

An IgE-dependent secretory response of mast cells can be induced by a glycosphingolipid-specific monoclonal antibody

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The signal transduction pathway of the type 1 Fcε receptor (FcεRI) has been proposed to be spatially constrained to plasma membrane microdomains enriched in glycosphingolipids and cholesterol. These domains are proposed to serve as platforms that enhance the efficiency of the antigen-receptor stimulus-response coupling process. Here we describe a monoclonal antibody (mAb) designated 2B5, raised by immunizing mice with rat mucosal-type mast cell (line RBL-2H3) membranes, which binds to glycosphingolipids and causes a dose-dependent secretory response of these cells. This secretory response to mAb 2B5 requires binding of IgE to the FcεRI on these cells, although direct interactions between IgE and mAb 2B5 are excluded. The bound IgE- or FcεRI-specific mAb did not affect binding of mAb 2B5 or its Fab fragments to the RBL-2H3 cells and only a limited interference with the binding of IgE to the FcεRI by mAb 2B5 was observed. Binding of mAb 2B5 to the RBL-2H3 cells induced a distribution of fluorescently labeled IgE similar to that produced by antigen-induced aggregation of the IgE-FcεRI. Thus we suggest that mAb 2B5 binds to cell surface glycosphingolipids that are probably associated with the FcεRI-IgE complexes and causes their aggregation, thereby initiating the cascade leading to the cell's secretory response.

Key words: Rat mucosal mast cell / RBL-2H3 line / Lipid raft / Endocytosis

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1 Introduction

Mast cell stimulation via its type 1 receptor for IgE (FcεRI) constitutes a paradigm for cell activation by immunoreceptors [2]. In the process of pursuing plasma mem-

[1 18532]

Abbreviations: **GSL:** Glycosphingolipid **RBL-2H3:** Rat basophilic leukemia cells, subline 2H3 **Forssman glycolipid:** GalNAc(α1 → 3)GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)-GlcCer **Lactosyl ceramide:** Gal(β1 → 4)-GlcCer **Ceramide trihexoside:** Gal(α1 → 4)Gal(β1 → 4)-GlcCer **Globoside:** GL4, GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)-GlcCer **GM3:** NeuAc(α2 → 3)Gal(β1 → 4)-GlcCer **GM2:** GalNAc(β1 → 4)Gal[(3 ← 2α)NeuAc](β1 → 4)-GlcCer **GM1:** Gal(β1 → 3)GalNAc(β1 → 4)Gal[(3 ← 2α)NeuAc](β1 → 4)-GlcCer **GD1a:** NeuAc(α2 → 3)Gal(β1 → 3)GalNAc(β1 → 4)Gal[(3 ← 2α)NeuAc](β1 → 4)-GlcCer **GD3:** Gal[(3 ← 2α)NeuAc(8 ← 2α)NeuAc](β1 → 4)GlcCer **GD1b:** Gal(β1 → 3)GalNAc-(β1 → 4)Gal[(3 ← 2α)NeuAc(8 ← 2α)NeuAc](β1 → 4)GlcCer **GT1b:** NeuAc(α2 → 3)Gal(β1 → 3)-GalNAc(β1 → 4)Gal[(3 ← 2α)NeuAc(8 ← 2α)NeuAc](β1 → 4)GlcCer **Gal:** Galactose **GlcCer:** Glucosylceramide **GalNAc:** N-acetylgalactosamine **NeuAc:** N-acetylneuraminic acid **SL:** Sphingolipid.

Gangliosides are named according to Svennerholm [1].

brane components of these cells involved in the cascade coupling the FcεRI stimulus to the secretory response, mAb were raised by immunization with these membranes. Several such components have so far been identified by specific mAb. While some are proteins, such as the α subunit of the FcεRI [3–6], other are low-molecular weight membrane components identified as gangliosides (glycosphingolipids [GSL] which contain one or more sialic acid residues), and the specific mAb were capable of modulating the cell's response. For example, mAb B17 binds the ganglioside GD_{1b} [7] on the rat mucosal-type mast cells (RBL-2H3 line) and was shown to enhance or suppress their secretory response to FcεRI stimulation in a dose-dependent manner. mAb AA4 binds an α-galactosyl derivative of the ganglioside GD_{1b} and inhibits IgE binding to the FcεRI [8]. While it does induce cellular protein tyrosine phosphorylation, it does not produce any secretory response [9]. The gangliosides are enriched in particular plasma membrane domains (recent literature therefore names these operationally detergent-insoluble GSL-enriched complexes [DIG], or detergent-resistant membranes [DRM] or rafts [10–24]). The molecular mechanism(s) by which these

lipids modulate cell functions is still largely unknown. Recent studies have shown the tendency of sphingolipids (SL) to be concentrated in the DIG together with cholesterol as well as doubly acylated Src family kinases (e.g. Lyn, Yes) and glycosylphosphatidylinositol (GPI)-anchored proteins and to affect protein segregation into them [10, 14–24]. While caveolae were implicated in some receptors' function and membrane transport, evidence for domains with similar lipid composition, yet distinct in morphology and lacking caveolin, has also been accumulated and these were shown to be enriched in SL and the other components indicated above [10, 18–20]. SL were suggested to interact with and affect protein segregation into these microdomains. This notion was first indicated by studies of artificial membranes, where GSL tend to segregate into clusters [16]. More recently, the importance of gangliosides compartmentalized into microdomains in the Fc ϵ RI stimulation of mast cells (RBL-2H3 line) was demonstrated by the fact that upon Fc ϵ RI clustering, it undergoes association with detergent-resistant fractions of the membranes [21–24]. It was hypothesized that these specialized plasma membrane domains are involved in and facilitate the Fc ϵ RI coupling with early steps of signal transduction. This was tested by analyzing these membranes' lipid composition, in particular their cholesterol content which was found to be critical for Lyn-mediated tyrosine phosphorylation of the Fc ϵ RI and the association of both with the DRM [23, 24].

Here we report the capacity of an IgM class mAb, designated 2B5, shown to bind specifically to GSL of RBL-2H3 cells, to induce a dose-dependent secretory response of these cells which, however, is strictly dependent upon IgE being bound to its Fc ϵ RI. This is apparently caused by Fc ϵ RI recruitment and clustering within lipid rafts.

2 Results

2.1 Production of mAb 2B5

The protocol employed for selection of mouse mAb specific for RBL-2H3 cell membrane components was based on the capacity of the mAb to modulate the Fc ϵ RI-induced secretory response [5, 7, 25]. From four different hybridizations 156 clones were obtained producing mAb capable of inducing secretory response. Clone 2B5 was selected for further study because mAb 2B5 caused a dose-dependent mediator secretion from IgE-primed RBL-2H3 cells. mAb 2B5 was then isolated, purified and characterized, as described below.

2.2 Binding of mAb 2B5 to RBL-2H3 cells

mAb 2B5 interactions with RBL-2H3 cells were studied by direct binding measurements. IgE-primed or non-primed RBL-2H3 cells were incubated with purified, ^{125} I-labeled mAb 2B5, and the extent of binding was determined as described below. mAb 2B5 bound to cells, with or without Fc ϵ RI-bound IgE, to the same extent (Fig. 1). mAb 2B5 binding was also monitored via fluorescence of a phycoerythrin-streptavidin conjugate interacting with biotin-labeled mAb 2B5 (not shown). Results of both methods showed no difference in the extent of mAb 2B5 binding to the cells in the presence or absence of Fc ϵ RI-bound IgE. Monovalent Fab fragments of the IgM 2B5 were produced in order to try and determine the intrinsic affinity and the number of binding epitopes per RBL-2H3 cell. However, Scatchard analysis of the Fab binding isotherm (data not shown) suggested that mAb 2B5 binds with a relatively low affinity ($K < 10^6 \text{ M}^{-1}$) which only allowed estimating a lower limit for the number of epitopes (ca. $10^5/\text{cell}$). Also, 2B5 Fab binding was unaffected by saturating the cells with either IgE, the glycolipid GD $_{1b}$ -specific mAb B17 or the glycoprotein MAFA-specific mAb G63.

We also investigated the possibility that mAb 2B5 affects IgE binding to the RBL-2H3 cells; a limited decrease in the binding of ^{125} I-labeled IgE ($\sim 25\%$) was observed, yet only by mAb 2B5 concentrations exceeding 20 nM.

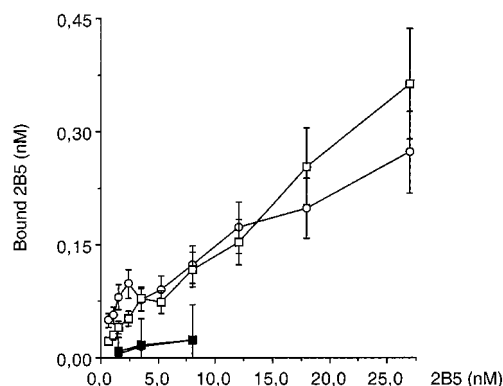


Fig. 1. Binding of mAb 2B5 to RBL-2H3 cells in the presence or absence of IgE. RBL-2H3 cells were washed and incubated in a first step with 20 nM IgE (or Tyrode's buffer), then an excess (60x) of non-radioiodinated mAb 2B5 or Tyrode's buffer was added and finally radioiodinated mAb 2B5 at the indicated concentrations. Each incubation step was for 2 h at 37 °C. Binding was determined as described in Sect. 4.9. Results are the mean of quadruplicates: without IgE (□), without IgE and an excess of non-radioiodinated mAb 2B5 (■), with IgE (○), with IgE and an excess of non-radioiodinated mAb 2B5 (●). Note, data points for both (■) and (●) are overlapping.

The possible direct mAb 2B5 binding to epitopes on the IgE or vice versa has also been excluded. Thus, the above direct binding measurements of mAb 2B5 to cells primed or non-primed with IgE showed no difference, as should have been expected if binding took place to the IgE. Additional extensive efforts to detect binding of mAb 2B5 to IgE were carried out by ELISA, as well as by Western blotting experiments and provided no evidence for the existence of any interactions.

2.3 mAb 2B5 binds to four different glycolipids of RBL-2H3 cells

The first indication that mAb 2B5 binds to a non-protein epitope was obtained from immunoprecipitation experiments: ^{125}I -surface labeled cells with or without Fc ϵ RI-bound IgE were incubated with mAb 2B5 for 2 h at 37 °C, and lysed in 1 % Triton lysis buffer. Lysates were absorbed on agarose beads conjugated with mouse IgM-specific antibodies. Beads were eluted with sample buffer and the eluate analyzed by SDS-PAGE. No bands of ^{125}I -labeled macromolecules were observed in the resultant autoradiograph made of samples derived from either IgE-primed or non-primed cells. Similarly, the insoluble fraction was solubilized by sonication and higher detergent concentrations still showed similar results. As a positive protein control, cell lysates were also immunoabsorbed with mAb G63, binding a membrane glycoprotein. This yielded as expected the protein MAFA bands [25]. The antigen(s) recognized by mAb 2B5 were therefore assumed to be of low molecular weight, possibly lipids. These were therefore extracted from RBL-2H3 membranes, separated by thin-layer chromatography (TLC) and mAb 2B5 binding was analyzed by TLC-immunostaining. No binding could be detected to the following standard glycolipids: Forssman glycolipid, lactosyl ceramide, ceramide trihexoside, globoside, sphingomyelin, sulfatide, or gangliosides GM3, GM2, GM1, GD1a, GD3, GD1b and GT1b (named according to Svennerholm [1]). mAb 2B5 did bind to four, so-far-unidentified glycolipids (Fig. 2). The major lipid detected by immunostaining migrated between GM2 and GM1. The migration properties of these glycolipids were further confirmed using two different solvent systems (see below). That these bands are indeed SL, was confirmed by metabolically labeling RBL-2H3 cells with [4,5- ^3H] dihydrosphinganine. Several labeled SL were observed, including the four lipids that had the same mobility as those detected by immunostaining. Moreover, phospholipids and 9-O-acetylated glycolipids were degraded by incubation with methanolic NaOH, which had no influence on the immunostaining.

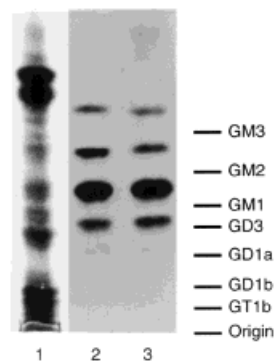


Fig. 2. TLC immunostaining of glycolipids from a lipid extract of RBL 2H3 cells. Lane 1 shows the radiogram of the SL profile after alkaline hydrolysis of [4,5- ^3H]-dihydrosphingosine-labeled RBL 2H3 cells. Four SL were detected with the mAb 2B5 by TLC immunostaining of lipid extracts of RBL 2H3 cells without (lane 2) and with alkaline hydrolysis (lane 3) as described in Sect. 4.5.

Possible binding of mAb 2B5 to cultured hippocampal neurons has been examined by immunofluorescence, as these cells are known to contain a wide variety of GSL [26]. No staining was however observed.

2.4 Secretory response to mAb 2B5

A dose-dependent secretory response to mAb 2B5 was observed for cells which had IgE bound to their Fc ϵ RI while non-IgE-primed cells yielded rather low response (Fig. 3). Still, it should be noted that the maximal secre-

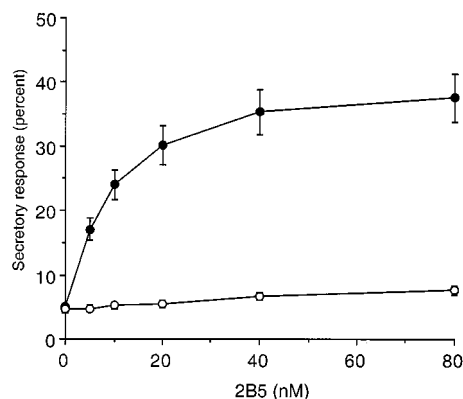


Fig. 3. Secretory response of RBL-2H3 cells to mAb 2B5. RBL-2H3 cells (1×10^5 /well) were cultured overnight with or without DNP-specific IgE. After washing, the cells were incubated with the indicated concentration of mAb 2B5 for 30 min at 37 °C. Secretion was assayed by the activity of β -hexosaminidase appearance in the supernatant. Results are the mean of six replicates [with (●) and without (○) IgE].

tory response to mAb 2B5 reached only about half that induced by the Fc ϵ RI-IgE stimulation with an optimal dose of a polyvalent antigen. Maximal response to mAb 2B5 was observed in the ~ 60 – 80 nM range and was independent of either specificity or clone of the following different rat or mouse IgE molecules employed for Fc ϵ RI saturation: the DNP-specific mAb [27] A2, 26.82 and SPE7 as well as the dimethyl amino naphthalene sulfonate (dansyl)-specific mAb 27–74.3: all cells binding these IgE yielded a similar, mAb 2B5 dose-dependent secretory response. While the mAb 2B5-induced secretion depended on IgE being bound to the Fc ϵ RI, binding to these cells' receptors of Fab fragments of the Fc ϵ RI- α -subunit-specific mAb H10 did not affect the secretory response to mAb 2B5 as IgE did. Hence, results of these experiments suggest that mAb 2B5 induces the secretory response in the presence of Fc ϵ RI-bound IgE and show that this response required 30–40 % Fc ϵ RI occupancy while most experiments were done with 50 % Fc ϵ RI saturation by IgE.

To examine possible mutual effects of antigen and mAb 2B5 on their respective stimulatory capacity, different concentrations of both reagents were employed. The concentrations of antigen and mAb 2B5 were chosen so as to include those used for each of the reagents alone. Comparison of the sums of the secretory response to each separate reagent with that caused by their mixtures showed that only an additive stimulus is caused by the two agents.

To check whether mAb 2B5 initiates the same coupling cascade as that triggered by the Fc ϵ RI, we examined the effect of mAb G63, previously shown to bind to the MAFA (a membranal C-type lectin present on RBL-2H3 cells) and to inhibit the secretory response to Fc ϵ RI clustering [25]. Significantly, mAb G63 was also found to inhibit the secretory response caused by mAb 2B5 and to a similar extent. Inhibition of the cells' secretory response to mAb 2B5 by MAFA clustering with mAb G63 suggested that the coupling of mAb 2B5 to the secretory response proceeds via the Fc ϵ RI-activated cascade [2]. To investigate whether mAb 2B5 induces activation of this pathway, we have examined the changes it causes in the tyrosine phosphorylation of the protein tyrosine kinase Syk known to be an essential early step in that pathway [2]. Cells were incubated as described below with or without IgE and were activated by the addition of either Ag or mAb 2B5. Following this, cells were lysed and Syk was immunoprecipitated, separated on SDS-PAGE, transferred to a nitrocellulose membrane, and Western blotted with a phosphotyrosine-specific mAb (PY99). As illustrated in Fig. 4, pronounced tyrosine phosphorylation is caused, as expected, by Ag activation and a more limited, yet clear tyrosine-phos-

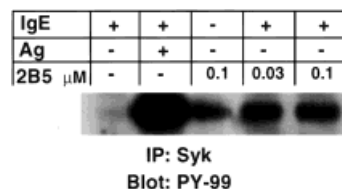


Fig. 4. mAb 2B5 activation of PTK Syk phosphorylation. RBL-2H3 cells (17×10^6 /sample in 15-cm petri dish) were incubated with or without 10 nM A2IgE at 37 °C for 1 h. The indicated samples were then treated either with Ag (30 ng/ml) or with the mAb 2B5 at 37 °C for 5 min. The cells were lysed in 1 % Triton X-100 lysis buffer, and immunoprecipitated with rabbit Syk-specific antibodies carried on protein G-Sepharose beads. The beads were eluted with sample buffer and resolved by SDS-PAGE, followed by Western blotting with the phosphotyrosine-specific mAb PY-99.

phorylated band was induced by mAb 2B5. Interestingly, weak Syk tyrosine phosphorylation is also observed upon treating the cells with mAb 2B5 alone (*i.e.* without IgE), yet this is apparently below the threshold required for stimulating the secretory response.

2.5 mAb 2B5 induces aggregation of cell surface IgE-Fc ϵ RI complexes

To examine whether mAb 2B5 affects the cell surface distribution of the IgE-Fc ϵ RI complexes, we have carried out confocal fluorescence microscopy studies using fluorophore (Cy3)-labeled IgE on RBL-3H3 cells, resting or treated either with mAb 2B5 or with antigen. Cells were primed with 1×10^{-8} M labeled IgE, as indicated below. Subsequently, the cells were incubated either with 30 ng/ml of antigen (DNP₁₁-BSA) or with 3×10^{-8} M mAb 2B5, at room temperature for 3 min. Treatment with antigen Fig. 5 a (C + D) or with mAb 2B5 (E + F), induced very similar and marked changes in the surface distribution of Cy3-IgE compared with the control resting cells (A + B). Moreover, we have also carried out experiments where both the IgE and mAb 2B5 were labeled by distinct fluorescent probes and investigated their respective cell surface distribution. As shown (Fig. 5 b, A + E) F-2B5 is uniformly distributed on the resting cell's surface. Binding of Cy3-IgE causes minimal changes in the F-2B5 surface distribution (Fig. 5 b, B + F), yet that of Cy3-IgE is punctuated, indicating aggregation (Fig. 5 b, C + G). Superposition of the emissions of the Cy3 and F probes suggests the co-localization of the IgE with that of the largely in excess and hence dispersed mAb 2B5 (Fig. 5 b, D + H).

We have also examined the cell surface distribution of Cy3-IgE and the GPI-anchored protein Thy-1 known to

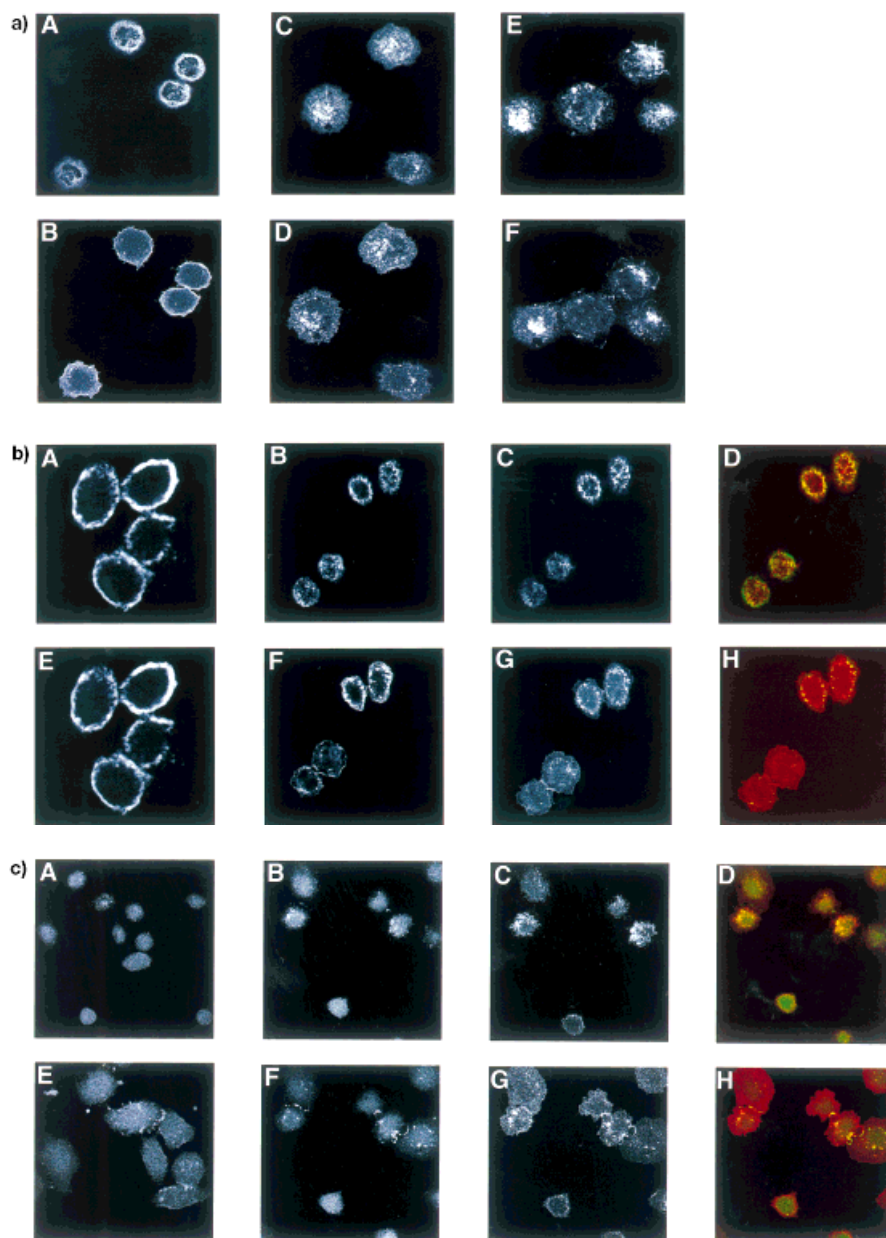


Fig. 5. (a) Cell surface distribution of Cy3-labeled IgE on resting and antigen or mAb 2B5 treated RBL-2H3 cells. A,B – Resting RBL-2H3 cells treated with (10^{-8}) Cy3-labeled, DNP-specific monoclonal IgE. Fluorescence patterns were examined first by a Zeiss Axiovert 35 and all the presented confocal fluorescence images were taken using a Bio-Rad MRC-1024 confocal system using an Ar/Kr mixed-gas laser for excitation via an Applitech MSV-700 CCD camera. C,D – Above cells activated by antigen (DNP₁₁-BSA; 30 ng/ml) for 3 min at room temperature followed by washing and fixation. E,F – Cells treated as in A and B with Cy3-labeled IgE, then with mAb 2B5 (0.03 μ M) followed by the above protocol and measurements. Shown is emission of fluorescein. A,C, E Upper focal plan, B,D,F lower focal plan. (b) Cell surface co-localization of fluorescein-labeled (F-) mAb 2B5 and Cy3-labeled IgE. A,E – RBL-2H3 cells treated with F-2B5 (3×10^{-8} M) only, followed by the above protocol and measurements. B,F – Cells were treated with both F-2B5 and Cy3-IgE by the above protocol, yet only emission of F-2B5 was monitored. C,G – RBL-2H3 cells were treated with F-2B5 and Cy3-IgE, yet only emission of Cy3-IgE was monitored. D,H – RBL-2H3 cells treated with both F-2B5 and Cy3-IgE and fluorescence of both F-2B5 and Cy3-IgE is presented. A,B,C,D Upper focal plan, E,F,G,H lower focal plan. (c) Cell surface localization of a F-Thy-1-specific antibody and Cy3-labeled IgE. A,E – RBL-2H3 cells treated with the F-Thy-1-specific mAb. B,F – RBL-2H3 cells were treated with both F-Thy-1 and Cy3-IgE, yet only the emission of F-Thy-1 is shown. C,G – RBL-2H3 cells were treated with both F-Thy-1 and Cy3-IgE, yet only the emission of Cy3-IgE is shown. D,H – As above, yet emissions of both F-Thy-1 and Cy3-IgE is presented with above respective colors. A,B,C,D Upper focal plan, E,F,G,H lower focal plan.

be expressed by RBL-2H3 cells [28, 29]. The latter was monitored using a commercially available fluorescein-labeled specific antibody. As Fig. 5c A + E shows, Thy-1 is distributed all over the cell surface essentially as uniformly as IgE on the resting cells (Fig. 5a, A + E). Only limited change in the Thy-1 distribution is observed upon $\text{C}\gamma 3$ -IgE binding (Fig. 5c, B + F). However, upon binding of the Thy-1-specific antibodies the distribution of the $\text{C}\gamma 3$ -IgE is changing indicating its aggregation (Fig. 5c, C + G). The $\text{C}\gamma 3$ -IgE fluorescence is at least partly superimposed on the excessive Thy-1 fluorescence (Fig. 5c, D + H).

3 Discussion

Considerable attention has recently been drawn to the diverse experimental evidence for microdomains in the plasma membranes of cells [10–24]. Such domains are assumed to have a distinct composition and the fact that specific lipid-conjugated or glycolipid-anchored proteins are associated with them, has turned out to imply rather important consequences for cellular function in general and that of antigen receptors in particular. Early evidence for such structures emerged when GSL were incorporated into artificial lipid bilayers of glycerolipids and they were found to form separate domains within these bilayers' plane [16]. Further evidence for such domains in cellular membranes emerged from a range of different experiments, mainly those where cell lysis was affected with the non-ionic detergent Triton X-100 and followed by sucrose gradient centrifugation [21–24, 28–33]. The Triton X-100-insoluble fractions were found to be enriched in GSL and cholesterol. Moreover, analysis of their protein content exhibited an enrichment in GPI-anchored proteins and various protein kinases. Baird et al. provided strong support for specific association of the $\text{Fc}\epsilon\text{RI}$ with such specialized membrane domains and its potential role in the secretory response mediated by this receptor [21–23]. These authors further suggested that the earliest step in the $\text{Fc}\epsilon\text{RI}$ stimulus-response coupling cascade is this receptor's association with membrane domains in which the src-family member protein tyrosine kinase (PTK) Lyn is activated. While the $\text{Fc}\epsilon\text{RI}$ and Lyn were found to be enriched in the detergent-insoluble fractions isolated from resting cells, $\text{Fc}\epsilon\text{RI}$ clustering induced significant changes in this association and the activation of Lyn [21–23]. It was suggested that a selective interaction between the $\text{Fc}\epsilon\text{RI}$ and specialized domains of the mast cells membrane, which include the PTK Lyn, induced by the $\text{Fc}\epsilon\text{RI}$ clustering is the first event in the coupling cascade. Related to these findings is the observation that the GPI-anchored Thy-1, also present in the RBL-2H3 membranes, is causing, upon being clustered, a dose-dependent mediator secretion of RBL-2H3 cells [28, 29].

Results of the current study show that mAb 2B5 is specific for GSL present in the outer leaflet of RBL 2H3 cells and induces their dose-dependent secretory response that requires $\text{Fc}\epsilon\text{RI}$ being occupied by IgE. This cellular response is markedly different from the effects which have so far been reported for GSL-specific antibodies such as mAb B17 or AA4 [7, 9]. Namely, the former cause either enhancement or suppression of the $\text{Fc}\epsilon\text{RI}$ -induced secretory response of RBL-2H3 cell depending on the dose. In contrast, mAb 2B5 apparently causes the cells' response by a different mechanism, because it is capable of inducing a dose-dependent secretion, though it is binding to lipid components of the cell plasma membrane, rather than to a membrane macromolecule. The results of this study lead us to assume that mAb 2B5 binds to GSL associated with the $\text{Fc}\epsilon\text{RI}$ -IgE complex (Fig. 6). Being an IgM class mAb, its decavalency significantly promotes GSL clustering and thereby also aggregation of the $\text{Fc}\epsilon\text{RI}$ -IgE complexes which are associated with these GSL. Support for this notion is lent by results of the fluorescence microscopy experiments. These show that treatment of the IgE-primed cells with either antigen or mAb 2B5 yields very similar, if not identical, IgE distribution patterns, *i. e.* typical for $\text{Fc}\epsilon\text{RI}$ -IgE aggregation by antigen and similar to those produced by Thy-1 clustering. We therefore propose that the mAb 2B5 action mechanism is based on an induced aggregation of the IgE- $\text{Fc}\epsilon\text{RI}$ complexes mediated by its associated GSL (Fig. 6).

The dependence of the mAb 2B5-induced secretory response on IgE binding to the $\text{Fc}\epsilon\text{RI}$ is a rather surprising result. A possible rationale for it may be that structural changes in the $\text{Fc}\epsilon\text{RI}$ and/or its annular lipids are induced upon IgE binding to the $\text{Fc}\epsilon\text{RI}$. Such a change may affect the interaction between the membrane lipids

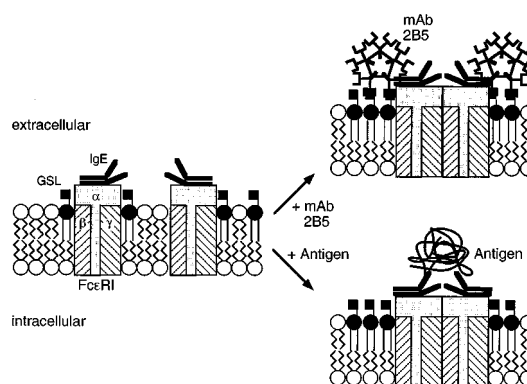


Fig. 6. Schematic diagram of DNP_{11} -BSA and mAb 2B5 $\text{Fc}\epsilon\text{RI}$ -IgE complex aggregation.

and the Fc ϵ RI-IgE complex and be responsible for the IgE dependency of the secretory response caused by mAb 2B5. This notion is supported by the strict requirement for IgE binding while binding of the Fab fragment of the α -chain specific mAb H10 to the Fc ϵ RI failed to cause a similar mAb 2B5-dependent secretory response, and by the independence of the secretory response to mAb 2B5 on the specificity or source of the Fc ϵ RI-bound IgE. The possibility that mAb 2B5 may bind directly to the IgE and cause the observed cell response has been excluded by several different experiments ranging from immunoprecipitation and Western blotting to direct binding assays. The additive secretion caused by treatment with mAb 2B5 and antigen-induced Fc ϵ RI-IgE clustering also indicates separate, but Fc ϵ RI-mediated, induction of the cell response. In line with this is also the observation that the response to mAb 2B5 is inhibited by mAb G63-specific MAFA, a C-type animal lectin interfering with the Fc ϵ RI cascade and inhibiting the secretory response [25, 34]. This is further supported by the tyrosine phosphorylation of the PTK Syk, induced upon binding of mAb 2B5.

In conclusion, the results described above suggest that the recently reported co-aggregation of membranal lipids with clustered cell surface proteins, notably antigen receptors like the TCR and the Fc ϵ RI [10, 31] also has its inverse counterpart: namely, clustering of SL by mAb 2B5 is apparently causing co-aggregation of the Fc ϵ RI-IgE complexes, *i.e.* these interactions can work in both directions. Furthermore, costimulation via a raft-localized protein can also enhance antigen receptor activity as recently illustrated by the effect of CD28 on the TCR activity [33]. The usually fast internalization of small receptor aggregates which would normally counteract the maintenance of such clusters, is apparently less effective when the decavalent IgM-2B5 is the clustering agent. Hence, the net outcome is the initiation of the Fc ϵ RI stimulatory cascade.

4 Materials and methods

4.1 Culture media and reagents

Culture media were purchased from GIBCO (Grand Island, NY). Fetal calf serum, glutamine, penicillin-streptomycin mixture, and sodium pyruvate as tissue culture supplements were from Bio-Lab (Jerusalem). The hybridoma IGELa2, secreting the IgE mAb A2 (DNP specific) [27] was grown in ascitic fluid and purified by affinity chromatography on DNP-Sepharose-4B. mAb G63 was produced and purified as described elsewhere [25]. BSA (fraction V, sigma St. Louis, MO) was derivatized with an average of eleven 2,4-DNP residues (DNP₁₁-BSA) in our laboratory by 1-fluoro-2,4-DNP-

benzene. Sphingolipid standards were obtained from Matreya Inc. (Pleasant Gap, PA) and Sigma (St. Louis, MO). Silica Gel-60 plates were from Merck. [4,5-³H]Sphinganine was synthesized as previously described [26]. [³H]Serotonin was purchased from Amersham, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) were obtained from Molecular Probes Inc. (Eugene, OR), Cy3 from Biological Detection Systems Inc. (Pittsburgh, PA) and phycoerythrin-streptavidin from Biomed (Foster City, CA). Fluorescein-labeled mAb specific for rat Thy-1 was from Caltag Labs. Inc., San Francisco, CA. All other solvents and chemicals used were at least of analytical grade.

4.2 Cell culture

Rat mucosal type mast cells of the RBL-2H3 line [3, 5] were obtained from Dr. R. Siraganian, NIH, Bethesda, MD and maintained in Eagle's minimal essential medium with Earle's salts (MEM) supplemented with 10 % FCS, 2 mM glutamine, and antibiotics in a humidified atmosphere with 7 % CO₂ at 37 °C. For binding and secretion experiments, the cells were in Tyrode's buffer: 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 20 mM Hepes, and 0.1 % BSA (pH 7.4).

4.3 Production of hybridomas

Hybridoma cell lines were generated as described elsewhere [3–7]. Positive clones were selected on the basis of the capacity of the secreted antibodies to modulate the secretory response of RBL-2H3 cells. The latter cells were plated and allowed to adhere to 96-well plates in the presence of [³H]serotonin for 16 h with or without IgE. After washing the monolayers, 50 μ l of hybridoma supernatant were added per well, and the plates were incubated for 30 min at room temperature. Secretion was then induced by adding antigen (DNP₁₁-BSA) for 40 min at 37 °C; 100 μ l of the supernatants were then taken for determining the amount of released [³H]serotonin.

4.4 Production and purification of mAb 2B5 and its Fab fragments

Supernatants of the hybridoma designated 2B5 were consistently found to cause secretion of the cells that were pre-incubated with IgE under the above assay conditions without antigen. mAb 2B5 was found to belong to the IgM class, as determined by double immunodiffusion against class-specific antisera and exhibited properties of a cryoprotein. Hence it was purified from culture-supernatants according to the method of Garcia-Gonzalez [35] by euglobulin precipitation. For mAb 2B5 precipitation, culture-supernatants were extensively dialyzed against double-distilled water at 4 °C for about 3 days. The precipitate was resuspended at

1/20 of the original volume of the cell culture in 0.1 M Tris-HCl, 1M NaCl (pH 8.0) and dialyzed against water. Pure mAb 2B5 (analyzed by SDS-PAGE/Coomassie brilliant blue staining) was obtained from the supernatant after two such precipitation steps. For production of Fab fragments, mAb 2B5 at a concentration of about 10 mg/ml in 0.1 M Tris-HCl, pH 8.2, was incubated with 2 % trypsin TPCK, 1 mg/ml CaCl_2 and 1.2 mg/ml cysteine at 37 °C, for 2 h. The digest was centrifuged and Fab fragments were isolated through Sephacryl S-100 gel filtration.

4.5 Identification of mAb 2B5 antigens

Since no RBL cell protein component could be immunoprecipitated by mAb 2B5 from RBL-2H3 lysates, we assumed that it binds a low-molecular weight membrane component. This was analyzed as follows: RBL cells were centrifuged ($420 \times g$, 5 min, room temperature) and washed twice with 5 ml Hanks' balanced salt solution (HBSS). The cells were resuspended in 5 ml distilled water and lyophilized, resuspended in 4 ml of $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{pyridine}$ (60:30:6:1 v/v/v/v), and incubated at 48 °C for 48 h with stirring. For degradation of phospholipids and 9-O-acetylated glycolipids, samples were incubated 2 h at 37 °C with 100 mM methanolic NaOH. Subsequently, the samples were desalted by reverse-phase (RP) chromatography on an RP-18 column and SL were separated by TLC using either $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{aqueous } 9.8 \text{ mM } \text{CaCl}_2$ (60:35:8 v/v/v) or $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (60:35:8 v/v/v) as the developing solvents. For metabolic labeling of GSL, RBL cells were grown for 2 days in 500 ml Falcon culture flasks and incubated with 20×10^6 cpm of $[4,5\text{-}^3\text{H}]\text{-dihydrosphinganine}$ (10 Ci/mmol) for two days. $[^3\text{H}]\text{SL}$ were separated as described above and visualized using a BAS-TR2040S imaging plate, a FUJIX BAS 1000 phosphorimager and Mac BAS v2.0 software (Fuji, Japan). The TLC immunostaining was performed as described before [36].

4.6 mAb 2B5 immunoprecipitation from surface radioiodinated RBL cells

Lysates in 1 % Triton lysis buffer were immunoprecipitated by either G63 mAb agarose beads as a positive control, or 2B5-agarose beads. This was performed on both the soluble fraction and a sonicated dispersed precipitate fraction of the lysates. The immunoprecipitates were eluted by boiling in SDS sample buffer and analyzed by SDS-PAGE on a 10 % gel followed by autoradiography.

4.7 Radioactive or fluorescent labeling of antibodies

mAb 2B5 or affinity-purified mouse IgE, were iodinated with ^{125}I using the chloramine T method [37]. Specific activities in the range of 5–10 mCi/mg protein were obtained and employed in the binding assays. Fluorophore conjugation to

proteins was usually performed by reacting $\sim 15:1$ w/w fluorophore: protein. Fluorophore was dissolved in 20 μl ethanol and added to 2 ml of protein solution in 0.1 M NaHCO_3 (pH 9.3). Reaction was allowed to proceed at room temperature for 2 h. The protein and free dye were separated through G25 gel filtration in 0.1 M NaHCO_3 pH 9.3.

4.8 Secretory response assays

Except for the hybridoma selection procedure, the secretory response was monitored by measuring the activity of the granular β -hexosaminidase in the cell supernatants after treatments indicated for each particular experiment [5]. From each sample assayed, supernatant aliquots (20 μl) were transferred to microtiter plates. Then, 40 μl of substrate solution (1.3 $\mu\text{g}/\text{ml}$ p-nitrophenyl-N-acetyl- β -D-glucosamine in 0.1 M sodium citrate, pH 4.5) were added and the plates incubated for further 60 min. at 37 °C. The reaction was then stopped and the color was developed by addition of 150 μl of 0.2 M glycine solution, pH 10.7. The light absorption by the substrate hydrolysis product was measured at 405 nm in an ELISA plate-reader. To determine the total cell content of this enzyme, an equivalent number of cells were lysed with 1 % Triton X-100 and a lysate aliquot was assayed in parallel to the other experimental samples. The extent of secretion is expressed as net fraction of the total β -hexosaminidase activity present in the cells.

4.9 Binding assays

RBL-2H3 cells ($3 \times 10^6 - 5 \times 10^6$) washed in Tyrode's buffer supplemented with 0.1 % NaN_3 were incubated in suspension with the indicated concentrations of ^{125}I -labeled either mAb 2B5 or IgE, for 1 h at 37 °C, with occasional shaking, in a total volume of 400 μl . Triplicate 100- μl samples from each test tube were layered on 200 μl of fetal calf serum in 400 μl microfuge polypropylene test tubes. The cells were sedimented for 1 min in a microfuge (15000 rpm, Heraeus, Germany). The bottom of the test tubes, which contained the cell pellet was cut and counted. Nonspecific binding was determined by a similar titration where each cells sample was preincubated with an excess of unlabeled mAb (mAb 2B5 or IgE), for 30 min.

4.10 Tyrosine phosphorylation of the PTK Syk

Cells were grown overnight at $\sim 10^6$ ml in 15-cm petri dishes, either with or without IgE, then washed three times with warm Tyrode's buffer and treated either by Ag (30 ng/ml) or by mAb 2B5 (0.03 and 0.1 μM) for 5 min at 37 °C. Activation was stopped by adding cold PBS. Cells were then lysed for 20 min, with ice-cold 1 % Triton lysis buffer, containing a mixture of protease (Calbiochem Cocktail Set III) and phosphatase inhibitors: β -glycerophosphate 50 mM and Na_3VO_4 1 mM. Specific Syk antibodies bound to protein

G-Sepharose beads, were then used to immunoprecipitate the Syk (2 h at 4 °C). The bound protein was then eluted with boiling sample buffer, separated by SDS-PAGE on a 10 % polyacrylamide gel, transferred to nitrocellulose membrane and Western blotted with mAb PY-99.

4.11 Fluorescence microscopy

Fluorescence microscopy studies were done monitoring: (1) the influence of antigen and of mAb 2B5 on the distribution of Cy3-labeled A₂IgE-FcεRI complexes; (2) the distribution of fluorescein-labeled mAb 2B5 (F-2B5); and (3) fluorescein-labeled mAb specific for rat Thy-1 on RBL cells. These were carried out as follows: 10⁵ RBL-2H3 cells/0.4 ml DMEM, were placed on glass cover slips, each placed in a 12-well Costar plate, and incubated for 2 h at 37 °C, allowing for cells to adhere. The cell supernatants were removed, and 0.4 ml of 1 × 10⁻⁸ M fluorescein- or Cy3-labeled IgE was added. Incubation was continued at room temperature for another 1 h and the IgE solution was washed out with Tyrode's buffer. Then the cells were reacted either with Ag (30 ng/ml) or with 3 × 10⁻⁸ M mAb 2B5 IgM, or with 1 × 10⁻⁷ M of rat Thy-1-specific mAb, at room temperature for 3 min. After rinsing with PBS, coverslips were fixed with 4 % paraformaldehyde (15 min, room temperature) and mounted in 50 % glycerol in PBS. The cell surface distribution of labeled IgE was first analyzed using a Plan Apochromat 40 X/1.3 n.a. oil objective of a Zeiss Axiovert 35. The presented fluorescence confocal images were then taken using a Bio-Rad MRC-1024 confocal system, utilizing an argon/krypton mixed gas laser, via an Applitec MSV-700 CCD camera transferred to a Macintosh 840AV computer, and fluorescence intensities analyzed using NIH image software.

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