Interaction of a Monoclonal IgE-Specific Antibody with Cell-Surface IgE−FcεRI: Characterization of Equilibrium Binding and Secretory Response†

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Received February 11, 2004; Revised Manuscript Received June 15, 2004

ABSTRACT: Aggregation of FcεRI, the high-affinity cell-surface receptor for IgE antibody, is required for degranulation of basophils and mast cells, but not all receptor aggregates elicit this cellular response. The stereochemical constraints on clusters of FcεRI that are able to signal cellular responses, such as degranulation, have yet to be fully defined. To improve our understanding of the properties of FcεRI aggregates that influence receptor signaling, we have studied the interaction of 23G3, a rat $IgG_{1\kappa}$ IgE-specific monoclonal antibody, with IgE−FcεRI complexes on rat mucosal-type mast cells (RBL-2H3 line). We find that 23G3 is a potent secretagogue. This property and the structural features of 23G3 (two symmetrically arrayed IgE-specific binding sites) make 23G3 a potentially valuable reagent for investigating the relationship between FcεRI clustering and FcεRI-mediated signaling events. To develop a mathematical model of 23G3-induced aggregation of FcεRI, we used fluorimetry and flow cytometry to quantitatively monitor equilibrium binding of FITC-labeled 23G3 intact Ab and its Fab′ fragment to cell-surface IgE. The results indicate that IgE bound to FcεRI expresses two epitopes for 23G3 binding; that 23G3 binds IgE resident on the cell surface with negative cooperativity; and that 23G3 appears to induce mostly but not exclusively noncyclic dimeric aggregates of FcεRI. There is no simple relationship between receptor aggregation at equilibrium and the degranulation response. Further studies are needed to establish how 23G3-induced aggregation of IgE−FcεRI correlates with cellular responses.

Mast cells trigger allergic reactions when IgE bound to FcεRI, the high-affinity cell-surface receptor for IgE, interacts with a multivalent antigen. Clustering of FcεRI plays an important role in this process (*1*). Receptor aggregation initiates a cascade of intracellular-signaling events (*2*, *3*), which lead to secretion of granule-stored mediators of inflammation, such as histamine, and the de novo synthesis and secretion of other mediators of allergic reactions.

Although the importance of aggregation in FcεRI signaling is well-known, the properties of FcεRI aggregates that influence signaling have yet to be fully defined (*4*). Considerable effort has been made to correlate properties of FcεRI aggregates with cellular responses, such as receptor phosphorylation and degranulation (*4*−*7*). An important element in these studies is the reagent used to induce FcεRI aggregation. Reagents that have been considered and have structural features that facilitate the characterization of receptor aggregation, include chemically cross-linked oligomers of IgE (*8*, *9*), chemically synthesized divalent haptens and antigens with two hapten groups (*10*−*13*), and monoclonal IgE- (*14*, *15*) and FcεRI- (*16*) specific antibodies.

Chemically cross-linked oligomers of IgE have been used extensively to study FcεRI (*17*−*20*). These oligomers are simple ligands in that the binding of IgE to FcεRI is monovalent. Thus, the number of receptors in aggregates induced by an IgE oligomer can be no greater than the number of IgE molecules in the oligomer, e.g., two for the case of IgE dimers. Although oligomers of IgE are useful tools, they have limitations (*6*). The kinetics of IgE binding to FcεRI (*21*) are atypical for antigen−antibody interactions, and compared to the time scale of early intracellular signaling events, IgE binding to FcεRI is slow and effectively irreversible (*20*). Thus, cellular responses elicited by IgE oligomers may differ, perhaps fundamentally, from responses elicited by antigens. Another drawback is that chemical cross linking of IgE antibodies is variable, and as a result, IgE oligomers are structurally heterogeneous, which makes these ligands unsuitable for investigating the stereochemical/orientational requirements of signaling-competent receptor aggregates.

† This work was supported by a Senior International Fellowship award to R.G.P. from the Fogarty Center (F06TW002308), Grants AI35977 and AI28433 from the National Institutes of Health, the Department of Energy, through contract W-7405-ENG-36, and a grant to I.P. from the Israel National Science Foundation.

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10.1021/bi049686o CCC: $27.50
Published on Web 08/12/2004

Symmetric divalent haptens and antigens with two epitopes (i.e., bivalent ligands) also have been used extensively to study FcεRI. A bivalent ligand is the simplest type of ligand capable of aggregating IgE−FcεRI complexes. The interaction of a bivalent ligand with the two antigen-combining sites of IgE can result in a spectrum of IgE−FcεRI aggregates. This spectrum can include receptor chains and rings of various sizes (*22*) but not aggregates with more complicated structures (see below). A major goal of studies involving bivalent ligands has been to determine or predict the types of receptor aggregates and the number of receptors in each type of aggregate on the cell surface, so that these quantities then can be compared and correlated with cellular responses (*12*, *22*−*29*).

However, this goal has proven elusive, because the bivalent ligands studied to date are either nonstimulatory or induce rather weak cellular responses. A variety of evidence suggests that these ligands are poor initiators of cellular signaling because they aggregate receptors predominantly in the form of stable cyclic dimers (*12*, *29*). Cyclic dimers, in which both binding sites of each of the two ligands bind two IgE−FcεRI complexes to form a closed ring, prevent receptor chain elongation, limiting the size of ligand-induced aggregates. They may also prevent signaling events from occurring for stereochemical reasons and/or promote inhibitory signals (*29*, *30*).

The appeal of bivalent ligands is the relative simplicity of the IgE−FcεRI aggregates they are capable of inducing. Bivalent ligands induce only chains and rings of receptors (*22*). As a result, the binding of a bivalent ligand is easier to characterize than the binding of a multivalent ligand, such as a densely haptenated protein. A ligand with more than two epitopes that interacts with surface IgE can induce not only chain- and ring-like structures but also tree- and network-like structures that are difficult to treat in mathematical models of receptor aggregation (*31*, *32*) or to study experimentally.

This property of divalent haptens and antigens with two epitopes, the ability to induce simple aggregates, is shared by antibodies with two antigen-combining sites (e.g., IgG class antibodies) that are specific for either IgE (*14*, *15*) or FcεRI (*16*). Such antibodies, if they stimulate cellular responses, can serve as valuable tools for defining the stereochemical properties of FcεRI aggregates that induce signal transduction. Stimulatory IgE-specific antibodies, in particular, are valuable, because the number of cell-surface receptors (i.e., the number of IgE antibodies bound to FcεRI) can, in principle, be manipulated in experiments involving the same cell line, and receptor number is an important parameter that influences ligand-induced aggregation. In contrast, with FcεRI-specific antibodies, receptor number is determined by cell type.

Here, we evaluate the potential of 23G3 (*33*), an IgE-specific rat monoclonal $IgG_{1\kappa}$ antibody, for use in studies of FcεRI signaling. We find that this antibody and its $(Fab')_2$ fragment stimulate significant degranulation of rat mucosal-type mast cells of the RBL-2H3 line. Thus, 23G3 can be used to investigate the properties of IgE−FcεRI aggregates that influence signaling by FcεRI. Because of the structural features of 23G3 (e.g., two symmetrically arrayed IgE-specific binding sites), it should be possible to develop a detailed, quantitative, and predictive characterization of the binding reactions of 23G3 with IgE−FcεRI, which can then be used to relate binding to downstream signaling events. As a first step in this direction, we have assayed the equilibrium binding of intact 23G3 Ab and its Fab′ fragment to cell-surface IgE. The results of these binding studies are well-characterized by a simple mathematical model, which we use to predict the equilibrium distribution of receptor aggregates over a range of ligand doses. There is no simple correlation between receptor aggregation and degranulation.

MATERIALS AND METHODS

Antibodies and Cross-Linking Reagents. The mouse monoclonal IgE antibody specific for 2,4-dinitrophenyl (DNP)[1] was isolated from hybridoma H1 26.82 (*34*) by affinity purification (*35*). The IgE was then subjected to gel filtration on a Sephacryl 300 column, and the monomeric fraction was collected. The rat monoclonal IgE-specific antibody 23G3, in forms labeled and unlabeled with FITC, and the FITC-labeled Fab′ fragment of 23G3 were purchased from Southern Biotechnology Associates (Birmingham, AL). Purification of these reagents included gel filtration on a Sephacryl 300 column to remove any aggregates and fragments, and the purity of the reagents was verified using SDS−PAGE and Elisa. Intact 23G3 was also isolated as previously described (*33*) and used to prepare $(Fab')_2$ fragments (*36*), which were then labeled with FITC (*10*). BSA coupled to DNP, DNP_{11}−BSA, was prepared as described previously (*37*).

Cells. RBL-2H3 cells (*38*) were grown adherent in 75-cm^2 flasks. Cultures were maintained at 37 °C and 5% CO_2 and used 4−6 days after passage. The culture medium consisted of MEM 1X with Earle's salts without glutamine (GIBCO BRL), 20% fetal bovine serum (HyClone, Logan, UT), 1% v/v 200 mM L-glutamine, 1% v/v penicillin, and 1% v/v streptomycin. Cells were sensitized by incubating with excess IgE (4 μg/mL) at 37 °C for 2 h.

Degranulation Assays. The secretion response of RBL cells was monitored by measuring the activity of the granule-stored enzyme β-hexosaminidase secreted into the supernatant (*39*). To allow time for ligand−receptor binding to reach equilibrium before induction of a secretory response, IgE-bearing RBL cells (10^6 cells/mL) were exposed to the indicated concentration of 23G3 for 1 h at 4 °C, a temperature at which degranulation does not occur. The temperature was then raised to 37 °C. After incubation for an additional hour, cells were centrifuged and 25 μL samples of supernatant were transferred to a microwell test plate and incubated for 60 min at 37 °C with 62.5 μL of substrate solution (1.3 mg/μL *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine in 0.1 M citrate at pH 4.5). The reaction catalyzed by β-hexosaminidase was stopped and developed by addition of 150 mL of 0.2 M glycine solution at pH 10.7. The color formed as a result of hydrolysis of the substrate, and production of *p*-nitrophenolate was measured at 405 nm. To assess the total amount of cellular β-hexosaminidase, cells of equal number were lysed with 0.1% Triton X-100 and an aliquot of this lysate was assayed at the same time as supernatant samples. The extent of secretion is expressed as a percentage of the total

[1] Abbreviations: DCT, ε-[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosyl; $(DCT)_2$-cys, *N*,*N*′-bis[DCT]-L-cystine; DNP, 2,4-dinitrophenol; RBL, rat basophilic leukemia.

β-hexosaminidase activity present after subtracting the activity in the supernatants of unstimulated cells.

Other experiments were carried out to determine whether the protocol for adding ligand affects the secretory response, which would be expected if secretion depends on the transient approach to the equilibrium for ligand−receptor binding or if signaling events occur to some extent at 4 °C. In these experiments, ligand was added not at 4 °C but at room temperature. The temperature was then immediately raised to 37 °C. After incubation for 1 h, the secretion response was assayed as described above. The different protocols for adding ligand yielded indistinguishable dose−response curves; the data, not shown, were collected on the same day.

Binding Assays. IgE-bearing cells (10^6 cells/mL) were incubated at 37 °C with FITC-labeled 23G3 intact Ab or Fab′ fragment in a suspension of freshly filtered (0.22-μm filters) buffered salt solution (BSS): 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 0.1% gelatin, and 20 mM Hepes (pH 7.8). Cell suspensions in BSS were supplemented with 10 mM sodium azide and 10 mM 2-deoxy-D-glucose (Sigma, St. Louis, MO) to inhibit receptor trafficking. After incubation for at least 1 h, a Becton Dickinson FACScan flow cytometer, controlled with Cell Quest software, was used to collect histograms of FITC fluorescence (520 nm), and the mean fluorescence, FL1, was recorded. To correct for nonspecific binding, a control experiment was performed with cells that lack surface IgE. The difference between FL1 and the mean fluorescence of the control is reported as the fluorescence because of specific binding, ΔFL1.

Binding assays were also performed in the absence of 2-deoxy-D-glucose and sodium azide. Consistent with an inability of 23G3 Fab′ to aggregate IgE−FcϵRI and trigger cell signaling, assays with and without these reagents were indistinguishable for Fab′ binding. However, because of the stimulatory capacity of intact 23G3 antibody, which presumably induces receptor internalization, assays differed for intact antibody binding to cell-surface IgE (the data, not shown, are quite noisy).

Stoichiometry of 23G3 Fab′ Binding to Cell-Surface IgE−FcϵRI Complexes. To determine the number of 23G3-epitopes on cell-surface IgE, equimolar samples of FITC-labeled IgE and 23G3 Fab′ were prepared, and the fluorescence of each sample was measured with a Spex Fluoromax fluorimeter. The fluorescence of FITC−IgE relative to that of FITC−Fab′, *X*, was recorded. Also, cells were saturated with either FITC−IgE or unlabeled IgE and incubated with excess FITC−Fab′ for 1 h at 4 °C. The mean FITC fluorescence of the two cell populations was then determined by flow cytometry. The mean fluorescence of cells bearing FITC−Fab′ relative to that of cells bearing FITC−IgE, *Y*, was recorded. The quantity *X*/*Y* indicates the number of 23G3-binding sites per IgE−FcϵRI complex provided that the fluorimetric measurements that determine *X* reflect the fluoresence intensities of the functional fractions of the labeled reagents; i.e., the fraction of FITC−IgE able to bind FcϵRI, and the fraction of FITC−Fab′ able to bind IgE−FcϵRI. If a significant amount of IgE or 23G3 Fab′ is nonfunctional, which seems unlikely, and in addition the functional and nonfunctional fractions are differentially labeled by FITC, then the ratio *X* could be either smaller or larger than the desired ratio that would be obtained in experiments with 100% functional FITC−IgE and FITC−Fab′. Thus, this method of determining the stoichiometry of 23G3 Fab′ binding to IgE−FcϵRI entails some uncertainty.

Mathematical Model for 23G3 Fab′ binding to Cell-Surface IgE. Scatchard plots for 23G3 Fab′ binding to surface IgE were found to be nonlinear (Figure 2b); therefore, in modeling the interaction of 23G3 Fab′ with IgE−FcϵRI complexes, we decided to allow for the possibility that binding is cooperative. Thus, we consider the following equilibrium equations:

$$R_1 = 2KFR_0 \quad \text{and} \quad 2R_2 = HKFR_1 \tag{1}$$

where F is the concentration of Fab′ in solution, R_0 is the surface density of unbound IgE, R_1 is the surface density of IgE attached to one Fab′ molecule, and R_2 is the surface density of IgE attached to two Fab′ molecules. The equilibrium constant K characterizes the affinity of Fab′ for a site on unbound surface IgE, and the cooperativity factor H characterizes the change in Fab′ affinity for a free site on IgE if one site is already bound. Cooperativity may arise if a Fab′ bound at one site sterically interferes with binding to the second site on IgE or if Fab′ binding to IgE induces a conformational change that affects further binding. In the absence of cooperativity, $H = 1$. Negative coopertivity is indicated by $H < 1$, and positive cooperativity is indicated by $H > 1$.

The equilibrium relations of eq 1 are coupled with the following conservation equations, which apply because experiments are performed under conditions that inhibit receptor trafficking:

$$F_T = F + C(R_1 + 2R_2) \quad \text{and} \quad R_T = R_0 + R_1 + R_2 \tag{2}$$

where F_T is the total concentration of Fab′ and R_T is the total surface density of IgE. The constant C is a factor that converts surface densities to concentrations; it is the cell concentration if surface densities are expressed in units of number per cell.

To calculate equilibrium quantities, we use

$$1 = f + (CR_T/F_T)\frac{2KF_T f + 2H(KF_T f)^2}{1 + 2KF_T f + H(KF_T f)^2} \tag{3}$$

which is obtained by combining eqs 1 and 2. The variable f is defined as the fraction of free Fab′, F/F_T. When values are specified for F_T, C, R_T, H, and K, the value of f can be determined by solving eq 3 (e.g., by bisection). Once f is known, other quantities can be determined by using eqs 1 and 2.

Mathematical Model for Intact 23G3 Binding to Cell-Surface IgE. To characterize 23G3 binding to surface IgE, we use the model of Wofsy and Goldstein (*40*) for cooperative binding of a bivalent ligand to a bivalent cell-surface receptor. Because we observed that degranulation responses to intact Ab and $(Fab')_2$ are similar (Figure 1) and because IgG interactions with Fcγ receptors are low affinity (*41*), we do not include these interactions in our model. If 23G3 binds surface IgE without intramolecular rearrangement

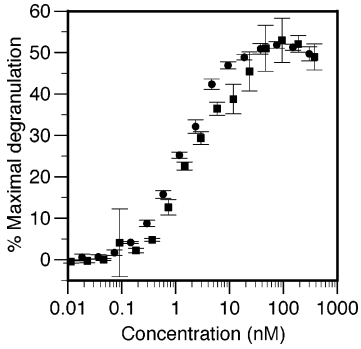

FIGURE 1: Degranulation of IgE-bearing RBL cells as a function of concentration of 23G3 $(Fab')_2$ fragment (●) or intact Ab (■). The activity of released β-hexosamidase is expressed as a fraction of the maximal total release that occurs upon solubilizing the cells with detergent. Each point represents the mean from six experiments, and a bar represents the standard error.

reactions (i.e., without forming ring-like aggregate structures), then the following equations apply at equilibrium:

$$1 = (1 - \eta)w + \eta\frac{w}{(1 - \delta w)^2} \tag{4}$$

and

$$1 = a + \frac{CR_T}{A_T}\left[1 + \left(\eta\frac{4\kappa a}{E} - 1\right)\frac{w}{1 - \delta w} + (1 - \eta)\frac{\delta w^2}{1 - \delta w}\right] \tag{5}$$

where $w = W/R_T$, $a = A/A_T$, $\kappa = HKA_T$, $\kappa_x = K_xR_T$, $E = 1 + 2\kappa a$, $\eta = E^2/[E^2 - (1 - H)]$, and $\delta = (\eta H)4\kappa_x\kappa a/E^2$. W is the surface density of unaggregated IgE−FcεRI, R_T is the total surface density of IgE−FcεRI, A is the concentration of free 23G3 Ab, and A_T is the total 23G3 Ab concentration. The parameter C is a factor that converts surface densities to concentrations. The equilibrium constant K is the microscopic single-site affinity of a 23G3 antigen-combining site for a free 23G3-epitope on surface IgE, the equilibrium cross-linking constant K_x is the microscopic single-site affinity of a membrane-anchored 23G3 antigen-combining site for a free site on surface IgE, and the constant H is the cooperativity factor by which K is multiplied when one of the two sites on surface IgE is bound.

To consider rings, we must include additional terms in eqs 4 and 5 (*40*). These terms introduce dimensionless ring-closure constants: J_n for $n = 1, ..., \infty$. Each J_n characterizes the propensity of a linear chain of n 23G3-aggregated molecules of IgE−FcεRI to close through binding of a free 23G3 antigen-combining site at one end of the chain to a free epitope on IgE at the other end of the chain. We assume $J_1 = 0$, because it seems unlikely that both arms of a 23G3 antibody could simultaneously bind both epitopes on a single molecule of IgE. We also assume $J_n = 2J_2/n$, which is consistent with ring closure depending on a random walk in two dimenions (*22*). Thus, our consideration of rings requires the introduction of only a single additional parameter, J_2.

After values for parameters are specified, eqs 4 and 5 involve only two unknowns (a and w), which can be determined numerically by using standard methods for solving systems of nonlinear algebraic equations. Once a and w are known, other equilibrium quantities can be determined by using equations of Wofsy and Goldstein (*40*). For

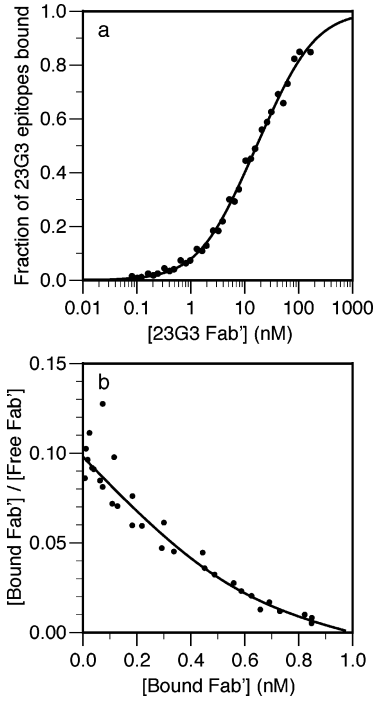

FIGURE 2: Flow cytometric measurement of the binding of FITC-labeled 23G3 Fab′ to cell-surface IgE. (a) Theoretical binding curve is derived from eqs 1−3 and our best-fit parameter estimates for the case without rings (Table 1). Fluorescence data are scaled by $\Delta FL1_{max}$, which is determined from the highest observed fluorescence and our estimate of Φ. (b) Scatchard plot. This plot is derived through transformation of the data presented in the panel above: $x_b = y_a$ and $y_b = y_a/(x_a - y_a)$, where $x_{a,b}$ and $y_{a,b}$ represent the coordinates in a or b as indicated by the subscript. Note that this transformation is based on a total concentration of 23G3 eptiopes, CS_T, of 1 nM (Table 1).

example, in the absence of rings, the fraction of receptors in aggregates of i receptors, f_i, is given by

$$f_i = i\eta\delta^{i-1}w^i \tag{6}$$

for $i \geq 2$. Note that $1 - w$ is the total fraction of receptors in aggregates of all sizes.

Estimation of Parameter Values. The values of A_T, F_T, and C are set by the concentrations of intact Ab, Fab′, and cells used in experiments. The value of R_T is set by the number of FcεRI per RBL cell ($\approx 3 \times 10^5$) (*42*). We estimate the values of H, K, K_x, and J_2 (when we do not set $J_2 = 0$) by using nonlinear least-squares fitting (*43*). In each fitting procedure, two sets of data (cf. Figures 2 and 3) are used simultaneously. Each set consists of measurements of ΔFL1 from a series of experiments in which different concentrations of FITC-labeled intact 23G3 or Fab′ were allowed to equilibriate with IgE-bearing cells. We relate measurements of ΔFL1 to quantities in the model by using the relation $\Delta FL1/\Delta FL1_{max} = (1 - F/F_T)F_T/(CS_T)$ for 23G3 Fab′ binding to surface IgE and the relation $\Delta FL1/\Delta FL1_{max} = (1 - A/A_T)A_T/(CS_T)$ for 23G3 antibody binding to surface IgE, where S_T, the surface density of 23G3-binding sites on IgE, is $2R_T$ and $\Delta FL1_{max}$ is the maximum FITC fluorescence because of specific binding. In addition to H, K, K_x, and J_2, the best fitting values of Φ for each data set were also determined, where Φ is defined as the theoretical $\Delta FL1_{max}$ divided by the largest ΔFL1 actually observed in experiments.

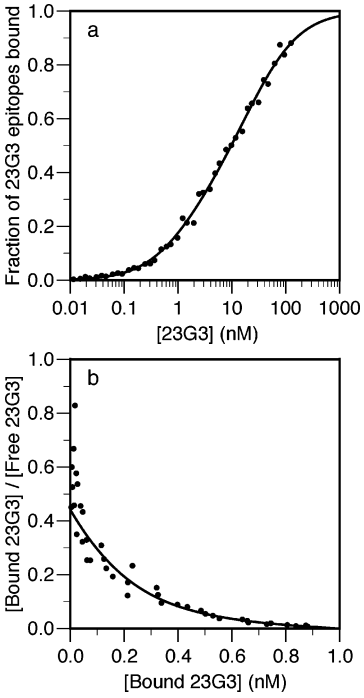

FIGURE 3: Flow cytometric measurement of the binding of FITC-labeled intact 23G3 antibody to cell-surface IgE. (a) Theoretical binding curve is derived from eqs 4 and 5 and our best-fit parameter estimates for the case without rings (Table 1). Fluorescence data are scaled by $\Delta FL1_{max}$, which is determined from the highest observed fluorescence and our estimate of Φ. (b) Scatchard plot. The data points of a are transformed as described in the caption of Figure 2. Thus, in making this transformation, we assume that 23G3 can be treated as a monovalent ligand, which is an approximation. The curve is derived by using our model to calculate the quantities indicated by the axis labels.

To statistically characterize uncertainty in parameter estimation, we used a bootstrap method (*44*, *45*). Best-fit parameter values were determined, as described above, for each of 1000 pairs of simulated data sets. Each simulated data set was generated by randomly drawing, N times, a data point from the corresponding set of N experimental data points. Initial estimates of parameter values were generated randomly in these fitting procedures to avoid bias (*46*). For each parameter, the 1000 values obtained from bootstrapping were sorted in rank order; we then used the 159th and 839th values to define a confidence interval at the 68% confidence level. Results are reported in Table 1.

The data reported in Figures 2 and 3 are representative of experimental results. Estimates of parameters using other data collected (not shown) are consistent with the estimate of K reported in Table 1 within a factor of about two. We observed greater data-set-dependent variability for estimates of H, K_x, and J_2, which we attribute to variability in cells and experimental conditions. In all cases, we estimated $H < 1$. Note that parameter estimation depends on a small difference between the association of 23G3 Fab′ (one IgE-specific epitope) and intact Ab (two IgE-specific epitopes) with cell-surface IgE (cf. Figures 2 and 3). The estimates presented in Table 1 were judged to be the most reasonable estimates obtained.

RESULTS

FcεRI-Bound IgE Expresses Two 23G3 Epitopes. Keegan et al. (*33*) determined that 23G3 binds to the $C_\epsilon3$ domain of IgE and that 23G3 does not block binding of IgE to FcεRI, which also interacts with $C_\epsilon3$ (*47*). Because each IgE constant region contains two $C_\epsilon3$ domains, we can expect IgE to contain two 23G3 epitopes, although it is conceivable that one of these epitopes may be concealed when IgE binds FcεRI. To determine the number of 23G3-epitopes actually expressed on surface FcεRI-bound IgE (one or two), we measured the fluorescence of equimolar concentrations of FITC-labeled IgE relative to that of FITC-labeled 23G3 Fab′ in solution ($X = 2.1$) and the fluorescence of RBL cells bearing saturating amounts of FITC-labeled IgE relative to that of RBL cells bearing an equal number of unlabeled IgE incubated with excess FITC-labeled 23G3 Fab′ ($Y = 1.1$). Under the assumption that reagents used in fluorimetric experiments are largely intact and functional, these measurements indicate that the ratio of bound 23G3 Fab′ to the total number of IgE molecules resident on the cell surface, which we equate with X/Y, is approximately 1.9 at saturating concentrations of Fab′. We conclude that IgE bound to FcεRI expresses two binding sites for 23G3.

23G3 Is a Potent Secretogue of IgE-Bearing RBL Cells. As shown in Figure 1, intact 23G3 stimulates degranulation in a dose-dependent manner. The maximum secretory response elicited by 23G3 is typically 40−50% of that observed with 0.5% Triton X-100 in BSS-solubilized extracts of RBL cells and 80% of that observed for sensitized cells exposed to optimal concentrations of hapten-derivatized protein (DNP_{11}−BSA). Thus, 23G3 is a potent secretogue.

Table 1: Parameter Estimates

	estimate[b]		bootstrap confidence limits (68%)[a] lower		upper	
parameter	− rings	+ rings	− rings	+ rings	− rings	+ rings
CS_T (nM)	1	1				
K (nM^{-1})	0.098	0.097	0.066	0.056	0.10	0.10
K_xS_T	2.5	1.9	1.8	1.4	2.9	2.3
H	0.39	0.39	0.095	0.056	0.46	0.44
J_1	0	0				
J_2	0	3.4		0.000 99		9.1
Φ (Fab′)	1.2	1.2	1.1	1.2	1.6	1.8
Φ (intact Ab)	1.1	1.1	1.1	1.1	1.6	1.7

[a] The fitting procedure is repeated 1000 times using randomly drawn ensembles of data from Figures 2 and 3. For each parameter, the 1000 best-fit values obtained are then ordered. The lower and upper confidence limits correspond to the 159th and 839th values in the ordered list. [b] The quality of fits is essentially the same with or without rings. The estimate of CS_T is based on $C = 10^6$ cells/mL, $S_T = 2R_T$, and $R_T = 3 \times 10^5$ IgE−FcεRI per RBL cell (*42*). We assume $J_n = 0$ for all n (− rings) or $J_1 = 0$ and $J_n = 2J_2/n$ (+ rings). Estimates of other parameters are the best-fit values consistent with the data of Figures 2 and 3, eq 3, which describes the interaction of 23G3 Fab′ with IgE−FcεRI, and eqs 4 and 5 as written or in extended forms appropriate for the consideration of rings (*40*). These latter equations describe the interaction of intact 23G3 antibody with IgE−FcεRI. The fitting procedure finds parameter values that minimize differences between predictions derived from eq 3 and data of Figure 2 and, simultaneously, differences between predictions derived from eqs 4 and 5 or the more complicated equations of Wofsy and Goldstein (*40*) and data of Figure 3. In the fitting procedure, an adjustable scaling factor, Φ (Fab′), is used to compare any given raw fluorescence measurement of Figure 2 with the corresponding predicted fraction of IgE-bound Fab′ obtained from eq 3, and a second adjustable scaling factor, Φ (intact Ab), is used to compare any given raw fluorescence measurement of Figure 3 with the corresponding predicted fraction of IgE-bound antibody obtained from eqs 4 and 5 or the more complicated equations of Wofsy and Goldstein (*40*).

Because the Fc region of 23G3 can potentially interact with Fcγ receptors on RBL cells (*48*), which can modulate FcεRI-mediated signal transduction (*49*), we also assayed the secretory response of IgE-bearing RBL cells to the $(Fab')_2$ fragment of 23G3. As can be seen (Figure 1), responses stimulated by $(Fab')_2$ and intact Ab are similar. The influence of Fcγ receptors on 23G3-stimulated secretion is subtle if not absent.

Quantitative Characterization of 23G3 Binding to Cell-Surface IgE−FcεRI Complexes. Because 23G3 possesses two antigen-combining sites and FcεRI-bound IgE expresses two 23G3 epitopes (see above), the interaction of 23G3 with surface IgE is that of a bivalent ligand with a bivalent cell-surface component. Consequently, 23G3 has the potential to induce a spectrum of receptor aggregates (*22*). It is impossible to directly observe the spectrum of aggregates, but receptor aggregation can be inferred using a mathematical model for the binding reactions of 23G3 and IgE−FcεRI.

To develop a model that can be used for predicting the distribution of FcεRI aggregates induced by 23G3, we used flow cytometry to monitor the equilibrium binding of FITC-labeled intact mAb 23G3 and its Fab′ fragment to receptor-bound IgE on RBL cells (Figures 2 and 3). The binding data of Figures 2 and 3 were used to test various mathematical models for 23G3 interaction with surface IgE. Theoretical binding curves, derived from eqs 1−6, are plotted in Figures 2 and 3. Scatchard plots are also shown. As can be seen, a simple model for 23G3 interaction with IgE−FcεRI (eqs 4−6) with the parameter values given in Table 1 for the case without rings is consistent with the results of our flow cytometric measurements. The model of eqs 4−6 is a special case of the model developed by Wofsy and Goldstein (*40*) for cooperative binding of a bivalent ligand to a bivalent cell-surface receptor. In this model, 23G3 is considered to bind surface IgE with negative cooperativity and aggregates of IgE−FcεRI induced by 23G3 are limited to linear noncyclic chains (i.e., $J_2 = 0$).

Alternative models were ruled out as follows. The simplest model for interaction of a bivalent ligand with a bivalent receptor is one without cooperative binding and without ring formation (*22*). If we set the cooperativity factor H in eqs 4 and 5 to unity, the model reduces to this case, but the quality of the fit that we find is significantly less than that illustrated in Figures 2 and 3 (results not shown), which suggests that a model without cooperative binding is inconsistent with the data. Also, the Scatchard plot for Fab′ binding to surface IgE is concave up (Figure 2b), which is consistent with negatively cooperative binding. Furthermore, our bootstrap analysis, summarized in Table 1, indicates that the value of H is less than 1 at the 68% confidence level given the data of Figures 2 and 3 (with or without ring formation in the model). Recall that $H < 1$ is indicative of negative cooperativity. The quality of fits is still less than that illustrated in Figures 2 and 3 (not shown) if, with $H = 1$, we modify the model to include ring-formation reactions (*40*). Also, fits are essentially unimproved if ring-formation reactions are included in the model (i.e., we remove the restriction that requires $J_2 = 0$ and allow the value of J_2 to vary during the fitting procedure). For example, the binding curve derived from a model with ring formation (not shown) and the curve shown in Figure 3a are overlapping and indistinguishable. The simplest model consistent with the data, from among the alternatives considered, is one with negative cooperativity and without ring formation. Of course, we cannot rule out the possibility of some ring formation, although the shape of the Scatchard plot for 23G3 binding to surface IgE (Figure 3b) is inconsistent with potent ring formation, which would cause the Scatchard plot to be concave down in the regime where 23G3 binding to surface IgE is low (*50*). Our fitting and bootstrap analysis (Table 1) indicates that J_2 has a value betweeen 0 (which indicates no ring formation) and 9. The value of J_2 is significant if it is much larger than $2K_xR$, which is estimated to be no greater than about 2 (Table 1).

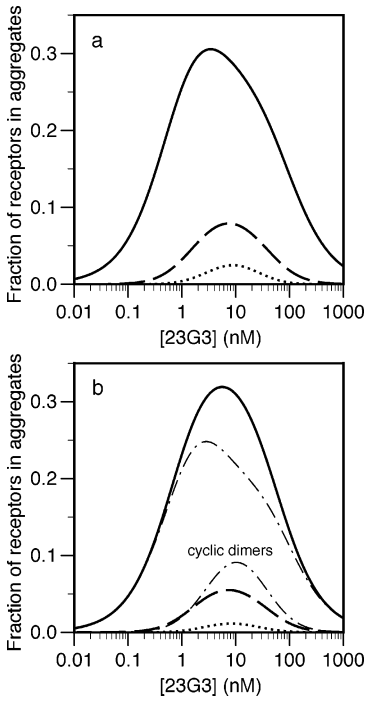

FIGURE 4: Predicted 23G3-induced clustering of IgE−FcεRI complexes. (a) Predictions of the simplest model consistent with the binding data of Figures 2 and 3, a model without ring formation. The solid line indicates the fraction of receptors in aggregates of two receptors (f_2), the broken line indicates the fraction of receptors in aggregates of three receptors (f_3), and the dotted line indicates the fraction of receptors in aggregates of four or more receptors ($1 - w - f_2 - f_3$). The curves are derived from eqs 4−6 and the parameter estimates of Table 1 for the case without rings. Note that, in the absence of cooperativity ($H = 1$), the quantities plotted here would peak at the same concentration of 23G3 (*22*). The curves peak at different ligand concentrations because of negative cooperativity in ligand−receptor binding (*40*). (b) Predictions of the model with ring formation. Calculations are based on the parameter estimates of Table 1 for the case with rings. Curves in this panel correspond to those in the panel above, but two additional curves are shown (dash−dot pattern). These curves indicate how receptor dimers are distributed among linear chains, the more prevalent form of receptor dimer at all ligand concentrations and cyclic rings.

Comparison of FcεRI Aggregation with the Degranulation Response. Using a model for 23G3 interaction with IgE−FcεRI complexes in which rings are omitted (eqs 4−6) or in which rings are included, we can predict, based on the parameter estimates of Table 1, the 23G3-mediated clustering of FcεRI (Figure 4) and compare receptor clustering with the observed degranulation response to different doses of 23G3 (Figure 1). The curves in Figure 4 indicate how receptors are distributed at binding equilibrium into clusters of two, three, and four or more receptors as a function of the 23G3 concentration. In addition, in Figure 4b, the

distribution of receptor dimers into chains (linear dimers) and rings (cyclic dimers) is shown. As can be seen by comparing the curves of Figures 1 and 4a, the formation of receptor dimers is predicted by the simplest model consistent with our binding data to peak at a concentration of 23G3 (≈3.5 nM) that stimulates less than maximal degranulation (≈60% of the peak response). This result suggests that trimers or larger receptor aggregates, which reach peak abundances at higher ligand concentrations than dimers, may play an important role in signaling degranulation. Interestingly, when ring formation is considered (Figure 1b), linear and cyclic dimers are predicted to reach maximal levels at different ligand concentrations and the formation of cyclic dimers, like the formation of of larger aggregates, is predicted to peak at a concentration of 23G3 (≈10 nM) that is close to the concentration that stimulates maximal degranulation. In any case, at very low and high doses of 23G3, for example, consider 0.2 and 200 nM, the number of receptor aggregates of any type is predicted by the model with or without rings to be about the same, but degranulation is minimal at 0.2 nM and nearly maximal at 200 nM. These results suggest a complex relationship between the concentrations of specific aggregate states (e.g., dimers and trimers) and degranulation even when a homogeneous and structurally defined clustering agent is employed.

DISCUSSION

Early studies with covalently cross-linked oligomers of IgE demonstrated that the number of receptors within an aggregate strongly affects the magnitude of the secretory response. IgE dimers were found to be weak secretogues and far less effective at stimulating secretion than IgE oligomers containing three or more antibodies (*9*, *15*, *51*). In later studies, Ortega et al. (*16*) showed that receptor dimers produced by some but not all examined FcεRI-specific monoclonal antibodies generate strong secretory signals. To explain these results, it was suggested that orientational properties of receptors within a dimer or the lifetime of a receptor within an aggregate affect the signaling capacity of FcεRI (*28*).

Additional support for the importance of stereochemical constraints in signaling has come from studies of bivalent DNP ligands (*29*, *30*, *52*). For RBL cells sensitized with DNP-specific IgE, the bivalent DNP ligand $(DCT)_2$-cys (*N*,*N*′-bis[[ε-[(2,4-dinitrophenyl)amino]caproyl]- L-tyrosyl]-L-cystine) triggers only weak calcium and secretory responses, even though it efficiently aggregates IgE−FcεRI complexes as monitored by binding and functional assays (*29*). The failure of $(DCT)_2$-cys to trigger a secretory response has been attributed to its tendency to induce cyclic receptor dimers, i.e., receptor aggregates containing two IgE−FcεRI complexes and two molecules of $(DCT)_2$-cys. Receptors in these cyclic dimers apparently have orientational properties that affect their ability to initiate signal transduction, because linear dimers have been observed to generate signals and stimulate degranulation (*30*, *52*).

Here, we have taken the first steps to characterize the degranulation inducing capacity and binding properties of a reagent that can be used to probe the stereochemical requirements of signaling-competent FcεRI clusters. We have shown that 23G3 is a potent secretagogue (Figure 1), and on the basis of quantitative binding assays (Figures 2 and 3), we have developed a mathematical model for the equilibrium distribution of IgE−FcεRI aggregates induced by any given concentration of 23G3 (eqs 4−6 and Table 1). The ability to quantify receptor aggregation, which initiates intracellular signaling reactions, is critical for model-guided quantitative studies of FcεRI-mediated signal transduction (*20*). 23G3 is a unique reagent for clustering FcεRI for the following reasons. It is a bivalent ligand that triggers FcεRI-mediated degranulation, like FcεRI-specific monoclonal antibodies but unlike synthetic bivalent ligands (which are weak secretogues) and that, in addition, is capable of inducing receptor clusters of different sizes (Figure 4). In contrast, FcεRI-specific antibodies are capable of inducing only dimers. Oligomers of IgE are also capable of generating clusters of FcεRI of different sizes, but IgE oligomers are structurally heterogeneous. Another advantage of 23G3 over FcεRI-specific antibodies is that, using a single cell line, one can vary the number of receptors (IgE−FcεRI complexes) on the cell surface available to interact with 23G3. This feature is attractive because the receptor number is a key parameter that affects ligand-induced receptor aggregation.

Because 23G3 has two antigen-combining sites and FcεRI-bound IgE was found in this study to express two 23G3 epitopes, the binding scheme for 23G3 interaction with IgE−FcεRI is the same as that for a symmetric bivalent DNP antigen interacting with DNP-specific surface IgE. 23G3 potentially induces a spectrum of receptor aggregates, but these aggregates are limited to chain- and ring-like structures. As is the case for any bivalent ligand, aggregates with branched structures are not possible. Our mathematical model for 23G3 interaction with IgE−FcεRI is a special case of a model developed for a bivalent ligand interacting cooperatively with a bivalent cell-surface receptor (*40*). The simplest model consistent with the binding data omits ring formation, which was found not to improve fits when included, and cooperativity in the model is negative, which we found to be necessary for consistency of the model with the binding data. The latter result suggests that once bound to IgE, 23G3 might limit access of a second 23G3 molecule to the remaining free epitope or induce a conformational change that affects further binding. However, we cannot rule out the possibility that the two 23G3 epitopes on FcεRI-bound IgE are instead distinct and independent, which would be the case if FcεRI sterically hinders access of 23G3 to one but not the other epitope. This mechanism of negative cooperativity is possible, because 23G3 and FcεRI both recognize sites within the $C_\epsilon3$ domain of IgE (*33*, *47*).

A variety of bivalent ligands that aggregate IgE−FcεRI complexes have been found to stimulate only weak signaling by FcεRI (*9*, *15*, *16*, *30*, *51*−*55*). In contrast, 23G3, a bivalent ligand, is a potent secretogue. There are several possible explanations, one or more of which can account for the observed degranulation response. It seems that, unlike DNP-carrying ligands, rings (e.g., cyclic dimeric aggregates containing two molecules of 23G3 and two molecules of IgE) are not the predominately favored aggregates. Cyclic dimers formed with DNP ligands are not stimulatory, and in fact, they may even be inhibitory (*30*). Analysis of the binding data for intact 23G3 and Fab′ fragment binding to cell-surface IgE (eqs 1−6) (Figures 2 and 3) suggests that 23G3 induces mainly noncyclic aggregates of IgE−FcεRI complexes. A

model consistent with the binding data predicts that 23G3-induced receptor aggregates consist mostly of linear dimers but also include trimers and larger aggregates (Figure 4). The configuration of FcεRI in 23G3-induced receptor dimers (and larger aggregates) may also play a role in accounting for the magnitude of the secretory response. The orientation of a receptor in an aggregate of FcεRI is likely to depend on various properties of the ligand used to cause receptor clustering. These properties might include spacing between ligand-binding sites (*7*), ligand flexibility, and the locations of sites on IgE or FcεRI that bind the ligand. The orientational constraints on receptors in aggregates induced by 23G3 are likely to differ from those imposed by other clustering reagents, particularly chemically synthesized ligands (*12*, *29*). Another possibility is that the dynamics of receptor clustering induced by 23G3 binding are simply favorable for stimulation of cellular responses. It is well-established that the kinetics of ligand−receptor binding can strongly influence receptor signaling (*20*, *26*−*28*, *56*), and it is possible that 23G3 produces aggregates that have ideal lifetimes for generating strong signals. Finally, a combination of the above explanations may account for the secretory capacity of 23G3.

Given the complexity of FcεRI signaling, we do not expect the correlation between equilibrium receptor aggregation and receptor signaling to necessarily be a simple one. A comparison of the data in Figures 1 and 4 suggests no simple relationship between 23G3-induced aggregation of FcεRI and degranulation. There are concentrations of 23G3 that produce relatively small numbers of aggregates (at equilibrium) yet give strong degranulation responses, while other concentrations of 23G3, which give similar numbers of receptor clusters, do not. We cannot explain these results. Previous work has shown that degranulation is sometimes characterized by a bell-shaped dose−response curve that does not parallel the aggregation curve but rather is shifted toward higher concentrations (*29*, *30*). For this reason, it is unsurprising that 23G3-induced clustering of FcεRI at equilibrium does not correlate directly with the degranulation response (Figures 1 and 4).

At present, we now have available a panel of IgE- and FcεRI-specific monoclonal antibodies that can be used to aggregate FcεRI. Some of these antibodies, like 23G3, trigger secretory responses of RBL cells but others do not. Comparative studies of these and other clustering reagents that also have well-defined structures may lead to a better understanding of the stereochemical properties of FcεRI aggregates that influence signaling. Although IgE-specific antibodies have been studied before, our study provides the first model-based characterization of the interaction of such an antibody with cell-surface IgE−FcεRI. This study is also important because 23G3 has a feature not shared by similarly well-characterized FcεRI-specific monoclonal antibodies; because IgE contains two 23G3 epitopes, 23G3 has the potential to induce receptor aggregates larger than dimers, wheras FcεRI-specific antibodies, which interact with a single epitope on the receptor, do not. Thus, in the future, it may be possible to use 23G3 to determine the relative contributions of receptor aggregate size and receptor orientation within an aggregate through model-guided manipulation of receptor clustering, particularly cluster size. Future work should also address the role of ligand−receptor-binding kinetics in FcεRI-mediated cellular responses to 23G3. Our equilibrium binding model provides a valuable reference point for studies of 23G3-binding kinetics, because rate constants must be consistent with the equilibrium constants estimated here.

ACKNOWLEDGMENT

This work is dedicated to the memory of Carla Wofsy, who was our colleague, collaborator, and friend.

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BI049686O