

Measurement of receptor crosslinking at the cell surface via multiparameter flow cytometry

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ABSTRACT

Many cellular responses, particularly in the immune system, are triggered by ligand binding to a cell-surface receptor. However, as indicated by bell-shaped dose-response curves, ligand binding alone is sometimes insufficient to trigger a response. Often, ligand binding must also induce the aggregation of cell-surface receptors through crosslinking, which occurs when a ligand binds simultaneously to two or more receptors. Thus, an important goal in cell biology has been to establish quantitative relationships between the amount of ligand present on a cell surface and the number of crosslinked ligand-specific cell-surface receptors. To better understand ligand-induced receptor aggregation, we have been investigating the binding of a model multivalent antigen (DNP₂₅PE) to cell-surface anti-DNP FITC-labeled IgE (FITC-IgE). To determine the kinetic and equilibrium parameters that characterize crosslinking in this system, we have developed a combined theoretical and experimental approach that is based on multiparameter flow cytometry. With this approach, we can measure both the average number of ligand molecules that are bound per cell and the average number of receptor binding sites that are bound per cell. The average number of DNP₂₅PE per cell is determined by measuring the fluorescence of phycoerythrin. The average number of occupied IgE sites per cell is determined by measuring the fluorescence of FITC, which is quenched upon ligand binding. This novel approach, together with conventional methods for changes in intracellular calcium, allows us to correlate for the first time the dynamics of IgE crosslinking with cell activation.

Key words: Receptor, Aggregation, Crosslinking, IgE, FcεRI, Flow Cytometry

1. INTRODUCTION

The binding of a ligand to a cell surface receptor is the first step in a cascade of events that leads to the generation of a transmembrane signal. In many cell systems, simple ligand binding is insufficient to initiate signal transduction, rather, receptor aggregation is a necessary first step. This is particularly evident in many cells of the immune system, where the amount of receptor crosslinking, as opposed to the number of ligands bound, determines the magnitude of the cellular response.

On the surface of mast cells, basophils and rat basophilic leukemia (RBL) cells are Fc receptors (Fc ϵ RI) that bind IgE with high affinity. An IgE bound to an Fc receptor on the surface of a cell acts as a bivalent receptor for the ligand it is capable of binding. The formation of aggregates of IgE, or equivalently of Fc ϵ RI receptors, triggers various cellular responses. However, it is not clear what physical and steric conditions must be met by the IgE-receptor aggregates in order to initiate signal transduction.

An important goal in immunology and cell biology has been to establish a quantitative relationship between the concentration of ligand in the solution surrounding a cell and the degree of receptor aggregation and cellular response that it induces. Theoretical models of receptor aggregation have been developed over the last 20 years (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) but quantitatively testing them has been difficult due to experimental limitations. As we show below, one way to determine the average number of receptor crosslinks present on a cell surface at equilibrium is by measuring the fraction of receptor sites and the total number of ligand molecules bound per cell at equilibrium. This has proved elusive because attempts at studying the complex interactions of multivalent ligands and cell surface receptors have been limited to experimental approaches that determine either the number of ligand molecules bound or the fractional receptor occupancy but not both.

In this study we employ a monoclonal IgE that recognizes the 2,4-dinitrophenol (DNP) hapten (11) that is bound to Fc ϵ RI as a cell surface receptor and phycoerythrin, conjugated with an average of 25 DNP moieties, as a multivalent antigen. Because each IgE has two DNP binding sites, it functions as a bivalent receptor for DNP ligands. Since multivalent DNP ligands can

simultaneously bind to multiple IgE molecules, they have the potential to form crosslinks between these receptors and trigger cellular responses.

We have developed a fluorescent technique that allows for determining both receptor occupancy and the average number of ligand molecules bound (DNP₂₅PE) per cell. To determine the binding parameters for DNP₂₅PE interacting with IgE receptor sites we sensitize RBL cells with fluorescein modified IgE (FITC-IgE). Occupancy of IgE binding sites is determined by measuring the fluorescence quenching that accompanies DNP binding to FITC IgE (FL1) (12). The number of bound ligand molecules (the number of ligand bound must be less than or equal to the number of receptor sites occupied) is proportional to phycoerythrin fluorescence (FL2) (13). FITC quenching and phycoerythrin binding are monitored simultaneously via multiparameter flow cytometry. Combining the information obtained from the two fluorescent channels allows one to determine the degree of crosslinking as a function of ligand concentration and or time.

2. MATERIALS AND METHODS

2.1 Reagents. Mouse monoclonal anti-DNP IgE was obtained from hybridoma H1 26.82 (11) and affinity purified. Final steps in the purification included ion exchange chromatography to remove bound DNP-glycine, then gel filtration to separate monomeric IgE from small amounts of IgE aggregates. IgE was labeled with FITC (FITC-IgE) as previously described (12).

B-Phycoerythrin (PE), conjugated with an average of 25 moles of DNP per mole of protein (DNP₂₅PE), was purchased via a custom synthesis from Molecular Probes (Eugene, Oregon).

Sodium azide, and 2-deoxy-D-glucose were purchased from Sigma Chemical Corporation (St. Louis, MO).

2.2 Cells. RBL-2H3 cells were grown adherent in 75 cm² flasks, kept at 37°C and 5% CO₂ and generally used 5 days after passage. Cell Media consisted of MEM 1X with Earle's salts, without glutamine (Gibco-BRL), 20% Fetal Bovine Serum (Hyclone), 1% v/v L-glutamine (Gibco-BRL) and 1% v/v penicillin and streptomycin(Gibco-BRL). Cells are harvested by first rinsing with trypsin-EDTA (Gibco-BRL) and then incubating with trypsin-EDTA for 5 minutes at 37°C. Cells in culture were incubated overnight at 37°C with 10 μg of anti-DNP FITC-IgE. After harvesting, the cells were washed and then resuspended in buffered salt solution (BSS: 135 mM NaCl, 5mM KCL, 1mM MgCl₂, 1.8 mM CaCl₂, 5.6 mM glucose, 0.1% gelatin, 20 mM Hepes, pH 7.4).

2.3 Flow Cytometric Binding Assays. Cytometric binding assays were performed on a Becton Dickinson FACScan flow cytometer interfaced with a Macintosh computer running Cell Quest software. A suspension of RBL-2H3 cells in freshly filtered (0.22 μm filters) BSS containing 10 mM sodium azide and 10 mM 2-deoxyglucose (to inhibit receptor internalization and cellular degranulation) was exposed to various concentrations of DNP₂₅-PE. Bound samples were those in which the cells were exposed to saturating concentrations of FITC-IgE at least 12 hours prior to harvesting while control samples were cells that did not have bound IgE on their surface. The number of Fab arms occupied by DNP groups was determined by measuring the decrease in FITC fluorescence (FL1) that occurs when DNP binds to FITC labeled anti DNP-IgE (12). Histograms (FL1) of DNP binding (occupancy of Fab arms) were acquired at room temperature in the cytometer and data were expressed as the mean fluorescence channel of the cell suspension. Simultaneously, histograms (FL2) that measure phycoerythrin binding/cell were acquired with the data expressed as the mean fluorescence channel of the cell suspension. Specific binding for each concentration of DNP₂₅-PE was calculated from mean fluorescence channel of the bound sample minus the mean of the control sample.

2.4 Spectrofluorometric measurement of changes in intracellular Ca²⁺ levels The calcium release assay uses the same suspension of RBL-2H3 cells as were used for the cytometric binding property experiment. Cells were loaded with the fluorescent Ca²⁺ indicator, indo-1 as previously described (14). After washing, the cells were resuspended at 1 x 10⁶ cells/ml in BSS. Fluorescence measurements were made with a SPEX Fluormax fluorescence spectrophotometer with excitation and emission wavelengths of 330 nm and 400 nm, respectively. In a typical experiment, 2 ml of the indo-1-loaded RBL cells were maintained at 37°C and stirred continuously in an acrylic cuvette. Fluorescence increases corresponding to increases in intracellular calcium levels were monitored following the addition of small amounts of DNP₂₅-PE.

3. RESULTS

In our studies we employ a highly fluorescent multivalent ligand, phycoerythrin with an average of 25 DNP molecules attached (DNP₂₅-PE), to interact with FITC labeled cell surface anti-DNP IgE. As shown in Figure 1, titrating DNP₂₅-PE into a solution of cells sensitized with FITC-IgE results in an increase in PE fluorescence (FL2) and a corresponding decrease in FITC fluorescence

(FL1). The decrease in FITC fluorescence is proportional to the occupancy of IgE binding sites (12), while the increase in PE fluorescence is proportional to the number of (DNP₂₅PE) molecules bound per cell (13). As we show below, we are then able to estimate the fraction of cell surface receptors crosslinked from measurements of the two fluorescence signals.

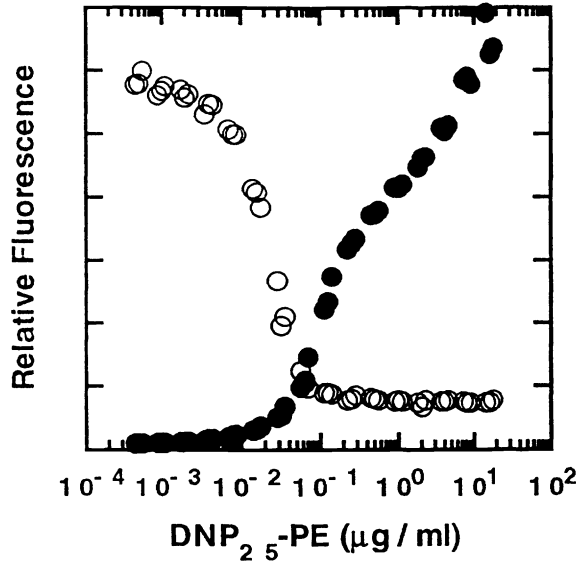


Figure 1. Representative data to illustrate the equilibrium binding of DNP₂₅-PE to FITC-IgE bound to Fc_εRI on the surface of RBL cells. Each data point represents the mean channel fluorescence as determined by flow cytometry for a given multivalent ligand concentration. With increasing ligand concentration, there is a decrease in FITC (FL1) fluorescence (O) and a corresponding increase in PE (FL2) fluorescence (•)

To begin our analysis we first determine the fraction of FITC-IgE sites that are occupied by DNP (f_{SB}) from the FL1 data. The fraction of sites bound (f_{SB}) is related to the relative fluorescence ($FL1$) by the expression

$$f_{SB} = (FL1_{max} - FL1) / (FL1_{max} - FL1_{min}) \quad (1)$$

Where $FL1_{max}$ is the value of relative fluorescein fluorescence before the addition of ligand and $FL1_{min}$ is the value of relative fluorescein fluorescence when all IgE binding sites are occupied.

We then determine the ratio of DNP₂₅PE to IgE binding sites (f_{LB}) from the FL2 fluorescence data. The fraction of ligand bound (f_{LB}) is related to the relative fluorescence ($FL2$) by the expression

$$f_{LB} = (FL2 - FL2_{min}) / (FL2_{max} - FL2_{min}) \quad (2)$$

Where $FL2_{min}$ is the value of relative phycoerythrin fluorescence when no ligand is bound and $FL2_{max}$ is the value of relative phycoerythrin fluorescence upon saturation (ligand bound = $2 \times [IgE]$).

3.1 Equilibrium properties of multivalent ligand binding. While the total number of ligand molecules bound to the cell surface as well as the fraction of receptor sites that are occupied increase monotonically with ligand concentration, most immunologic systems and many hormone and cytokine receptors exhibit a bell-shaped, dose response curve. Thus, the bell-shaped form of the dose response curve is clearly not a consequence of the dependence of simple receptor binding on ligand concentration. The theory of multivalent ligand binding to cell surface receptors, predicts that at equilibrium the fraction of receptor sites crosslinked will be a bell-shaped function of the logarithm of the ligand concentration. In fact, one generally observes bell-shaped dose response curves in systems in which the signal transduction pathways are initiated by the clustering of cell surface receptors (15).

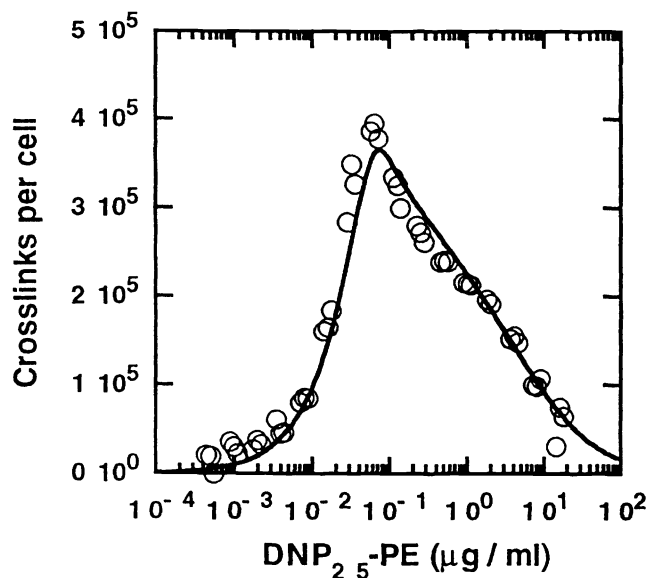


Figure 2. Plotted is the total number of IgE-Fc_εRI crosslinks per cell (at equilibrium) as a function of DNP₂₅-PE concentration. As expected, the crosslinking curve exhibits the generic bell-shaped form.

In order to understand the relationship between ligand concentration and receptor signalling it is important to quantify the amount of crosslinking on the cell surface. For a multivalent ligand that is bound at multiple sites, different notions of crosslinking are conceivable. Here we use the definition that the first bond does not establish a crosslink (it holds the ligand to the cell surface) and that each subsequent ligand-receptor bond adds exactly one more crosslink. This

means that an i -fold bound ligand establishes $i-1$ crosslinks. The number of crosslinks per cell can be calculated from measurements of the number of bound receptor sites and the number of bound ligand molecules as shown in ref. (10).

$$\text{Crosslinks per cell} = (f_{SB} - f_{LB}) \times (2 \times [IgE]) \quad (3)$$

Using this technique we have, confirmed experimentally (Figure 2) that the equilibrium crosslinking curve, i.e., the number of receptors that are crosslinked plotted versus the logarithm of the total ligand concentration, is in fact bell-shaped (Figure 2).

3.2 Kinetics of Ligand Binding. Using the approach described above we have measured the association kinetics for multivalent ligand (DNP₂₅PE) binding to fluorescein modified cell surface IgE (FITC-IgE).

The time course of fluorescein fluorescence (FL1) for the association and dissociation of DNP₂₅PE is illustrated in Figure 3. Initially only FITC-IgE is present (the first plateau), then DNP₂₅PE is added ($t = 0$) and the fluorescence is quenched as DNP groups bind to FITC-IgE receptor sites.

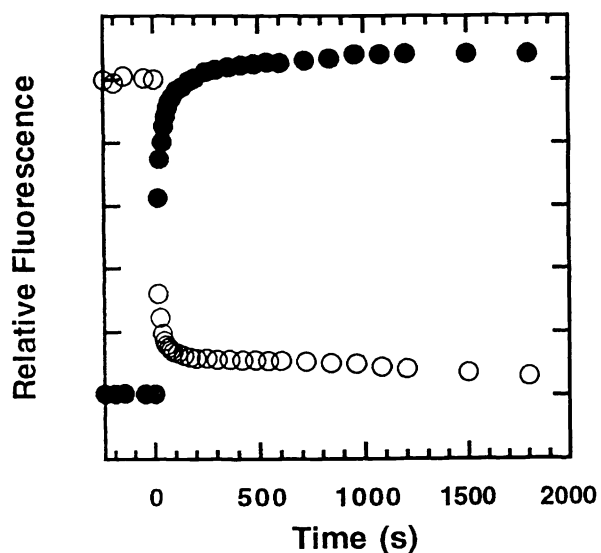


Figure 3. The time course of DNP₂₅-PE binding and receptor occupancy. At $t = 0$, DNP₂₅-PE is added resulting in quenching of FITC (FL1) fluorescence (O), and a corresponding increase in PE (FL2) fluorescence (•).

At a time t after the addition of DNP₂₅PE, the fraction of FITC-IgE sites that are occupied by DNP (f_{SB}) is related to the relative fluorescence F by equation (1). The number of phycoerythrin molecules bound per cell is proportional to the fluorescence intensity (FL2). The time course of phycoerythrin fluorescence for the of DNP₂₅PE is illustrated in Figure 3. As expected, one observes an increase in PE fluorescence intensity (FL2) as DNP₂₅PE binds to cell surface IgE

with the ratio of DNP₂₅PE to IgE binding sites given by equation (2). Thus, multiparameter flow cytometry allows for the simultaneous measurement of FITC quenching (FL1) and bound phycoerythrin (FL2).

Applying equation 3 to the data in Figure 3 allows for the determination of the number of crosslinks formed on the cell surface as a function of time (see Figure 4). Since cells of the immune system generally respond to receptor crosslinking, not receptor binding *per se* (15) we expect the time course of cellular activation pathways to be influenced by the dynamics of the crosslinking events that occur on the cell surface. In Figure 4 we demonstrate this effect by comparing the time course of changes in intracellular calcium levels with that for receptor crosslinking.

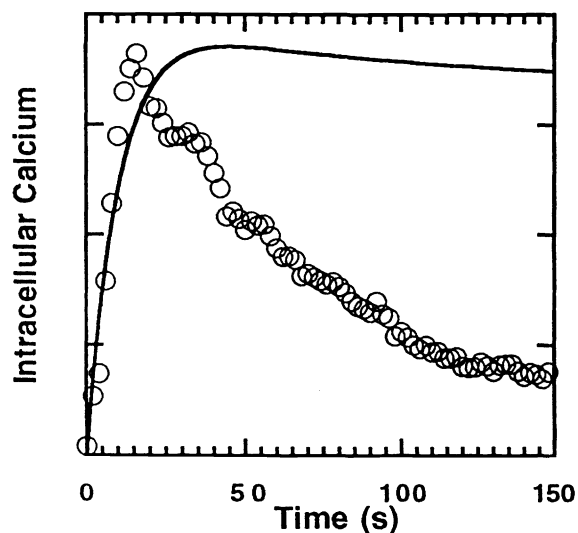


Figure 4. IgE-Fc_εRI crosslinks per cell (solid line) as a function of time plotted vs changes in intracellular calcium levels (O) when IgE sensitized RBL cells are exposed to 0.5 μg/ml DNP₂₅-PE. The level of intracellular Ca²⁺ ions reaches a peak at approximately the same time as the number of crosslinks becomes maximal.

4. DISCUSSION

There have been numerous attempts at developing a quantitative picture of receptor aggregation on cell surfaces. Fewtrell and Metzger (16) performed binding studies using covalently linked radiolabeled oligomers of IgE to aggregate Fc_εRI receptors. The use of radiolabeled IgE oligomers allowed for the determination of the number of oligomers bound but not of the number of Fc_εRI receptors occupied. Thus, it was not possible to experimentally measure the degree of Fc_εRI aggregation that resulted from exposure to a given concentration of IgE oligomer. Their studies also showed that IgE dimers were far less effective at triggering degranulation in RBL cells, than IgE trimers and higher order oligomers.

Another approach to determine the critical aspects of FcεRI aggregation has been to construct simple ligands of well defined structure. Since aggregation is required, the simplest possible signal is provided by a bivalent ligand. When such ligands bind to bivalent immunoglobulin molecules a spectrum of linear chains and rings of various sizes form in a fashion that is dependent in a complex way on concentration and time. The analysis of the response of cells to such a spectrum of polymeric products is an experimental and theoretical problem that we and others have worked on for some time (3, 7, 9, 12, 17, 18, 19, 20, 21, 22).

Mathematical models have been developed that describe bivalent (2, 9, 23, 24, 25) and multivalent (4, 6, 7, 10, 26) ligand interactions with cell surface Ig. To better understand the biological consequences of ligand binding to cell surface receptor systems, one would like to know the concentration of all reaction products present on the cell surface as a function of time and the concentration of the reactants. This requires the simultaneous measurement of the number of occupied receptor binding sites as well as the number of ligand molecules bound per cell. Several previous studies have measured either fractional receptor occupancy (27) or the total amount of ligand bound per cell (13) but not both.

Recently Woodard *et al.* (28) addressed this issue by employing two-color flow cytometry to attempt to determine both receptor occupancy and amount of ligand bound. Woodard *et al.* assess the number of receptor sites that are bound to the multivalent ligand indirectly, using a labeled, monovalent ligand to measure the number of receptor sites that are free. Data obtained from this indirect method are difficult to interpret, since the reactions are reversible and binding of multivalent ligand influences the binding of the monovalent ligand and vice versa. Our measurement of receptor occupation does not require an additional monovalent probe and, thus, avoids the complications arising from the competition between two different ligands binding to the same receptor. In a way, our approach is complementary to that of Woodard *et al.* (28). Our approach depends on the labeling of the receptors (in our case with FITC), which is not possible if the receptor is not available in a secreted form or cannot be reattached to the cell surface. The second condition is not fulfilled for many receptors and, consequently, an indirect method such as that employed by Woodard *et al.*, is required to measure aggregation in those systems.

In this study we have shown how the number of crosslinks can be experimentally determined when both the number of receptor sites occupied and the number of ligand bound per receptor are measured simultaneously. Using this technique we have confirmed experimentally that the equilibrium crosslinking curve, i.e., the number of receptors that are crosslinked plotted versus the total ligand concentration, is in fact bell-shaped. We have then, for the first time, measured the time evolution of receptor crosslinking on the cell surface.

Finally we have begun the next step in this quantitative approach which is to correlate the number of crosslinks with cellular responses (Figure 4). Our goal is to derive a quantitative relationship between the intensity of the response and the degree of receptor aggregation

5. ACKNOWLEDGEMENTS

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References

1. G. I. Bell. 1974. Model for the binding of multivalent antigen to cells. *Nature* 248:430.
2. M. Dembo and B. Goldstein. 1978. Theory of equilibrium binding of symmetric bivalent haptens to cell surface antibody: application to histamine release from basophils. *J. Immunol.* 121:345.
3. A. S. Perelson. 1981. Receptor clustering on a cell surface. III. Theory of receptor cross-linking by multivalent ligands: description by ligand states. *Math. Biosci.* 53:1.
4. F. W. Wiegel and A. S. Perelson. 1981. Configurations of a polymeric antigen absorbed to a B cell membrane. *J. Theor. Biol.* 88:533.
5. S. K. Dower, J. A. Titus, and D. M. Segal. 1984. The binding of multivalent ligands to cell surface receptors. In *Cell Surface Dynamics: Concepts and Models*, A. S. Perelson, C. DeLisi, and F. W. Wiegel, eds. Marcel Dekker New York. p. 277.
6. A. S. Perelson. 1984. Some mathematical models of receptor clustering by multivalent ligands. In *Cell Surface Dynamics: Concepts and Models*, A. S. Perelson, C. DeLisi, and F. W. Wiegel, eds. Marcel Dekker New York. p. 223.

7. C. A. Macken and A. S. Perelson. *Branching Processes Applied to Cell Surface Aggregation Phenomena*. volume 58 of *Lecture Notes in Biomathematics*. Springer. New York. 1985.
8. R. G. Posner and M. Dembo. 1994. Binding of bivalent ligand to cell surface ige: Can one detect ring formation?. *Mol. Immunol.* 31:1439.
9. R. G. Posner, C. Wofsy, and B. Goldstein. 1995. The kinetics of bivalent ligand-bivalent receptor aggregation: Ring formation and the breakdown of the equivalent site approximation. *Math. Biosci.* 126:171.
10. B. Sulzer and A. S. Perelson. 1996. Equilibrium binding of multivalent ligands to cells: effects of cell and receptor density. *Math. Biosci.* 135:147.
11. F. Lui, J. Bohn, E. Ferry, H. Yamamoto, C. Molinaro, L. Sherman, N. Klinman, and D. Katz. 1980. Monoclonal dinitrophenyl-specific murine IgE antibody: Preparation, isolation, and characterization. *J. Immunol.* 124:2728.
12. J. Erickson, P. Kane, B. Goldstein, D. Holowka, and B. Baird. 1986. Crosslinking of IgE-receptor complexes at the cell surface: a fluorescence method for studying the binding of monovalent and bivalent haptens to IgE. *Mol. Immunol.* 23:769.
13. J. Seagrave, G. G. Deanin, J. C. Martin, B. H. Davis, and J. M. Oliver. 1987. DNP-phycoobiliproteins, fluorescent antigens to study dynamic properties of antigen-IgE-receptor complexes on RBL-2H3 rat mast cells. *Cytometry* 8:287.
14. R. Posner, K. Subramanian, T. Feder, J. Thomas, D. Holowka, B. Baird, and B. Goldstein. 1995. Simultaneous crosslinking by two non triggering bivalent ligands causes synergistic signaling of IgE-FcεRI complexes. *J. Immunol.* 155:3601.
15. H. Metzger. 1992. Transmembrane signaling: the joy of aggregation. *J. Immunol.* 149:1477.
16. C. Fewtrell and H. Metzger. 1980. Larger oligomers of IgE are more effective than dimers in stimulating rat basophilic leukemia cells. *J. Immunol.* 125:701.
17. B. Vogelstein, R. Z. Dintzis. and H. M. Dintzis. 1982. Specific cellular stimulation in the primary immune response: a quantized model. *Proc. Natl. Acad. Sci. USA* 79:395.
18. R. Schweitzer-Stenner, A. Licht, I. Luscher, and I. Pecht. 1987. Oligomerization and ring closure of immunoglobulin e class antibodies by divalent haptens. *Biochem.* 26:3602.

19. R. Posner, J. Erickson, B. Goldstein, D. Holowka, and B. Baird. 1991. Dissociation kinetics of bivalent ligand-immunoglobulin e aggregates in solution. *Biochem.* 30:2348.
20. J. Erickson, R. Posner, B. Goldstein, D. Holowka, and B. Baird. 1991. Bivalent ligand dissociation kinetics from receptor bound IgE: evidence for a time dependent increase in ligand rebinding at the cell surface. *Biochem.* 30:2357.
21. R. Schweitzer-Stenner, I. Luscher, and I. Pecht. 1992. Dimerization kinetics of the IgE-class antibodies by divalent haptens i. the fab-hapten interactions. *Biophys. J.* 63:551.
22. R. Schweitzer-Stenner, I. Luscher, and I. Pecht. 1992. Dimerization kinetics of the IgE-class antibodies by divalent haptens ii. the interaction between intact IgE and haptens. *Biophys. J.* 63:563.
23. A. S. Perelson and C. DeLisi. 1980. Receptor clustering on a cell surface. I. Theory of receptor cross-linking by ligands bearing two chemically identical functional groups. *Math. Biosci.* 48:71.
24. A. S. Perelson. 1980. Receptor clustering on a cell surface. II. Theory of receptor cross-linking by ligands bearing two chemically distinct functional groups. *Math. Biosci.* 49:87.
25. C. Wofsy and B. Goldstein. 1987. The effect of co-operativity on the equilibrium binding of symmetric bivalent ligands to antibodies: theoretical results with application to histamine release from basophils. *Mol. Immunol.* 24:151.
26. C. A. Macken and A. S. Perelson. 1986. Renewal theory, Geiger counters, and the maximum number of receptors bound to a randomly haptenated polymer chain. *IMA Journal of Mathematics Applied in Medicine & Biology* 3:71.
27. J. Erickson, B. Goldstein, D. Holowka, and B. Baird. 1987. The effect of receptor density on the forward rate constant for binding of ligands to cell surface receptors. *Biophys. J.* 52:657.
28. S. L. Woodard, M. Aldo-Benson, D. A. Roess, and B. G. Barisas. 1995. Flow cytometric analysis of T-independent antigen binding to dinitrophenyl-specific cells. *J. Immunol.* 155:163.