

Cross-linking of Receptor-bound IgE to Aggregates Larger than Dimers Leads to Rapid Immobilization

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Abstract. Controlled cross-linking of IgE-receptor complexes on the surface of rat basophilic leukemia cells and mast cells has allowed a comparison of the lateral mobility and cell triggering activity of monomers, dimers, and higher oligomers of receptors. Addition of a monoclonal anti-IgE(Fc) antibody to IgE-sensitized cells in stoichiometric amounts relative to IgE produces IgE-receptor dimers with high efficiency. These dimers are nearly as mobile as IgE-receptor monomers and trigger cellular degranulation poorly, but in the presence of 30% D₂O, substantial immobilization of the dimers is seen and degranulation activity doubles. Addition of this monoclonal antibody in larger amounts results in the formation of larger oligomeric receptor clusters which are immobile and effec-

tively trigger the cells. Thus, small receptor clusters that are active in stimulating degranulation are immobilized in a process that is not anticipated by simple hydrodynamic theories. Further experiments involving cross-linking of receptor-bound IgE by multivalent antigen demonstrate that immobilization of receptors occurs rapidly (<2 min) upon cross-linking and is fully and rapidly reversible by the addition of excess monovalent hapten. The rapidity and reversibility of the immobilization process are entirely consistent with the possibility that immobilization represents a recognition event between clustered receptors and cytoskeleton-associated components that plays an important role early in the cell triggering mechanism.

THE aggregation of cell surface receptors for IgE on mast cells, basophils, or rat basophilic leukemia (RBL)¹ cells triggers cellular degranulation in a coupling process that exemplifies the general phenomena of recognition and transduction of biological signals at cell membrane. In the nonaggregated state these receptors are numerous ($\sim 3 \times 10^5$ receptors/cell [Metzger, 1978]), mobile (lateral diffusion coefficient $\sim 3 \times 10^{-10}$ cm²s⁻¹ [Schlessinger et al., 1976; Wolf et al., 1980; McCloskey et al., 1984; Menon et al., 1985]), and uniformly distributed ($\sim 10^3$ receptors/ μ m²). Each receptor can bind one molecule of IgE (Schlessinger et al., 1976; Mendoza and Metzger, 1976) with very high affinity ($K_A > 10^{10}$ M⁻¹ [Kulczycki and Metzger, 1974]). The addition of small oligomers of IgE to the cells and the concomitant production of small oligomers of receptors suffices to initiate the triggering sequence that leads to degranulation (Segal et al., 1977; Fewtrell and Metzger, 1980; Menon et al., 1984). Previous studies in this laboratory have shown that receptor oligomerization by this means is accompanied by a dramatic

reduction of receptor lateral mobility and under some conditions the formation of large visible patches after an extended time (Menon et al., 1984, 1985). The degree of immobilization seen with a particular oligomer preparation correlates positively with its relative ability to stimulate degranulation, suggesting that the immobilization event represents a binding or recognition step between the clustered receptors and other components associated with the plasma membrane and that this may be an important part of the triggering mechanism. This hypothesis has been examined in greater detail in the present study.

In the experiments described here fluorescence photobleaching recovery (FPR) and fluorescence microscopy have been used to examine the mobility of receptor oligomers formed by the binding of two monoclonal anti-IgE antibodies, A2 and B5 (Conrad et al., 1983), to receptor-bound IgE. A2 is a particularly valuable reagent because its binding properties allow receptor dimers to be tested separately from larger oligomers. This feature is useful to these studies because receptor dimers elicit a poor response from RBL cells that is enhanced in the presence of D₂O, whereas trimers and larger oligomers are considerably more active (Fewtrell and Metzger, 1980). The ability to separate receptor dimers from higher oligomers did not exist in previous FPR studies using stable IgE oligomers (Menon et al., 1985) because of the difficulties in obtaining pure dimers and the generally low probability

¹ Abbreviations used in this paper: BGG, bovine gamma-globulin; BSS, buffered salt solution; *D*, diffusion coefficient determined by FPR, $D_0 = 10^{-10}$ cm²s⁻¹; DNP, 2,4-dinitrophenyl; FITC, fluorescein isothiocyanate; FPR, fluorescence photobleaching recovery; [³H]5HT, [5-1,2-³H(N)] hydroxytryptamine binoxalate; IgE_M, murine monoclonal anti-DNP IgE; IgE_R, rat myeloma IgE; *R*, percent recovery determined by FPR; RBL, rat basophilic leukemia; TRITC, tetramethylrhodamine isothiocyanate.

that both Fc segments in a given dimer bind simultaneously to receptors (Fewtrell and Metzger, 1980). Results from mobility studies with A2-induced oligomers have been compared with results from degranulation assays carried out with the same preparations to establish a correspondence between the degree of immobilization and triggering ability.

Materials and Methods

Materials

Tetramethyl rhodamine isothiocyanate (TRITC), [5-1,2-³H(N)]-hydroxytryptamine binoxalate ([³H]5HT), colchicine, cytochalasin D, 2-deoxy-D-glucose, murine monoclonal anti-2,4-dinitrophenyl (DNP) IgE (IgE_M), and rat myeloma IgE (IR162; IgE_R) were obtained as described in the accompanying paper (Menon et al., 1985). DNP-L-lysine was from Sigma Chemical Co. (St. Louis, MO) and fluorescein-5-isothiocyanate (FITC) was from Molecular Probes Inc. (Junction City, OR). The anti-IgE_R monoclonal antibodies A2 and B5 were gifts from Dr. D. Conrad (The Johns Hopkins University), and ¹²⁵I derivatives were prepared by the chloramine T method (Holowka and Baird, 1983). Fluorescent derivatives of monomeric IgE_M, IgE_R, A2, and B5 were prepared as described previously (Menon et al., 1984) using either TRITC or FITC. The degree of fluorescence conjugation for the derivatives was as follows: fluorescein/IgE_R, ~10:1; rhodamine/IgE_M, ~2:1; fluorescein/IgE_M, ~6:1; and rhodamine/IgE_M, ~2:1.

RBL Cells

RBL cells (subline 2H3) were grown adherent to 75-cm² flasks and harvested with trypsin when confluent (4–7 d after passage) as described (Menon et al., 1984). The washed cells were suspended in supplemented medium (Eagle's minimum essential medium with Earle's balanced salt solution, 10% newborn calf serum (M.A. Bioproducts, Walkersville, MD), 20 mM Hepes, pH 7.4) and sensitized with monomeric IgE if required by incubation with a 5–10 molar excess of IgE for 1–1.5 h at 37°C. Excess IgE was removed by washing, and the cells were finally resuspended in supplemented medium or in a buffered salt solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5.6 mM glucose, 2–4% newborn calf serum, 20 mM Hepes, pH 7.4).

For degranulation assays [³H]5HT (15 μCi) was incubated overnight with cells growing adherent in culture. The cells were harvested and sensitized with IgE as described above and resuspended in BSS at 2.2 × 10⁶/ml. 100-μl aliquots of cells were added to wells in a U-bottom microtiter plate containing 100-μl dilutions of the monoclonals A2 or B5 in BSS modified as indicated with D₂O, colchicine, or cytochalasin D. The plate was incubated at 37°C for 1.5 h, and the supernatants were harvested and counted for ³H activity as described elsewhere (Baird et al., 1983).

¹²⁵I-Labeled Fab and F(ab')₂ Fragments of A2

A2 F(ab')₂ was produced by pepsin digestion of A2 and purified as previously described (Holowka et al., 1985) with minor changes. In brief, the peak fraction from a Sephacryl S-300 column (Pharmacia Fine Chemicals, Piscataway, NJ) of the pepsin digest was concentrated about twofold by Sephadex G-25 absorption of the borate-buffered saline solution through a dialysis bag [final F(ab')₂ concentration, ~0.18 mg/ml]. This was followed by ¹²⁵I-iodination with chloramine T and removal of free ¹²⁵I on centrifuge columns. After modification by FITC at pH 7.0 (Holowka et al., 1985) the ¹²⁵I-A2 F(ab')₂ fragment was reduced with 2 mM dithiothreitol and alkylated with 5 mM *N*-ethyl maleimide to produce ¹²⁵I-A2 Fab'. SDS PAGE analysis of this Fab' fragment showed the complete absence of any products with intact heavy chains or any F(ab')₂ fragments.

Binding Studies

RBL cells were sensitized with fluorescently modified or unlabeled IgE_R, washed, and suspended in supplemented medium at 2 × 10⁶/ml [A2 or A2 F(ab')₂ binding] or 2 × 10⁷/ml (A2 Fab' binding). Tubes containing 50 μl of ¹²⁵I-labeled A2 derivatives in serial dilutions were prepared in duplicate. 200 μl of a cell sample was added to each tube and incubated for 30–45 min at 37°C in a shaking water bath. At the end of the incubation, 100-μl aliquots were removed from each sample, layered over 200-μl phthalate oil in microfuge tubes, and microfuged at 13,000 rpm for 1.5 min. The supernatant and most of the oil were removed from each tube by suction, and the bottom of the tube was cut off, placed in a biovial, and measured in a Beckman Gamma 4000 radiation spectrophotometer (Beckman Instruments Inc., Palo Alto, CA) to

determine cell-bound ¹²⁵I counts. These values were corrected for nonspecific binding by subtracting counts obtained from parallel samples of cells not sensitized with IgE. ¹²⁵I counts were converted to molar quantities using the specific activity of the ¹²⁵I-A2 derivatives. The number of IgE molecules per cell was determined separately by saturating the cells with ¹²⁵I-IgE_M or ¹²⁵I-IgE_R, and the cells per sample were counted before and after the binding incubation.

Rat Peritoneal Mast Cells

Cell suspensions were obtained by peritoneal lavage of from two to four male Wistar rats using a buffer containing 135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.1% gelatin, 10 IU heparin/ml, 10 μg deoxyribonuclease I/ml, 10 mM Hepes, pH 7.4. The peritoneal washes were pooled, and the cells were washed by centrifugation and resuspension in 2 ml of the same buffer. Mast cells were purified by centrifugation for 15 min at 300 *g* through 2 ml of a 22.5% solution of metrizamide (Sigma Chemical Co.). The mast cell pellet was washed once, then resuspended at 5 × 10⁶/ml in the same buffer containing newborn calf serum added to 20%.

For degranulation assays, the cells were loaded with [³H]5HT and sensitized with monomeric TRITC-IgE_R (5 μg/ml) in the course of an incubation for 1 h at 37°C, then washed and resuspended at 10⁶/ml in BSS containing phosphatidyl serine (Sigma Chemical Co.) added to 10 μg/ml. Equal volumes of cells and prewarmed dilutions of the monoclonal antibodies A2 and B5 were combined, incubated for 10 min at 37°C, and quenched with an ice cold solution of 135 mM NaCl, 5 mM KCl, 20 mM Hepes, pH 7.4. Cells were pelleted by centrifugation, and aliquots of the supernatant were added to 5 ml Aqueous Counting Scintillant (Amersham Corp., Arlington Heights, IL) in vials then counted for ³H activity. Total counts per sample were determined by directly adding aliquots of quenched samples (without centrifugation) to scintillant.

Diffusion Measurements

The FPR technique yielding diffusion coefficients (*D*) and percent recovery or mobile fraction (*R*) for fluorescently labeled cell surface receptors used for these experiments is described in the accompanying report (Menon et al., 1985). The radius of the cell surface laser-illuminated spot was 0.55–0.65 μm. All measurements were made at ambient temperature on RBL cells or mast cells sensitized with column-purified, TRITC-labeled, monomeric IgE_R or IgE_M. Cell suspensions (5 × 10⁶/ml) were rocked at ambient temperature, and for experiments involving A2 or B5, cells were exposed to the antibodies for 0.5 h before any measurements were made. For the experiments shown in Fig. 6 the cells were maintained in medium modified as indicated while they were being sensitized with IgE as well as in subsequent steps. Most of the data are presented as histograms, each block in a histogram representing an average of five photobleaching sweeps from five different cells. Summarized data are given as mean $R(\bar{R}) \pm \text{SEM}$, mean $D(\bar{D})$, or $\log(\bar{D}/D_0) \pm \text{SEM}$, where $D_0 = 10^{-10}$ cm²s⁻¹.

Bleb Formation

Blebbing was induced by either of two methods as follows. (a) Cells were allowed to adhere to wells in a six-well polystyrene plate (Becton Dickinson Labware, Oxnard, CA), washed with a buffer containing 150 mM NaCl, 2 mM CaCl₂, 10 mM Hepes, pH 7.4, and then exposed to 2 mM dithiothreitol and 25 mM formaldehyde in the same buffer for 0.5 h at 37°C. The cells were then washed with phosphate-buffered saline, pH 7.4, before exposure to the multivalent antigen DNP₁₆ bovine gamma-globulin (DNP₁₆BGG). All maneuvers were carried out as gently as possible to avoid dissociating the blebs from the cells. (b) Cells were incubated at 37°C in BSS containing 10 mM sodium azide and 10 mM 2-deoxy-D-glucose for 30 min in the presence or absence of A2 or B5, and aliquots were dropped onto glass slides and overlaid with coverslips. Cells begin blebbing as they started to adhere to the slide.

Results

Previous measurements of the binding of monoclonal antibodies A2 and B5 to IgE_R on RBL cells showed that A2 binds at a single tight site in the Fc region such that at up to 10-fold molar excess over IgE, no more than one A2 binds to two receptor-bound IgE_R, whereas the corresponding ratio for B5 binding to IgE at sites in the Fab regions is one to one (Conrad et al., 1983). These results indicated that A2 might be used to

form receptor dimers on the cell surface in a controlled and efficient manner that cannot be achieved with multivalent antigen or even with dimeric IgE. Furthermore, triggering of degranulation was only achieved at much larger ratios of A2 to IgE (Conrad et al., 1983), suggesting the presence of a weaker binding site for A2 that permitted more extensive cross-linking of the IgE-receptor complexes. This presented an opportunity to test the correlation we had observed previously between the immobilization of oligomerized receptors and stimulation of degranulation (Menon et al., 1985). To this end we investigated the binding of B5 and A2 derivatives to IgE_R bound to receptors on RBL cells corresponding to samples that were also examined by degranulation assays, fluorescence microscopy, and FPR measurements.

Binding Studies

Fig. 1 shows curves for binding of monovalent A2 Fab' (a) or bivalent A2 and A2 F(ab')₂ (b) to cell-bound IgE_R, FITC-IgE_R, or TRITC-IgE_R. The major feature to be noted is that all the curves require at least two association constants for complete characterization. The highest association constant corresponds to the tight binding site measured by Conrad et al. (1983): $K_A = 3.4 \times 10^7 \text{ M}^{-1}$ for A2 Fab' (Fig. 1a) and $K_A = 2.8 \times 10^9 \text{ M}^{-1}$ for A2 F(ab')₂ or A2 (Fig. 1b). These tight binding sites are saturated by the addition of roughly stoichi-

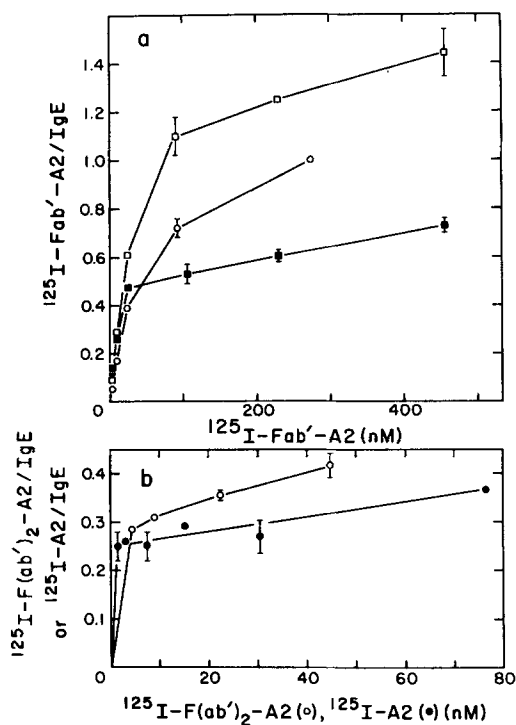


Figure 1. (a) Binding of ¹²⁵I-A2 Fab' to RBL cells sensitized with FITC-IgE_R (■), TRITC-IgE_R (○), or unlabeled IgE_R (□). Concentrations of cell-bound IgE_R were 5.6 nM (FITC-IgE_R), 5.4 nM (TRITC-IgE_R), and 6.4 nM (unlabeled IgE_R). (b) Binding of A2 F(ab')₂ to cell-bound FITC-IgE_R (0.91 nM) (○), and binding of A2 to cell-bound TRITC-IgE_R (0.69 nM) (●). Binding incubations were carried out for 0.5 h at 37°C, and appropriate cell samples without IgE_R were used to correct for nonspecific binding (10–15% of total counts bound). Data are presented as the molar ratio of cell-bound A2 derivatives to IgE_R as a function of total concentration of the A2. Bars represent the range of duplicate determinations.

ometric amounts of bivalent A2 relative to IgE_R (Fig. 1b) or by the addition of a small excess of monovalent A2 Fab' over IgE_R (Fig. 1a). As seen in Fig. 1a fluorescence modification of IgE_R lowers the tight binding stoichiometry without substantially altering K_A . The gradually rising portions of the binding curves in Fig. 1, a and b at higher concentrations of A2 derivatives correspond to a much weaker interaction not characterized previously. These weaker association constants can be estimated by assuming that the tight sites are saturated before the weak sites are bound appreciably and taking the slope of the apparently linear increase beyond this first saturation point. Values for the weaker affinity constant obtained in this manner are $\sim 3 \times 10^6 \text{ M}^{-1}$ for A2 and $\sim 6 \times 10^5 \text{ M}^{-1}$ for A2 Fab'. It is reasonable that each IgE_R has two identical A2 binding sites (one per heavy chain) and that occupation of the first site substantially weakens binding to the other site for steric reasons. The presence of these weak binding sites would allow the receptors dimerized by tight A2 binding to be extended into oligomeric chains or aggregates. Thus by varying the concentration of A2 it is possible to control the extent of receptor cross-linking and compare these distinct situations: receptor monomers only (no A2 added), receptor clusters no larger than dimers (1–10-fold excess A2 added relative to IgE_R), and receptor oligomers including clusters larger than dimers (10–100-fold excess A2 added). In similar experiments B5 binding to IgE_R-sensitized cells was found to be similar to that measured by Conrad et al. (Conrad et al., 1983) and could be characterized by a single association constant, $K_A \sim 5 \times 10^8 \text{ M}^{-1}$, and a binding stoichiometry of B5/IgE_R $\sim 1:1$. No evidence for a lower affinity site for B5 was obtained when binding was carried out at up to a 30-fold excess (300 nM) of ¹²⁵I-B5 over cell-bound IgE (data not shown).

Degranulation

For further characterization the monoclonal antibodies A2 and B5 were tested for their ability to trigger [³H]5HT release from RBL cells and rat peritoneal mast cells. Fig. 2a shows data for RBL cells in the presence or absence of 30% D₂O,

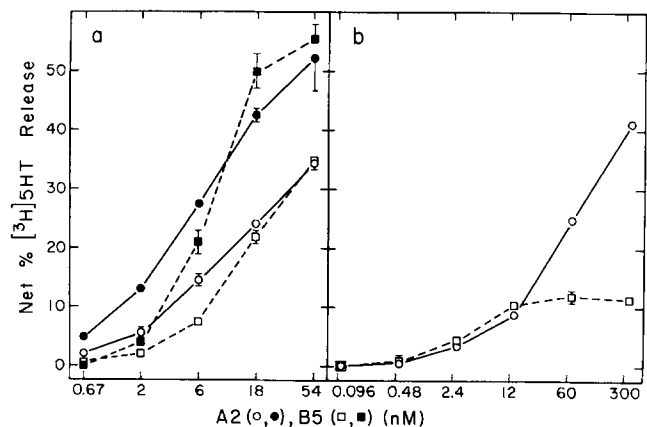


Figure 2. (a) Net percent [³H]5HT release from RBL cells elicited by A2 and B5 in the presence (●, ■) or absence (○, □) of 30% D₂O. The cells had been sensitized with FITC-IgE_R and were suspended at $1.1 \times 10^6/\text{ml}$ (cell-bound IgE_R, $\sim 0.55 \text{ nM}$). (b) Net percent [³H]5HT release from rat peritoneal mast cells elicited by A2 (○) and B5 (□). The cells ($10^5/\text{ml}$; cell-bound IgE_R, $\sim 0.05 \text{ nM}$) had been sensitized with TRITC-IgE_R. Each point represents the mean of duplicates with the range shown by the bars.

and it can be seen that release triggered by each of the monoclonals is enhanced by including D₂O in the assay buffer. Significantly, release triggered by concentrations of A2 in the range 0.66–2 nM is approximately doubled in the presence of D₂O (compare ○ to ●, Fig. 2*a*). At these concentrations A2 binding would be confined almost exclusively to the tight-binding, dimer-forming interaction described above. This combination of results is consistent with studies involving dimethyl suberimidate-cross-linked covalent dimers of IgE_R in which it was shown that the low level of dimer-induced release was considerably enhanced by 30–40% D₂O (Fewtrell and Metzger, 1980). B5 triggers release similar to A2 at large doses (>15 nM), but at concentrations where the stoichiometry of B5/IgE ranges from 0.2 to 0.5 (0.67–2.0 nM B5), almost no release is seen even with D₂O present (Fig. 2*a*, □ vs. ■).

With dimethyl suberimidate-cross-linked oligomers of IgE_R, RBL cells are triggered much less effectively by dimers than by larger oligomers, but mast cells were triggered by dimers almost as well as by larger oligomers (Fewtrell and Metzger, 1980). However, sensitized mast cells tested with A2 showed a large difference in response to A2-induced dimers as compared with larger oligomers, (Fig. 2*b*) similar to the RBL cell results (Fig. 2*a*). B5-induced secretion by mast cells was poor even at high doses (>50 nM, Fig. 2*b*).

Microscopy/Internalization

Under most conditions tested, A2 and B5 did not perturb the uniform surface distribution of fluorescence observed microscopically when TRITC-IgE_R was bound to RBL cells. At high concentrations of A2 (100-fold molar excess over cell-bound IgE_R), some slight patchiness of fluorescence was seen, and this patchiness was considerably enhanced by lengthy incubation (5 h) at 4°C or by maintaining the cells in BSS without Ca²⁺ and with 1 mM EGTA. The enhanced clustering seen at 4°C is consistent with earlier work describing the oligomeric IgE-induced patching of receptors (Menon et al., 1984, 1985). No internalization was seen by microscopy with either A2 or B5 even when cells were examined after incubation at 37°C for 0.5–1 h or at ambient temperature for up to 4 h.

A more quantitative assay for internalization is shown in Fig. 3. The assay makes use of the quenching of fluorescein

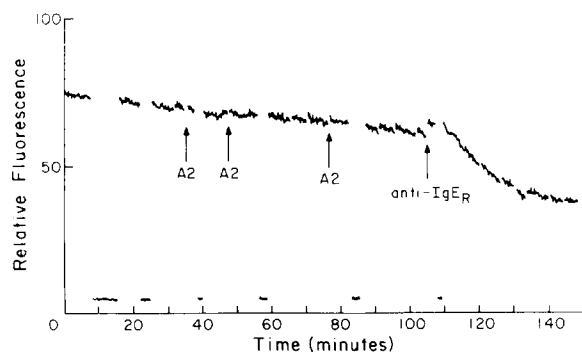


Figure 3. Fluorescence assay for internalization. FITC-IgE_R-labeled cells (10⁷/ml; cell-bound IgE_R, ~5 nM) at 37°C were exposed to A2 in three successive doses (arrows) of 6.7, 6.7, and 133.3 nM (total A2, 146.7 nM) and finally exposed to 80 nM polyclonal rabbit anti-IgE_R. FITC fluorescence was excited at 495 nm, and emission was monitored at 520 nm. The intermittent lower trace is from an unlabeled cell sample monitored in parallel.

fluorescence when the fluorophores are transferred to the acidic environment of internal vesicles (Okhuma and Poole, 1978; Tycko and Maxfield, 1982). Fig. 3 shows that the addition of A2 (>30-fold excess) has no effect on FITC fluorescence from FITC-IgE_R-sensitized cells maintained at 37°C but that the addition of polyclonal rabbit anti-IgE_R antibodies causes rapid internalization (half-time, ~10 min), which can be visualized microscopically by the appearance of round, fluorescent dots scattered within the cell boundary (not shown).

The procedure whereby receptor-bound IgE on RBL cells can be dissociated from the cell surface by exposure to a pH 3 buffer (Kulczycki and Metzger, 1974) was also used to assay internalization. Cells labeled with ¹²⁵I-IgE were exposed to 0, 2, 40, and 100-fold molar excesses of A2 and incubated at 37°C for up to 45 min. At all concentrations ~80% of the total counts could be dissociated by the pH 3 treatment, and this remained constant over the incubation period, indicating that almost all the IgE_R remained on the cell surface, accessible to the buffer (data not shown). That the monoclonals did not induce internalization under most conditions was very useful for the FPR measurements described below: it guaranteed fluorescence confined to the cell surface and permitted experiments to be carried out conveniently for extended periods at ambient temperature.

Diffusion Measurements

FPR experiments provided diffusion coefficients (*D*) and percent recovery (*R*) values for TRITC-IgE_R-labeled receptors on RBL cells, and these are presented as histograms in Fig. 4. The average results obtained for receptor monomers labeled with TRITC-IgE_R (Fig. 4*a*: $\bar{D} = 2.6 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ [$\log(D/D_0) = 0.41 \pm 0.03$] $\bar{R} = 88 \pm 2\%$) are similar to those reported previously (Wolf et al., 1980; Menon et al., 1985) for monomeric TRITC-IgE. With the addition of a roughly stoichiometric amount of A2, expected to produce primarily receptor dimers, *R* remained essentially unchanged although the average *D* was reduced by a factor of 1.6 (Fig. 4*b*: $\bar{D} = 1.6 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ [$\log(D/D_0) = 0.20 \pm 0.02$] $\bar{R} = 85 \pm 3\%$). With additional amounts of A2 (Fig. 4, *c–e*) and the concomitant production of larger oligomers of receptors *R* was increasingly reduced, but *D* did not change appreciably. After the addition of a 100-fold excess of A2 over cell-bound IgE_R most of the receptors were immobilized ($\bar{R} = 13\%$, Fig. 4*e*). In contrast, a 100-fold excess of B5 did not cause substantial receptor immobilization ($\bar{R} = 77 \pm 3\%$, $\bar{D} = 1.5 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ [$\log(D/D_0) = 0.16 \pm 0.06$]; histograms not shown).

Fig. 5 summarizes the RBL cells data described above and compares these with those from experiments on rat peritoneal mast cells. The trends in *R* (Fig. 5*c*) and *D* (Fig. 5*d*) for TRITC-IgE_R-labeled mast cells as a function of A2 concentration were similar to the results for RBL cells (Fig. 5, *a* and *b*). In both cases, the most striking trend was the reduction in *R* that occurred with the addition of A2 in excess of stoichiometric amounts. Another notable common feature was that *D* for the mobile IgE-receptors after addition of stoichiometric or larger amounts of A2 (or large amounts of B5) was approximately half the value measured for unperturbed receptor monomers (Fig. 5, *b* and *d*).

The observation by Furuichi et al. (1985) of the co-endocytosis of up to 30% of free monomeric IgE-receptor com-

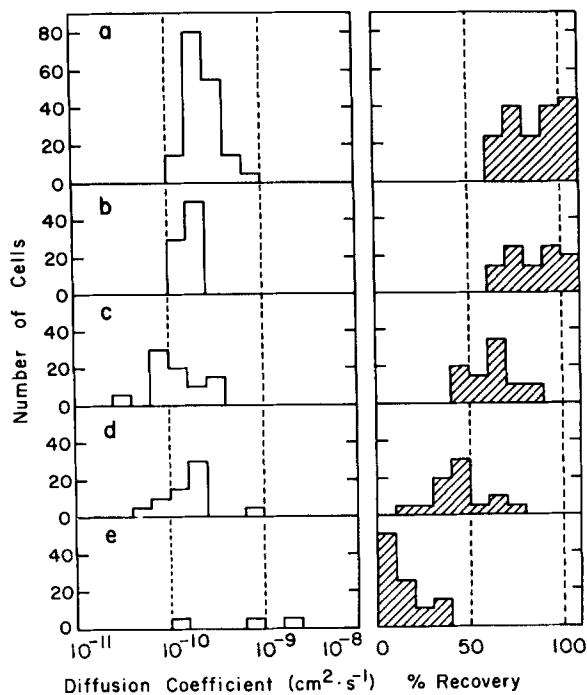


Figure 4. Histograms of diffusion coefficient (D) and percent recovery (R) values from FPR measurements on RBL cells sensitized with TRITC-IgE_R and exposed to various concentration of A2: (a) 0, (b) 6.7, (c) 33.3, (d) 133.3, and (e) 333.3 nM. Cell concentration for the incubation with A2 was $\sim 5 \times 10^6$ /ml. Five measurements made on five different cells were averaged, and numerous such averages were used to construct the histograms. Each average constituting a unit block in the histograms is counted as five cells on the vertical axis.

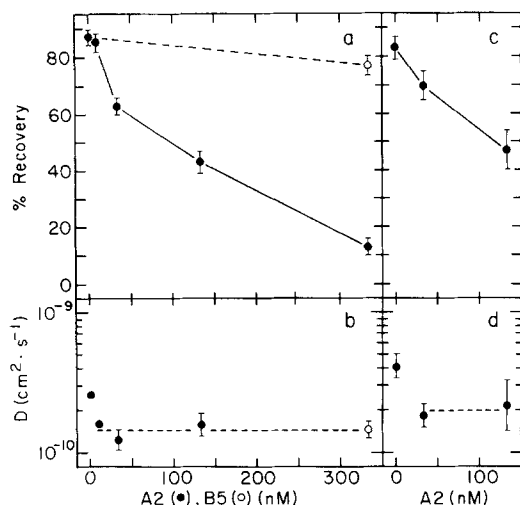


Figure 5. Mean percent recovery (R) and diffusion coefficient (D) values for TRITC-IgE_R-labeled RBL cells (a and b) and rat peritoneal mast cells (c and d) exposed to indicated concentration of B5 or A2. Dashed lines are drawn through all points corresponding to non-zero concentrations of A2 and B5. Bars represent SEM. The RBL cell data (a and b) correspond to the histograms in Fig. 4, a-e.

plexes with cross-linked receptors suggested the possibility that monomeric receptors might in some way be associated on the cell surface with cross-linked and immobilized receptors with concomitant constraints on lateral mobility. This possibility was explored in the following experiment. RBL

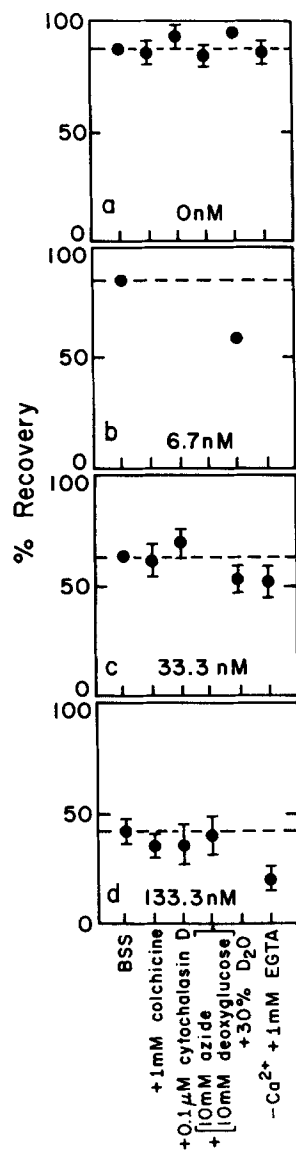


Figure 6. Mean percent recovery (R) values for TRITC-IgE_R-labeled RBL cells (5×10^6 /ml; cell-bound IgE_R, ~ 2.5 nM) exposed to various concentrations of A2 (indicated at the bottom of each frame) and incubated in BSS containing the reagents indicated on the horizontal axis. The dashed line in each frame corresponds to R measured at each A2 concentration for cells in unmodified BSS. Bars represent SEM.

cells were simultaneously sensitized with unlabeled IgE_R and TRITC-IgE_M monomers in equal proportion to see what effect immobilizing IgE_R-receptor complexes with 60–100-fold excess A2 would have on the mobility of TRITC-IgE_M-labeled receptors. With IgE_R-receptor complexes immobilized, TRITC-IgE_M-receptors had $\bar{R} = 79 \pm 5\%$ and $\bar{D} = 5.3 \times 10^{-10} \text{ cm}^2\text{s}^{-1}$ [$\log(D/D_0) = 0.72 \pm 0.12$]. In a parallel control experiment in which cells were labeled only with TRITC-IgE_M and exposed to 60-fold excess A2 (A2 cross-reacts only very weakly with IgE_M [Conrad et al., 1983]), $\bar{R} = 88 \pm 7\%$ and $\bar{D} = 4.7 \times 10^{-10} \text{ cm}^2\text{s}^{-1}$ [$\log(D/D_0) = 0.67 \pm 0.14$]. Thus, even when half the receptors on the cell surface become immobilized after cross-linking, the mobility characteristics of the other non-cross-linked half remained consistent with monomer mobility, although it was not possible to rule out co-immobilization of $<20\%$ of the monomeric receptors in these experiments. This apparent lack of large scale association between clustered and unclustered receptors on the RBL cell supports the conclusions of Schlessinger et al. (1976), who carried out a similar test on mast cells.

Various reagents were tested for their effects on the mobility (D and R) of monomeric receptors or oligomers of receptors

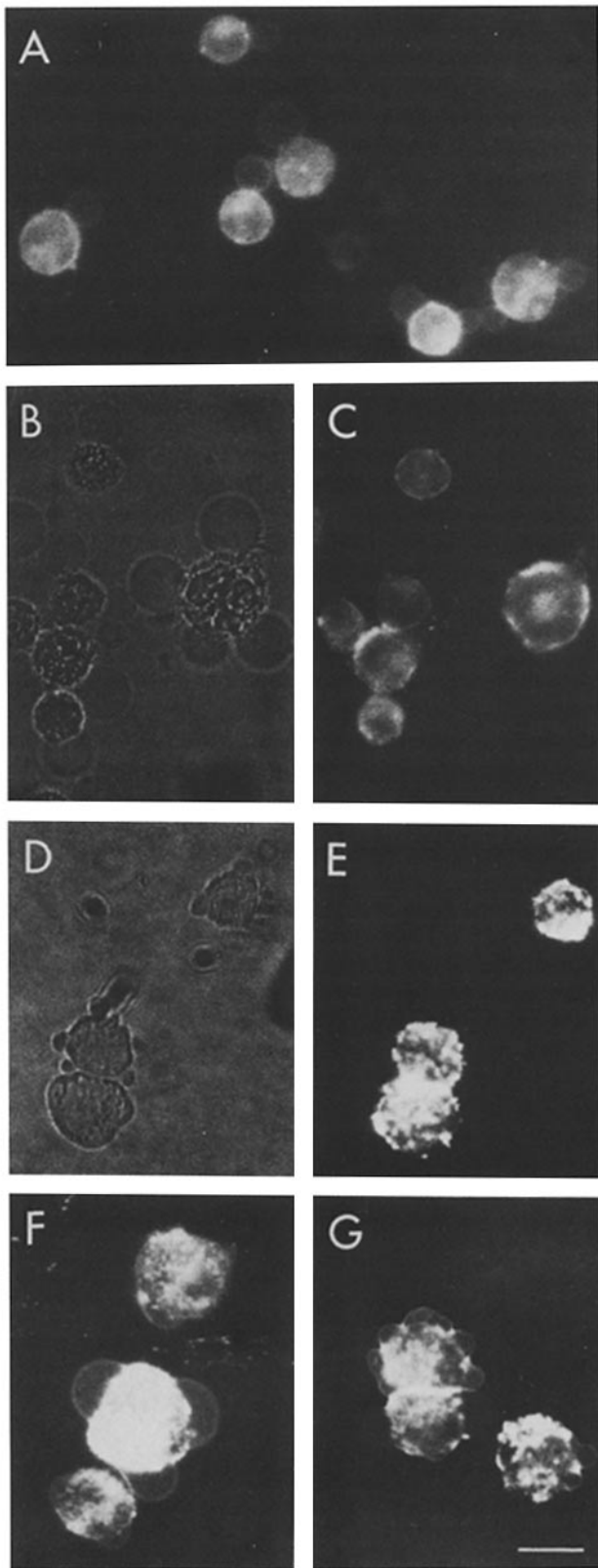


Figure 7. Experiments with cell-attached blebs showing that receptor clusters larger than dimers are selectively immobilized on the cell surface: (A) blebbing RBL cells labeled with FITC-IgE_M; (B) bright-field and (C) fluorescence micrographs of FITC-IgE_M-labeled blebbing cells treated with the multivalent antigen DNP₁₆BGG at 4°C, then fixed in 3.7% formaldehyde to preserve the fluorescence distribution; (D) brightfield and (E) fluorescence micrographs of IgE_R-labeled blebbing cells treated with a 100-fold excess FITC-A2; (F) IgE_R

produced by A2 binding: 1 mM colchicine, 0.1 μM cytochalasin D, 10 mM sodium azide with 10 mM 2-deoxy-D-glucose, 30% D₂O, and 1 mM EGTA (Ca²⁺ free). At the concentrations tested, colchicine causes a 48% inhibition of net [³H]5HT release and cytochalasin D enhances release 1.7-fold for RBL cells triggered with 33 nM A2 (data not shown). No systematic effects on *D* were seen with any of the chemical treatments (not shown). As presented in Fig. 6, none of the reagents significantly affected *R* for receptor monomers (Fig. 6*a*), and the cytoskeleton disrupting agents (colchicine and cytochalasin D) did not affect *R* for receptors that had been oligomerized to various degrees with A2. The finding is consistent with the lack of effect of these drugs on surface immunoglobulin immobilization on B lymphocytes (Dragsten et al., 1979). That external Ca²⁺ is not required for the reduction of *R* (Fig. 6) indicates that the immobilization process is not dependent on Ca²⁺ influx, nor is it dependent on cellular degranulation, which does not occur in Ca²⁺-free medium. It is notable that 30% D₂O, which enhances secretion particularly under poor triggering conditions (Fig. 2*a*), also enhances reduction of *R* under similar conditions (Fig. 6, *b* and *c*). This is most evident in Fig. 6*b* where the amount of A2 added is expected to result in the formation of dimers of receptors. These receptor dimers trigger release ineffectively (Fig. 2*a*: A2 in the range 0.66–2 nM) and have approximately the same mobility characteristics as receptor monomers (Fig. 4, *a* and *b*), but in the presence of 30% D₂O secretion doubles (Fig. 2*a*) and the mobile fraction is substantially reduced (Fig. 6*b*: $\bar{R}(-D_2O) = 85 \pm 3\%$, $\bar{R}(+D_2O) = 59 \pm 3\%$).

Bleb Experiments

An independent, visual confirmation of the mobility changes measured by FPR was provided by observations involving cell-attached blebs. These structures are spherical protrusions of the cell membrane (Fig. 7*B*), largely devoid of filamentous F-actin (Barak and Webb, 1982) and containing highly mobile membrane proteins (Tank et al., 1982) including the receptor for IgE (Holowka and Baird, 1983). They can be induced to form on cells by several methods as described in Materials and Methods. Fig. 7*A* shows FITC-IgE_M-labeled cells, and both the bleb membrane and the cell membrane appear uniformly fluorescent. The bleb fluorescence is somewhat dimmer than that on the adjacent cell, a feature that may be attributed to differences in receptor density or to an impression of high density on the cell surface created by the convoluted, villous structure of the cell membrane as compared with the smooth bleb membrane. The addition of a multivalent antigen DNP₁₆BGG to such blebbed cells results in the loss of almost all the bleb fluorescence (Fig. 7, *B* and *C*), suggesting the selective immobilization of FITC-IgE_M-labeled receptors on the cell surface. It appears that structures necessary to immobilize antigen-induced clusters of receptors are absent from or impotent on the bleb membrane, and receptor

labeled blebbing cells treated with a stoichiometric amount of FITC-A2; (G) IgE_R labeled blebbing cells treated with ~100-fold excess TRITC-B5. Blebbing cells in *A*–*C* were produced by method *a* and in *D*–*G* by method *b* (see Bleb Formation, Materials and Methods). The profuse fluorescent spots associated with the cells in micrographs *E*–*G* are due in part to cellular autofluorescence, and some cell surface patchiness of FITC-A2 contributes to the fluorescence image in *E*. Bar, 10 μm.

clusters diffusing freely on the bleb are immobilized and trapped when they encounter the cell membrane at the cell-bleb line of contact. The experiment shown in Fig. 7C was carried out at 4°C, and the cells were fixed to preserve the fluorescence distribution, showing that immobilization of receptor clusters can occur at 4°C as well as at higher temperatures.

Experiments involving cells labeled with IgE_R and treated with either FITC-A2 or TRITC-B5 are shown in Fig. 7, D–G. Fig. 7, D and E show cells treated with a 100-fold excess FITC-A2 and then caused to bleb. Several small blebs are visible in Fig. 7D, but none of these are fluorescently stained (Fig. 7E), suggesting that the fluorescent A2-cross-linked receptors are confined to the cell surface and unable to move into the bleb membrane. This is not the case in Fig. 7F, where a stoichiometric amount of FITC-A2 was added, or in Fig. 7G, where cells were treated with a 100-fold excess TRITC-B5. In Fig. 7, both F and G, the bleb membranes show uniform fluorescence, implying that most receptors can diffuse between cell and bleb membranes and are not trapped on the cell. These results correlate well with FPR measurements of the mobile fraction of receptors shown in Figs. 4 and 5a: in measurements of samples with A2 concentrations corresponding to Fig. 7E, only 13% of the receptors were mobile ($\bar{R} = 13\%$); for samples corresponding to Fig. 7F, $\bar{R} = 85\%$, and for samples corresponding to Fig. 7G, $\bar{R} = 77\%$.

The data shown in Fig. 7, taken in conjunction with FPR measurements (Figs. 4 and 5) show that clustered receptors, although free to diffuse in the bleb membrane, are substantially immobilized on the cell surface. The origin of this immobilization cannot be purely hydrodynamic but must involve a structure or mechanism special to the cell membrane that is lost to the membrane when it is lifted away from the cell body to produce a bleb.

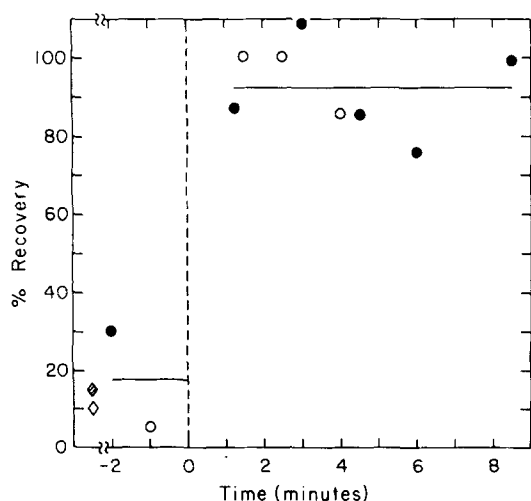


Figure 8. Rapid, reversible immobilization induced by multivalent antigen. TRITC-IgE_M labeled cells were exposed to DNP₁₆BGG at 4°C ~1.5 min before the start of each of two separate series of FPR measurements (O, ●). The first measurement in each series is the data point at time $t < 0$ min. DNP-lysine, a monovalent competitor for the multivalent DNP₁₆BGG, was added at $t = 0$ min, and successive FPR measurements on different cells were made in the $t > 0$ min period. Each point represents the beginning of a 25-s FPR sweep. Additional measurements of DNP₁₆BGG-induced immobilization observed in separate experiments are shown as five cell averages at the left of the figure (◊, ◊).

Reversibility of Immobilization

As shown above, high R values (~85%) are obtained from FPR measurements on cells labeled with fluorescent IgE and maintained on ice, and immobilization occurs upon addition of agents that cause the receptors to cluster. The rate at which this immobilization occurs and the extent to which it is reversible were tested by the experiment shown in Fig. 8. TRITC-IgE_M-labeled cells (5×10^6 /ml) were incubated on ice for 5 min before the addition of the multivalent antigen DNP₁₆BGG (1 μ g/ml), and the cells were mixed for 1 min before making FPR measurements. After a single photobleaching sweep and as rapidly as possible, a large excess of DNP-lysine (20 μ M) was added to the cell suspension, a slide was prepared, and FPR measurements were made successively on different cells over the next several minutes. Fig. 8 indicates that the receptors were almost totally immobilized within 1.5 min after adding the DNP₁₆BGG ($R = 30$ and 5% for the two measurements shown), but that these constraints were released <1.25 min after the addition of the monovalent competitor DNP-lysine. This reversibility of antigen-induced immobilization by monovalent hapten resembles the reversibility observed with degranulation activity (Fewtrell, 1985). Since earlier measurements were not possible it is conceivable that both immobilization and remobilization occur much more rapidly than indicated by these results.

Discussion

The primary conclusion from these studies is that formation of clusters of IgE receptors larger than dimers on the surface of RBL cells leads to their rapid immobilization, an event that is rapidly reversible and appears to correlate with the delivery of a triggering signal. Highly suggestive in this context is the selective effect of 30% D₂O in reducing the mobility of IgE-receptor dimers (Fig. 6b) which correlates with the previously unexplained enhancing effect of D₂O on the degranulation of both RBL cells (Fewtrell and Metzger, 1980) and human basophils (Gillespie and Lichtenstein, 1972). The rapidity of immobilization observed with cross-linking by multivalent antigen (Fig. 8) together with the apparent lack of a requirement for influx of external Ca²⁺ (Fig. 6, c and d) is consistent with the involvement of this immobilization process in early signaling events.

Since some of the important conclusions drawn from these experiments assume that addition of a stoichiometric amount of A2 relative to cell-bound IgE_R results in the cross-linking of receptors to form dimers it is useful to summarize the primary evidence for this. (a) The association constant for A2 binding to cells is ~100 times greater than for A2 Fab' binding, and the tight binding stoichiometries on cells are A2/Fab' IgE_R, ~1:1 (Fig. 1a and Conrad et al., 1983) and A2/IgE_R, 0.2–0.4:1 (Fig. 1b and Conrad et al., 1983). (b) Poor [³H]5HT release triggered by a stoichiometric addition of A2 (Fig. 2a: A2, 0.67–2 nM; release, 2–6%) is more than doubled in the presence of 30% D₂O (Fig. 2b: release, 5–13%). Such an enhancing effect was also observed with dimethyl suberimidate cross-linked covalent dimers of IgE_R (Fewtrell and Metzger, 1980). (c) Although FPR measurements of percent recovery (R) remain essentially unchanged with the addition of a stoichiometric amount of A2 [$\bar{R}(-A2) = 88 \pm 2\%$, $\bar{R}(+A2) = 85 \pm 3\%$], a substantial difference is seen if the cells are exposed to 30% D₂O [Fig. 6, a and b: $\bar{R}(-A2, +D_2O)$

= 95 ± 3%, $\bar{R}(+A2, +D_2O) = 59 \pm 3\%$] which evidently reveals the A2 cross-linking activity. In addition the D₂O effect cannot obviously accommodate the possibility that despite the tight binding of A2 to IgE_R, the length of time any pair of receptors remains cross-linked by A2 is insufficient to cause immobilization or trigger degranulation. The reduction in diffusion coefficient (*D*) upon addition of A2 is also consistent with stable A2 cross-linking [Fig. 5*b*: $\bar{D}(-A2) = 2.6 \times 10^{-10} \text{ cm}^2\text{s}^{-1}$, $\bar{D}(+A2) = 1.6 \times 10^{-10} \text{ cm}^2\text{s}^{-1}$]. (*d*) Chemical cross-linking by membrane impermeable bis(sulfo-succinimidyl)suberate (Staros et al., 1982) of cell-bound ¹²⁵I-IgE_R that has been aggregated to various extents with different concentrations of A2 results in the formation of covalent complexes containing oligomeric IgE_R. Analysis of cell extracts by autoradiography after the cross-linking reaction and electrophoresis on SDS polyacrylamide gels showed that bands corresponding to IgE_R and IgE_R cross-linked to the α-subunit of the IgE receptor present in control samples were substantially depleted in the presence of stoichiometric amounts of A2, whereas higher molecular weight bands appeared that had the mobility expected for complexes containing A2 and two IgE_R. With a 50–100-fold molar excess of A2 over IgE_R a large fraction of the ¹²⁵I label did not enter the gel, suggesting that larger oligomers had been formed (unpublished results).

FPR measurements showed notable differences in the lateral mobility of IgE-receptor monomers and variously sized oligomers produced by A2 binding. The addition of roughly stoichiometric amounts of A2 that results in the dimerization of 50–60% of the receptors (Fig. 1) leaves *R* unchanged but causes a reduction in *D* by a factor of ~1.6 (Figs. 5, *a* and *b*, and 4*b*). The addition of more than stoichiometric amounts of A2 to receptor-bound IgE causes substantial reductions in *R* without further reducing *D* for the mobile fraction (Figs. 4, *c–e*, and 5, *a* and *b*). Thus, immobilization of receptors accompanies the production of receptor oligomers larger than dimers. The same results appear with mast cells, although the data are not as complete (Fig. 5, *c* and *d*). Studies with chemically-produced stable oligomers of IgE of limited size (from two to six IgE monomers) showed similar trends in receptor immobilization (Menon et al., 1985), indicating that the results obtained with A2 can be generalized to other means of cross-linking. Previous FPR measurements that used anti-fluorescein to cross-link FITC-labeled IgE receptor complexes on mast cells did not detect significant immobilization (Schlessinger et al., 1976), but the extents of cross-linking and/or possible internalization of cross-linked complexes were not directly assessed in this study.

FPR experiments with the anti-IgE_R(Fab) monoclonal antibody, B5, showed that a 100-fold excess of the antibody over cell-bound IgE_R did not cause extensive receptor immobilization (Fig. 5, *a* and *b*). Although this result, together with binding studies and fluorescence microscopy of blebbed cells (Fig. 7*g*), suggests that B5 does not cross-link receptor-bound IgE_R to a large extent even at high concentrations, the degranulation results (Fig. 2*a*) suggest that the limited clusters so formed must be very efficient at delivering a triggering signal. Relative receptor orientation may be crucial in determining the triggering capability of a given receptor aggregate. For example, it may be that the orientation of receptors dimerically bridged by A2 is not optimal for a triggering signal on RBL cells or mast cells, whereas the bridging by B5 that does

occur is more effective on RBL cells (Fig. 2, *a* and *b*) and receptor dimers formed by binding of dimethylsuberimidate-IgE dimers are more effective on mast cells (Fewtrell and Metzger, 1980). The experiments with B5 also reflect a major distinction in the character of information available from FPR measurements and from [³H]5HT release assays: a maximal secretion response from a cell can be elicited by a small fraction of receptors appropriately clustered (Fewtrell, 1985), but the mobility characteristics of a small number of clusters would be substantially diluted and obscured in FPR experiments that measure average receptor mobility.

The immobility of receptor oligomers is operationally defined here as $D < 5 \times 10^{-12} \text{ cm}^2\text{s}^{-1}$, i.e., a reduction in *D* for monomers by at least two orders of magnitude. This cannot be explained by a comparison of the hydrodynamic properties of receptor monomers and receptor chains or by mechanical interactions among such chains diffusing in a lipid bilayer. Both these possibilities are ruled out because immobilization occurs only on cell membranes and not on attached bleb membranes (Fig. 7). Furthermore, hydrodynamic theories of protein diffusion in viscous membranes predict a weak (logarithmic) size dependence of the lateral diffusion coefficient (Saffman and Delbrück, 1975) in contrast to the extreme sensitivity of *D* to cluster size observed in these experiments.

The immobilization mechanism revealed in these experiments must be peculiar to the cell membrane and dissociated from or inactive in the bleb membrane. Several possible models may be considered which are also compatible with the finding that immobilization is both rapid and reversible (Fig. 8):

(*a*) The immobilization of receptor oligomers occurs because at least one receptor in the chain is part of the original immobile fraction (15%) seen with FPR measurements of receptor monomers (Figs. 4*a* and 5*a*). This is not a satisfactory mechanism because it would predict that the range of *R* seen for monomers (Fig. 4*a*, *R*, ≈60–110%) would be expanded to ~30–110% when ~60% of the receptors are dimerized, whereas the observed range is quite different, ~60–103% (Fig. 4*b*). It also does not simply account for the increased immobilization seen in the presence of 30% D₂O (Fig. 6) or for the large-scale clustering of immobilized oligomers seen at 4°C (Menon et al., 1984).

(*b*) Lateral diffusion is controlled by a labile, cytoskeletal matrix underlying the plasma membrane (Koppel et al., 1981). Such a matrix would obstruct the diffusion of proteins that project into it on the cytoplasmic face of the membrane, and this could provide a plausible mechanical reason for the anomalously slow diffusion ($D \approx 10^{-10} \text{ cm}^2\text{s}^{-1}$) of many membrane proteins as compared with membrane lipids ($D \approx 10^{-8} \text{ cm}^2\text{s}^{-1}$). Since the basis of matrix control of lateral diffusion is steric, the effect could be strongly dependent on size and would account for the immobilization of receptor oligomers such that *D* would be below the detection limit of an FPR measurement. However, the matrix model cannot provide an obvious explanation for the extensive redistribution of oligomers into visible patches nor can it explain the observation that cross-linking of receptors renders them insoluble in non-ionic detergents and associated with the insoluble cytoskeletal matrix (Baird et al., 1984, and Robertson, D., D. Holowka, and B. Baird, manuscript in preparation). These features demand more specific and permanent interactions between clustered receptors and immobilizing structures.

(c) Immobilization occurs because of attachments formed by receptor oligomers with a cytoplasmic or integral membrane protein, X, which in turn is directly or indirectly linked to the cytoskeleton (Bourguignon and Singer, 1977). In one variation of this model X is mobile and contains two receptor binding sites, such that interaction of X with receptor monomers or dimers would produce only linear chains, whereas interaction of X with trimers and larger oligomers would result in the formation of branched lattices with the possibility of gel formation (DeLisi, 1979; Goldstein and Perelson 1984). In this case, if the X molecules bind to some immobile cytoskeletal structure in such a way that a lattice structure is required for the interaction, then only oligomeric receptor-X complexes would be immobilized and become detergent insoluble.

In a second variation of model c X is multivalent and stably attached to the cytoskeleton, so that the strength of the interaction of X with receptors would increase with the size of the receptor oligomers, and the stability of the whole complex would depend on surviving disruptive collisions with other membrane proteins. In this view, a dimer-X interaction would not be strong enough to survive for a sufficient length of time, but larger oligomers would associate tightly with X and thereby with the detergent insoluble cytoskeleton.

(d) Receptor immobilization occurs in specialized regions such as coated pits. The density of coated pits per cell (random distribution, ~ 0.2 pits/ μm^2 ; Stump, R. F., and J. Oliver, personal communication) and an oligomer diffusion coefficient of $D \sim 10^{-10}$ cm^2s^{-1} would permit diffusion to and trapping in coated pits within the observed upper limit period of ~ 1.5 min (Fig. 8; Goldstein et al., 1984). However, the total area of coated pits would have to accommodate enough A2-clustered receptors such that the immobilization is nearly complete (Fig. 4e), and these must be unable to internalize without the addition of a second antibody (Fig. 3). The absence of microscopically detectable clusters of fluorescent IgE under some conditions of immobilization is contrary to what would be expected if all of these receptors had been localized in coated pits (Pastan and Willingham, 1981). Furthermore, Furuichi et al. (1985) have noted that in the cold, antigen-cross-linked IgE-receptor complexes are not associated with coated pits and that this association occurs only after warming the cells to 37°C . Since immobilization of antigen-induced IgE-receptor clusters occurs at 4°C (Fig. 7C), it appears to happen to a large extent outside coated pits, i.e., receptors are immobilized at the positions on the cell surface at which they become part of a small cross-linked cluster.

Although available data favor model c described above, the details of the mechanism by which immobilization of clustered IgE receptors occurs remains to be elucidated, and to this end an investigation of the direct protein-protein interactions by the use of chemical cross-linking reagents is being undertaken. The rapidity, extent, and reversibility of the immobilization process are completely consistent with its being part of the cell triggering signal. From the results obtained in this and previous studies we would predict that functionally important interactions such as possible associations of the receptor with either Ca^{2+} channels (Mazurek et al., 1984) or GTP-binding proteins (Fernandez et al., 1984) occur in an immobilized complex on the cell surface. Interaction with the cytoskeleton or immobilization of cell surface receptors have been observed with B cells after cross-linking

of surface immunoglobulin (Dragsten et al., 1979; Braun et al., 1982) and also with neutrophils after chemotactic peptide binding (Jesaitis et al., 1984). Thus these events may be involved in a type of mechanism that is quite general in initiating cellular responses.

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