

A Bispecific Antibody Against Human IgE and Human Fc γ RII that Inhibits Antigen-induced Histamine Release by Human Mast Cells and Basophils

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Abstract

Background: Fc γ RIIB are low-affinity IgG receptors that we previously demonstrated to negatively regulate IgE-induced mast cell activation when coaggregated with Fc ϵ RI. Here, we engineered and characterized a bispecific reagent capable of coaggregating Fc γ RIIB with Fc ϵ RI on human mast cells and basophils.

Methods: A bispecific antibody was constructed by chemically crosslinking one Fab' fragment against human IgE and one Fab' fragment against human Fc γ R2. This molecule was used to coaggregate Fc ϵ RI with Fc γ R2 on human mast cells and basophils sensitized with human IgE antibodies, and the effect of coaggregation was examined on mediator release upon challenge with specific antigen.

Results: When used under these conditions, this bispecific antibody not only failed to trigger the release of histamine by IgE-sensitized cells, but it also prevented specific antigen from triggering histamine release. Comparable inhibitions were observed with mast cells and basophils derived *in vitro* from cord blood cells and with peripheral blood basophils.

Conclusions: The bispecific antibody described here is the prototype of similar molecules that could be used in new therapeutic approaches of allergic diseases based on the coaggregation of activating receptors, such as Fc ϵ RI, with inhibitory receptors, such as Fc γ RIIB, that are constitutively expressed by mast cells and basophils.

Key words:

Basophils, Mast cells, Fc ϵ RI, Fc γ RIIB, Negative regulation, Therapeutic antibody

Introduction

Although many other cells participate in the complex network of inflammatory processes that lead to the development of allergies, mast cells and basophils play a critical role in the initiation of IgE-dependent allergic reactions. When activated by IgE and allergen, mast cells and basophils secrete inflammatory mediators and cytokines that act on vascular and muscular cells and recruit inflammatory cells. These, in turn, secrete inflammatory mediators and recruit other inflammatory cells, and the process goes on. Following an IgE-induced, mast cell-dependent immediate-type reaction, waves of inflammatory cells thus become sequentially attracted and activated that altogether generate a long-lasting inflammation. As a consequence, new means of controlling mast cell activation would provide a novel therapeutic approach of allergic diseases by preventing the initiation of the inflammatory response. It happens that mast cells and basophils are constitutively equipped with regulatory receptors that could be used in therapeutics, provided that appropriate ligands be engineered.

The activation of mast cells is positively regulated by several membrane receptors, the prototype of which is the high-affinity IgE receptor $Fc\epsilon RI$ (1). These receptors possess one or several intracytoplasmic molecular motifs named *Immunoreceptor Tyrosine-based Activation Motifs* (ITAMs) (2). When these receptors are aggregated at the cell surface by multivalent ligands (e.g. a multivalent allergen that binds to receptor-bound IgE antibodies in the case of $Fc\epsilon RI$), their ITAMs are phosphorylated by protein tyrosine kinases of the src family (3). Phosphorylated ITAMs provide docking sites for a variety of SH2 domain-containing cytosolic molecules (4) which can interact with each others in a spatially and temporally organized fashion. These molecules include enzymes, substrates and adapters which, altogether, build up a dynamic signaling complex under aggregated receptors (5). Intracellular signals generated in this complex trigger an array of metabolic pathways that lead to an increase in the cytosolic Ca^{2+} concentration and the activation of transcription factors

(6). Ultimately, the exocytotic machinery is activated, leading to the release of granular preformed mediators, and cytokine genes are transcribed, leading to the synthesis and secretion of their products.

The activation of mast cells is negatively regulated by several membrane receptors the prototypes of which are the low-affinity IgG receptors Fc γ RIIB (7). These receptors possess one or several intracytoplasmic molecular motifs named *Immunoreceptor Tyrosine-based Inhibition Motifs* (ITIMs) (8, 9). When these receptors are coaggregated with ITAM-bearing receptors (e.g. by an allergen-IgG antibody complex that binds simultaneously to Fc ϵ RI-bound IgE antibodies *via* the allergen and to Fc γ RIIB *via* the Fc portion of IgG antibodies (10), their ITIMs are phosphorylated by the same src protein tyrosine kinases that phosphorylate ITAMs (11). Phosphorylated ITIMs also provide docking sites for SH2 domain-containing cytosolic molecules. The specificity of phosphorylated ITIMs, however, is different from that of phosphorylated ITAMs: they recruit selectively phosphatases. These are either SH2 domain-containing phosphatidylinositol 5-phosphatases (SHIPs), in the case of Fc γ RIIB (12-15), or SH2 domain-containing protein tyrosine phosphatases (SHPs), in the case of other inhibitory receptors (16, 17). When recruited in the signaling complex, these phosphatases interfere with activation signals. As a consequence, the Ca²⁺ response is impaired (18, 19) and signals that lead to the activation of transcription factors are extinguished (20). Ultimately, both the release of preformed mediators and the secretion of cytokines are inhibited (10). The validity of negative regulation of mast cell activation by Fc γ RIIB demonstrated *in vitro* was confirmed *in vivo* by using genetically modified mice. Indeed, Fc γ RIIB-deficient mice exhibited enhanced IgG- and IgE-induced local and systemic anaphylactic reactions (21).

Although well established in mice, Fc γ RIIB-dependent negative regulation of mast cell activation is poorly documented in humans. One reason is that there are several types of Fc γ R in humans, which are usually coexpressed on the same cells (7).

These are the inhibitory $Fc\gamma RIIB$, as in mice, and the activating $Fc\gamma RIIA$ and/or C that are unique to humans (22). $Fc\gamma RIIA/C$ are capable of activating mast cells, as shown when transfected and aggregated in the rat mast cell model RBL-2H3 (8). They indeed possess an ITAM in their intracytoplasmic domain (2), instead of an ITIM. Another difficulty is that no mAb specific for human $Fc\gamma RIIB$ is available. As a consequence, which type(s) of $Fc\gamma RII$ are expressed by human mast cells and basophils, and in which proportions, is unclear. In a previous work, we showed that human $Fc\gamma RIIB$ had the same inhibitory properties on IgE-induced mast cell activation as murine $Fc\gamma RIIB$, when transfected in RBL-2H3 cells (8). In addition, we showed that $Fc\gamma RII$ constitutively expressed by human blood basophils also inhibited IgE-induced histamine release by these cells (8). Finally, we showed that a bispecific molecule capable of coaggregating mouse IgE with mouse $Fc\gamma RIIB$ profoundly inhibited IgE-induced secretory responses in RBL-2H3 transfectants (10). Based on these previous results, we constructed a bispecific molecule capable of coaggregating human IgE with human $Fc\gamma RII$. We report here that this molecule inhibited antigen-induced secretory responses of human mast cells and basophils sensitized with human IgE antibodies. Our findings provide the grounds of a new immunotherapeutic approach for allergic diseases.

Methods

Cells

1. *Cultured human cord blood-derived mast cells (CBMCs)*. Human CBMCs were derived from human cord blood $CD34^+$ cells grown in the presence of stem cell factor, IL-6, and IL-10 (PeproTech, Rocky Hill, NJ), as modified from the procedure of Saito *et al.* (23) and described in Cho *et al.* (24). Briefly, heparin-treated umbilical cord blood was purchased from Advanced Bio-technologies (Columbia, MD). The cord blood

sample was diluted 1:4 in PBS supplemented with 2% BSA (Sigma-Aldrich, St. Louis, MO) and 0.6% citrate, loaded onto Ficoll-Histopaque (Sigma-Aldrich), and centrifuged twice at 200 g. The interface containing mononuclear cells was mixed with Dynabeads M-450 CD34 according to manufacturer's protocol (Dyna, Lake Success, NY). The CD34⁺ cells were magnetically separated and later detached from the beads. The washed mononuclear cells were resuspended at $1-5 \times 10^5$ cells/ml in culture medium consisting of RPMI 1640 (Life Technologies/GIBCO/BRL, Rockville, MD), 20% FBS (Sigma-Aldrich), 2 mM L-glutamine, 50 mM 2-ME, 100 U/ml penicillin, 100 mg/ml streptomycin and 10 mg/ml gentamicin (GIBCO/BRL), supplemented with 80 ng/ml stem cell factor, 50 ng/ml IL-6, and 5 ng/ml IL-10. The cytokine-supplemented medium was replaced on a weekly basis. Cells were harvested after 8-9 wk of culture. Over 94% of these cells were mast cells as assessed by staining with a mAb directed against mast cell tryptase (Chemicon International, Temecula, CA).

2. *Cultured human cord blood-derived basophils (CBBs)*. Human CD34⁺ cord blood cells were purchased from Poietics Inc. (BioWhittaker, Walkersville, MD). The thawed mononuclear cells were resuspended $1-5 \times 10^5$ cells/ml in culture medium supplemented with 1 ng/ml recombinant human IL-3 and 10 ng/ml recombinant human TGF- β 1 (Peprotech Inc.). The cytokine-supplemented medium was replaced on a weekly basis. Cells were harvested after 4 wk of culture.

3. *Human basophil-containing PBMCs*. Peripheral blood lymphocytes were purified from heparinized human blood buffy coat from healthy donors as described (25). The heparinized blood was mixed 1:4 part of RPMI media (Gibco) and centrifuged at 200 g for 10 min at 10°C to remove the red cells. The supernatant was mixed 1:4 part of histopaque (Sigma-Aldrich) and the mixture was centrifuged at 500 g for 45 min at 10°C to separate the lymphocyte layer. Cells were washed twice at 200 g for 10 min at 10°C in RPMI, and cell viability was assessed by trypan blue staining. About 1-2% of PBMCs were basophils.

4. *Daudi cells*. The human Burkitt lymphoma cell line, Daudi, was maintained in RPMI 1640 (Life Technologies) supplemented with 10% FBS (Sigma-Aldrich), 2 mM L-glutamine, 50 mM 2-mercaptoethanol (2-ME), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO/BRL).

Antibodies

The mouse anti-human Fc γ RII AT10 mAb (26) was purified from ascitic fluid, harvested from (BALB/c x CBA) F1 mice injected i.p. with the AT10 hybridoma cells, using ammonium sulphate precipitation and ion-exchange chromatography. The mouse anti-human IgE mAb E10.10.3, generated at Tanox, was purified from culture supernatant from hybridoma cells. Purified monoclonal mouse IgE (IgE-3) was purchased from BD PharMingen (San Diego, CA). The human IgE anti-gp120 mAb SE44 was generated in a B cell transfectoma, containing hybrid epsilon and kappa genomic DNA of human C ϵ region and κ chains and the H and L chain V regions of the murine mAb BAT123, which reacts with the gp120 envelope protein of HIV-1 (27). Goat anti-mouse (GAM) F(ab')₂ was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Bispecific antibody

AT10 F(ab')₂ fragments were prepared by pepsin (Sigma-Aldrich) digestion of AT10 IgG antibodies at pH 4.1 according to Lamoyi and Nisonoff (28). E10.10.3 F(ab')₂ fragments were prepared from E10.10.3 IgG antibodies by pepsin digestion in 20 mM sodium acetate pH 4.5 for 48 hr at 37°C using immobilized pepsin beads (Pierce Inc. Rockford, IL). A bispecific molecule containing Fab' fragments of the two mouse mAbs was prepared by linking half-cysteine residues via thioether bonds using the bifunctional cross-linking agent, o-phenylenedimaleimide (o-PDM) (Sigma-Aldrich) as described (26, 29). F(ab')₂ fragments from the two antibodies diluted at 10 mg/ml in 0.2 M Tris-HCl

buffer, pH8.0, containing 10 mM EDTA were reduced by the addition of 20 mM 2-ME for 30 min at 30°C. The samples were chilled to 4 °C and reduced F(ab')_{SH} were separated from the reducing agent by gel filtration through a G25 column in 50 mM sodium acetate bufer, pH 5.3, containing 0.5 mM EDTA. A half volume of 12 mM o-PDM dissolved in chilled dimethylformamide was then added to one of the two mouse F(ab')_{SH} preparations. After 30 min, the maleimidated F(ab')_{mal} were separated from other solutes by passage through Sephadex G-25 column. They were then added immediately to the other F(ab')_{SH} preparation in a molar ratio of 1:1.3 of F(ab')_{mal} : F(ab')_{SH} and concentrated to 5 mg/ml by ultrafiltration under nitrogen using a Diaflo membrane in a chilled Amicon chamber (Amicon, Beverly, MA). After incubation for 18 hr, the pH of the reaction mixture was adjusted to 8.0 using 1 M Tris-HCl, before reducing with 20 mM 2-ME for 30 min at 30°C and alkylating with 25 mM iodoacetamide. Finally, the bispecific antibody was separated from other products by passage through Ultrogel AcA44 (IBF Biotechnics, Paris, France) equilibrated in 0.2 M Tris-HCl, 10 mM EDTA, pH 8.0.

HPLC analysis

The bispecific antibody was checked for purity by rapid size fractionation in a high-pressure liquid chromatography (HPLC) system (Amersham Pharmacia, Piscataway, NJ). The fractionation was performed at 0.5 ml / min in a Superdex 200 column (Amersham Pharmacia) equilibrated in 0.2 M phosphate, pH 7.0, containing 10 mM sodium azide. Each assessment requires approximately 30 minutes, monitored with a 280 nm interference filter (Amersham Pharmacia). The profile of the bispecific antibody was then compared to a molecular weight standard (Amersham Pharmacia) run to approximate the protein size.

ELISA

ELISA plates (Immulon 2, VWR, So. Plainfield, NJ) were coated by an overnight incubation at 4°C with 1 µg/ml human IgE SE44. Plates were saturated with Blotto (5 g dry milk, 0.05% Tween 20 in PBS buffer) for 1 hr at room temperature. The wells were then washed 4 times in 0.05% Tween 20 diluted in PBS. Serial dilutions of AT10, E10.10.3 F(ab')₂, or bispecific antibody were added to wells in duplicates and plates were incubated at room temperature for 2 hr. Plates were washed in 0.05% Tween 20, and horseradish peroxidase-conjugated goat anti-mouse kappa antibodies (Jackson ImmunoResearch) were added for 1 hr at room temperature. Plates were washed again in 0.05% Tween 20, and the presence of enzyme-conjugated antibodies was revealed by adding TMB substrate (BD PharMingen, San Diego, CA) for 15 min at room temperature. The reaction was stopped with 2 M H₂SO₄, and the optical density of the reaction mixture was measured using an ELISA spectrophotometer at 450 nm.

Indirect immunofluorescence

Daudi cells were incubated for 30 min at 0°C with 25 µg/ml bispecific antibody, AT10 F(ab')₂, or F(ab')₂ fragments of an irrelevant isotype control antibody (26). The cells were washed and incubated for 30 min at 0°C with 0.3 mg/ml FITC-labeled polyclonal rabbit anti-mouse Ig antibodies (BD PharMingen, San Diego, CA). After a final wash, fluorescent cells were analyzed in the presence of 1 mM NaN₃ (to prevent internalization) using a fluorescence-activated cell sorter (FACS III; Becton Dickinson, Mountain View, CA).

Histamine release

Cells sensitized with murine IgE. Aliquots of 1 x 10⁵ human CBMCs were resuspended in a 1:1 mixture of RPMI medium: histamine release ionic buffer

(Immunotech / Beckman Coulter, Fullerton, CA). They were incubated for 1 hr at 37°C with 10 µg/ml mouse IgE SE44 and with or without various concentrations of AT.10. Cells were washed twice and challenged for 1 hr at 37°C with 10 µg/ml pre-warmed (15 min at 37°C) GAM F(ab')₂.

Cells sensitized with human IgE. Aliquots of 1×10^5 9-wk old CBMCs, of 5×10^4 4-wk old CBBs or of 1×10^5 PBMCs were incubated for 1 hr at 37°C with 10 µg/ml human IgE anti-gp120 (mAb SE44), washed and incubated for 30 min at 37°C with or without indicated concentrations of bispecific antibody, 10 µg/ml E10.10.3 or 10 µg/ml AT10. Cells were washed again and challenged for 1 hr at 37°C with 100 ng/ml gp120-conjugated ovalbumin (27).

Histamine measurements. At the end of the reaction, supernatants were collected by centrifugation and acylated according to the Immunotech protocol. Histamine was measured in supernatants using the Immunotech human histamine competitive ELISA kit.

Results

Fc γ RII inhibit IgE-induced histamine release when coaggregated with Fc ϵ RI on human cord blood-derived mast cells

In preliminary immunofluorescence studies, we found that, in addition to high-affinity IgE receptors (Fc ϵ RI), human CBMCs express low-affinity IgG receptors. Indeed, F(ab')₂ fragments of the mouse mAb AT10 (26), which recognize both Fc γ RIIA/C and Fc γ RIIB (8), bound to CBMCs (our unpublished results). F(ab')₂ fragments of the Fc γ RIIA/C-specific mouse mAb IV.3 (8) also bound to CBMCs. These observations indicated that human CBMCs express the cell-activating receptors Fc γ RIIA/C. They neither indicated nor excluded that these cells express the inhibitory receptors Fc γ RIIB. As a consequence, one could not predict whether aggregating Fc γ RII on human CBMCs would trigger cell activation, or not; and whether coaggregating Fc ϵ RI with Fc γ RII would result in a potentiation, or an inhibition of IgE-induced secretory responses.

To answer this question, CBMCs were sensitized with mouse IgE or without, and incubated with AT10 or without. After being washed, they were challenged with Goat anti-Mouse Ig (GAM) F(ab')₂ fragments, and histamine released in the supernatant was measured by ELISA. Mouse IgE alone, AT10 alone or the combination of both induced no histamine release over background. Fc ϵ RI aggregation, induced by GAM F(ab')₂ in CBMCs sensitized with IgE, triggered histamine release, whereas Fc γ RII aggregation, induced by GAM F(ab')₂ in CBMCs incubated with AT10, did not. The coaggregation of Fc ϵ RI with Fc γ RII, induced by GAM F(ab')₂ in CBMCs sensitized with IgE and incubated with AT10, triggered no histamine release either (Fig. 1).

That histamine release was induced neither upon Fc γ RII aggregation nor upon coaggregation of Fc γ RII with Fc ϵ RI suggests that human CBMCs do express Fc γ RIIB and that Fc γ RIIB could inhibit both Fc γ RIIA/C-dependent activation (induced by

AT10 + GAM F(ab')₂), and Fc_γRIIA/C+Fc_εRI-dependent activation (induced by IgE + AT10 + GAM F(ab')₂). The same was previously observed using the same experimental protocol on human peripheral blood basophils (8). Human cultured mast cells and blood basophils could therefore be used to assess the potential inhibitory properties of a molecule capable of coaggregating Fc_εRI with Fc_γRII.

Construction of a monovalent bispecific anti-Fc_γRII/anti-IgE antibody

In order to construct a reagent capable of coaggregating Fc_εRI with Fc_γRII on human mast cells and basophils, Fab' fragments were prepared from the mouse mAb anti-human IgE E10.10.3 and from the mouse mAb anti-human Fc_γRII AT10. The resulting two Fab' fragments were chemically linked to each other using *o*-phenylenedimaleimide as described (26, 29). Under the experimental conditions used, only heterodimers could be generated. The hybrid molecule was purified by gel chromatography and characterized by HPLC, by ELISA and by indirect immunofluorescence.

When analyzed by HPLC, this molecule eluted as a predominant peak with a m.w. of about 100 kDa (Fig. 2 A). When analyzed by ELISA, it bound onto human IgE-coated plates almost as efficiently as F(ab')₂ fragments of the parental mAb E10.10.3. Under the same conditions, AT10, the other parental mAb, did not bind at all to IgE (Fig. 2B). When analyzed by indirect immunofluorescence on the Fc_γRII-positive Burkitt lymphoma cell line Daudi, the hybrid molecule bound as efficiently as F(ab')₂ fragments of the parental mAb AT10 (Fig. 2C).

A 100-kDa molecule, capable of binding both to human IgE and to human Fc_γRII was therefore generated. Considering the preparation conditions used and its apparent m.w., this bispecific molecule should be composed of one AT10 Fab' fragment and of one E10.10.3 Fab' fragment. This monovalent bispecific molecule, referred to as

(AT10XE10) bsAb, was used to coaggregate Fc ϵ RI and Fc γ RII on human mast cells and basophils sensitized with human IgE.

(AT10XE10) bsAb prevents IgE-induced histamine release by human mast cells and basophils

The effect of (AT10XE10) bsAb on IgE-induced histamine release was examined on three types of human cells. CBMCs, obtained by culturing human CD34⁺ cord blood cells in the presence of SCF, IL-6 and IL-10, cord blood-derived basophils (CBBs), obtained by culturing the same CD34⁺ cells in the presence of IL-3 and TGF- β , and basophil-containing PBMC, prepared from human buffy coat, were sensitized with human IgE anti-gp120 (clone SE44). After being washed, they were incubated with various concentrations of (AT10XE10) bsAb or with one of the parental antibodies (E10.10.3 or AT10), washed again, and challenged with gp120-conjugated ovalbumin or not. Histamine released into the supernatant was monitored by ELISA.

None of the three cell types released significant amounts of histamine when incubated with (AT10XE10) bsAb and challenged with gp120-ovalbumin or not. As expected, all three cell preparations released histamine when sensitized with IgE anti-gp120 and challenged with gp120-ovalbumin (Fig. 3A, B, C) or with parental anti-IgE mAb E10.10.3, but not with (AT10XE10) bsAb (Fig. 3A, B). Histamine release was inhibited when cells sensitized with IgE anti-gp120 were incubated with (AT10XE10) bsAb before challenge with gp120-ovalbumin (Fig. 3A, B, C). Inhibition was observed in all three cell types, for all concentrations of (AT10XE10) bsAb tested. An incubation of cells with the parental antibody AT10,

instead of (AT10XE10) bsAb, induced no significant inhibition (not shown). The coaggregation, by (AT10XE10) bsAb, of Fc ϵ RI-bound IgE antibodies with Fc γ RII on cultured mast cells, on cultured basophils or on peripheral blood basophils, therefore prevented specific antigen from inducing histamine release.

Discussion

The present paper describes the construction of a bispecific molecule able to coaggregate Fc ϵ RI and Fc γ RII on human mast cells and basophils sensitized with IgE (Fig. 4). This molecule was found to be monovalent and to retain the specificity of both parental antibodies. When used to coaggregate Fc ϵ RI-bound human IgE and Fc γ RII, it prevented antigen from eliciting histamine release. Comparable inhibitions were induced in cultured human mast cells and basophils, as well as in peripheral blood basophils. It follows that, although both ITAM-containing activating Fc γ RII and ITIM-containing inhibitory Fc γ RII are expressed on human mast cells and basophils, Fc γ RIIB-dependent inhibition is dominant and can be exploited, using bispecific antibodies, for therapeutic purposes. Based on these results, similar bispecific antibodies could be tailored aiming at coaggregating Fc ϵ RI with other inhibitory receptors expressed on human mast cells and basophils.

Fc γ RIIB-dependent negative regulation of cell activation is difficult to assess in human mast cells and basophils. First, these cells were not formally demonstrated to express Fc γ RIIB. Second, they express cell-activating Fc γ RIIA/C, as revealed by the binding of the Fc γ RIIA/C-specific mAb IV.3 (8). Ideally, one would need antibodies that would specifically recognize Fc γ RIIB on the cell surface in order to 1) ascertain whether this inhibitory receptor is expressed by mast cells and basophils, and 2) coaggregate Fc ϵ RI selectively with Fc γ RIIB. Unfortunately, such antibodies are not

available and are unlikely to be generated in view of the highly conserved sequences of the extracellular domains of $FC_{\gamma}RIIA$, $FC_{\gamma}RIIB$ and $FC_{\gamma}RIIC$. There is indeed no extracellular amino acid present in $FC_{\gamma}RIIB$ that is not also present in $FC_{\gamma}RIIA$ or $FC_{\gamma}RIIC$. Under these conditions, one can only coaggregate $FC_{\gamma}RII$ (A/C+B, if any) with $FC_{\epsilon}RI$, hoping that $FC_{\gamma}RIIB$ -dependent inhibition will be dominant over $FC_{\gamma}RIIA/C$ -dependent activation. Supporting this possibility, IgG immune complexes are, in general, unable to activate basophils (30), and indeed, we could not induce histamine release when aggregating $FC_{\gamma}RII$ with the mAb AT10, which binds equally well to $FC_{\gamma}RIIA/C$ and to $FC_{\gamma}RIIB$ (8), on human peripheral basophils or on CBMCs. Moreover, IgE-dependent histamine release was inhibited when $FC_{\gamma}RII$ were coaggregated with $FC_{\epsilon}RI$ on the same cells. These data suggest that human CBMCs probably express $FC_{\gamma}RIIB$, and that $FC_{\gamma}RIIB$ -dependent negative regulation is dominant not only over $FC_{\gamma}RIIA/C$ -dependent cell activation but also over ($FC_{\gamma}RIIA/C + FC_{\epsilon}RI$)-dependent cell activation. Similar results were previously observed in peripheral human basophils (8). One may therefore envision the coaggregation of $FC_{\epsilon}RI$ with $FC_{\gamma}RII$, on cells which express the two receptors, as a possible new therapeutic approach of allergies.

Based on this strategy, a recently published paper reported that a bifunctional fusion protein could inhibit $FC_{\epsilon}RI$ -dependent human mast cell and basophil activation (31). The inhibitory properties of this molecule, made of a human IgG1 Fc portion (hinge- $C_{\gamma H}2-C_{\gamma H}3$) fused to a human IgE Fc portion ($C_{\epsilon H}2-C_{\epsilon H}3-C_{\epsilon H}4$) *via* a 15-aminoacid linker, were proposed to result from the coaggregation of $FC_{\epsilon}RI$ with $FC_{\gamma}RII$. The requirement of prolonged incubation periods, the inhibitory effect on syk phosphorylation and the intrinsic inability of the bifunctional molecule to coligate $FC_{\gamma}RII$ with those $FC_{\epsilon}RI$ that were sensitized with specific IgE and engaged by antigen made a competitive effect likely to account for inhibition, rather than a coaggregation of $FC_{\epsilon}RI$ with $FC_{\gamma}RII$. In the present work, we constructed a molecule made of one Fab' fragment of an anti- $FC_{\gamma}RII$ antibody (AT10) and one Fab' fragment of an anti-IgE antibody known

not to interfere with the binding of IgE to Fc ϵ RI (E10.10.3). An anti-human IgE antibody was chosen, instead of an anti-Fc ϵ RI antibody, for the following reasons. Allergic patients are expected to have already synthesized allergen-specific IgE antibodies that are bound to Fc ϵ RI and, due to the low dissociation rate (32), remained bound to these receptors. More importantly, one anticipates an efficient inhibition of allergen-induced reactions to preferentially target receptors occupied by specific IgE antibodies, rather than unoccupied receptors, since we previously demonstrated that inhibition requires that Fc γ RIIB be coligated with Fc ϵ RI that are aggregated by antigen (10) and thereby provide the protein tyrosine kinase which phosphorylates Fc γ RIIB (11). One possible problem resulting from this strategy was that an anti-IgE molecule might activate mast cells sensitized with IgE (both specific and nonspecific). This was not the case since cultured mast cells, cultured basophils or blood basophils did not release significant amounts of histamine when exposed to (AT10XE10) bsAb, whether sensitized or not with human IgE. This molecule is therefore most likely monovalent for both IgE and Fc γ RII. Indeed, as judged by its apparent m.w., it only contained two Fab' fragments and, because of the preparation conditions used (one partner only was maleimidated), only two Fab' with different specificities could be chemically linked. (AT10XE10) bsAb is bispecific, as demonstrated by its ability to bind to human IgE, as assessed by ELISA, and by its ability to bind to the Fc γ RII-expressing human Burkitt lymphoma cells Daudi, as assessed by indirect immunofluorescence. Being both monovalent and bispecific, it was used to coaggregate Fc ϵ RI with Fc γ RII on IgE-sensitized human mast cells and basophils and, as a result, it significantly inhibited antigen-induced histamine release. Inhibition indeed similarly affected all three cell types used, *i.e.* cord blood-derived mast cells, cord blood-derived basophils and peripheral blood basophils, and as little as 1 μ g/ml bsAb could inhibit histamine release induced by 10-fold higher concentrations of IgE.

As a consequence of these observations, we would like to propose that bispecific molecules such as the (AT10XE10) bsAb described herein could be humanized and possibly used as therapeutic tools. As demonstrated here, such molecules would be expected to prevent IgE-induced mast cell activation, which determines the onset of allergic diseases. One should point out that, due to the difficulties discussed above, direct evidence that human mast cells and basophils do express Fc γ RIIB remains to be obtained. It should confirm our functional data. Not all mast cell populations may possibly express Fc γ RIIB, and in any case with the same Fc γ RIIB/Fc γ RIIA ratio. If so, Fc γ RII-dependent negative regulation may not affect identically these cells, as the ratio of activating and inhibitory receptors can be anticipated to be critical. Interestingly, due to their bispecificity, they would target not only mast cells and basophils, but also monocytes/macrophages (33, 34) and eosinophils (35, 36) which also express both Fc γ RII and Fc ϵ RI, and which are thought to be major effectors in diseases such as asthma and atopic dermatitis. Following the same rationale, we also propose that other bispecific molecules be constructed, that would coaggregate Fc ϵ RI with other inhibitory receptors. Three such molecules, gp49B1/HM18 (37), PIR-B (38) and SIRP α (39) were found to be expressed on mast cells and to inhibit Fc ϵ RI-dependent mast cell activation (39-41). gp49B1 possesses two ITIMs, PIR-B and SIRP α four ITIMs and, by contrast with Fc γ RIIB which recruits the single-SH2 domain-containing phosphatases SHIP1 and SHIP2 (14), these three inhibitory receptors were shown to recruit the two-SH2 domain-containing phosphatases SHP-1 and/or SHP-2 (39, 42, 43). Having different inhibitory mechanisms (44), bispecific molecules that would coaggregate Fc ϵ RI with Fc γ RII and bispecific molecules that would coaggregate Fc ϵ RI with gp49B1, PIR-B or SIRP α could have additive, if not synergistic, inhibitory effects on IgE-induced mast cell activation, when used in combination.

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Figure Legends

Fig. 1: Inhibition of histamine release upon coaggregation of Fc ϵ RI with Fc γ RII on human cord blood-derived mast cells. Aliquots of 1×10^5 human CBMCs were incubated for 1 hr at 37°C with mouse IgE and/or mouse mAb anti-human Fc γ RII antibody AT10. Cells were washed and challenged or not challenged with goat anti-mouse (GAM) F(ab')₂. All reagents were used at 10 μ g/ml, final concentration. Histamine released into the supernatant was measured by ELISA. Histograms show the concentration of histamine (mean \pm SE from 3 independent experiments) in cell supernatants following treatments indicated below the graph.

Fig. 2: Characterization of the (anti-IgE x anti-Fc γ RII) bispecific antibody. A) *HPLC analysis of (AT10XE10) bsAb.* The graph represents the elution profile of (AT10XE10) bsAb, measured by the absorbance at 280 nm, as a function of time. The major peak eluted together with a m.w. marker of about 100 kDa. **B)** *ELISA analysis of the binding of (AT10XE10) bsAb to human IgE.* The binding of serially diluted (AT10XE10) bsAb, E10.3.10 F(ab')₂ (anti-IgE) and AT10 (anti-Fc γ RII) onto human IgE-coated plates was measured by ELISA using horseradish peroxidase-conjugated anti-mouse κ antibodies. The curves represent the absorbance at 250 nm as a function of the dilution of antibodies. **C)** *Immunofluorescence analysis of the binding of (AT10XE10) bsAb to human Fc γ RII.* The Fc γ RII-expressing Burkitt lymphoma cells Daudi were incubated with 25 μ g/ml (AT10XE10) bsAb, AT10 or an irrelevant isotype control. Cell-bound antibodies were revealed using FITC-rabbit anti-mouse antibodies. Histograms show the fluorescence intensity measured by flow cytometry.

Fig. 3: Inhibition of histamine release by (AT10XE10) bsAb on human mast cells and basophils. Aliquots of **A)** 1×10^5 cord blood-derived mast cells, **B)** 5×10^4 cord blood-derived basophils and **C)** 1×10^5 basophil-containing PBMCs were sensitized with human IgE anti-gp120 (IgE SE44), washed, incubated or not with indicated concentrations of (AT10XE10) bsAb, washed again, and challenged with medium, parental anti human IgE mAb (E10103) or gp120-conjugated albumin (gp120-ova) as indicated under the graph. Histamine released in the supernatant was measured by ELISA. The histograms represent the concentration of histamine (mean \pm SE from 3 independent experiments) in cell supernatants following indicated treatments.

Fig. 4. Schematic representation of (AT10XE10) bsAb when coaggregating $Fc\epsilon RI$ with $Fc\gamma RII$ on human mast cells or basophils sensitized with human IgE and challenged with specific antigen. $Fc\epsilon RI$ and $Fc\gamma RII$ are coexpressed by human mast cells and basophils. The two receptors are coaggregated on the same cells when (AT10XE10) bsAb binds simultaneously to $Fc\epsilon RI$ -bound IgE via its E10 Fab' and to $Fc\gamma RII$ via its AT10 Fab' .

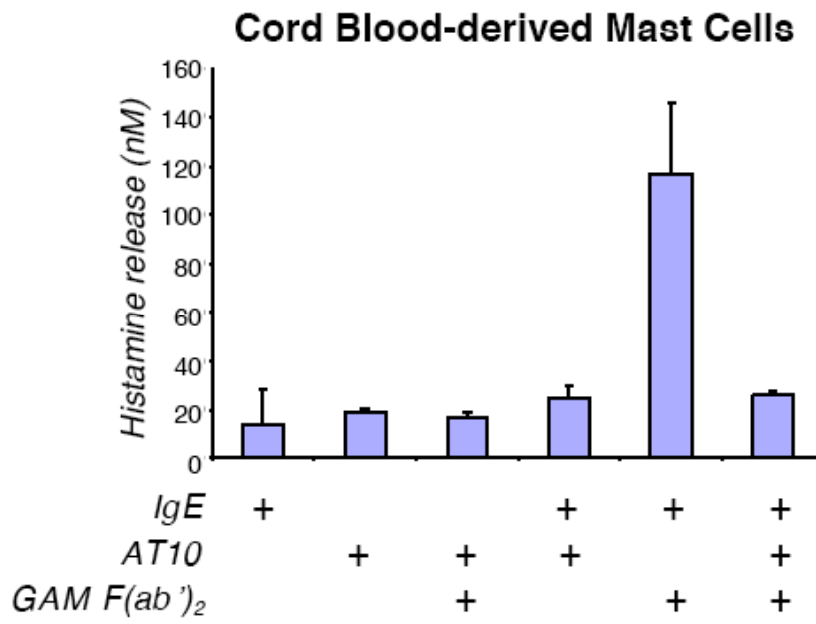


Fig. 1

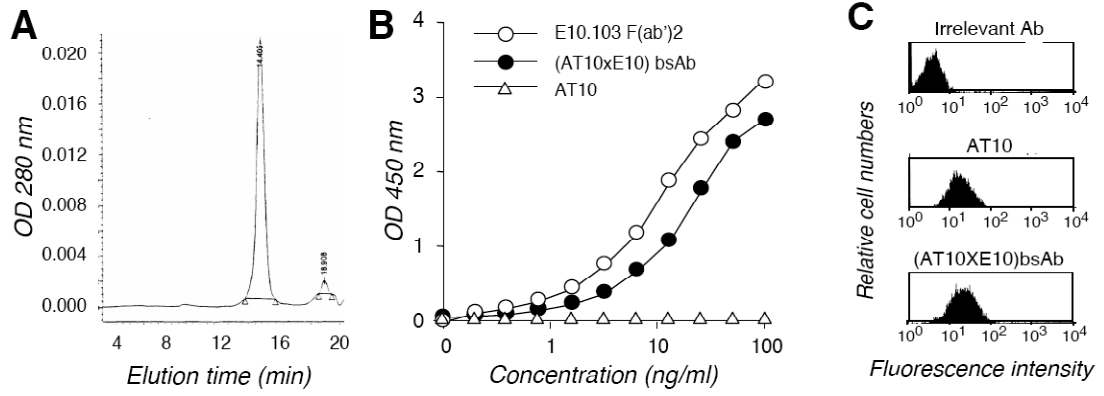


Fig. 2

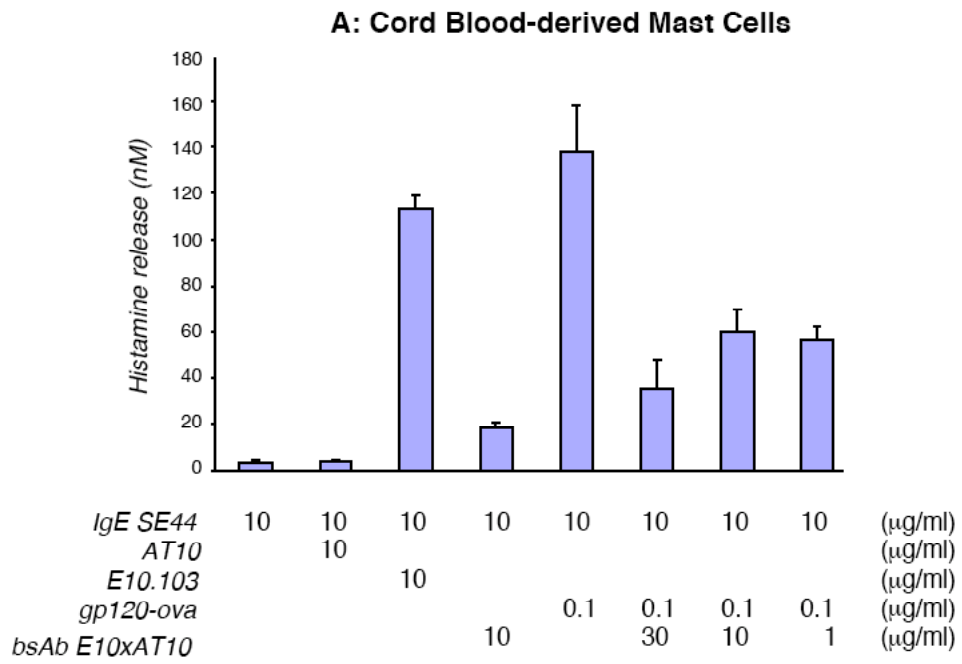


Fig. 3

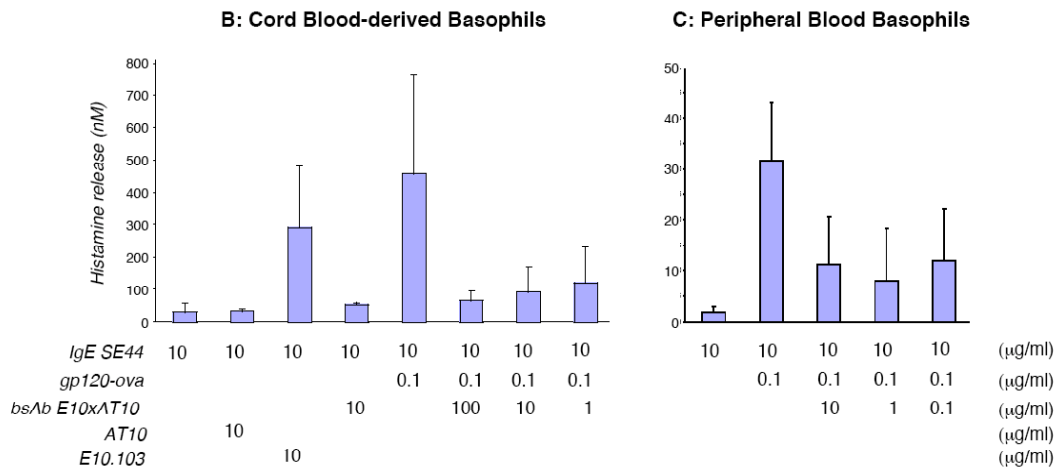


Fig. 3

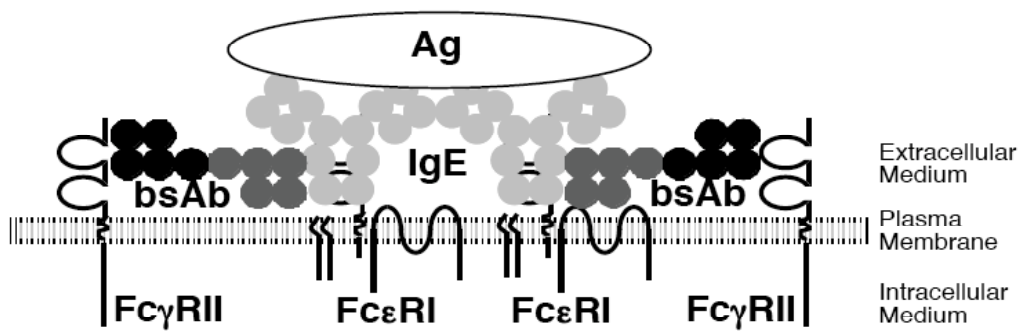


Fig. 4