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Possible orientational constraints determine secretory signals induced by aggregation of IgE receptors on mast cells

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Three biologically active monoclonal antibodies (mAbs) specific for the monovalent, high-affinity membrane receptor for IgE ($Fc_\epsilon R$) were employed in analysing the secretory response of mast cells of the RBL-2H3 line to crosslinking of their $Fc_\epsilon R$. All three mAbs (designated F4, H10 and J17) compete with each other and with IgE for binding to the $Fc_\epsilon R$. Their stoichiometry of binding is 1 Fab:1 $Fc_\epsilon R$, hence, the intact mAbs can aggregate the $Fc_\epsilon Rs$ to dimers only. Since all three mAbs induce secretion, we conclude that $Fc_\epsilon R$ dimers constitute a sufficient 'signal element' for secretion of mediators for RBL-2H3 cells. The secretory dose–response of the cells to these three mAbs are, however, markedly different: F4 caused rather high secretion, reaching almost 80% of the cells' content, while J17 and H10 induced release of only 30–40% mediators content. Both the intrinsic affinities and equilibrium constants for the receptor dimerization were derived from analysis of binding data of the Fab fragments and intact mAbs. These parameters were used to compute the extent of $Fc_\epsilon R$ dimerization caused by each of the antibodies. However, the different secretory responses to the three mAbs could not be rationalized simply in terms of the extent of $Fc_\epsilon R$ dimerization which they produce. This suggests that it is not only the number of crosslinked $Fc_\epsilon Rs$ which determines the magnitude of secretion-causing signal, but rather other constraints imposed by each individual mAb are also important. These constraints are most probably configurational ones, yielding differently oriented $Fc_\epsilon Rs$ in the produced dimers. These results provide, to our knowledge, the first evidence for a possible role of the relative orientation of receptors being crosslinked in a stimulatory aggregation process.

Key words: Fc_ϵ receptors/secretion of mediators/RBL-2H3 cells/transmembrane signalling

Introduction

Clustering of plasma-membrane receptors provides for transduction of a diverse range of signals into cells. In particular, immunological stimuli are characterized by this requirement of multivalent interactions leading to aggregation of cell surface components. Thus, for example, activation of resting T lymphocytes is initiated through multiple interactions of the heterodimer T cell antigen receptor with a complex consisting of an antigen and proteins of the major histocompatibility complex (Marrak and Kappler, 1986). Aggregation caused by monoclonal antibodies (mAbs) to either this receptor or to its associated CD3 complex mimic cellular activation by antigen (Meuer *et al.*, 1983). Moreover, synergism has been observed upon crosslinking the T cell antigen receptor with the CD8 differentiation antigen (Emmerich *et al.*, 1986). In mast cells, aggregation of the monovalent, high affinity membrane receptor for IgE ($Fc_\epsilon R$) is known to provide the initial signal for secretion of stored granular and *de novo* synthesized mediators of inflammation. While the requirement for aggregation is well recognized, its quantitative, structural and mechanistic aspects are still essentially unresolved (Ishizaka and Ishizaka, 1984; Metzger *et al.*, 1986). Several studies have suggested that small oligomers of IgE provide a sufficient signal for initiating the secretory process (Ishizaka and Ishizaka, 1968). Early experiments aimed at a quantitative analysis of the crosslinking requirements employed divalent haptens as the simplest model antigen amenable to such a treatment (Siraganian *et al.*, 1975). Divalent haptens would, however, crosslink the $Fc_\epsilon R$–IgE complex to form oligomeric chains of unknown multiplicity. Later, covalently crosslinked oligomers of IgE were employed and led to the proposal that even dimers can serve as the 'unit signal' for mast cell degranulation (Segal *et al.*, 1977). More recent studies, however, did cast some doubts on this relatively simple notion. These studies employed essentially two types of experimental approaches: the first extended the use of covalent IgE oligomers to another type of mast cell (Fewtrell and Metzger, 1980), namely the rat basophilic leukemia line RBL-2H3 (Barsumian *et al.*, 1981; Seldin *et al.*, 1985). These cells were found to be virtually unresponsive to purified fractions of covalent IgE dimers (Fewtrell and Metzger, 1980). In addition, rat peritoneal mast cells did not degranulate significantly upon treatment with an anti-IgE mAb, which is assumed to form only dimeric IgE under the employed protocol (Menon *et al.*, 1986). The second experimental approach examined, by physical methods, the fate of the initially formed small $Fc_\epsilon R$ oligomers (Menon *et al.*, 1984). These studies revealed that large scale coalescence of IgE–$Fc_\epsilon R$ complexes takes place following the initial formation of small $Fc_\epsilon R$ oligomers. Though these observations were interpreted in a way that seems to resolve the apparent conflict, the question as to the signalling capacity of $Fc_\epsilon R$ dimers, at least in the widely employed RBL-2H3 cells, still appears unsettled (Metzger *et al.*, 1986).

The mAbs directed against membrane receptors have been established as effective reagents for both the isolation and for investigating their mode of action (e.g. Fernandez-Pol, 1985; Defize *et al.*, 1988). We have raised several mAbs to the $Fc_\epsilon R$ present on RBL-2H3 cells. Three of these mAbs (of the IgG class) were now further characterized in terms of their binding and secretory response which they induce in these cells. These mAbs are homogeneous and structurally defined crosslinking agents, enabling a rigorous quantitative analysis of the relationship between $Fc_\epsilon R$ aggregation and secretion.

Theoretical background

Binding of IgE and monovalent Fab to the cells. The intrinsic affinity of each Fab to the $Fc_\epsilon R$ and the number of ligands bound at saturation were derived from analysis of binding data of the monovalent Fabs prepared from the three mAbs. The overall reaction can be formally described by:

$$n\ \text{Fab} + \text{Fc}_\epsilon\text{R} \overset{K_r}{\rightleftharpoons} \text{Fab}_n\text{–Fc}_\epsilon\text{R}$$

where n denotes the number of Fab-fragments which can bind to one $Fc_\epsilon R$. The law of mass-action, then, allows to calculate the concentration of bound Fab-fragments $[Fab]_b$ in terms of free epitopes concentration $[Fc_\epsilon R]_f$ as follows:

$$[\text{Fab}]_b = K_r[\text{Fc}_\epsilon\text{R}]_f\,[\text{Fab}]_f. \quad (1)$$

Where K_r denotes the intrinsic binding constant and $[Fab]_f$ is free Fab-concentrations. These are calculated using the mass-conservation law which leads to the following equations:

$$[\text{Fab}]_f = -\alpha + \sqrt{\{\alpha^2 + \beta\}} \quad (2)$$

where $\alpha = \{[\text{Fab}]_t + [\text{Fc}_\epsilon\text{R}]_t - 1/K_r\}/2$ and $\beta = [\text{Fab}]_t/K_r$.

$$[\text{Fc}_\epsilon\text{R}]_f = [\text{Fc}_\epsilon\text{R}]_t - [\text{Fab}]_t + [\text{Fab}]_f. \quad (3)$$

Inserting these expressions for $[Fab]_f$ and $[Fc_\epsilon R]_f$ into equation (1), one obtains the concentration of bound Fab-fragments as a function of the total Fab-concentration $[Fab]_t$ and total epitopes concentration $[Fc_\epsilon R]_t$. This equation was used in a program designed to fit the experimentally obtained binding isotherms of the different Fabs. The equilibrium constant K_r and the total concentration of receptor binding epitopes $[Fc_\epsilon R]_t$ are used as free parameters in this fitting procedure.

In order to determine the stoichiometry of the Fab-binding to the $Fc_\epsilon R$-receptor, we have measured in parallel experiments the binding of IgE to the very same cell batches. The respective reaction is given by:

$$\text{IgE} + \text{Fc}_\epsilon\text{R} \overset{K'_r}{\rightleftharpoons} \text{IgE–Fc}_\epsilon\text{R}.$$

Employing the law of mass-action and the established 1:1 stoichiometry, the concentration of bound IgE is obtained by:

$$[\text{IgE}]_b = K'_r\,[\text{IgE}]_f\,[\text{Fc}_\epsilon\text{R}]'_f \quad (4)$$

where K'_r denotes the corresponding equilibrium constant. $[IgE]_f$ and $[Fc_\epsilon R]'_f$ are the free concentrations of IgE and receptors respectively. These can be calculated in terms of the total receptor and IgE concentrations by employing equations similar to equations (2) and (3). The equilibrium constant K'_r and the total receptor concentration $[Fc_\epsilon R]'_t$ were used as free parameters to fit the IgE-binding isotherms. The unknown stoichiometry of the Fab + $Fc_\epsilon R$-reaction can now be calculated: $n = [Fc_\epsilon R]/[Fc_\epsilon R]'$ at binding saturation.

The results of binding different $Fc_\epsilon R$ specific Fabs or IgE to the cells were also presented using the Scatchard formalism:

$$Y/[\text{Fab}]_f = K_r\,(n-Y) \quad (5)$$

for the Fabs, and

$$Y/[\text{IgE}]_f = K'_r\,(1-Y) \quad (6)$$

for the IgE-binding results where Y denotes the mole fraction of occupied epitopes or binding sites respectively (i.e. the $[Fc_\epsilon R]_b/[Fc_\epsilon R]_t$ calculated for the respective Fab or for IgE).

Analysis of the binding of intact anti-$Fc_\epsilon R$ mAbs to the cells

The binding of intact anti-$Fc_\epsilon R$-mAbs to their respective epitopes on the surface of RBL-2H3-cells was analysed in terms of a simple model which is based on the determined 1:1 stoichiometry of the Fab–$Fc_\epsilon R$-reaction (i.e. $n = 1$). Hence, the mAbs can crosslink the $Fc_\epsilon R$ into dimers only. The first step in each antibody–receptor reaction is the binding of one Fab-binding site to its respective epitope:

$$\text{mAb} + \text{Fc}_\epsilon\text{R} \overset{K_r}{\rightleftharpoons} \text{mAb–Fc}_\epsilon\text{R}.$$

The equilibrium constant K_r is given by:

$$K_r = [\text{mAb}]_b/2[\text{mAb}]_f\,[\text{Fc}_\epsilon\text{R}]_f. \quad (7)$$

The second step is described by the following reaction:

$$\text{mAb–Fc}_\epsilon\text{R} + \text{Fc}_\epsilon\text{R} \overset{K_d}{\rightleftharpoons} \text{Fc}_\epsilon\text{R–mAb–Fc}_\epsilon\text{R}$$

which leads to $Fc_\epsilon R$-dimer formation on the cell surface. Therefore, the concentration of the $Fc_\epsilon R$–mAb–$Fc_\epsilon R$ and the $Fc_\epsilon R$–mAb complexes must now be expressed in terms of the number of species per volume V^* (Reynolds, 1979; see also Dower *et al.*, 1984 for an alternative analysis). This is defined for a given cell by its surface area A and an arbitrarily chosen surface layer thickness δ, i.e.

$$V^* = A \cdot \delta. \quad (8)$$

Hence, the respective equlibrium constant K^*_d is given by:

$$K^*_d = D^*/M^*\,[\text{Fc}_\epsilon\text{R}]^*_f, \quad (9)$$

where $D^* = [Fc_\epsilon R–mAb–Fc_\epsilon R]^*$ is the number of receptor dimers, $M^* = [Fc_\epsilon R–mAb]^*$ is the number of ligated receptor monomers and $[Fc_\epsilon R]^*_f$ is the number of free receptors, all per volume element V^* averaged over all cells.

To facilitate the numerical calculation, we formulate equation (9) in terms of molar concentrations by considering the adjusted cell concentration C (cells/l). This yields:

$$K_d = D/M[\text{Fc}_\epsilon\text{R}]_f, \quad (10)$$

where

$$K_d = K^*_d\text{L}/CV^*\text{L}$$
$$D = CV^*D^*/\text{L}$$
$$M = CV^*M^*/\text{L}$$
$$[\text{Fc}_\epsilon\text{R}]_f = CV^*[\text{Fc}_\epsilon\text{R}]^*_f/,$$

where L denotes the Avogadro constant.

Now we can calculate the total concentration of bound mAbs which is the observed quantity in our experiments:

$$[\text{mAb}]_b = 2K_r[\text{Fc}_\epsilon\text{R}]_f\,[\text{mAb}]_f + 2K_rK_d[\text{mAb}]_f\,[\text{Fc}_\epsilon\text{R}]^1_f. \quad (11)$$

Equation (11) expresses $[mAb]_b$ as a function of free mAb and receptor concentrations. In order to relate these quantities to the respective total concentrations $[mAb]_t$ and $[Fc_\epsilon R]_t$, the conservation of mass once again leads to the equations:

$$[\text{mAb}]_t = [\text{mAb}]_f + 2K_r\,[\text{mAb}]_f\,[\text{Fc}_\epsilon\text{R}]_f + 2K_rK_d\,[\text{mAb}]_f\,[\text{Fc}_\epsilon\text{R}]^2_f \quad (12)$$

$$[Fc_\epsilon R]_t = [Fc_\epsilon R]_f + 2K_r\,[mAb]_f\,[Fc_\epsilon R]_f + 4K_rK_d\,[mAb]_f\,[Fc_\epsilon R]^2_f. \quad (13)$$

Rearranging these equations, one obtains the quantities $[mAb]_f$ and $[Fc_\epsilon R]_f$. Since the equations are not linear, we employed a two-dimensional version of the Newton–Raphson method (Morgenau and Murphy, 1956) in a numerical zero-search program.

In order to present the extent of dimer formation, we calculated χ_{dim} the mole-fraction of receptors per cell incorporated into dimers:

$$\chi_{dim} = 4K_rK_d\,[mAb]_f\,[Fc_\epsilon R]^2_f/[Fc_\epsilon R]_t. \quad (14)$$

Equations (11–14) now represent the basis for the fitting procedure in which the equlibrium constant K_d is the only free parameter. The intrinsic binding constant K_r is obtained from the analysis of the binding results of the respective Fab-fragments, and $[Fc_\epsilon R]_t$ is determined independently by titration with [^{125}I]IgE.

In order to quantify the quality of the fits, we calculated the error value according to the equation:

$$f = \sqrt{\sum_{i=1}^{n} \{([mAb]_{b(t,i)} - [mAb]_{b(e,i)})^2/N\cdot\sigma_i^2\}}$$

where $[mAb]_{b(t)}$ and $[mAb]_{b(e)}$ denote the theoretical and experimental concentrations of bound mAb. N is the number of data points and σ_i is the standard deviation of the respective experimental data from their mean value.

All computer fits were carried out with a fitting routine called MINUITL obtained from the CERN-library and described by James and Ross (1975).

Results

The mAbs secreted by the hybridomas designated F4, J17 and H10 were selected for their ability to induce mediators secretion from RBL-2H3 cells. That these three mAbs bind epitopes on the $Fc_\epsilon R$ is supported by the following observations. (i) Both the intact mAbs (not shown) and their Fab fragments (Figure 1) compete with IgE for binding to RBL-2H3 cells. Reciprocally, IgE can also fully prevent the binding of both intact and Fab fragments of each of these mAbs. (ii) The secretion induced by the three mAbs is qualitatively similar to that induced by IgE and multivalent antigen, in its extent, dependence on extracellular Ca^{2+} ion and temperature. (iii) IgE inhibits the secretion induced by the three mAbs. (iv) F4 and H10 immunoprecipitate from extracts of surface radioiodinated RBL-2H3 cells, a single labelled component, which appears on SDS–PAGE as a broad band of 50–70 kd, and is indistinguishable from that isolated by IgE. This component is known as the α chain of the $Fc_\epsilon R$ (Froese, 1984; Metzger *et al.*, 1986). Furthermore, incubation of the labelled cell extract with immobilized IgE prior to the immunoprecipitation significantly reduces the amount of immunoprecipitated material, thus corroborating the notion that the component immunoprecipitated by F4 and H10 is indeed the α chain of the $Fc_\epsilon R$. Using the same experimental protocol, J17 failed to immunoprecipitate any labelled component.

Taken together, these observations indicate that these three mAbs recognize epitopes on the $Fc_\epsilon R$. Furthermore, the ability of the Fab fragments of each of the three mAbs to inhibit the binding of IgE and of each other (Figure 1),

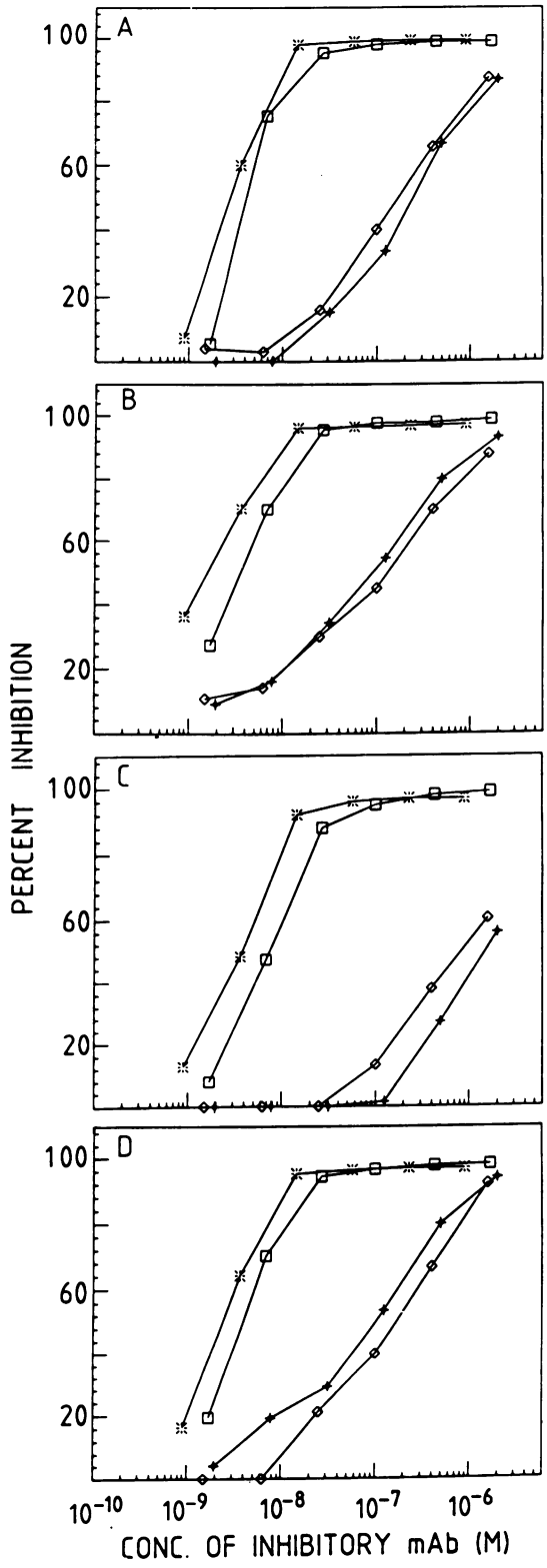

Fig. 1. Mutual competition among IgE, H10-Fab, J17-Fab and F4-Fab for binding to RBL-2H3 cells. Cells (3×10^6) were incubated for 60 min at 4°C with different concentrations of unlabelled IgE (★), H10-Fab (□), J17-Fab (◇), or F4-Fab (◆), in a total volume of 350 μl. After this incubation, 50 μl of ^{125}I-labelled protein was added (without washing the unlabelled competitor). The samples were incubated for a further 60 min at 4°C, and the binding of the radioactive protein was determined as described under Materials and methods. The ^{125}I-labelled probes were: **A**: IgE (2×10^{-9} M); **B**: J17-Fab (1.3×10^{-8} M); **C**: H10-Fab (4×10^{-9} M); and **D**: F4-Fab (1.6×10^{-8} M). Points are the mean of triplicates. Variation within triplicates was usually <5% of the bound c.p.m.

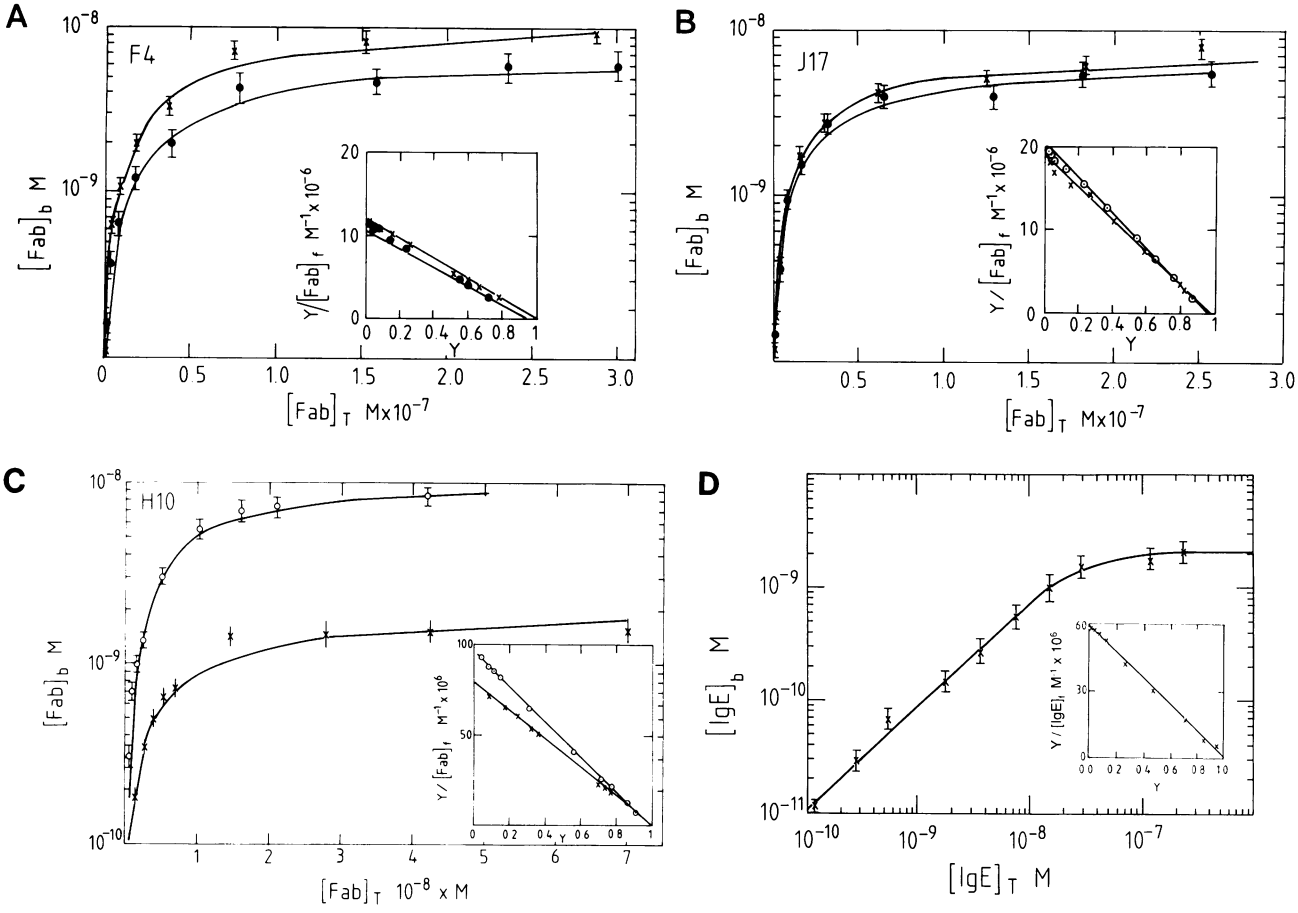

Fig. 2. Binding of Fab fragments of H10, F4 and J17 to RBL-2H3 cells. Increasing concentrations of ^{125}I-labelled Fab fragments or IgE were incubated for 60 min at 25 or 37°C with cells in suspension. After this time, the cell-bound radioactivity was determined as described in Materials and methods. Insets show Scatchard plots of the same data. Points are the mean of triplicates. The solid lines were calculated from the fitting to the data (see text). **A**: F4-Fab (● 25 and × 37°C); **B**: J17-Fab (● 25 and × 37°C); **C**: H10-Fab (● 25 and × 37°C); **D**: IgE (37°C).

suggests that these three mAbs recognize $Fc_\epsilon R$ epitopes which are closely located, and overlap, at least in part, with the IgE binding site.

To determine the intrinsic binding affinity, and the number of binding sites per cell for each mAb, we measured the binding of ^{125}I-labelled Fab fragments derived from each of them to RBL-2H3 cells. Figure 2 shows representative saturation curves for each of the Fabs and for IgE determined at 25 and 37°C. Practically for all Fab-binding measurements, parallel titrations with [^{125}I]IgE were performed using cells of the same respective batch in order to compare the concentrations of bound Fab and IgE at saturation. Equations (1−4) of the Theory section were employed to calculate the intrinsic binding constants (K_r and K'_r) and the concentrations of receptor binding sites from the experimental data.

The binding curves of the three Fabs and IgE were all well fitted by assuming single binding constants. The Scatchard plots (insets in Figure 2) also show a good linear fit, thus confirming that a single affinity and homogeneous epitopes are involved in the binding reactions. Table I summarizes the parameters derived from several experiments using different preparations of Fab fragments of each mAb. Two features are noteworthy. (i) In all experiments performed on one given cell batch, the concentrations of binding sites obtained from the binding isotherm for each Fab and of IgE are practically identical. Since the high affinity binding of IgE is assumed to have a stoichiometry of 1:1 to the $Fc_\epsilon R$ (Metzger *et al.*, 1986), the data suggest that for each of the mAbs the binding stoichiometry is 1 Fab per $Fc_\epsilon R$. This directly implies that the maximal size of $Fc_\epsilon R$ aggregates, that each of the mAbs can form, is a dimer. (ii) The Fabs binding constants, K_r, were found to be practically invariant between 25 and 37°C.

The binding constants obtained for IgE are significantly lower than those derived earlier from kinetic measurements Kulczycky and Metzger, 1974; Wank *et al.*, 1983). This discrepancy is probably only in part due to differences in IgE species or in the cell populations employed since, using the very same reagents as those used in binding studies, we performed kinetic measurements similar to those described by Kulczycky and Metzger, and obtained a value for $K_r' = k_1/k_{-1} = 5.9 \times 10^9\ M^{-1}$, which is in excellent agreement with the published values. The disagreement between the $Fc_\epsilon R$ affinity for IgE determined by direct binding studies, and that calculated from k_1/k_{-1} has been previously noticed, yet was assigned to possible incomplete equilibration (Metzger, 1977). However, it could also indicate that the reaction between IgE and $Fc_\epsilon R$ proceeds by a more complex mechanism than the assumed single step equilibrium. Therefore, further experimental work would be required in order to justify use of the rate constant ratios as the equilibrium binding constants.

We measured the binding of intact ^{125}I-labelled mAbs F4,

Table I. Binding of monoclonal $Fc_\epsilon R$ specific antibodies, their Fab fragments or of IgE to RBL-2H3 cells

Reagent bound	Experiment no.	Temperature (°C)	$[Fc_\epsilon R]_t$ (nM)	K_r^a (M^{-1} to 10^{-7})	K_d M^{-1}	f
F4 (Fab)	1	25	7.2	1.8		
F4 (Fab)	7	25	6.0	1.1		
F4 (Fab)	2	37	12.0	1.2		
F4	7	25	5.5	1.0	7.5×10^3	1.0
F4	8	37	2.0	1.0	3.8×10^4	2.5
H10 (Fab)	4	25	9.7	19.0		
H10 (Fab)	5	37	1.8	12.0		
H10 (Fab)	6	37	9.0	25.0		
H10	4	25	9.7	20.0	1.8×10^9	0.74
H10	8	37	2.0	10.0	1.8×10^9	36.0
J17 (Fab)	1	25	6.6	1.8		
J17 (Fab)	3	37	7.6	2.0		
J17	1	25	6.0	1.8	1.6×10^5	0.81
J17	8	37	2.0	1.0	2.6×10^5	1.6
IgE	1	25	6.7	6.8		
IgE	4	25	11.0	6.8		
IgE	7	25	6.0	7.0		
IgE	2	37	12.0	9.0		
IgE	3	37	9.0	12.0		
IgE	6	37	8.5	16.0		
IgE	8	37	2.0	6.0		

[a]K'_r for IgE-binding.

H10 and J17 to RBL-2H3 cells. Results were analysed using a model which considers the binding of bivalent antibodies to monovalent, freely mobile cell surface receptors (see Theory section). This binding is described by two parameters. (i) K_r, the intrinsic binding constant of a single Fab to an $Fc_\epsilon R$ epitope on the cell. (ii) K_d, the equlibrium constant for the dimerization step, i.e. the formation of a complex consisting of one antibody bound to two $Fc_\epsilon Rs$.

In analysing our experimental data, the values of total $Fc_\epsilon R$ concentration ($[Fc_\epsilon R]_t$) and of K_r were used as fixed parameters. $[Fc_\epsilon R]_t$ was determined for each mAb binding experiment by a parallel titration of cells from the same batch with [^{125}I]IgE, and K_r was obtained from titrations with the corresponding Fab. Thus, the only free parameter in the fitting procedure was the equilibrium constant of dimer formation, K_d.

Figure 3 shows results of binding measurements of the three mAbs at 25 and 37°C. The lines were calculated using the parameters obtained from the fitting procedure. All data sets measured at 25°C may be well fitted by our model. This is also reflected in the f-values which are smaller than 1. The f-values for the titrations performed at 37°C are, however, significantly larger. The lower quality of the fits is particularly evident in the case of mAb H10. The difficulty in fitting the model and the binding data of the intact mAbs at 37 but not at 25°C, can be most probably assigned to the drastic changes in the RBL-2H3 cells plasma membrane, that take place upon crosslinking their $[Fc_\epsilon R]_f$ (Phillips *et al.*, 1985).

The secretory response of RBL-2H3 cells to mAbs F4, J17 and H10 was measured on cell monolayers treated with a range of concentrations of purified mAbs. After 30 min at 37°C, secretion was determined by monitoring the release of either [^{3}H]serotonin or β-hexoaminidase. Time-course experiments showed that the secretion induced by the three mAbs occurs at similar rates, approaching a plateau after 20 min at 37°C. Typical secretory dose–response curves for the three mAbs are shown in Figure 4A. Very marked differences are observed in the secretion patterns induced by each of the three mAbs. (i) The maximal secretion induced by F4 is by far higher than that induced either by J17 or by H10, and is similar in extent to that obtained under optimal IgE-antigen stimulation. (ii) The dose–response curves of F4 and J17 both show a bimodal shape, unlike that obtained for H10. (iii) The maximal secretion induced by mAb H10 occurs at relatively high concentrations, even though its affinity is an order of magnitude higher than that of F4 or J17.

Practically the same dose–response patterns were obtained for the three mAbs in several similar experiments ($n > 20$), irrespective of whether the secretion was followed by the release of [^{3}H]serotonin or β-hexosaminidase. Since we have consistently employed one and the same batch of cells in each set of secretion experiments, the different response patterns to each of the mAbs cannot be assigned to differences in the cell populations. Furthermore, the same results were obtained using several different preparations of the three mAbs. Thus, the differences in the secretory dose–responses must be due to intrinsic properties of each of the mAbs.

In exploring the origin of the different cellular dose–response patterns to the mAbs, one possibility entertained was that already the binding of each Fab to its particular epitope causes structural changes in the $Fc_\epsilon R$ that would affect the receptors capacity to provide stimulatory signals upon aggregation. To examine this possibility, we performed experiments in which monolayers of RBL-2H3 cells were first incubated for 30 min at 37°C with a range of concentrations of each of the different Fabs. After washing away the unbound Fabs, secretion was induced by crosslinking the cell-bound Fab fragments by the same constant amount of an anti-mouse Ig antiserum. Secretion was allowed

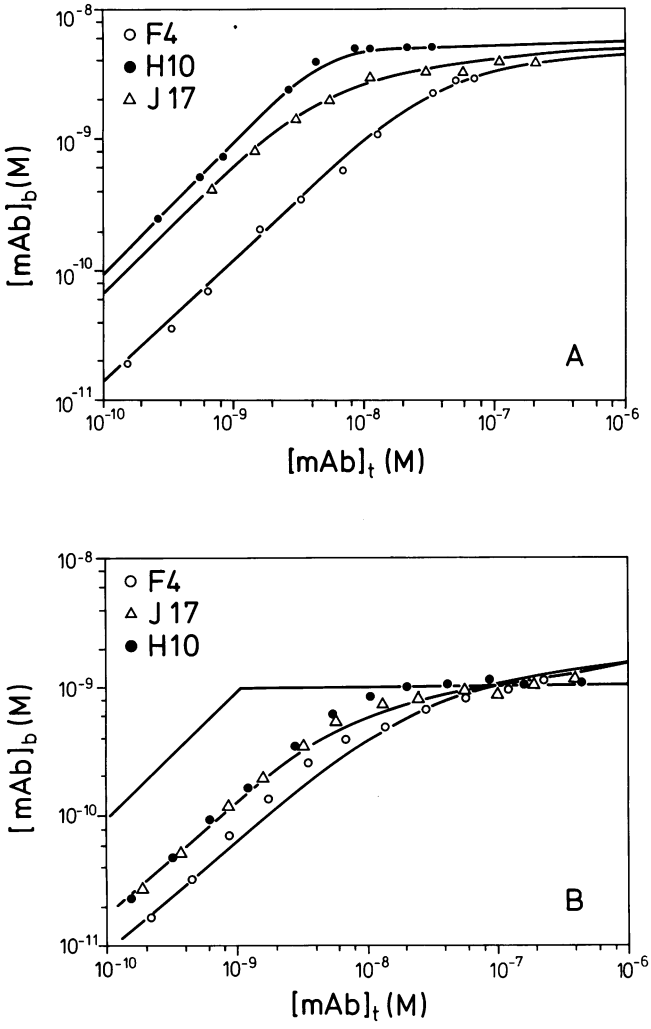

Fig. 3. (**A**) Binding of the intact $Fc_\epsilon R$ specific mAbs (F4, J17 and H10) to RBL-2H3 cells at 25°C. Increasing concentrations of ^{125}I-labelled mAbs were incubated for 60 min at 25°C with cells in suspension. After this time the cell-bound radioactivity was determined as described. Points are the mean of triplicates and the variation within each was always $<10\%$. The solid lines are drawn according to the parameters obtained from the non-linear fitting procedure. F4 (○), J17 (△), H10 (●). (**B**) Same as in (A) but at 37°C, and on cells in monolayers, so as to enable assessment of secretion (see Materials and methods).

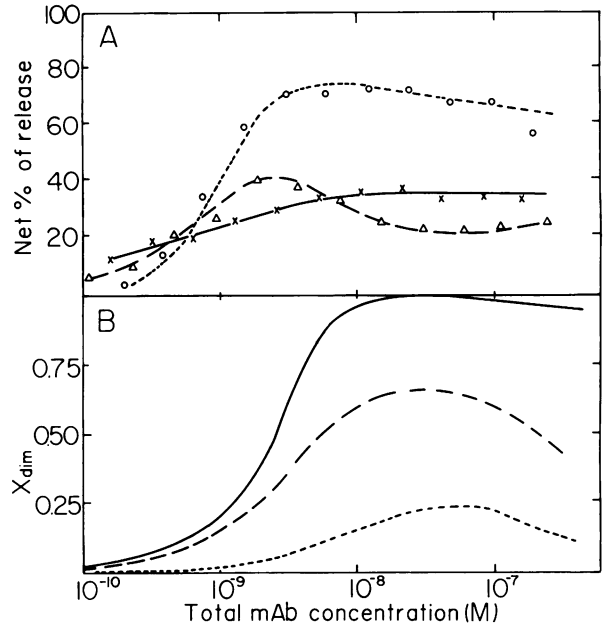

Fig. 4. (**A**) RBL-2H3 cells secretion induced by mAbs F4 (○), J17 (△) and H10 (×). Increasing concentrations of mAbs were added to monolayers of RBL-2H3 cells. After 30 min at 37°C, secretion was determined by following β-hexosaminidase activity in the supernatants as described in the text. Points are the means of triplicates. Similar secretory patterns to these shown here were observed in a large number of experiments ($n>20$). (**B**) Mol fraction of $Fc_\epsilon R$ per cell incorporated in dimers (χ_{dim}) as function of the concentration of mAbs F4 (- - -); J17 (— —) and H10 (——). The curves were calculated as described in the text.

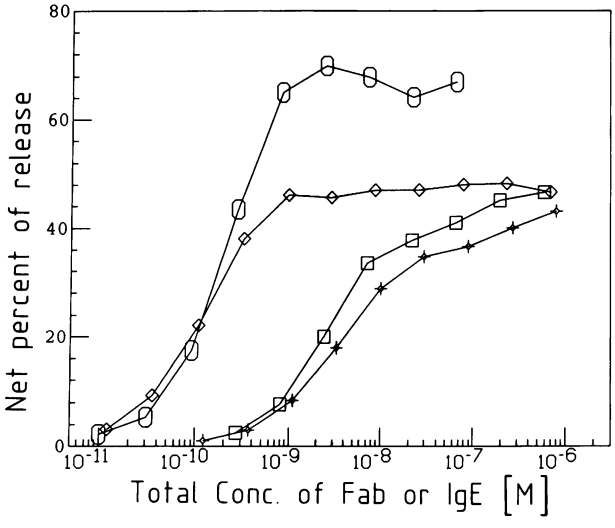

Fig. 5. RBL-2H3 mediator secretion induced upon crosslinking cell-bound Fab fragments of the three mAbs by goat anti-mouse Ig. Monolayers of [^{3}H]serotonin loaded RBL-2H3 cells in 96-well plates, were incubated with the given concentrations of J17-Fab (+), F4-Fab (□), H10-Fab (◇), or IgE (○); for 45 min at 25°C. The monolayers were washed and 150 μl of goat anti-mouse Ig (at 15 μg/ml) were added per well. After 30 min at 37°C, supernatant samples were taken to measure the [^{3}H]serotonin secreted. Points are the means of triplicates. Essentially the same results were obtained in three independent similar experiments.

to proceed for 30 min at 37°C, and was assessed by the release of [^{3}H]serotonin. The concentration of anti-mouse Ig antiserum used, was that which induced optimal secretion from similar cell monolayers which were saturated with IgE. Control experiments demonstrated that neither the Fabs alone nor the anti-mouse Ig by itself induced secretion. The results of these experiments showed (Figure 5) that when cross-linked in the way described above, all three different $Fc_\epsilon R$-specific Fabs induced secretion reaching practically the same maximal level. The observed concentration dependence of the secretory responses reflects the Fabs relative affinities. These results rule out the possibility that Fab-binding by itself induce specific structural or configurational changes in the $Fc_\epsilon R$ which would affect the subsequent secretory response.

Thus, the differences in the dose−response to the three intact mAbs arise from properties for which the intact (bivalent) structure of the mAbs is responsible. Since the stoichiometry of binding of the three mAbs is 1 Fab:1 $Fc_\epsilon R$, it follows that the size of the $Fc_\epsilon R$ aggregates that each one of them can form is only dimers. A simple rationale explaining the different dose−response patterns could be that they are reflecting the different amounts of $Fc_\epsilon R$ dimers that

each mAB is producing, due to their respective binding parameters. To examine this possibility we computed the degree of $Fc_\epsilon R$ dimerization induced by the range of concentrations of each of the mAbs, and compared it to the respective dose–secretory response curves. The values used to calculate the curves shown were obtained as follows: (a) K_r from the fits of the binding of Fab fragments of the three mAbs to RBL-2H3 cells (Figure 2); (ii) K_d was obtained from fits of binding curves of intact mAbs to RBL-2H3 cells (Figure 3), measured at 25°C; and (iii) $[Fc_\epsilon R]_t$ was obtained from a titration with [^{125}I]IgE of identical monolayers of RBL-2H3 cells as those used for obtaining the secretion curves shown in Figure 4A. Figure 4B shows the calculated fraction of $Fc_\epsilon R$ incorporated in dimers (χ_{dim}) as function of each mAb concentration.

Comparing the dose–response curves for the mAbs induced secretion (Figure 4A) and $Fc_\epsilon R$ dimerization (Figure 4B) one observes that: (i) the degree of secretion does not correlate in a simple way with the extent of $Fc_\epsilon R$ dimerization, and (ii) the maximal dimerization caused by each of the mAbs is very different. Thus, for example, mAb F4 which induces the highest levels of secretion is the least effective in producing dimers, while H10 induces virtually 100% of $Fc_\epsilon R$ dimerization, yet causes relatively low levels of secretion. These results indicate that it is not only the number of $Fc_\epsilon R$ dimers formed per cell that determines the extent of secretion evoked. Rather, they suggest that other properties of the $Fc_\epsilon R$ dimers formed by distinct mAbs are as important in this capacity to provide the transmembranal signal.

Discussion

Aggregation of the high affinity monovalent $Fc_\epsilon R$ present in the plasma membrane of mast cells and basophils is recognized as the initial signal for the immunologically stimulated mediator release from these cells. In spite of extensive efforts made in different laboratories, a satisfactory quantitative description of this initiating step is not yet available. One major reason for this situation is the lack of structurally well defined crosslinking agents. In the present study, we have employed monoclonal antibodies specific for $Fc_\epsilon R$ epitopes as homogeneous, structurally defined crosslinking agents to study the relationships between $Fc_\epsilon R$ dimerization and secretion. Our results have yielded several conclusions. First and prominent, all three mAbs employed cause mediator secretion, indicating that $Fc_\epsilon R$ aggregates as small as dimers are capable of providing an effective stimulus. The capacity of $Fc_\epsilon R$ dimers to provide RBL-2H3 cells with the initial stimulatory signal has been a matter of some debate, and the present results seem to finally resolve this issue. Earlier studies have shown that $Fc_\epsilon R$ dimers produced by binding of covalently linked IgE-dimers, were relatively ineffective in causing RBL-2H3 cells to secrete (Fewtrell and Metzger, 1980). The use of covalent IgE oligomers as triggering agents, however, also has its inherent drawbacks: these reagents, though apparently homogeneous in terms of molecular mass, suffer from being structurally ill-defined because the chemical random crosslinking yields different points of attachment which would markedly influence the structure and flexibility of the IgE oligomers. This may introduce critical (and uncontrolled) differences in their actual capacity to cause $Fc_\epsilon R$ dimerization. The mAbs used in the present study are structurally homogeneous and, by virtue of their binding stoichiometry (1 Fab:1 $Fc_\epsilon R$) and bivalency, can produce, at least initially, only $Fc_\epsilon R$ dimers. That they all cause secretion demonstrates that, indeed, $Fc_\epsilon R$ aggregates as small as dimers are sufficient to generate the activatory signal for mast cell secretion of the RBL-2H3 line as well.

The second and more striking observation is the substantial differences in the dose–response patterns displayed by the three mAbs. Not only are there large differences in the maximal secretory response to each of the mAbs, but also no simple correlation could be discerned between the extent of $Fc_\epsilon R$ dimerization and the secretion that each of the mAbs provokes. These findings clearly indicate that $Fc_\epsilon R$ dimers formed by each of the three mAbs differ in their capacity to induce secretion.

The possibility that already the Fab-binding would cause distinct structural changes in the $Fc_\epsilon R$ leading to the disparate secretory response is inconsistent with the results of experiments where practically the same maximal secretion was attained when Fab fragments of the three mAbs were crosslinked with anti-murine Ig antibodies (Figure 5). The rationale for the observed behaviour must therefore be based on different properties of the $Fc_\epsilon R$ dimer formed by each of the three mAbs. Such differences could lie in their structural properties and/or in the dynamics of mAb–$Fc_\epsilon R$ interactions. Thus, if the average lifetime of a given $Fc_\epsilon R$ dimer is too short, the secretory signal which it causes may be ineffective. Kinetic studies have been initiated to examine this possibility, and the results obtained so far do not support the notion that the different rates of binding or dissociation of the mAbs with the cells could rationalize the differences in secretory responses.

If one assumes that each of the employed mAbs may confer, on the $Fc_\epsilon Rs$ in the dimer it produces, distinct configurational relationships this, in turn, could cause different secretory signals for the cell. Two elements may play a role in providing an optimal spatial configuration: (i) the distance, and (ii) the relative orientation of the receptors with respect to each other in the dimer. Though the present results do not enable an unambiguous resolution between the two constraints (which are not necessarily independent), we are inclined to suggest that the dominant factor affecting the efficacy of $Fc_\epsilon R$ dimers in providing the secretory stimulus is the orientational one. This notion is supported by the fact that all three mAbs bind to epitopes which are relatively proximal, at least within the cross-section of Fv domains. Furthermore, the proximity limits into which each of the three mAbs would bring the $Fc_\epsilon Rs$ are also dictated by the physical dimensions and flexibility of these mAbs. However, since both F4 and J17 are IgG1 molecules and show such disparity in secretory response, one has to resort to the role of orientation as the crucial one.

Even the current limited knowledge of the $Fc_\epsilon R$ topology provides a good reason to expect marked differences in the possible relative orientation of two such membrane protein assemblies when brought together. The recent cloning and sequencing of the cDNA coding for the IgE-binding α-chain of the receptor (Kinet *et al.*, 1987; Shimizu *et al.*, 1988) predicts a structure having a single transmembrane stretch and an extracellular portion containing two Ig-like domains. The $Fc_\epsilon R$ comprises two further membrane subunits (β and γ) non-covalently associated with the α-chain (Rivnay *et al.*,

1984), thus clearly yielding an asymmetrical structure. One can therefore expect that different mAbs would yield dimers with differently oriented $Fc_\epsilon Rs$. The interaction of the receptors with each other and with membranal or cellular components which couple the initial signal to the continuing cascade could, thus, be affected by the spatial configuration of the signalling dimer.

Finally, the quantitative aspect of the secretory stimulus applied to RBL-2H3 cells by the three mAbs is noteworthy. Two of the three mAbs (F4 and J17) exert their maximal secretory effect already when a minute fraction of $Fc_\epsilon Rs$ has been incorporated in dimers. This is particularly evident for F4, where maximal secretion is attained when only 5% of the $Fc_\epsilon Rs$ are crosslinked. This pattern agrees with earlier reports (Fewtrell *et al.*, 1979) where reagents less amenable to such a quantitative analysis were employed, yet it was still shown that a rather small fraction of $Fc_\epsilon Rs$ need to be crosslinked in order to yield an optimal secretory response.

Materials and methods

Culture media and reagents

Powdered culture media were purchased from GIBCO, Grand Island, NY, USA. Fetal calf serum, as well as glutamine, penicillin–streptomycin mixture and sodium pyruvate as tissue culture supplements were from Bio-Lab, Jerusalem, Israel. Specific antisera for mouse Ig classes and subclasses were from Meloy Laboratories, Springfield, VA, USA. Papain, dithiothreitol, iodoacetamide, and Triton X-100 were from Sigma Chemical Co., St Louis, MO, USA. Sepharose–Protein A and SDS–PAGE mol. wt standards were obtained from Pharmacia, Uppsala, Sweden. DNP-specific monoclonal murine IgE (IGEL a2) (Rudolph *et al.*, 1981), was kindly provided by Dr V.T.Oi, Stanford University; and was affinity-purified on DNP–Sepharose in our laboratory. Goat anti-mouse Ig antibodies were purchased from Sigma Chemical Co. (Prod. M8019).

Cells

Rat Basophilic Leukemia cells, subline 2H3 (Barsumian *et al.*, 1981), were obtained from Dr H.Metzger, NIH, Bethesda, MA. They were maintained in Eagle's minimal essential medium with Earle's salts (MEM) supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics in a humidified atmosphere with 7% CO_2 at 37°C. For binding and secretion experiments, cells were maintained in Tyrode's buffer: 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM Hepes, 0.1% bovine serum albumin, pH 7.4. NSO myeloma cells were obtained from Dr Z.Eshhar, The Weizmann Institute of Science. Myeloma and hybridoma cells were maintained in Dulbecco's modified essential medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 2 mM sodium pyruvate and antibiotics, in a humidified atmosphere with 7% CO_2 at 37°C.

Production of hybridomas

C57/bl mice were obtained from Olac, Bicester, UK. They were immunized intraperitoneally with 2×10^7 washed RBL-2H3 cells for a total of four times at 2 week intervals. At least 6 weeks after the last injection, two additional immunizations with the same dose were given, 4 and 3 days prior to the fusion. Spleen cells from one immunized mouse were fused with NSO myeloma cells (ratio 5:1) using 41% polyethylene glycol 1500 (Serva, Heidelberg, FRG) essentially as described with Eshhar (1985). Two weeks after the fusion, supernatants of the hybridomas were tested for their capacity to induce secretion from RBL-2H3 cells. This was done with cells plated in 96-well plates, labelled with [^{3}H]serotonin as described below. After washing the monolayers, 100 μl of MEM and 50 μl of hybridoma supernatant were added per well, and the plates were incubated for 30 min at 37°C. Aliquots of 100 μl of the supernatant were then taken for measuring the amount of [^{3}H]serotonin released. The assay was done in duplicate, on days 14 and 17 after the fusion. Positive clones were selected, expanded, retested and cloned by limiting dilution. The selected clones were grown in tissue culture for production of mAb.

Production and purification of mAbs

All mAbs used were purified from hybridoma culture supernatants by chromatography on Protein A–Sepharose. The mAbs were eluted from the column using 0.2 M glycine pH 2.9. The eluate was collected directly into tubes containing 2 M Tris buffer pH 8.2, dialysed against PBS and stored frozen at −20°C. mAbs F4 and J17 belong to the IgG_1 subclass, and H10 to the IgG_{2b} subclass, as determined by double immunodiffusion against specific antisera for mouse Ig subclasses. Fab fragments of the three mAbs were prepared by digestion with papain at a 1:50 (w/w) ratio, in 20 mM Tris buffer pH 8.2, containing 0.1 mM dithiothreitol and 2 mM EDTA. Digestion was carried out for 2 h at 37°C, after which iodoacetamide (10 mM) was added for 1 h at 0°C. Fc fragments and undigested antibody were eliminated by passing the preparation twice through a Protein A–Sepharose column. The Fab preparations did not contain intact or partially digested molecules, as judged both by SDS–PAGE stained with Coomassie Blue, and by their inability to induce secretion for RBL-2H3 cells. Antibody concentrations were determined by abosorbance at 280 nm using an $OD_{0.1\%} = 1.4$. For conversion to molecular concentrations, mol. wt of 150 kd was assumed for H10, F4 and J17 mAbs; of 50 kd for their Fab fragments, and of 180 kd for IgE.

Iodination of antibodies

Intact mAbs, their Fab fragments, or affinity-purified mouse IgE were iodinated with ^{125}I using the chloramine T method (Hunter and Greenwood, 1962). Specific activities in the range of 5–10 μCi/μg protein were usually employed. After iodination, >95% of the labelled protein maintained its capacity to bind to the cells.

[^{3}H]Serotonin release from RBL-2H3 cells

Cells were incubated overnight with 1 μCi of [^{3}H]hydroxy-tryptamine creatinine sulphate ([^{3}H]serotonin) (Amersham, UK) per 10^6 cells in 96-well tissue culture plates (10^5 cells in 100 μl MEM/well). On the following day, the plates were washed three times with Tyrode's buffer. Following the treatments indicated for each experiment, secretion was allowed to proceed for 30 min at 37°C. 100 μl of the supernatant were then taken from each well to count the amount of [^{3}H]serotonin released. Secretion is expressed as percent of the total serotonin taken up by the cells, which was determined by lysing them in several control wells with 1 M NaOH, and counting an equivalent aliquot. Spontaneous release, i.e. that observed in absence of secretagogue was substracted from each experimental value to yield the net percent of release. Spontaneous release was usually between 3 and 6% of the total serotonin incorporated into the cells.

β-Hexosaminidase release

Where indicated, secretion from RBL-2H3 cells was monitored by following the activity of the granular enzyme β-hexosaminidase. For this end, cells were plated in 96-well plates (10^5 cells in 100 μl MEM/well). On the following day, the monolayers were washed three times with Tyrode's buffer. After the treatments indicated for each experiment, secretion was allowed to proceed for 30 min at 37°C. From each well, two aliquots of 20 μl were transferred to a separate plate. To these samples, 50 μl of substrate solution (1.3 mg/ml *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine in 0.1 M citrate, pH 4.5) were added and the plates incubated for 90 min at 37°C. The reaction was stopped with 150 μl of stop solution (0.2 M glycine, pH 10.7). The colour formed due to substrate hydrolysis was measured in 405 nm in an ELISA reader. To evaluate the total content of enzyme, cells in several control wells were lysed by 0.5% Triton X-100. The results are expressed as percent of the total β-hexosaminidase present in the cells. The release observed in absence of secretagogue (spontaneous release) was subtracted from each experimental value to yield the net percentage release.

Binding assays

Two different types of binding assays were employed, depending on whether the cells were in suspension or in monolayers. For experiments with suspended cells, $3-4 \times 10^6$ washed RBL-2H3 cells were incubated with different concentrations of ^{125}I-labelled mAb or their Fab fragments for 60 min at the indicated temperature, with occasional shaking, in a total volume of 400 μl. For assaying the binding, triplicate samples of 100 μl taken from each test tube were layered on 200 μl of fetal calf serum in 400 μl microfuge polypropylene test tubes. These preparations were centrifuged for 1 min in a microfuge (Beckman, Palo Alto, CA, USA). Each test tube was cut and the bottom part containing the cell pellet was counted in a gamma counter. Non-specific binding was determined by carrying out a parallel titration in which each sample of cells was pre-incubated with excess unlabelled IgE for 30 min before addition of the ^{125}I-labelled probe. The non-specific binding was subtracted from each experimental value to obtain the specifically bound radioactivity. This correction would also eliminate any possible contribution from the binding of the ^{125}I-labelled mAbs through its Fc domain to the $Fc_\gamma R$ on the cells. For experiments done with monolayers, RBL-2H3 cells were grown for 16 h in 96-well plates, washed four times with Tyrode's buffer and different concentrations of the ^{125}I-

labelled probe were added in a total volume of 150 μl. After the specified incubation time the monolayers were washed four times with Tyrode's buffer. When necessary, two 20 μl samples were withdrawn from each well to measure the release of β-hexosaminidase before the monolayers were washed. After washing, the cells in the monolayers were lysed by adding 100 μl of 1% Triton X-100 and incubated for 15 min at room temperature. The contents of each well were then transferred to vials for gamma counting. Each experimental point was done in triplicate. The non-specific binding was determined by carrying out a parallel titration in which the monolayers were pre-incubated with excess unlabelled IgE for 30 min before addition of the ^{125}I-labelled probe. This procedure was employed in binding assays of intact mAbs and of IgE when parallel measurements of secretion were done on identical monolayers of cells.

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