage of total radioactivity released in supernatants. (b) Membrane FcγR were precipitated from ^{125}I-labeled BMMC and P815 cells with 2.4G2 and examined by SDS-PAGE under reducing conditions, before and after deglycosylation with endoglycosidase F.
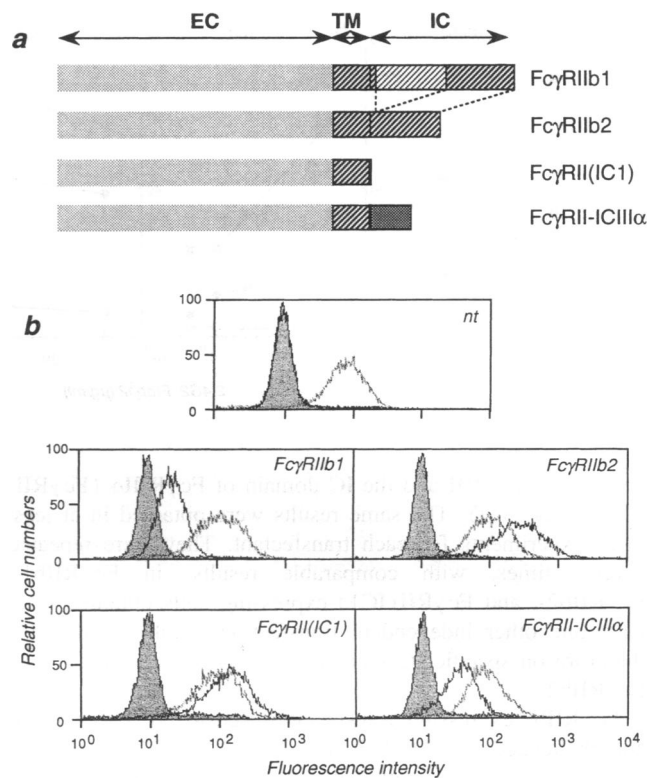
Figure 2. Structure and expression of wild-type and mutant murine FcγRII in RBL-2H3 cells. (a) The figure schematizes the structure of recombinant murine FcγRIIb1, FcγRIIb2, IC domain-deleted FcγRII (IC1), or chimeric FcγRII-ICIIIα encoded by the cDNAs transfected into RBL-2H3 cells. EC, extracellular; TM, transmembrane; and IC, intracytoplasmic domains. (b) Clones of stable transfectants were examined by immunofluorescence for the expression of FcεRI and murine FcγR. The binding of the rat monoclonal IgE LO-DNP-30 (open gray histograms) and of 2.4G2 (open black histograms) to nontransfected RBL-2H3 cells (nt) and to RBL-2H3 cells transfected with cDNA encoding wild-type FcγRIIb1 and FcγRIIb2, deleted FcγRII(IC1) or chimeric FcγRII-ICIIIα was examined by indirect immunofluorescence with FITC-labeled (MAR) F(ab')2. Shaded histograms represent the fluorescence of cells incubated with FITC-MAR F(ab')2 only.

served in three other experiments of the same design, on BMMC sensitized with monoclonal IgE anti-DNP and challenged with DNP-BSA complexed to IgG1 anti-DNP (not shown). Antigen complexation to IgG antibodies therefore decreased IgE-mediated serotonin release by mouse mast cells.

Low-affinity IgG receptors expressed by BMMC were immunoprecipitated by the rat anti-mouse FcγRII/III mAb 2.4G2 and examined by SDS-PAGE analysis before and after endoglycosidase-F treatment. Deglycosylated polypeptides with an apparent m.w. of 37, 32, and 28 kD were precipitated from the control mouse mastocytoma cells P815. They were previously identified as FcγRIIb1, FcγRIIb2, and FcγRIIIα respectively (6, 31). Confirming our previous results (6), large amounts of FcγRIIb1 were precipitated from BMMC, but only traces of FcγRIIb2 and FcγRIIIα (Fig. 1 b). These results altogether raised the possibility that the crosslinking of FcγRIIb1 to FcεRI by immune complexes could inhibit IgE-induced mast cell activation and the subsequent secretory response.

Inhibition of IgE-induced secretory responses by cross-linking FcεRI and FcγRII in RBL-2H3 transfectants. The latter hypothesis was tested using Rat Basophilic Leukemia cells

(RBL-2H3) stably transfected with cDNAs encoding wild-type, deleted or chimeric murine FcγRII (Fig. 2 a). The surface expression of murine FcγRII and of endogenous FcεRI was assessed by indirect immunofluorescence with 2.4G2 and with rat monoclonal IgE respectively (Fig. 2 b). The expression of FcγRII was checked again, systematically, in all subsequent experiments. When sensitized with rat IgE, all transfectants released serotonin upon challenge with MAR F(ab')2 (Fig. 3). When preincubated with 2.4G2 F(ab')2 fragments, no transfectant released serotonin above background upon challenge with MAR F(ab')2 (Fig. 3, open circles). The effect of cross-linking FcγRII to FcεRI was searched for by studying serotonin release triggered by MAR F(ab')2 in RBL transfectants sensitized with rat IgE, and incubated with increasing concentrations of F(ab')2 fragments of the rat mAb 2.4G2 (Fig. 3, closed symbols). IgE-induced serotonin release was inhibited by crosslinking FcεRI to FcγRIIb1 (Fig. 3 a) or FcγRIIb2 (Fig. 3 b), but not to FcγRII deleted of all but one intracytoplasmic aminoacids (FcγRII[IC1], Fig. 3 c) or to chimeric receptors having the extracellular and transmembrane
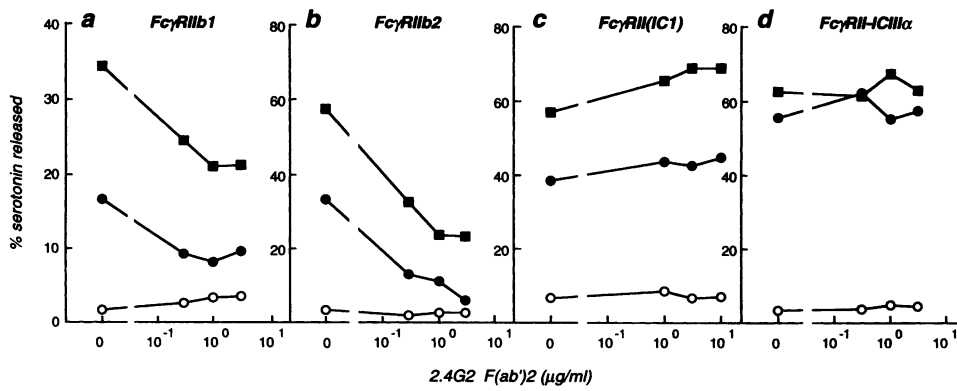
Figure 3. Inhibition of IgE-induced serotonin release by wild-type FcγRII in RBL transfectants. RBL transfectants expressing wild-type FcγRIIb1 (*a*) or FcγRIIb2 (*b*), deleted FcγRII-(IC1) (*c*) or chimeric FcγRII-ICIIIα (*d*) were incubated for 1 h at 37°C with 0 (*open circles*), 0.1 (*closed circles*), or 0.3 (*squares*) μg/ml rat IgE LO-DNP-30 and with indicated concentrations of 2.4G2 F(ab')2. After being washed, cells were challenged for 30 min at 37°C with 50 μg/ml MAR F(ab')2, and serotonin released in supernatants was measured.

domains of FcγRII and the IC domain of FcγRIIIα (FcγRII-ICIIIα, Fig. 3 *d*). The same results were obtained in at least three experiments for each transfectant. They were repeated several times, with comparable results, in FcγRIIb1-, FcγRIIb2-, and FcγRII(IC1)-expressing cells obtained in at least one other independent transfection. Inhibition depends therefore on specific IC sequences common to FcγRIIb1 and FcγRIIb2.

FcγRIIb2-expressing transfectants, sensitized with rat IgE and challenged with MAR F(ab')2 for 3 h at 37°C, produced TNFα that could be quantitated by assaying cytotoxicity of cell-free supernatants on L929 cells. Cytotoxicity depended on the concentration of IgE used for sensitization (Fig. 4, *open circles*). It was markedly reduced if cells were incubated with 2.4G2 F(ab')2 before challenge with MAR F(ab')2 (Fig. 4, *closed symbols*). Like inhibition of serotonin release, inhibition of TNF production was proportional to the dilution of IgE used for sensitization. IgE-induced mediator release and cytokine production were therefore both inhibited by crosslinking FcεRI to FcγRII.

Inhibition of IgE-induced mediator release requires FcεRI-FcγRII cross-linking. In order to determine whether the decreased serotonin release might result from a change in the kinetics of the release process, FcγRIIb2-expressing cells were sensitized with rat IgE in the presence or absence of 2.4G2

F(ab')2 and challenged with MAR F(ab')2 for 15, 30, 45, or 60 min. Inhibition of serotonin release was of the same magnitude whatever the duration of challenge, when cells had been preincubated with 2.4G2 F(ab')2 (data not shown). The cross-linking of FcεRI to FcγRII therefore did not slow down secretion but indeed decreased the amount of mediator released.

In order to determine whether FcγRII occupancy would affect IgE-induced serotonin release, FcγRIIb2-expressing transfectants were incubated with or without 2.4G2 F(ab')2, sensitized with mouse IgE anti-DNP, and challenged with DNP-BSA. Serotonin release was of the same magnitude in cells preincubated or not with 2.4G2 F(ab')2 (data not shown). Ligand binding to FcγRII (binding of 2.4G2 was checked by indirect immunofluorescence) therefore had no inhibitory effect. To determine whether an independent FcγRII aggregation would affect IgE-induced serotonin release, FcγRIIb2-expressing transfectants were incubated with 2.4G2 F(ab')2 and sensitized with mouse IgE anti-DNP. They were washed, preincubated for various periods of time at 37°C with MAR F(ab')2, and finally challenged with DNP-BSA (Fig. 5). Compared to cells not preincubated with MAR F(ab')2 (*open symbols*), serotonin release induced by DNP-BSA was unaffected in the presence of MAR F(ab')2, whatever the duration of the preincubation with MAR F(ab')2 (*closed symbols*). The aggregation of FcγRII therefore had no effect on IgE-induced serotonin
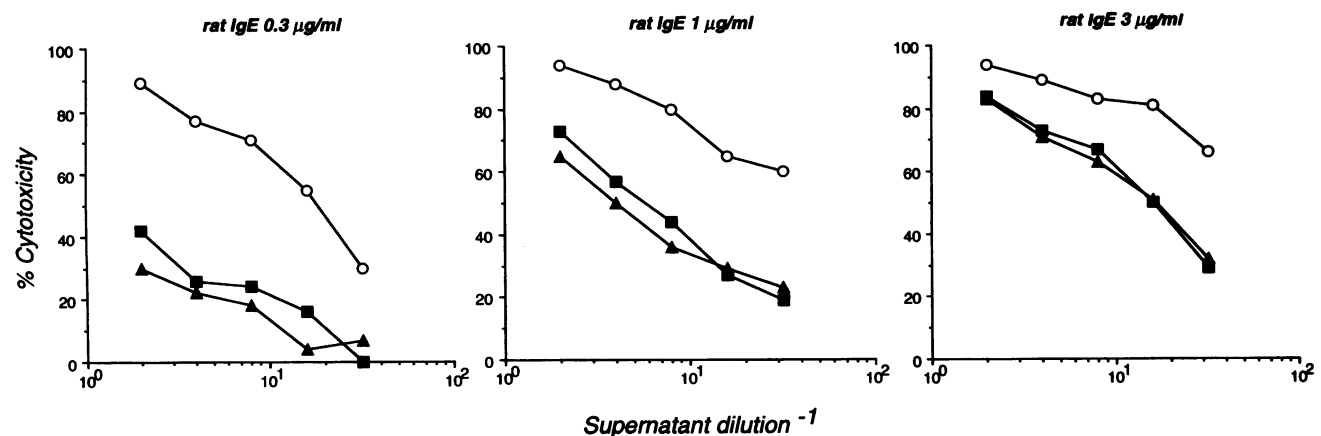

Figure 4. Inhibition of IgE-induced TNFα production by RBL transfectants expressing FcγRIIb2. FcγRIIb2-expressing transfectants were sensitized with 0.3, 1, or 3 μg/ml rat IgE LO-DNP-30, in the presence of 0 (*open circles*), 1 (*closed squares*), or 10 (*closed triangles*) μg/ml 2.4G2 F(ab')2. They were challenged with 50 μg/ml MAR F(ab')2 for 3 h at 37°C. Cell-free supernatants were harvested and twofold dilutions were tested for cytotoxicity on L929 cells. The figure represents the percentage of cytotoxicity as a function of the dilution of supernatants.
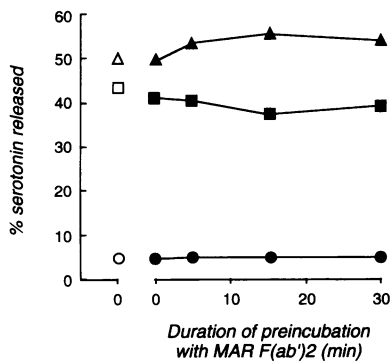
Figure 5. Crosslinking requirement for FcγRIIb2-mediated inhibition of IgE-induced serotonin release. FcγRIIb2-expressing transfectants were sensitized with 0 (*circles*), 0.03 (*squares*), or 0.1 (*triangles*) μg/ml mouse IgE anti-DNP 2682-I in the presence of 3 μg/ml 2.4G2 F(ab')2. Cells were then preincubated with 0 (*open symbols*) or 50 (*closed symbols*) μg/ml MAR F(ab')2 for indicated times at 37°C before they were washed and challenged with 3 μg/ml DNP-BSA for 30 min.

release, even if achieved 30 min before challenge with antigen. No effect on IgE-induced serotonin release was seen following an independent FcγRII aggregation in two other experiments.

In above experiments, FcεRI were cross-linked to FcγRII by the same reagent that was used also for challenge (DNP-BSA or MAR F[ab']2, depending on the IgE). Under these conditions, FcεRI-FcγRII cross-linking and FcεRI aggregation were simultaneous. To dissociate the two events, FcγRIIb2-expressing transfectants were sensitized with a dinitrophenylated mouse monoclonal IgE, not directed against DNP, 14.205 A1, (DNP-mIgE) that could be subsequently cross-linked to FcγRII with heteroaggregates made of 2.4G2 Fab fragments and rabbit anti-DNP Fab fragments (referred to as [2.4G2 × a-DNP] Fab2), without being aggregated. Cells were then challenged with rat anti-mouse Ig (RAM) F(ab')2 to aggregate FcεRI. Serotonin release induced by RAM F(ab')2 was abolished, whatever the concentration of DNP-mIgE used for sensitization, if cells were preincubated with as little as 0.3 μg/ml (2.4G2 × a-DNP) Fab2 (Fig. 6 *a*).

Nontransfected cells and transfectants expressing FcγRIIb1 or FcγRII(IC1) were sensitized by the same DNP-mIgE, incubated with or without (2.4G2 × a-DNP) Fab2, and challenged with RAM F(ab')2. When not exposed to (2.4G2 × a-DNP) Fab2, all three cells released serotonin upon challenge. Serotonin release was abolished if FcγRIIb1-expressing transfectants, but not FcγRII(IC1)-expressing transfectants or nontransfected cells, were preincubated with (2.4G2 × a-DNP) Fab2. Under the same conditions, (2.4G2 × a-DNP) Fab2 did not inhibit serotonin release by FcγRIIb1-expressing transfectants sensitized with the same IgE, but not dinitrophenylated (Fig. 6 *b*). Identical results were seen in at least three experiments of the same design performed with each transfectant. FcεRI-FcγRII crosslinking, prior to FcεRI aggregation, therefore abrogated subsequent serotonin release.

Inhibition reversibly affects only FcεRI cross-linked to FcγRII. The possibility to dissociate FcεRI-FcγRII crosslinking from FcεRI aggregation enabled to examine whether inhibition would affect only crosslinked FcεRI or whether it would affect also other FcεRI. This was achieved by sensitizing mast cells with two types of IgE which could be cross-linked to FcγRII or aggregated independently. When sensitized with dinitrophenylated mouse IgE 14.205 A1 (DNP-mIgE), FcγRIIb2-expressing transfectants released serotonin upon challenge with RAM F(ab')2 (Fig. 7 *a, open circles*), but not with streptavidin

(not shown). Serotonin release was inhibited if cells were preincubated with (2.4G2 × a-DNP) Fab2 (Fig. 7 *a, closed circles*). When sensitized with biotinylated rat IgE IR162 (Biot-rIgE), FcγRIIb2-expressing transfectants released serotonin upon challenge with streptavidin (Fig. 7 *b, open circles*), but not with RAM F(ab')2 (not shown). Serotonin release was not affected if cells were preincubated with (2.4G2 × a-DNP) Fab2 (Fig. 7 *b, closed circles*). When sensitized with a mixture of DNP-mIgE and Biot-rIgE, FcγRIIb2-expressing transfectants released serotonin upon challenge with either RAM F(ab')2 or streptavidin, and a dose-dependent competition between the two IgE used for sensitization could be seen (Fig. 7 *c–f, open circles*). Serotonin release induced by RAM F(ab')2 (Fig. 7 *c* and *e, closed circles*), but not serotonin release induced by streptavidin (Fig. 7 *d, and f, closed circles*), was abolished if cells were incubated with (2.4G2 × a-DNP) Fab2 prior to challenge. It follows that inhibition was restricted to FcεRI crosslinked to FcγRII and left non–cross-linked FcεRI fully capable of triggering mediator release upon aggregation. The same conclusion could be drawn from two additional experiments of the same design performed with the same reagents.

Finally, we wondered whether inhibition observed upon FcεRI-FcγRII cross-linking would be reversed upon cross-linking disengagement in the presence of an excess of monovalent hapten. FcγRIIb2-expressing transfectants were sensitized with dinitrophenylated mouse IgE 14.205 A1 (DNP-mIgE) that was subsequently cross-linked or not cross-linked to FcγRIIb2 with (2.4G2 × a-DNP) Fab2, as in above experiments. Cells were then incubated for increasing periods of time at 37°C with an excess of DNP-lysine or, as a negative control, of lysine, before they were challenged with RAM F(ab')2. As expected, serotonin release was inhibited when cells not exposed to hapten were incubated with (2.4G2 × a-DNP) Fab2 (Fig. 8, *open circles*). DNP-lysine (*black circles*) had no effect on serotonin release induced by the aggregation of DNP-mIgE not cross-linked to FcγRIIb2 (broken line), but it reduced by 50% inhibition of serotonin release induced by aggregating DNP-mIgE crosslinked to FcγRIIb2 (*solid line*). The same molar concentration of lysine (*grey circles*) had no effect on inhibition (*solid line*). Comparable results were obtained in two other experiments. This indicates that FcεRI whose triggering ability was previously abolished recovered from inhibition upon cross-linking disengagement. Inhibition therefore is reversible and requires that FcεRI-FcγRII cross-linking be maintained.

Discussion

Our results demonstrate that mast cell activation via high-affinity receptors for IgE may be controlled by low-affinity receptors for IgG. This control was effective whenever FcεRI were cross-linked to FcγRII with an intact IC domain, irrespectively of the cross-linking ligand. It required FcεRI-FcγRII cross-linking, affected only FcεRI crosslinked to FcγRII and was reversible upon cross-linking disengagement. Such a regulation might be of physiological and of clinical relevance.

Inhibition was observed in RBL transfectants, when endogenous rat FcεRI were cross-linked to recombinant murine FcγRIIb1 or to FcγRIIb2. Inhibition was not due to a competition between IgE and 2.4G2 for MAR F(ab')2 or to extracellular steric hindrance for the following two reasons: (*a*) the MAR F(ab')2 concentration used for crosslinking IgE and 2.4G2 F(ab')2 was 25-fold higher than the concentration sufficient
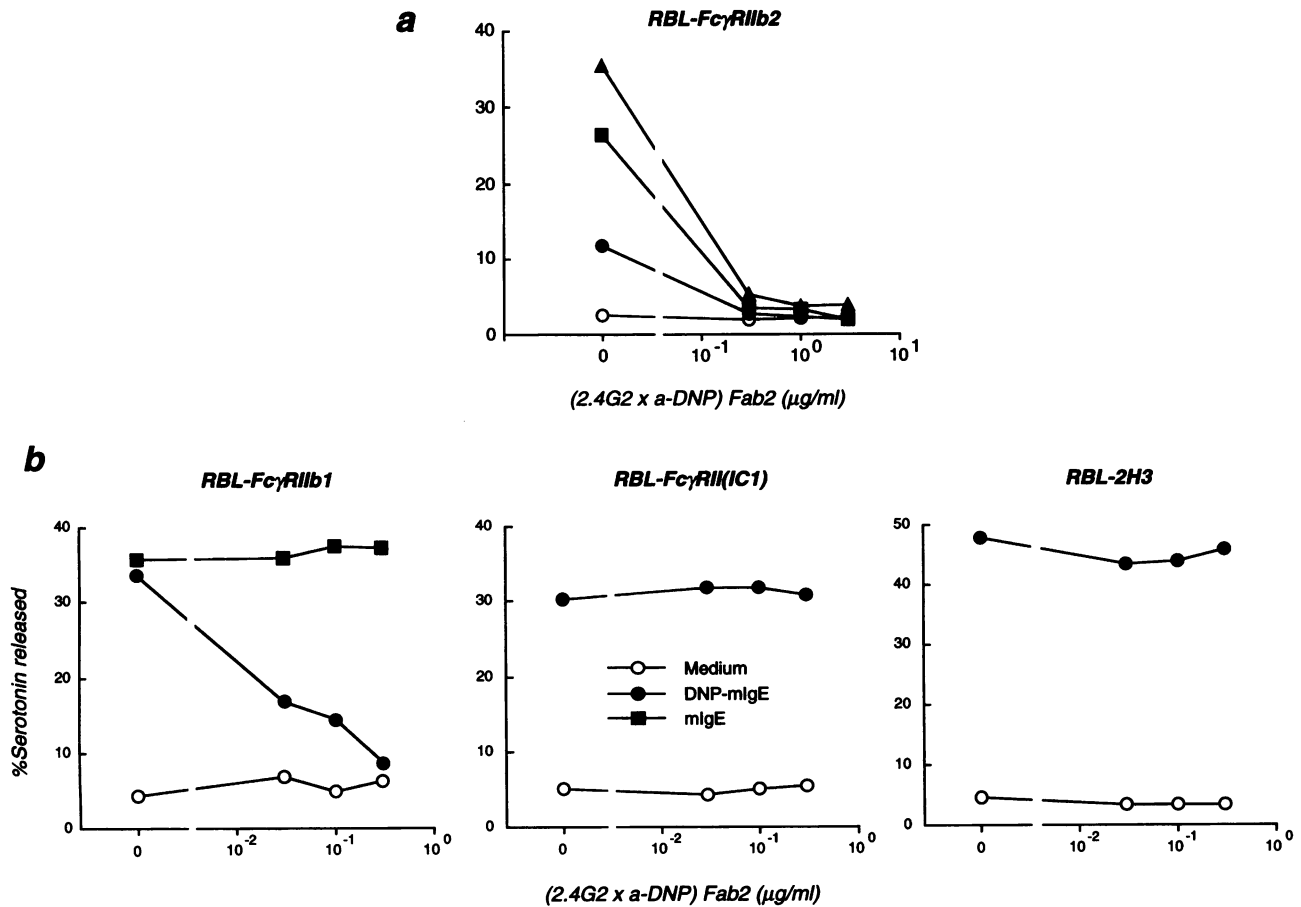
Figure 6. Inhibition of IgE-induced serotonin release by cross-linking FcεRI to FcγRII before challenge. (*a*) FcγRIIb2-expressing transfectants were sensitized with 0 (*open circles*), 1 (*closed circles*), 3 (*closed squares*), or 10 (*closed triangles*) μg/ml dinitrophenylated mouse IgE 14.205 A1 (DNP-mIgE). After being washed, cells were incubated for 1 h at 37°C with indicated concentrations of (2.4G2 × a-DNP) Fab2, washed again, and challenged with 50 μg/ml RAM F(ab′)2 for 30 min. (*b*) FcγRIIb1- or FcγRII(IC1)-expressing transfectants and nontransfected RBL-2H3 cells were sensitized with 0 (*open circles*) or 3 μg/ml IgE 14.205 A1, dinitrophenylated (DNP-mIgE, *closed circles*) or not (mIgE, *closed squares*). After being washed, cells were incubated for 1 h at 37°C with indicated concentrations of (2.4G2 × a-DNP) Fab2, washed again, and challenged with 50 μg/ml RAM F(ab′)2 for 30 min.

for triggering an optimal serotonin release upon aggregation of IgE, and comparable inhibitions were seen when 50, 25 or 12.5 μg/ml MAR F(ab′)2 were used for challenge (data not shown); (*b*) Inhibition was not seen in cells expressing FcγRII deletants lacking IC domain, which bound comparable amounts of 2.4G2 F(ab′)2 fragments as wild-type FcγRII as judged by immunofluorescence with FITC-conjugated MAR F(ab′)2. Steric hindrance of IC domains could neither account for inhibition because it was not seen in transfectants expressing chimeric receptors having the extracellular and transmembrane domains of FcγRII and the IC domain of FcγRIIIα. Inhibition therefore depends on specific IC sequences shared by FcγRIIb1 and FcγRIIb2. Murine FcγRII have been known for long to inhibit B cell activation triggered by B cell receptor (BCR) aggregation, and IC sequences required for FcγRII to inhibit BCR-mediated B cell activation have recently been identified (32, 33). Whether the same FcγRII IC sequences are responsible for FcγRII-mediated inhibition of IgE-induced mast cell activation is currently under study. BCR, like FcεRI and FcγRIII, is composed of a ligand-binding subunit, associated with two subunits whose IC domains possess consensus tyrosine-con-

taining activation motifs similar to those of FcεRIγ and β (34), and it may be a general property of FcγRII to regulate tyrosine-containing activation motif-dependent cell activation.

Inhibition affected secretory responses of mast cells as different as the release of preformed mediators and the de novo synthesis of cytokines. It is therefore susceptible to control both the initiation of allergic reactions and the late phase reaction in which cytokines play a major role (35). Inhibition, however was not the consequence of a general desensitization of mast cells since non–cross-linked receptors could function normally and triggered serotonin release upon aggregation while cross-linked receptors could not. Inhibition was not an all-or-nothing phenomenon. Experiments on FcγRIIb2-expressing transfectants incubated with lower concentrations of 2.4G2 F(ab′)2 than those used in Fig. 3 showed a clearcut dose-dependent inhibition (not shown). Inhibition required the crosslinking of FcεRI to FcγRII. MAR F(ab′)2 could indeed crosslink rat IgE LO-DNP-30 and rat 2.4G2 F(ab′)2 inasmuch as (*a*) the same MAR F(ab′)2 conjugated to FITC bound to both rat reagents in indirect immunofluorescence experiments (see Fig. 2), and (*b*) MAR F(ab′)2 were functionally divalent since they trig-
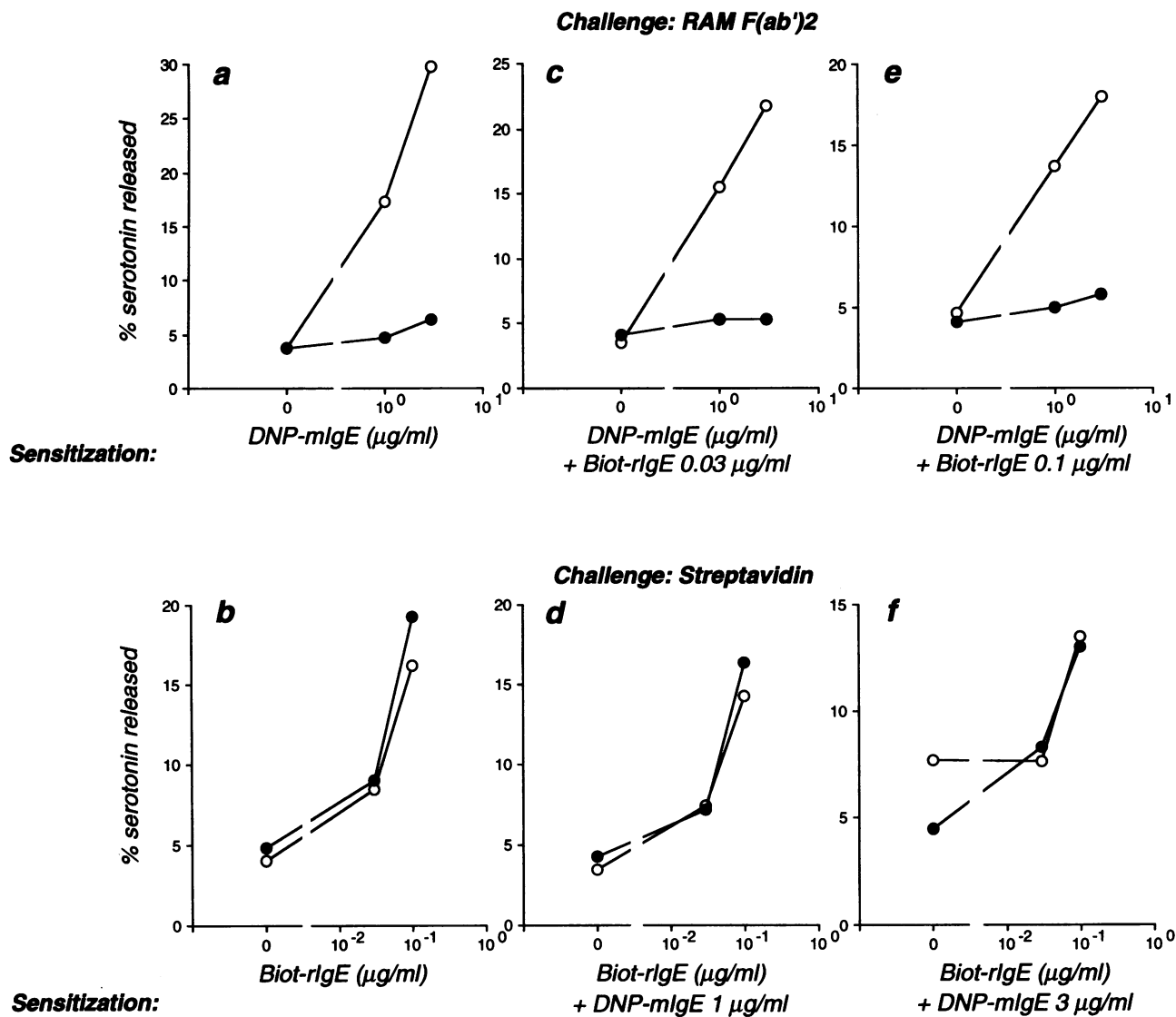
Figure 7. Inhibition of IgE-induced serotonin release affects only FcεRI crosslinked to FcγRII. FcγRIIb2-expressing transfectants were sensitized with dinitrophenylated mouse IgE 14.205 A1 (DNP-mIgE) and/or biotinylated rat IgE IR162 (Biot-rIgE) at concentrations indicated in abscissa, and preincubated for 1 h at 37°C with (closed circles) or without (open circles) 1 μg/ml (2.4G2 × a-DNP) Fab2. Cells were washed and challenged with either 50 μg/ml RAM F(ab')2 (upper panel) or 10 μg/ml streptavidin (lower panel) for 30 min at 37°C.

gered serotonin release and TNF secretion by RBL cells sensitized with rat IgE (this manuscript) and by transfectants expressing FcγRIII preincubated with 2.4G2 F(ab')2 (12, 13). Neither FcγRII occupancy by 2.4G2 F(ab')2 nor FcγRII aggregation by 2.4G2 F(ab')2 and MAR F(ab')2, by themselves, affected serotonin release. This was confirmed with (2.4G2 × a-DNP) Fab2 which had no effect when bound only to FcγRII (in FcγRIIb1-expressing cells sensitized with nondinitrophenylated IgE), or to IgE only (in nontransfected cells sensitized with DNP-IgE), but abolished serotonin release when cross-linking IgE to FcγRII with an intact IC domain (in FcγRIIb1- and FcγRIIb2-, but not in FcγRII(IC1)-expressing cells, sensitized with DNP-IgE). Moreover, inhibition could be reversed when DNP-mIgE previously cross-linked to FcγRII by (2.4G2 × a-DNP) Fab2 were disengaged from cross-linking with monovalent DNP-Lysine. One may notice that a prolonged exposure to DNP-Lysine was required for inhibition to be reduced. Whether

this reflects a low dissociation rate of multiple interactions between (2.4G2 × a-DNP) Fab2 and DNP$_{7.5}$-mIgE or whether FcεRI required time to recover from cross-linking is not known. We are currently investigating the intracellular consequences of receptor cross-linking.

Inhibition was not seen only in RBL transfectants, when recombinant murine FcγRII were artificially cross-linked to rat FcεRI with monoclonal anti-FcγR antibodies and MAR F(ab')2 or with (2.4G2 × a-DNP) Fab2 heteroaggregates. IgE-induced serotonin release was also inhibited in nontransformed mast cells, which express constitutive FcγRII, sensitized with polyclonal serum IgE, when receptor cross-linking was achieved by IgG immune complexes. To rule out a possible competition of antibodies for the same epitope, we used IgE and IgG antibodies of different specificities. The complexation of DNP-BSA to IgG1 anti-DNP mAb also inhibited the release of serotonin by BMMC sensitized with mouse monoclonal IgE
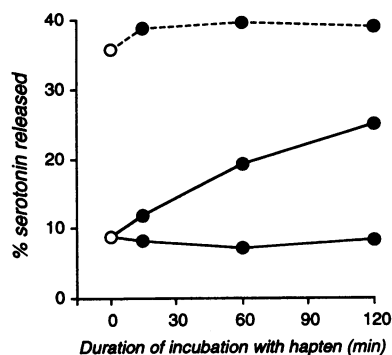
Figure 8. Reversal of inhibition of IgE-induced serotonin release upon disengagement of FcεRI–FcγRII cross-linking. FcγRIIb2-expressing transfectants were sensitized with 10 μg/ml dinitrophenylated mouse IgE 14.205 A1, washed, and preincubated with (*solid lines*) or without (*broken line*) 1 μg/ml (2.4G2 × a-DNP) Fab2 for 1 h at 37°C. They were washed and incubated at 37°C for indicated periods of time with 100 μM DNP-Lysine (*black circles*) or lysine (*gray circles*), added at various times (all cells were incubated for a total of 2 h). Cells were washed and challenged with 50 μg/ml RAM F(ab')2 for 30 min at 37°C.

anti-DNP, as well as the production of TNFα by the murine mastocytoma cells MC9 sensitized with IgE anti-DNP (data not shown). Under these conditions, competition between IgG and IgE antibodies for the same epitopes cannot be excluded. An inhibitory effect of FcγRII, rather than a competition for antigen or an extracellular steric hindrance, however, was strongly suggested by the finding that inhibition of the TNF response was abolished if MC9 cells were preincubated with with 2.4G2 F(ab')2 before challenge with immune complexes (data not shown). 2.4G2 F(ab')2, indeed, block the Fc-binding site of FcγRII (24). Immune complex-induced inhibition mimics physiologic conditions, when IgG antibodies directed against identical or different epitopes of the same antigen as IgE antibodies interact with FcγRII by their Fc portions, while antigen binds FcεRI-bound IgE on the same cell. A reasonable hypothesis would be that, during the course of a normal immune response, physiologic IgE-mediated mast cell activation might occur in the presence of antigen, which would be normally maintained at low levels by IgG antibodies bound to the same antigen, so that no sign of hypersensitivity is detectable. If so, one can speculate that allergic manifestations might arise when, for any reason, this control is not efficient enough.

This also reminds the situation when allergic patients are treated by the injection of increasing amounts of allergen. Such an immunotherapy is usually successful after high titers of IgG antibodies have been induced against allergen (21), but no satisfactory mechanism was yet proposed. Human FcγRIIB are the human equivalent of murine FcγRII, and they were recently reported to inhibit murine B cell activation (36). One might therefore expect that FcγRIIB could be capable of regulating FcεRI in human basophils. If so, inhibition described here might not only explain the success of desensitization, it might also explain its relative inefficacy. When FcεRI aggregation and crosslinking to FcγRII were simultaneously produced by MAR F(ab')2 reacting with FcγRII-bound 2.4G2 F(ab')2 and FcεRI-bound rat IgE, or by antigen–IgG immune complexes, inhibition was partial and inversely proportional to the concentration of IgE used for sensitization. Under these conditions, one cannot prevent adjacent IgE molecules from being aggregated without being cross-linked to 2.4G2, and chances of IgE aggregation can be expected to increase with the density of IgE at the cell surface.

It was recently reported that asthmatic patients injected with IgG immune complexes made with allergen and affinity-purified anti-allergen IgG antibodies had markedly reduced symptoms (37, 38). The mechanism of this effect was not investigated and we propose the inhibition described here as a likely explanation. This suggests that manoeuvres aiming at crosslinking FcεRI to FcγRII might be successful for controlling hypersensitivity manifestations in allergic patients. One advantage of FcγRII-mediated inhibition of IgE-induced mast cell activation, in terms of therapeutics, is that it can operate after mast cells were already sensitized with IgE, as it is the case in allergic patients. Our model where DNP-IgE are crosslinked to FcγRII by (2.4G2 × a-DNP) Fab2, simulates such a therapeutic approach. Under these conditions, the inhibitory effect was deep enough for enabling less than 1 μg/ml (2.4G2 × a-DNP) Fab2 to abolish serotonin release triggered by 50 μg/ml MAR F(ab')2 in RBL cells sensitized with 10 μg/ml IgE. The higher efficacy of bispecific heteroaggregates in inhibiting IgE-induced serotonin release, compared with that of 2.4G2 F(ab')2 and MAR F(ab')2, may be explained because the preincubation of transfectants with (2.4G2 × a-DNP) Fab2 enabled FcεRI to be cross-linked to FcγRII before FcεRI-bound IgE were aggregated by RAM F(ab')2. Appropriate ligands capable to reproduce the same cross-linking in humans are currently under investigation.

Acknowledgments

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