Analysis of FcεRI-Mediated Mast Cell Stimulation by Surface-Carried Antigens

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ABSTRACT Clustering of the type I receptor for IgE (FcεRI) on mast cells initiates a cascade of biochemical processes that result in secretion of inflammatory mediators. To determine the FcεRI proximity, cluster size, and mobility requirements for initiating the FcεRI cascade, a novel experimental protocol has been developed in which mast cells are reacted with glass surfaces carrying different densities of both antigen and bound IgE, and the cell's secretory response to these stimuli is measured. The results have been analyzed in terms of a model based on the following assumptions: 1) the glass surface antigen distribution and consequently that of the bound IgE are random; 2) FcεRI binding to these surface-bound IgEs immobilizes the former and saturates the latter; 3) the cell surface is formally divided into small elements, which function as a secretory stimulus unit when occupied by two or more immobilized IgE-FcεRI complexes; 4) alternatively, similar stimulatory units can be formed by binding of surface-carried IgE dimers to two FcεRI. This model yielded a satisfactory and self-consistent fitting of all of the different experimental data sets. Hence the present results establish the essential role of FcεRI immobilization for initiating its signaling cascade. Moreover, it provides independent support for the notion that as few as two FcεRIs immobilized at van der Waals contact constitute an "elementary stimulatory unit" leading to mast cell (RBL-2H3 line) secretory response.

INTRODUCTION

Aggregation of the type I Fcε-receptor (FcεRI) on mast cells and basophils initiates a cascade of biochemical processes resulting in the secretion of inflammatory mediators (Siraganian, 1988). This process is of intrinsic interest and provides a useful paradigm for studying the mechanism of multichain immuno-recognition receptor (MIRR)-induced signaling (Ravetch and Kinet, 1991; Jorgensen et al., 1992; Schatz et al., 1992; Holowka and Baird, 1996). FcεRI aggregation may be accomplished by a variety of protocols, e.g., clustering of receptor-bound IgE by multivalent antigens or divalent haptens (Schweitzer-Stenner et al., 1987), covalent cross-linkers (Menon et al., 1984, 1986a), IgE specific antibodies (Menon et al., 1986a,b), or directly, by FcεRI-specific polyclonal or monoclonal antibodies (mAb) (Basciano et al., 1986; Ortega et al., 1988). Despite numerous studies using the rat mucosal mast cell line RBL-2H3 as a model system, the structural and physical parameters governing the stimulatory efficiency of FcεRI clusters are not fully resolved (Holowka and Baird, 1996). The difficulty in obtaining quantitative insights is due partly to the fact that the dose response of these cells strongly depends on the way the FcεRI clusters were formed and cannot be simply correlated with their number or size (Perelson and DeLisi, 1980; Fewtrell, 1985; Oliver et al., 1988; Ortega et al., 1988; Posner et al., 1995). Moreover, recent studies indicate that the stimulatory efficiency of FcεRI clusters depends on their lifetime (Schweitzer-Stenner et al., 1994) as well as their configuration (Ortega et al., 1988; Pecht et al., 1991). Further problems arise from desensitization processes that terminate the stimulatory signal of FcεRI clusters (Dembo et al., 1979; Fewtrell, 1985; Oliver et al., 1988; Weetal et al., 1993; Schweitzer-Stenner et al., 1994). Some studies proposed that maintenance of FcεRI signaling requires continuous de novo formation of FcεRI clusters (Oliver et al., 1988; Posner et al., 1995). This notion was mainly supported by the observation that the stimulus resulting from clustering IgE-carrying FcεRI by polyvalent antigens is abrogated by the subsequent addition of monovalent haptens, whereas a considerable fraction of antigen is still bound to the cell surface IgE (Oliver et al., 1988).

Recently we developed and employed an experimental protocol to study FcεRI density and motion requirements for initiating and sustaining a secretory response of mast cells (Tamir et al., 1996). Our protocol employed IgE bound either to soluble photoactivatable antigens or to flat glass surfaces onto which these antigens were covalently attached. RBL-2H3 cells were reacted with these reagents via their FcεRI, yielding a secretory response. Results of these studies unambiguously showed that no recruitment of new FcεRI into clusters is required for sustaining the secretory response. Moreover, it was found that these cells degranulate in response to their binding to matrix-immobilized IgE, provided that the IgE surface density is higher than 800–1000 μm^{-2}. It was therefore concluded that sustained FcεRI signaling requires solely that the average density of immobilized FcεRI exceed a distinct threshold value for a period of time that allows stimulus-secretion coupling to take place. Thus receptor clustering by soluble, polyvalent

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antigens may be considered one specific stimulation procedure.

The present study is intended to assess how many immobilized FcεRI constitute a stimulatory element, and within what distance. To this end we have developed and employed a statistical model that allows a quantitative and self-consistent analysis of the RBL-2H3 cells' secretory dose response to surfaces carrying immobilized, randomly and nonrandomly distributed IgE. IgE immobilization was achieved by binding DNP-specific IgEs to surfaces fully covered by the antigen DNP_{11}-BSA and confirmed by FRAP (fluorescence recovery after photobleaching) measurements. Nonrandom IgE distributions were produced by using an IgE-specific monoclonal antibody (mAb) to dimerize them before binding to these surfaces (see below). Moreover, we also changed the surface distribution of IgE by altering the antigen density. In each case the cells' secretory dose response to their interaction with these surfaces was measured as a function of IgE surface density. All of the data were subjected to a global fitting procedure based on a model that assumes that two FcεRI immobilized in close proximity serve as a triggering unit for the secretory stimulus of RBL-2H3 cells.

MATERIALS AND METHODS

Cell cultures

RBL-2H3 cells (Barsumian et al., 1981), originally obtained from Dr. R. Siraganian (NIH, Bethesda, MD), were grown in Dulbecco's minimum essential medium (DMEM) (Bio-Lab, Israel) supplemented with 10% fetal calf serum (Gibco-BRL, USA), 2 mM glutamine, and antibiotics (Bio-Lab, Israel) in a humidified atmosphere with 7% CO_2 at 37°C. The adherent RBL-2H3 cells were harvested by incubation with 10 mM EDTA (in DMEM) for 15 min at room temperature.

Antibodies and soluble antigens

The mouse monoclonal 2,4-dinitrophenyl (DNP)-specific IgE clone A2 (Rudolph et al., 1981) and the IgE-specific rat monoclonal antibody clone 95.3 (Baniyash and Eshhar, 1984) were a kind gift of Dr. Z. Eshhar (Weizmann Institute of Science, Rehovot, Israel). The mAb A2 was purified by affinity chromatography on DNP-Sepharose and centrifuged before its use at 100,000 × *g* for 1 h at 4°C to remove any oligomeric forms. Only the upper two-thirds of the supernatant were collected, kept at 4°C, and employed within a week after centrifugation. The mAb 95.3 was used without further purification. Bovine serum albumin (BSA) carrying an average of 11 DNP groups (DNP_{11}-BSA) was prepared by reacting 2,4-dinitrofluorobenzene with BSA as described previously (Eisen et al., 1959; Hardy, 1986). Iodination of DNP_{11}-BSA with ^{125}I was performed on ~100 μg protein samples by the chloramine-T method (Hunter and Greenwood, 1962). Specific activities in the range of 5–10 Ci/g protein were obtained.

Unless otherwise stated, all other reagents were purchased from Sigma. All organic solvents used were high-performance liquid chromatography grade.

Preparation of antigen-carrying surfaces

Antigen-carrying glass surfaces were prepared essentially as described earlier (Tamir et al., 1996). Briefly, glass coverslips were reacted with 1% (w/w) 3-aminopropyltriethoxysilane (APTES) (Aldrich) by sonication in dichloromethane, washed several times with the latter solvent, and air-dried. The resultant 3-aminopropylsilyl (APS)-carrying coverslips were further derivatized by glutaraldehyde (10 mM in phosphate-buffered saline (PBS) for 1 h at room temperature), followed by reaction with 1–1000 nM DNP_{11}-BSA overnight at 4°C. The remaining free aldehyde groups on the resultant surfaces were blocked by reaction with BSA (1 mg/ml in PBS) for 3 h at room temperature. The amount of DNP_{11}-BSA bound to the glass surfaces was determined by performing the above reaction with ^{125}I-labeled DNP_{11}-BSA. The obtained DNP_{11}-BSA surface density varied between experiments and hence was always determined in quadruplicate. The standard deviation of this density among different surfaces within a single experiment was ±10%. Maximum surface coverage of ~2.3×10^4 μm^{-2} was obtained by using 500 nM DNP_{11}-BSA in the above protocol.

Preparation of IgE complexes with antigen-carrying surfaces

Coverslips with DNP_{11}-BSA covalently bound to the thin glass surface were placed in 24-well plates, and IgE binding was allowed to take place at 22°C overnight with 0.1–1000 nM ^{125}I-labeled IgE in PBS, followed by extensive washings with PBS. On average, the obtained IgE surface density (σ_{IgE}) was related to the reacting IgE molar solution concentration by $\sigma_{IgE} \approx 3 \times 10^7$ [IgE].

The IgE surface density was determined separately for each set of coverslips before the secretory response induced by the reaction of RBL-2H3 cells with these surfaces was measured (see below).

Unlike the experiments performed in our earlier study (Tamir et al., 1996), the present study did not employ photoactivable haptens as surface-carried epitopes. This is justified by the facts that 1) the secretory response to coverslips carrying covalently bound IgE was found to be indistinguishable from that induced by nonphotoactivated (and thus noncovalently bound) hapten-IgE complexes and 2) IgE dissociation from DNP_{11}-BSA-carrying surfaces is negligible on the time scale of the experiment.

For studies involving IgE dimerized by the specific mAb (clone 95.3), the former was either first diluted in Tyrode's buffer to the required final concentrations (0.1–100 nM) and then reacted with 10 nM of the mAb 95.3 at 22°C for 2 h, or first reacted with 100 nM of the IgE-specific mAb and then diluted. The dimeric IgE-containing solutions were next incubated overnight with the antigen-carrying surfaces at 22°C, followed by extensive washings with PBS.

Secretion assays

Harvested RBL-2H3 cells were washed twice with Tyrode's buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA) and suspended in this buffer at 4 × 10^6 cells/ml. Next the suspended cells were allowed to adhere to IgE-carrying glass surfaces placed in 24-well plates (2 × 10^6 cells/well) for 1 h at 10°C, followed by washing of the cell-carrying glass surfaces twice in Tyrode's buffer to remove nonadherent cells. These glass coverslips were then transferred to fresh wells and further incubated in 250 μl Tyrode's buffer per well at 37°C for 45 min to allow for secretion. After withdrawal of supernatant samples for β-hexosaminidase assay, the cell-carrying coverslips were transferred to Tyrode's buffer containing 1% Triton X-100 for 1 h at room temperature to extract and determine the remaining β-hexosaminidase activity. Next the glass surfaces were removed and taken to determine their radioactivity with a gamma-counter. The β-hexosaminidase enzymatic activity in supernatant samples and in 1% Triton X-100 cell extract was assayed in triplicate, essentially as described earlier (Ortega-Soto and Pecht, 1988). The determined absorption was background corrected, and the net percentage secretory response was expressed as (100 × secreted)/(secreted + extracted) and is presented in the dose-response curves with its standard error.

RESULTS AND ANALYSIS

In the experiments described below, the induced secretory response of the RBL-2H3 cells was measured as a function of both antigen and IgE densities on the glass surface. In the first type of experiment, the DNP-specific IgE class mAb (clone A2) was reacted overnight at room temperature with glass surfaces covalently derivatized with $2.3 \times 10^4/\mu m^2$ DNP_{11}-BSA molecules (i.e., a full monolayer coverage). IgE surface density was varied over three orders of magnitude between 8 and $5 \times 10^3/\mu m^2$ by altering the reactant IgE solution concentration. The cellular dose response observed upon reacting RBL-2H3 cells with these surfaces was very similar to that observed in our earlier study (Tamir et al., 1996), i.e., the secretion steeply increased above a density threshold value of $\sim 8 \times 10^2/\mu m^2$ IgE (Fig. 1, *filled symbols*).

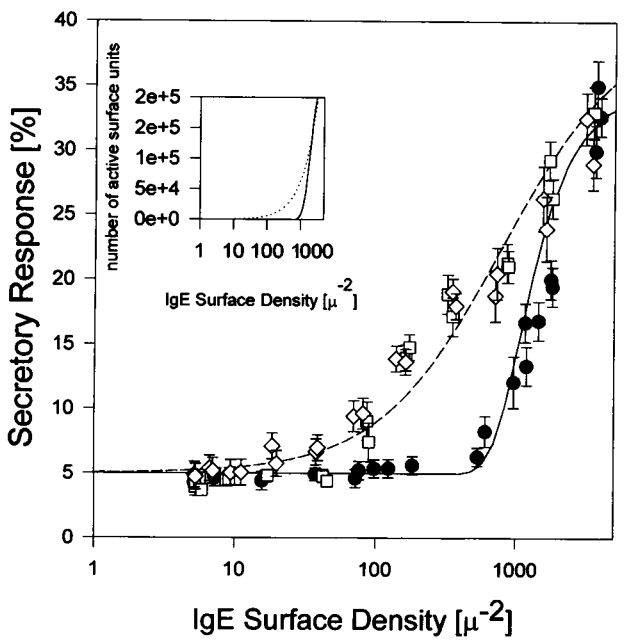


FIGURE 1 Dependence of the secretory response of RBL-2H3 cells on the density of surface-immobilized monomeric (●) and dimeric (□, ◇) IgE. The DNP-specific IgE mAb (clone A2) was bound to coverslips fully coated with DNP_{11}-BSA (2.3×10^4 μm^{-2}) as described under Materials and Methods. IgE dimerization was performed in solution by the IgE-specific mAb clone 95.3, either by diluting the IgE in Tyrode's buffer to the required final concentrations (0.1–100 nM) and then reacting it with 10 nM of the mAb 95.3 at 22°C for 2 h (□), or first reacting the IgE with 100 nM of this mAb and then diluting to the required final concentration (◇). The dimeric IgE-containing solutions were next incubated overnight with the antigen-carrying surfaces at 22°C, followed by extensive washings with PBS. RBL cells were allowed to settle on these surfaces for 1 h at 10°C, followed by removal of nonadherent cells. The glass surface-carried cells were then incubated in Tyrode's buffer for 45 min at 37°C, and their secretory response was measured. The solid and dashed lines result from fitting the data obtained with monomeric and dimeric IgE according to the model described in the text. The inset shows the computed number of stimulating surface units (n_{au}) as a function of the IgE surface density σ_{IgE} for reactions with monomeric (*solid line*) and dimerized (*dashed line*) surface-bound IgE. The error bars represent the standard deviation of the secretory response determined in triplicate.

The above protocol leads to immobilization of the IgE-FcεRI complexes for the following reasons: The dissociation of cell-bound IgE from DNP-haptens has a time constant of ~ 10–10^2 s (Goldstein et al., 1989), whereas the lateral diffusion of free IgE-FcεRI proceeds in the millisecond time domain (DeLisi, 1981). Hence the cells' reaction with IgE-carrying surfaces yields immobilized IgE-FcεRI complexes. Indeed, FRAP experiments performed with glass surface carrying $\sim 10^3$ fluorescently labeled IgE/μm^2 noncovalently bound to DNP_{11}-BSA have shown immobilization of the former.

For the second type of experiment, the IgE was dimerized by its specific mAb (95.3) (Baniyash and Eshhar, 1984) before its binding to the above DNP_{11}-BSA-carrying surfaces by employing two different dimerization protocols (cf. Materials and Methods). The cells' secretory dose response to stimulation by the dimerized IgE-carrying surfaces was then determined. As can be seen (Fig. 1, *open symbols*), the data exhibit a considerably lower density threshold for the onset of degranulation (~ 10–60 IgE/μm^2) as compared with that observed on surfaces carrying randomly distributed monomeric IgE. The steepness of the dose-response curve is also significantly lower in the former case and is qualitatively similar to that observed when conventional soluble FcεRI clustering agents are employed (Oliver et al., 1988). (It should be noted that both experiments, i.e., those employing surface-bound monomeric and dimeric IgE, were performed with the same batch of cells. Hence these data are amenable to a rigorous and consistent theoretical analysis.) Data obtained at very high IgE surface densities (e.g., $>10^3$ μm^{-2}), were however at the level where saturation could not be determined with sufficient accuracy.

The above results show that RBL-2H3 cells respond differently to these two distinct forms of immobilized IgE. We have previously analyzed the dose response to surface-bound monomeric IgE by a relatively simple model which assumes that an onset of secretion occurs when the average density of immobilized IgE-FcεRI exceeds a distinct threshold value. To simultaneously account for the qualitatively and quantitatively different secretory response to the different immobilized IgE distributions, a more elaborate theoretical model has now been developed, which is based on the following principles and assumptions:

1. The cell's surface is formally subdivided into j ($j = 1, 2, \ldots, N_p$) squares of a side length s_j.
2. All patches become active signal-transducing units upon occupation with immobilized FcεRI exceeding a distinct threshold density value Θ.
3. These triggering units are assumed to be nonhomogeneous with respect to their side length, i.e., s_j exhibits a Gaussian distribution.

$$P(s_j) = \frac{1}{\delta s \sqrt{2\pi}} e^{-(s_j - \bar{s})^2/[2(\delta s)^2]} \qquad (1)$$

where $\bar{s}$ denotes the expectation value for the patches' side length and δs their distribution's half-width. The

number of patches per cell with side lengths in the interval $s + \Delta_s$ can be calculated by

$$N(s) = \frac{2A_c r_{cell}^2}{s^2}\left[\mathrm{erf}\left(\frac{s-\bar{s}}{\sqrt{2}\delta s}\right) - \mathrm{erf}\left(\frac{s-\bar{s}+\Delta_s}{\sqrt{2}\delta s}\right)\right] \quad (2)$$

where r_{cell} (5 μm) is the average radius of RBL-2H3 cells (Oliver et al., 1988). A_c is the average fraction of the cells' surface in contact with the coverslip.

4. Each IgE at the cell-glass contacts is occupied by a FcεRI, so that the density of immobilized FcεRIs matches that of IgE on the glass surface.
5. The complete coverage of the coverslip with DNP_{11}-BSA causes a random distribution of IgEs on it. The number n_c of FcεRI-IgE complexes within patches with side length s can therefore be expressed as a function of the IgE density at the glass surface by

$$n_c = s^2\sigma_{IgE} \quad (3)$$

where σ_{IgE} denotes the surface density of IgE in μm^{-2}. The average number of active units can now be written as

$$n_{au} = \begin{cases} 0 & n_c < \Theta \\ \displaystyle\int_{-\infty}^{\infty} \frac{N(s)(n_c-1)}{\Theta - 1}\,ds & n_c \geq \Theta \end{cases} \quad (4)$$

The integral in Eq. 4 was truncated for practical reasons, assuming $\bar{s} - 2\delta s$ and $\bar{s} + 2\delta s$ as lower and upper values, respectively. The integration was carried out numerically.

6. Following the concept of Dembo and co-workers (Dembo et al., 1979), the relationship between secretory response $R(n_{au})$ and the number of triggering units per cell (n_{au}) is described by a Hill function (Schweitzer-Stenner et al., 1994), namely,

$$R(n_{au}) = \eta\frac{(K_{stim}n_{au})^h}{1 + (K_{stim}n_{au})^h} \quad (5)$$

where K_{stim} is defined as the efficiency of the stimulus-secretion coupling per secretion stimulatory unit, η denotes the maximum secretion at high n_{au} values, and h is the Hill coefficient.

The above model was now employed to fit the dose response to randomly distributed surface bound IgE (*solid data points* in Fig. 1), using k_{stim}, η, h, s, and δs as free parameters. The threshold Θ was fixed to 2, i.e., only two FcεRI-IgE complexes within the same patch were assumed to constitute a secretion stimulus unit. This fit yielded the solid curve depicted in Fig. 1 for the dose response to the randomly distributed IgEs. The parameter values obtained are listed in Table 1. The average side length of the triggering surface units was found to be 30 ± 10 nm, which is about twice the estimated diameter of the FcεRI-IgE complex (i.e., ~15 nm; Holowka et al., 1985; Zheng et al., 1992; Kubitscheck et al., 1993). The fitting parameters therefore indicate a requirement for closest (i.e., van der Waals) proximity of two immobilized FcεRIs for forming a signal-transducing unit. (The variance of the side length is the full half-width of the Gaussian distribution $P(s_j)$ described by Eq. 1.)

The above analysis only deals with the dose response to randomly distributed surface-bound IgE. To also account for the secretion observed with coverslips carrying dimerized, i.e., nonrandomly distributed IgE (*open symbols* in Fig. 1), we assume that each IgE dimer constitutes a secretion stimulus unit upon its binding to two FcεRIs on the cells' surface. Hence the number of signaling units per cell is now given by

$$n_{au} = \frac{\sigma_{IgE}\cdot A_s}{2\cdot n_{cell}} \quad (6)$$

where A_s is the total surface of the coverslip and n_{cell} is the number of cells on the coverslip. The dose response is now obtained by inserting Eq. 6 into Eq. 5.

To fit the thus modified model to the dose-response curve, we used the very same parameter values as obtained above from the analysis of the secretion induced by surfaces with randomly distributed IgE (cf. Table 1). Hence we assume that IgE-FcεRI dimers produced by clustering IgE with mAb 95.3 exhibit the same triggering efficiency K_{stim} as two immobilized IgE-FcεRI in close proximity. Despite these restrictions, a satisfactory fitting of the experimental data is obtained, as judged from the dashed line in Fig. 1. This strongly supports the validity of the model and the obtained parameter values.

The reason for the observed distinct patterns of dose response to monomeric and dimeric surface-bound IgE can be understood as follows. At low σ_{IgE} two of the randomly distributed IgE are unlikely to be in close proximity, so that no stimulating surface units are created by IgE-FcεRI binding. The situation changes when σ_{IgE} exceeds a distinct threshold. Above this value the number of doubly occupied surface units increases significantly. As a consequence, one observes a steep increase in secretion. The situation is qualitatively different when the cells react with surfaces carrying IgE dimers; because each of them creates a stimulating unit upon binding to two FcεRIs, stimulation is caused at very low σ_{IgE}. This is demonstrated by the inset in Fig. 1, which displays the computed number of secretion-stimulating surface units as a function of IgE surface density for cells reacting with monomeric or dimeric IgE. These curves are very similar to the corresponding experimentally observed dose-response curves.

TABLE 1 Parameters obtained from the fits to the dose-response curves in Figs. 1 and 2.

Experiment	s (nm)	δs (nm)	K_{stim}	A_c	η (%)	Θ
I	30	10	0.5	0.3	30	2
II	50	15	0.5	0.3	35	2

The above results provide strong evidence that the stimulus induced upon immobilizing FcεRI by randomly distributed or by dimerized surface-bound IgEs can be described in terms of the very same biophysical parameters (i.e., K_{stim} and η). To further support this notion, we measured the cells' secretory response to surfaces carrying different densities of either antigen or monomeric IgE. To this end, we employed glass surfaces with three different antigen surface densities, i.e., $\sigma_{Ag} = 308 \pm 30$, 1860 ± 130, and 2718 ± 570 μm^{-2}. The dose response was then measured as a function of IgE surface density in the range of 10–1000 μm^{-2} for each of these glass surfaces. As shown in Fig. 2, the observed secretion decreased significantly with decreasing σ_{Ag}, whereas the onset of response occurred at essentially the same IgE density, although with a different slope.

To analyze the above data, we adopted a model based on the following general considerations. The concentration $[C_k]$ of DNP_{11}-BSA bound to k IgE can be calculated by using the mass-action law to yield

$$[C_k] = 2 \cdot \left(\frac{4 - k + 1}{k}\right) K_1^k [\mathrm{IgE}]^k [Ag]_f \qquad (7)$$

where $[Ag]_f$ is the number of free epitopes on the glass surface, [IgE] is the concentration of IgE in the solution used to produce the examined surface, and K_1 is the affinity of DNP binding to a Fab-combining site. The latter is an effective parameter, because steric factors may affect this value differently. $[Ag]_f$ depends on the total concentration of sterically exposed haptens, and thus on the surface density of the epitopes ($[Ag]_T$), and can be calculated by utilizing the law of mass conservation:

$$[Ag]_f = \frac{[Ag]_T}{1 + \sum_{k=1}^{m} [2 \cdot (4 - k + 1)/k] K_1^k [\mathrm{IgE}]^k} \qquad (8)$$

It should be noted that the simultaneous binding of a single IgE to BSA with both of its Fab-binding sites (cf. Fig. 3) is not accounted for by Eqs. 7 and 8. (Binding of a single IgE to two DNP-haptens on the same BSA-DNP_{11} molecule is unlikely, because the minimum distance between its Fab-combining sites is ~13 nm (Schweitzer-Stenner et al., 1987), whereas the estimated size is of BSA is ~12 nm.) All species concentrations in these equations are thus far expressed as molarity. To convert $[C_k]$ to surface densities, we employ

$$\sigma_{C_k} = \frac{[C_k] \cdot L \cdot V}{A_s} \qquad (9)$$

where L is Avogadro's constant, and V is the sample's bathing buffer volume.

We now divide the number of IgE-bound antigen into two subsets. One contains all antigen molecules occupied by a single IgE (σ_{C_1}). At high antigen density, this subset constitutes a random distribution of IgEs. For low densities, however, only those IgEs bound to antigens in close proximity can produce a signaling unit upon reaction with FcεRI. The fraction $P(\sigma_{Ag})$ of antigens fulfilling this requirement can be calculated by solving the integral

$$P(\sigma_{Ag}) = \int_0^{r_s} w(r)\, dr \qquad (10a)$$

where

$$w(r) = 2\pi r \sigma_{Ag} \exp(-\pi r^2 \sigma_{Ag}) \qquad (10b)$$

and r is the distance between two antigens on the glass surface. (Equation 10a,b can be derived by employing the

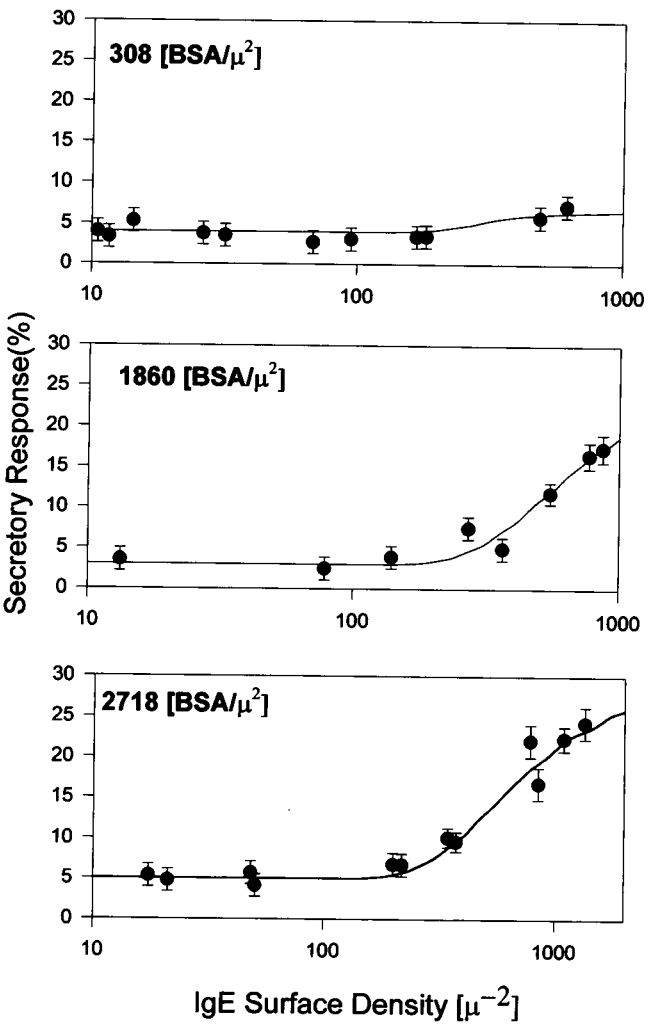
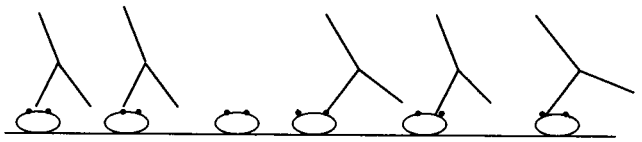


FIGURE 2 Dependence of secretory response of RBL-2H3 cells on the densities of both surface-carried antigen and immobilized monomeric IgE. IgE was bound to coverslips coated with the indicated surface densities of DNP_{11}-BSA, as described in Materials and Methods. RBL-2H3 cells were next allowed to settle on these surfaces for 1 h at 10°C, followed by removal of nonadherent cells. The glass surface-carried cells were then incubated in Tyrode's buffer for 45 min at 37°C, and their secretory response was measured and presented as a fraction of the total granular contents. The solid lines result from fitting all data points according to the model described in the text. The error bars represent the standard deviation of the secretory response determined in triplicate.



•: DNP-groups

FIGURE 3 Schematic representation of IgE binding to the antigen (DNP_{11}-BSA) carried on a glass surface. It is assumed that only two DNP groups per antigen molecule are accessible for IgE binding to BSA. Simultaneous binding of the two Fab combining sites of the same IgE to one DNP_{11}-BSA molecule is excluded for steric reasons.

procedure of Chandrasekhar (1943) for random distributions of particles on a two-dimensional surface. The antigen distribution is thereby treated as an ideal gas, thus neglecting the excluded volume effect.) We assume that to stimulate, two antigen-IgE-FcεRI complexes must fill the square patches of a triggering unit, so that $r_s = \bar{s}$. After this is inserted into Eq. 10, a straightforward calculation yields

$$P(\sigma_{Ag}) = 1 - \exp(-\pi r_s^2 \sigma_{Ag}) \tag{11}$$

The secretory response to the subset C_1 can be described by Eqs. 2–7, substituting σ_{IgE} by $(\sigma_{C_1}) \cdot P(\sigma_{Ag})$.

The second subset comprises all antigen molecules carrying more than one IgE. We assumed therefore that irrespective of the number k of IgEs bound to the same BSA-DNP_{11}, steric hindrance prevents the simultaneous binding of more than two of them to FcεRIs (m = 2). The contribution of the thus formed FcεRI dimers (n'_{au}) to the number of stimulatory units (n_{au}) on the cells' surface can be written as

$$n'_{au} = \frac{A_s \sigma_{c_k}}{n_{cell}} \tag{12}$$

The equilibrium constant K_1 for IgE binding to surface-bound DNP_{11}-BSA was assumed to be equal to the solution equilibrium constant of $10^7\ M^{-1}$ (Schweitzer-Stenner et al., 1987).

The above model was employed to simultaneously fit the secretory dose-response curves depicted in Fig. 2, by allowing k_{stim}, η, h, s, and δs to be free parameters. This fitting procedure yielded the solid curves shown in Fig. 2. The parameter values are listed in Table 1.

It should be noted that for σ_{Ag} values of 2718 and 1860 μm^{-2} (*middle* and *lower panels* in Fig. 2), the parameter values do not depend on the number m of sterically accessible DNP epitopes, because the secretory response is determined by species of the subset C_1. In contrast, C_2 is of relevance for $\sigma_{Ag} = 308\ \mu m^{-2}$ (Fig. 2, *upper panel*), and $m \geq 3$ is now required to account for the maximum IgE surface density still achieved in this experiment (i.e., 700 μm^{-2}). However, the data do not allow us to determine the exact stoichiometry of the IgE-antigen reaction, because the dose response is still insensitive to variations of k in the region covered by the experimental data.

To determine the uncertainty of the above parameters, we have performed calculations with detuned parameters to determine the extent to which their values can be varied without a deterioration of the fit's quality. This leads to an estimate of their statistical error, showing that h, s, and δs have an uncertainty between 10% and 15%. In contrast, strong correlation effects (Grinvald and Steinberg, 1973) were observed for K_{stim} and η, so that multiple pairs of these parameters could be found that produce reasonable fits to the same dose-response curves. This is mostly due to the fact that our dose-response data do not reach the region of saturation. Their relative error is eventually found to be between 30% and 40%.

It should be mentioned that some parameter values (i.e., s, δs, and, to a minor extent, η) obtained from the second set (Fig. 2) of experiments are somewhat different from those emerging from the analysis of the first one (Fig. 1; cf. Table 1). However, these differences are far from being significant and may well be explained by the fact that different cell batches were used for these experiments.

The above model is based on the assumption that all IgEs at cell-glass contacts are saturated by surface-bound FcεRI. This is supported by experiments (McCloskey and Poo, 1986) in which cell-size phospholipid vesicles containing DNP derivatized lipids were allowed to react with RBL-2H3 cells carrying DNP-specific, fluorescently labeled IgE on their FcεRI. It was found that upon cell-vesicle contact, the IgE-FcεRI complexes undergo a lateral redistribution to accumulate at the site of cell-vesicle contacts. That can also be expected to occur upon cell binding to the antigen-carrying glass surfaces. This will provide access of the FcεRI to the surface carrying IgE. After this reaction has reached equilibrium, however, the total concentration of FcεRI does not significantly exceed that of immobilized IgE-FcεRI on the cells (cf. Tamir et al., 1996).

Finally, to check the influence of possible incomplete IgE-FcεRI saturation on the fitting parameters, we have fitted the data in Fig. 1, assuming that only 60% of the surface-bound IgEs have reacted with FcεRI. The fit obtained was still satisfactory and yielded $\bar{s} = 40$ nm and $K_{stim} = 0.3$. Thus the difference from the parameters obtained for complete saturation is insignificant and would not affect the implications of our analysis.

DISCUSSION

The mechanism by which members of the MIRR family initiate the cascades that couple them to the respective cellular response is still not clearly resolved. FcεRI has been one of the more extensively studied paradigms for MIRR action. Numerous studies have indicated that producing an effective immunological stimulus in RBL-2H3 cells requires clustering of their FcεRI into dimers or larger oligomers (Ortega et al., 1988; Holowka and Baird, 1996).

However, no knowledge about the requirement for FcεRI immobilization is available. Earlier theoretical considerations have led DeLisi (1980) to suggest that bringing two FcεRI into close proximity for a time period longer than a given threshold would allow appropriate downstream signal transmission. Thus this time period has to be considerably longer than the lifetime of FcεRI encounter complexes, which is determined by the receptors' lateral diffusion. To examine the above predictions, we have devised a new experimental approach. It involves immobilization of the FcεRI upon their reaction with DNP-specific IgE antibodies bound to DNP_{11}-BSA-carrying glass surfaces (Tamir et al., 1996). The secretory response of the cells reacting with these surfaces was measured as a function of IgE surface density and found to exhibit a rather sharp onset of secretion above a density threshold of ~800 IgE/μm^2 (Fig. 1). This observation indicates that a secretory stimulus production requires only the surface density of immobilized FcεRI to exceed a distinct threshold value at which a sufficient number of them are in close proximity. Moreover, we have measured the cells' secretory response to surfaces that carried altered distributions of immobilized IgE. This was attained either by IgE dimerization in solution before its surface immobilization or by varying the immobilizing antigen density on the glass surface. Both of these protocols were expected to change the probability of immobilizing the required number of FcεRIs within signaling proximity and therefore the onset of secretory response and the shape of its dose dependence. This was indeed observed. First, the onset of the secretion induced by glass surfaces with predimerized IgE was observed at much lower IgE surface densities (10–60 IgE/μm^2), and the slope of the dose-response curve is less steep than that observed for surfaces carrying monomeric IgEs (Fig. 1). Second, reducing the antigen surface density significantly decreased secretion and the slope of its initial dose-response phase (Fig. 2).

The implications of these results were examined by using a novel theoretical model based on statistical considerations. The finding that this model consistently reproduces all experimentally observed dose-response data sets (Figs. 1 and 2) supports the assumptions on which it is based, namely, that monomeric IgE are randomly distributed on the glass surfaces, whereas predimerized IgE yields a nonrandom distribution. Furthermore, the IgE glass surface density is the same as that of the immobilized IgE-FcεRI complexes at the glass-cell interface.

To characterize the clusters that initiate the stimulus, the cell's surface is formally divided into the smallest square patches that are capable of triggering secretion upon their occupancy by at least two immobilized FcεRI. These FcεRI clusters would then trigger secretion autonomously (Oliver et al., 1988). Using the patches' side length as a free parameter, a remarkably good fit to the experimental data was obtained. Hence, in spite of its simplicity, our model fully accounts for observed variations in the dose response to qualitative and quantitative changes in IgE distribution on the glass surface. The result of the fit provides evidence that an elementary signaling unit requires only two FcεRI in such proximity, that they are accommodated into patches of 30 ± 10 nm side length, i.e., they are essentially in van der Waals contact.

The model also accounts for results of earlier studies by McConnel and associates (Cooper et al., 1981; Balakrishnan et al., 1982). They have investigated the interaction of RBL-2H3 cells carrying DNP-specific IgE mAb with liposomes containing DNP-derivatized lipids. Different types of liposomes were employed, i.e., "solid" and "liquid" ones with lipid diffusion coefficients of 10^{-10} and 10^{-7} to 10^{-8} cm^2/s, respectively. Both were found to be capable of stimulating the secretion of RBL-2H3 cells, but the "solid" liposomes were up to threefold more effective. In view of our results, one would indeed expect the "solid" liposomes to be effective in triggering secretion, because they are likely to immobilize the FcεRI on the reacting cells' surfaces. Indeed, the dose responses to these liposomes are very much like that shown in Fig. 1, inasmuch as they both display a steep increase above a certain threshold value of hapten surface density (cf. Figure 3 in Balakrishnan et al., 1982), thus indicating that the employed protocol immobilizes a sufficient number of FcεRI in close proximity. The relatively low dose response to the "liquid" liposomes was rationalized by invoking the accumulation of IgE-FcεRI complexes at cell-liposome contact regions that would indeed significantly increase the effective FcεRI density. Such molecular crowding on membrane surfaces was shown by simulations (Minton, 1989; Saxton, 1989, 1992, 1993) to impede lateral diffusion, so that the effective lifetime of a given FcεRI pair may become sufficiently long to produce a stimulus. Therefore, the model developed and employed here also rationalizes the dose response to both "solid" and "liquid" hapten-carrying liposomes.

Ortega et al. (1988) have already shown that FcεRI dimerization by different specific mAbs caused effective RBL-2H3 degranulation. Moreover, the marked differences in response to the FcεRI dimers produced by the three employed mAbs suggested that the triggering capacity of FcεRI dimers depends on their intrinsic properties, e.g., their lifetimes and/or the relative orientation of the receptors within a dimer (Ortega et al., 1991; Schweitzer-Stenner et al., 1994). The present study provides an independent quantitative characterization of the smallest functional triggering units, i.e., two FcεRI occupying a patch with a side length of approximately 30 nm, which brings them into van der Waals contact. The major new insight gained by the current protocols is the decisive role that receptor immobilization plays in initiating the signaling cascade. As the number of FcεRI copies per cell of the RBL-2H3 line is high ($3–6 \times 10^5$), the probability of immobilizing them at the required proximity is considerable, and hence the stimulus is produced. For other multichain immunoreceptors that are present at considerably lower density (e.g., T-cell receptors), stimulation indeed requires protocols that cause both clustering and immobilization (cf. also McKeithan, 1995).

Why do the very early biochemical coupling processes set these requirements on a stimulatory FcεRI cluster? The earliest known process in the RBL-2H3 cells is activation of the FcεRI-associated protein tyrosine kinase (PTK)-Lyn (Ravetch and Kinet, 1991; Eiseman and Bolen, 1992; Adamczewski et al., 1992; Adamczewski and Kinet, 1994) that is bound with low affinity to the NH_2 tail of the resting cell's FcεRI β-chain (Lin et al., 1996). Upon FcεRI clustering, Lyn undergoes activation and phosphorylates tyrosyl residues of the adjacent FcεRI's β- and γ-subunits on their immunoreceptor tyrosine activation motifs (ITAMs). The phosphorylated γ-chains then recruit the PTK Syk, which upon binding to the phosphorylated tyrosyls of the ITAMs, also undergoes activation. This mechanism obviously requires that the clustered FcεRIs remain immobile at close proximity for the time span required by these processes to take place.

Finally, it should be mentioned that physiologically, mast cell stimulation is attained under conditions very different from those employed in most studies using the RBL-2H3 line (including the present one). Whereas for the latter cells, the FcεRI are saturated by mAbs with a single binding specificity in the living animal, mast cells would carry polyclonal IgEs with the whole repertoire of specificities and affinities present, few of which are the same. In contrast, the situation is different for B- and T-cells, each of which carries on its surfaces MIRRs with only one specificity. Therefore the requirements for activating the latter cells are similar to those established for the FcεRI on mast cells, i.e., clustering and immobilization of their MIRRs. Hence the model developed in the present study and its results may be of wider applicability to other immunological systems.

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