Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 2993–2997, July 1977
Immunology

Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation

(histamine release/exocytosis/crosslinking reagent/Fc receptor)

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Communicated by Elvin A. Kabat, April 20, 1977

ABSTRACT **Rat immunoglobulin E (IgE) was treated with a crosslinking reagent, dimethyl suberimidate, and fractionated by gel filtration into monomers, dimers, trimers, and higher polymers. The fractions retained substantial ability to bind specifically to mast cells. About one-third of the cell-bound dimers appeared to bind bivalently. The fractions were assayed *in vivo* by passive cutaneous anaphylaxis in rats, and for histamine or serotonin release *in vitro* using normal or tumor mouse mast cells. The monomers showed no activity, while the dimers and higher polymers gave excellent and approximately equivalent responses. We conclude that IgE that has been crosslinked to form dimers prior to the addition to mast cells can serve as a unit signal for triggering IgE-mediated exocytosis.**

Mast cells and basophils bind monomeric immunoglobulin E (IgE) to their surface via the Fc portion of the antibody molecule. When such cells are exposed to antigens capable of reacting with the Fab portion of the bound IgE, the cells are stimulated to release histamine, serotonin, and other active components enclosed in the basophilic granules. A majority of studies suggests that in IgE-mediated exocytosis, intermolecular crosslinking of the cell-bound antibody is required for triggering (1). Thus, multivalent antigens are usually capable of eliciting a physiological response whereas univalent antigens in general cannot. Moreover, crosslinking does not have to be mediated by antigen; bivalent antibody against IgE (2) or artificially aggregated IgE or Fc fragments of IgE are fully capable of inducing a reaction (3).

The purpose of the present study was to determine the minimal degree of crosslinking required. Toward this end, we treated IgE with the crosslinking reagent dimethyl suberimidate and separated the mixture into monomers, dimers, trimers, and higher polymers. We found that preformed dimers were capable of triggering exocytosis when added to mast cells.

MATERIALS AND METHODS

Materials. Rat IgE was isolated from the ascitic fluid of Lou/M rats carrying the immunocytoma IR162 (4) by methods previously described (5). It was iodinated with ^{131}I or ^{125}I by the chloramine-T method (6). The preparations of fluoresceinated IgE and rabbit antibody against fluorescein have also been described (7). Rat basophilic leukemia cells (8) were grown to the stationary stage of growth (9). Unfractionated mouse peritoneal cells were used as a source of normal mast cells. For a few experiments, cells from the mouse mastocytoma AB-CBF$_1$-MCT-1 (10) were used.

Preparation of IgE Oligomers. IgE (27 mg/ml, 52 mg) was covalently crosslinked in 0.2 M Tris·HCl, pH 8.5, using a 30-fold molar excess (2.1 mg) of dimethyl suberimidate (Pierce Chemicals). The solution was incubated for 2 hr at 30°, and the oligomers were separated by fractionation on sequential Sephadex G-200 (Pharmacia) and Ultrogel AcA22 (LKB) columns as described (11). Elution was carried out in 0.15 M NaCl/0.01 M Tris·HCl, pH 7.5.

Oligomers that were trace labeled with ^{125}I were prepared from the radio-labeled native protein. In this case, approximately 3 mg of ^{125}I-labeled IgE in 1 ml of phosphate-buffered saline was precipitated by addition of an equal volume of 30% polyethylene glycol 6000. After centrifugation the precipitate was redissolved in 0.25 ml of 0.2 M Tris·HCl, pH 8.5, and a 30-fold molar excess of dimethyl suberimidate in 30 μl of H_2O was added. After 2 hr at 30°, the oligomers were fractionated as described above.

Fractions were analyzed by polyacrylamide gel electrophoresis using a Pharmacia GE-4 gel electrophoresis apparatus and PAA 2/16 gradient slab gels. Electrophoresis was carried out for 8 hr in Tris·borate buffer, pH 8.4, at 20°. Gels were stained with 0.05% Coomassie blue in aqueous 25% isopropanol/10% acetic acid, and were destained in 10% acetic acid.

Binding Studies. The binding of derivatized IgE was studied indirectly by its capacity to inhibit the binding of untreated radioiodinated IgE in preliminary tests, but principally by direct studies using iodinated derivatized IgE (5).

Assays *In Vivo*. The capacity of derivatized IgE to cause mast cell degranulation was measured by passive cutaneous anaphylaxis (PCA) (3). Sprague–Dawley rats were injected intravenously with 2.5 mg of Evans blue dye in 0.7 ml of 0.15 M NaCl, the shaved skin of the back was injected intradermally with 0.05 ml of the test sample (diluted in 0.01 M $NaPO_4$ buffer, pH 7.2/0.15 M NaCl/0.01% bovine serum albumin), and the animals were killed after 25 min. The interior surface of the skin was displayed and the injection sites were scored by multiplying the color intensity (1+ to 4+) by the square of the radius (in mm).

Assays *In Vitro*. Cells were reacted with derivatized IgE and the release of mediators was monitored. For normal mast cells, male LAF$_1$ or SJL mice were killed by cervical dislocation, and peritoneal cells were harvested in a medium containing 123 mM NaCl/2.7 mM KCl/25 mM Tris/5.6 mM glucose/0.1% (wt/vol) bovine serum albumin/10 units of heparin per ml at pH 7.4 at 37°. The cells were washed twice at 0–4° and resuspended at 1×10^6 cells per ml in the above medium without heparin but containing 1.0 mM $CaCl_2$ and 0.4 mM $MgCl_2$.

The warmed cell suspension (0.2 ml) containing approximately 2.5% mast cells (as determined by staining with 0.1% toluidine blue) was added to 12 × 75-mm polypropylene tubes containing 0.3 ml of warm medium with the material to be tested and 7.5 μg of phosphatidylserine (12). After incubation for 60 min at 37° with intermittent shaking, the tubes were chilled and centrifuged. Histamine in the supernatants was assayed by the automated fluorimetric technique of Siraganian

Abbreviations: IgE, immunoglobulin E; PCA, passive cutaneous anaphylaxis.

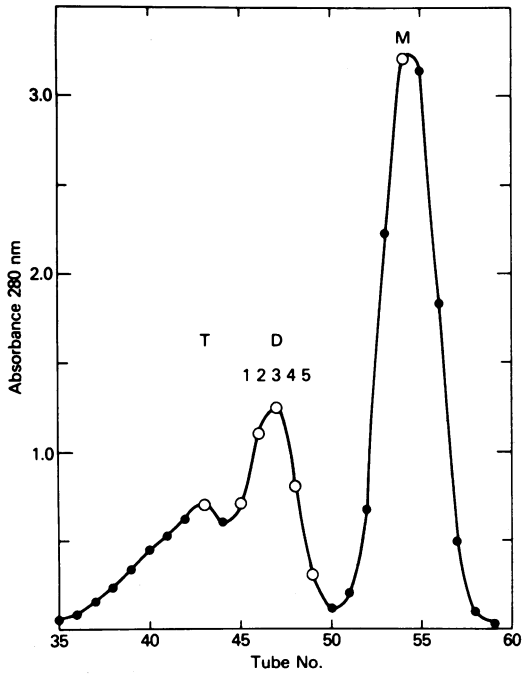

FIG. 1. Elution of crosslinked IgE on sequential 1.6 × 95-cm Sephadex G-200 and Ultrogel AcA 22 columns in Tris-buffered saline, pH 7.5. T, D, and M refer to trimer, dimer, and monomer fractions, respectively. Five fractions (D_1–D_5) in the dimer peak were taken. The volume of each fraction was 3.8 ml.

(13). Total histamine was determined on perchloric acid lysates of the cells. For a few experiments, release of incorporated [^{3}H]serotonin (14, 15) from cultured mouse AB-CBF$_1$MCT-1 mastocytoma cells (16) was studied in an analogous manner but in the absence of phosphatidylserine (since the latter does not seem to affect release from these cells).

RESULTS

Characterization of IgE oligomers

In order to determine the degree of crosslinking required to elicit an allergic response, IgE was covalently polymerized with dimethyl suberimidate, and oligomers of defined size were isolated as described in *Materials and Methods*. A typical elution pattern of the dimethyl suberimidate-treated IgE is shown in Fig. 1. Fractions indicated in the figure were analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions, and the results are given in Fig. 2.

It was previously shown (11) that the peaks labeled M, D, and T in Fig. 1 correspond to monomeric, dimeric, and trimeric immunoglobulin, respectively. The gel pattern in Fig. 2 shows that the fractions are homogeneous with respect to size, except for a trace of tetramer contaminating the trimer fraction and the small amount of trimer observed in the first dimer fraction (D_1). In a separate experiment in which 6.7 μg of D_3 and varying amounts of the trimer fraction were examined on the same gel, we found that the contamination of D_3 with trimer must have been less than 4%.

Electrophoresis of IgE oligomers with buffers containing sodium dodecyl sulfate confirmed that the crosslinking was indeed covalent. The extent of crosslinking depends upon the ratio of dimethyl suberimidate to protein used. In the experiment shown in Fig. 1, trimer, dimer, and monomer accounted for approximately 13, 23, and 60% of the recovered UV absorbance, respectively. Comparable results were obtained in two other experiments.

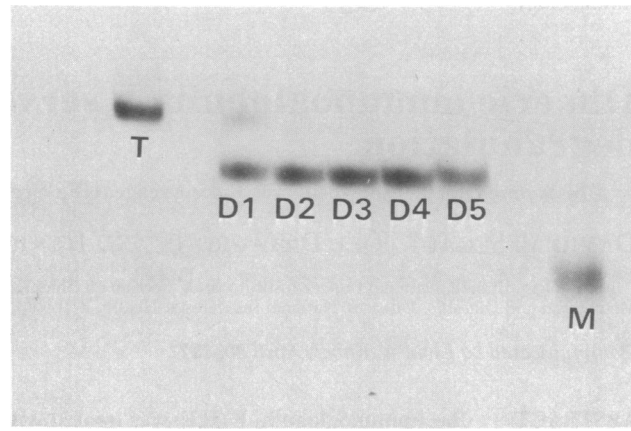

FIG. 2. Polyacrylamide gel electrophoresis of fractions indicated in Fig. 1. Electrophoresis was performed under nondenaturing conditions at pH 8.4, with a 2–16% gradient slab gel.

Binding properties of derivatized IgE

The binding properties of the derivatized IgE were assessed on rat basophilic leukemia cells in several ways. The fraction of protein capable of binding to cells was measured by incubating excess cells with limiting amounts of ^{125}I-labeled IgE (5). Routinely, 80% or more of native, underivatized IgE will bind specifically. The derivatized IgE was somewhat less active: in two separate preparations 63 ± 4, 49 ± 4, and 36 ± 5% (mean ± SD), respectively, of the monomer, dimer, and trimer fractions bound to the cells. All preparations showed negligible nonspecific binding when exposed to cells in the presence of a large excess of unlabeled IgE (e.g., see footnote in Table 1) (5). When cells that had been incubated with any of the labeled fractions were then exposed to an excess of unlabeled IgE, no significant release of cell-bound radioactivity occurred over several hours. This indicates that the bound IgE dissociated very slowly (5).

In order to estimate the fraction of bound dimers that were bound divalently (i.e., with both Fc regions in the dimer engaging the cell surface receptors), we performed the following experiment (Table 1). ^{125}I-Labeled monomer or dimer was incubated with cells such that the *bindable* counts were present in stoichiometric or substoichiometric amounts relative to the binding capacity of the cells. [A portion of the same cells had been assayed for their receptor number (5) in a preliminary test.] After suitable incubation at 37° the cells were washed twice during a 20-min period, a portion of them was assayed, and the remainder was re-incubated with excess underivatized ^{131}I-labeled IgE. (In preliminary experiments we had determined that all the incubations were sufficiently long so that the results would not be time dependent.)

Table 1 shows the data from one such experiment. During the first incubation the cell receptors appeared to be approximately 75% saturated with ^{125}I-labeled monomer and dimer IgE (specimens A and C). During the second incubation a larger number of ^{131}I-labeled IgE became bound to the cells that had been preincubated with the dimer fraction, so that the total molecules of bound IgE on those cells exceeded the number of receptor sites (defined by specimens A and E) by almost 25%. Clearly the excess number of bound molecules in specimen C must have arisen from IgE molecules within the dimers which did not occupy receptor sites. The excess bound molecules divided by half the number of IgE molecules bound in the dimer sample defines the fraction of dimers bound monomerically; 1 minus this fraction, the amount bound dimerically. The latter

Table 1. Evaluation of binding of monomer in contrast to dimer*

Specimen	First incubation	Molecules/cell ($\times 10^{-5}$)	Second incubation	Molecules/cell ($\times 10^{-5}$)		
				^{125}I-IgE	^{131}I-IgE	Total IgE
A	^{125}I-Monomer	6.24 ± 0.18	^{131}I-IgE	6.06 ± 0.08	3.69 ± 0.13	9.75 ± 0.21
B	^{125}I-Monomer (excess)	8.70 ± 0.37				
C	^{125}I-Dimer	6.89 ± 0.03	^{131}I-IgE	7.12 ± 0.57	4.94 ± 0.23	12.06 ± 0.81
D	^{131}I-IgE (excess)	9.03 ± 0.35				
E			^{131}I-IgE		9.86 ± 0.31	9.86 ± 0.31

* All results are averages of duplicates (±SD) and are corrected for nonspecific binding calculated by subtracting the values obtained for control samples incubated with a large excess of unlabeled IgE. The average control value for the 14 determinations performed in this experiment was 2.7 ± 1.9%. During the second incubation the underivatized ^{131}I-labeled IgE (^{131}I-IgE) was in approximately 20-fold molar excess over receptor sites for all specimens. Calculations of molecules/cell for both the monomer and dimer are based on a molecular weight of 200,000.

was calculated as 42% in the experiment shown. The average from three separate experiments (from two different preparations) was 33 ± 9%. Since only about 50% of the added dimeric IgE bound at all, about 17% of the added dimer bound as dimer and another 33% bound monomerically.

PCA tests

In some initial experiments the principal fractions of the monomer, dimer, and trimer peaks were pooled, concentrated, and diluted to the same 280-nm absorbance. After intravenous injection of dye, 150 ng (in 0.05 ml) of each pool was injected in the skin in duplicate and the injection sites were then scored (*Materials and Methods*). A typical result is illustrated in Fig. 3. Only the dimer and trimer fractions gave reactions that exceeded that of the solvent alone. In the experiment shown the scores were: solvent 16 ± 4, monomer 16 ± 0, dimer 130 ± 0, and trimer 162 ± 8. Data from a more complete dose response test are given in Fig. 4. It is evident that the minimal dose required for a positive response was approximately the same for the dimer and trimer fractions and that at higher doses the trimer was only slightly more effective. A detectable response was observed when 3 ng of dimer was administered (equivalent to 0.5 ng of dimer binding bivalently, see above). A 20,000-fold greater amount of monomer, from the same reaction mixture which produced the oligomer, gave no detectable response.

In additional tests, selected unpooled, unconcentrated fractions obtained promptly after gel filtration were examined. In this experiment, using fractions from the elution pattern shown in Fig. 1, the minimal effective dose was 14 ng or less of added protein and was the same for all the crosslinked fractions tested. Similarly, the magnitudes of the minimal positive responses were approximately the same (Table 2). The fraction containing monomer only was inactive.

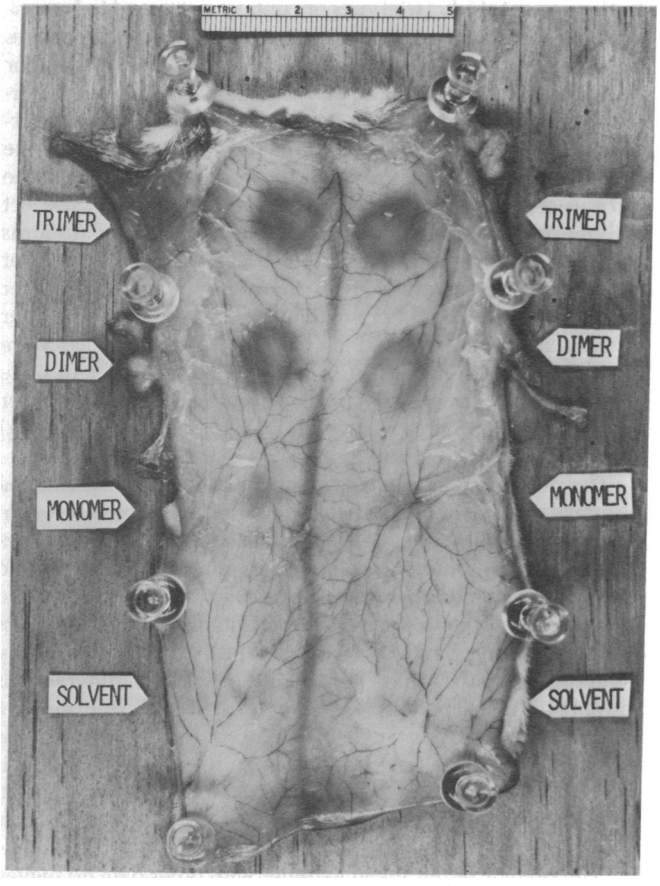

FIG. 3. PCA results obtained by injecting the skin of a rat with 150 ng of monomer, dimer, and trimer fractions of derivatized IgE. The photograph was taken approximately 3 hr after completion of the test, the skin having been stored at 4°. A modest amount of fading occurred in the interval.

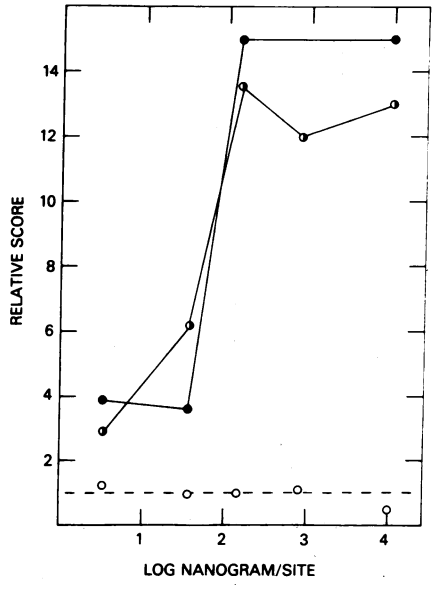

FIG. 4. Results of dose response to PCA test of monomer (○), dimer (◑), and trimer (●) fractions of derivatized IgE. The injection sites were scored and the ratio of the scores for the fraction relative to the scores for the solvent alone (on the same animal) is plotted. All relative scores are the averages of duplicates.

Table 2. Assay of gel filtration fractions by PCA analysis

Tube*	Approximate M_r† ($\times 10^{-6}$)	Relative score‡
25	3.2	<1
27	2.6	1.7
30	2.0	<1
35	1.2	1.7
38	0.93	1.0
43	0.56	1.1
45	0.47	1.7
46	0.43	1.2
47	0.40	1.0§
48	0.36	0.8
49	0.32	0.8
54	0.20	0

* See Figs. 1 and 2.

† A linear plot of log molecular weight (M_r) against elution volume was constructed based on the assumption that tubes 43, 46.5, and 54.5 represented the elution position of trimer (6×10^5), dimer (4×10^5), and monomer (2×10^5), respectively (Figs. 1 and 2). This plot was then used to calculate the average M_r expected for material eluting maximally in the respective fraction.

‡ Dimeric IgE (D_3, tube 47) was titrated and the two lowest doses that gave a positive response were determined. The same amount of material from the other tubes was then tested. The average net PCA scores relative to those given by D_3 on the same animal are shown.

§ By definition.

Assays *in vitro*

Histamine release from mouse peritoneal mast cells exposed to 0.08–50 μg/ml of derivatized IgE was measured. The net release in the presence of monomer never exceeded 1.4% at any concentration tested, but the release elicited by dimeric and trimeric IgE was significant and dose dependent. Fig. 5 shows the percent released at two different doses of the unpooled, unconcentrated fractions of derivatized IgE obtained by gel filtration (Fig. 1). As a positive control the same cells were passively sensitized with fluoresceinated IgE and incubated with 50 μg/ml of antibody against fluorescein. In this way only the newly bound IgE, and not that present endogenously, would react with antibody. The cells released 10 ± 0.5% of their histamine—values comparable to those observed with the cells treated with the crosslinked IgE.

Release of incorporated [^{3}H]serotonin from mouse mastocytoma cells was qualitatively similar to the histamine release from normal mouse mast cells. In the presence of 1 μg/ml of monomeric, dimeric, or trimeric IgE, 10^6 cells per ml released 1.1 ± 0.9, 8.1 ± 2.1, and 11.1 ± 0.1% of the total incorporated serotonin, respectively.

DISCUSSION

Our results suggest that the bridging of two molecules of IgE is sufficient to generate a unit signal; the summation of such signals leads to degranulation of mast cells. This finding is consistent with two types of observations made previously: (*a*) that bridging of IgE molecules by antigen (17–19) or other means (2, 3) is a necessary and apparently sufficient event for IgE-mediated mast cell degranulation, and (*b*) that the bridging does not have to be, indeed must not be, too extensive (20–22).

It has been previously suggested that dimers of IgE (23, 24), or in analogous experiments, IgG (25–27), might be or were likely to be sufficient to trigger mast cell degranulation.

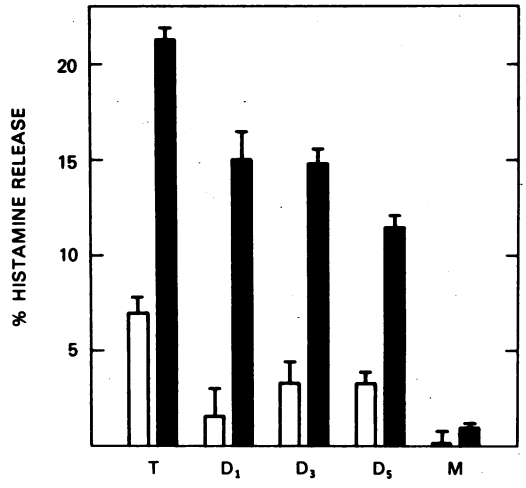

FIG. 5. Histamine release from mouse peritoneal cells mediated by derivatized IgE. T, D_1, D_3, D_5, and M refer to the corresponding fractions from the elution pattern shown in Fig. 1. Open bars indicate dose of 0.5 μg/ml; solid bars, 50 μg/ml. Each result represents the mean and standard deviation of triplicate values.

However, since the amount of bound aggregated immunoglobulin required to generate a detectable response is uncertain (below), it is critically important to rule out the possibility that small amounts of contaminants (particularly higher polymers) rather than the dimeric IgE were responsible for the effect. In previous studies this possibility could not be rigorously excluded. In the present study two types of contaminant need to be considered. Molecules that have undergone intramolecular changes due to derivatization seem highly unlikely to be responsible for the effect. The derivatization is carried out under mild conditions and the similarly treated monomer did not show detectable activity. Polymeric contaminants were excluded by the results of experiments in which the specific activities of the various column fractions were measured. One would expect polymeric contaminants to be present in increasing amounts in the dimer fractions that eluted first (see D_1 in Fig. 2). Yet there was no substantial difference in activity across the dimer peak. Although the trimer fraction(s) showed a slightly higher activity in both *in vitro* and *in vivo* assays, the differences were much too small (Table 2) for contaminating trimer to have accounted for the activity in the dimer fractions. The latter were only minimally (fraction D_1) or undetectably (D_2–D_5) contaminated with higher polymers (Fig. 2).

The present experiments were not designed to determine the minimal number of unit signals required to obtain an observable response. The binding analyses indicated that only about one-third of the bound dimers were interacting strongly with two receptor sites simultaneously. However, we could not exclude the possibility that a greater proportion of the bound dimers were interacting weakly with two receptor sites such that newly added IgE could compete for the sites. Such weak interactions might still be sufficient to generate a signal. Furthermore, a variety of observations suggest that a positive response may be damped by a simultaneous inhibitory reaction (28, 29). It is possible that the somewhat artificial nature of the stimulus applied in the present experiments may influence the balance between stimulation-inhibition.

The present results taken together with other data do suggest a plausible mechanism for the early events in IgE-mediated mast cell degranulation: IgE binds reversibly but tightly to the cell-surface receptors for IgE. These receptors are monovalent (7, 30) and more or less freely and independently mobile in the

plane of the membrane (7, 30). When the molecules are (or become) crosslinked, two or more receptors become apposed. The apposition of two receptors (or receptor–IgE complexes) represents the minimal unit signal for triggering. Progress in the isolation of soluble, active, unaggregated receptors (G. Rossi, J. Kanellopoulos, and H. Metzger, unpublished data) now makes it feasible to search for molecular consequences of such an appositional signal.

We thank Dr. Reuben Siraganian for considerable assistance in performing the experiments on normal mouse mast cells. Dr. Chaviva Isersky and Mr. John Rivera supplied repeated samples of rat basophilic leukemia cells; Mr. George Poy isolated and iodinated the IgE and assisted in the PCA experiments.

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