Clustering, Mobility, and Triggering Activity of Small Oligomers of Immunoglobulin E on Rat Basophilic Leukemia Cells

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Abstract. We have recently shown that small oligomers of IgE bound to univalent receptors for IgE on the surface of rat basophilic leukemia cells induce extensive aggregation of the receptors at 4°C into patches resolvable by fluorescence microscopy and that this does not occur with monomeric IgE (Menon, A. K., D. Holowka, and B. Baird, 1984, *J. Cell Biol.* 98:577–583). Here we use fluorescence photobleaching recovery measurements to show that receptor oligomerization by this means is accompanied by a dramatic reduction of receptor lateral mobility, and that this immobilization occurs even when the clustering is not microscopically detectable. Furthermore, the degree of immobility induced by a particular oligomer fraction from a gel filtration column correlates positively with its ability to trigger cellular degranulation, whereas receptors labeled with monomeric IgE have no triggering activity and exhibit typical membrane protein mobility. The slow, large-scale oligomer-induced clustering appears to be a long term consequence of earlier selective interactions that result in receptor immobilization, and this highly clustered state provides a competent, noninhibitory triggering signal resulting in cellular degranulation upon warming to 37°C. We conclude that even limited clustering of IgE receptors on rat basophilic leukemia cells induces interactions with other cellular components that constrain receptor mobility and eventually cause massive coalescence of the clusters. These primary selective interactions occurring at the level of receptor oligomers or small clusters of oligomers that result in immobilization may play a role in triggering cellular degranulation.

THE mobility and interactions of cell surface receptors and ligand-receptor complexes are of considerable interest in studies of the transduction and regulation of biological signals. These signals provoke a variety of cellular responses including the degranulation of mast cells, basophils, and the tumor analogue rat basophilic leukemia (RBL)[1] cells, which result in the release of mediators of immediate hypersensitivity. Degranulation is triggered in these cells by the aggregation of cell-surface Fc receptors for IgE by various cross-linking ligands. It is known that each of these receptors binds one molecule of IgE (Mendoza and Metzger, 1976; Schlessinger et al., 1976*b*) and that the receptors are laterally mobile (Schlessinger et al., 1976*b*; Wolf et al., 1980; McCloskey et al., 1984; and present work) and diffusely distributed on the cell surface (Sullivan et al., 1971; Lawson et al., 1975; Mendoza and Metzger, 1976; Schlessinger et al., 1976*b*; Menon et al., 1984) with a density of $\sim 10^3$ m^{-2} (Metzger, 1978). Small clusters of receptors formed by the binding of small oligomers of IgE appear to be sufficient to initiate the triggering sequence that leads to degranulation (Segal et al., 1977; Fewtrell and Metzger, 1980; Sobotka et al., 1981; Menon et al., 1984).

In recent studies (Menon et al., 1984) we observed that binding of small fluorescently labeled IgE oligomers (IgE_n, $n \leq 6$) to RBL cells at low temperatures (4–10°C) initially resulted in a uniform distribution of surface fluorescence, the receptor density being too high to resolve individual clusters. At higher temperatures (25–37°C) the receptor clusters were internalized, appearing as round, fluorescent spots within the cell boundary. If internalization was suppressed by continued incubation at low temperature, the diffuse cell-surface fluorescence initially observed was transformed over several hours into a pattern of patchy fluorescence in which clusters containing 10^3–10^4 receptors were clearly resolved. This large-scale clustering did not occur significantly with monomeric IgE. These results suggested that limited cross-linking of IgE receptors triggered a selective interaction between the oligomeric receptors and other cellular components. The large scale clustering is probably a long term manifestation of earlier interactions that may be important in signal transduction.

In this report we have continued our study of the cell-imposed constraints on oligomeric IgE bound to receptors on

[1] *Abbreviations used in this paper:* BSS, buffered salt solution; *D*, diffusion coefficient determined by FPR, $D_0 = 10^{-10}$ cm^2s^{-1}; DNP, dinitrophenyl; FPR, fluorescence photobleaching recovery; [^{3}H]5HT, [5-1,2-^{3}H(N)] hydroxytryptamine binoxalate; IgE_M, murine monoclonal anti-DNP IgE; *R*, percent recovery determined by FPR; RBL, rat basophilic leukemia; $RIgE_M$ and $RIgE_R$, forms of TRITC-labeled IgE; TRITC, tetramethylrhodamine isothiocyanate.

RBL cells. To test the hypothesis that selective cellular interactions with oligomeric receptors occur much earlier than that represented by the large scale clusters, we have used the method of fluorescence photobleaching recovery (FPR) to monitor constraints on receptor mobility before visible patches are formed. We have also explored the relationship between constrained oligomer mobility and degranulation activity.

Materials and Methods

Materials

Tetramethyl rhodamine isothiocyanate (TRITC) was obtained from Research Organics Inc. (Cleveland, OH). 5-[1,2-^{3}H(N)]hydroxytryptamine binoxalate ([^{3}H]5HT) was from New England Nuclear (Boston, MA), and cytochalasin D, 2-deoxy-D-glucose, and 2,4-dinitrophenyl (DNP)-L-lysine were from Sigma Chemical Co. (St. Louis, MO). Cytochalasin D was stored at 4°C as a stock solution (1 mg/ml, 2 mM) in dimethyl sulfoxide. Rat IgG was from Jackson Immuno Research Laboratories, Inc. (Avondale, PA). Murine monoclonal anti-DNP (IgE_M) was obtained and purified as previously described (Liu et al., 1980; Holowka and Metzger, 1982). Rat myeloma IgE (IR162, IgE_R) was a gift from Dr. Henry Metzger (National Institutes of Health). ^{125}I-IgE was prepared by the chloramine T method (Holowka and Baird, 1983a).

Cells

RBL cells (2H3 subline) were maintained in stationary culture (Barsumian et al., 1981; Taurog et al., 1979) and used 4–7 d after passage. Cells were typically grown adherent to 75-cm^2 flasks and harvested when confluent by treatment for 5–10 min with trypsin-EDTA (Microbiological Associates, Walkersville, MD). Trypsin activity was quenched by the addition of supplemented medium (Eagle's minimum essential medium with Earle's balanced salt solution, 10% newborn calf serum, 20 mM Hepes, pH 7.4), and the cells were washed by centrifugation and resuspension. If the cells were to be sensitized with IgE they were resuspended in supplemented medium at a concentration of 5×10^6–10^7/ml. For [^{3}H]5HT release assays they were suspended at 2.5×10^6/ml in a buffered salt solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 2–4% newborn calf serum, 20 mM Hepes, pH 7.4), and for microscopy or FPR measurements they were suspended at 10^7 ml in BSS after sensitization with IgE. In some experiments modifications to the BSS or supplemented medium were made as indicated.

IgE Binding

In a few experiments the receptors on RBL cells were saturated with monomeric IgE by the addition of a 5–10-fold molar excess of IgE and incubation at 37°C for 1–1.5 h. More typically for experiments involving oligomeric IgE and appropriate controls with monomeric IgE, binding was achieved in the course of two or three successive incubations at 4°C. Each step involved the addition of a 1–2-fold molar excess of bindable IgE monomer units followed by 1–1.5-h period during which the cells were rocked on a Labquake rocker (Labindustries, Berkeley, CA). In this way multivalent binding of oligomers was maximized (Menon et al., 1984). After the binding incubations the cells were washed by centrifugation and resuspension at 4°C in BSS. Using this protocol for sensitization cells first became available for experiments 4 h after the start of the binding. Binding in the presence of 10 mM sodium azide and 10 mM 2-deoxy-D-glucose, was carried out in 0.75–1 h steps at ambient temperature. For all other experiments, unless otherwise stated, cell suspensions were maintained at 4°C, and aliquots were removed for microscopy or FPR.

[^{3}H]5HT Release Assays

[^{3}H]5HT (15 μCi) was incubated overnight with cells growing adherent in culture. The cells were harvested as described and sensitized with IgE if required. For experiments concerned with the kinetics of [^{3}H]5HT release, cells were rapidly warmed to 37°C by immersion in a water bath. At each time point, a 100-μl aliquot was removed and quenched by the addition of 100 μl ice-cold BSS. 150 μl of the quenched aliquot was layered onto 200 μl phthalate oil (Segal and Hurwitz, 1977) in a microfuge tube and microfuged for 1.5 min. 100 μl of the supernatant was then added to 2.5 ml Aqueous Counting Scintillant (Amersham Corp., Arlington Heights, IL), vortexed, and counted in a Beckman LS 250 counter (Beckman Instruments, Inc., Palo Alto, CA). Total counts were determined by directly adding 100 μl of the quenched aliquot to scintillant and counting.

Dose-response curves were obtained using a microtiter plate assay, the wells of the plate containing [^{3}H]5HT loaded cells and dilutions of the IgE derivatives or antigens to be tested. The assay is described in detail elsewhere (Menon et al., 1984; Baird et al., 1983).

Fluorescence Conjugation

Rhodamine derivatives of IgE were prepared using a dialysis method described elsewhere (Holowka and Baird, 1983*b*; Research Organics Inc. Bulletin 95) and summarized here. IgE at a concentration 1–3 mg/ml (total 1.5–5 mg) was dialyzed overnight against borate-buffered saline, pH 9.3, at 4°C. TRITC (4 mg in 0.2 ml dimethyl sulfoxide) was added to the dialysis buffer (50 ml), and the reaction was allowed to proceed for 38 h. The buffer was then replaced by borate-buffered saline, pH 8, and the dialysis was continued for several days with regular changes of buffer. By matching of absorption spectra of rhodamine-IgE prepared by this method to the data of Selwyn and Steinfeld (1972), the average degree of conjugation was found to be rhodamine/IgE monomer, 2:1.

Characterization of Fluorescently Labeled IgE

Fluorescently labeled IgE preparations were chromatographed on a 1 cm^2 × 77 cm gel filtration column of Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) with a flow rate of 0.07 ml/min. The eluting buffer was Na^+/K^+ Hepes (135 mM NaCl, 5 mM KCl, 3 mg/ml gelatin, 3 mM NaN_3, 10 m Hepes, pH 7.4). 0.5-ml fractions were collected after the first 15 ml of eluent was discarded.

Gel filtration profiles for TRITC-labeled IgE (R-IgE_M and R-IgE_R) and for ^{125}I-IgE_M are shown in Fig. 1. R-IgE_R and ^{125}I-IgE_M appeared as reasonably narrow peaks corresponding to monomeric IgE, and peak fractions had no activity when assayed for their ability to trigger degranulation (not shown). In contrast, R-IgE_M preparations showed a certain degree of non-covalent aggregation, and molecular weight standards (Bio-Rad Laboratories, Richmond, CA) indicated aggregates of hexamer size or less. The stability of these aggregates was indicated by two criteria: (*a*) gel filtration chromatography of a single preparation gave almost identical profiles in runs one month apart; and (*b*) the ability of different fractions to trigger [^{3}H]5HT release was commensurate with their positions in the elution profile; this was true several months after the fractions had been collected (Fig 4*a*). Thus it appears that the TRITC modification by the procedure described above induces limited non-covalent aggregation of IgE_M, resulting in stable, functional, small oligomers of IgE_M. These

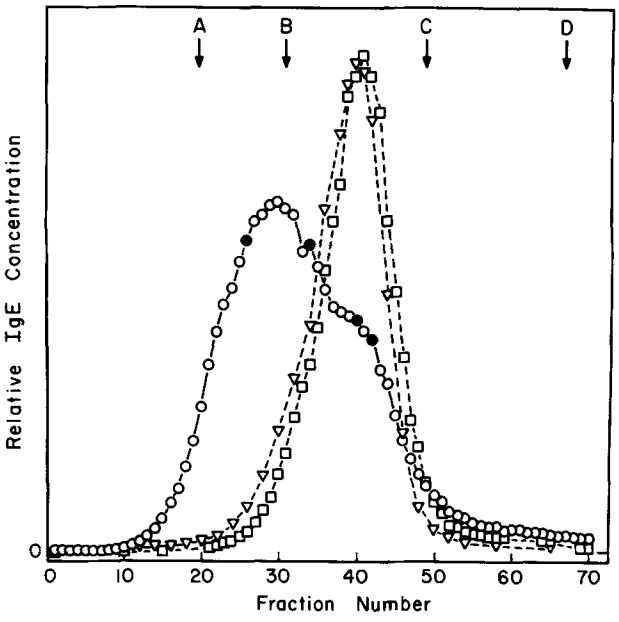

Figure 1. Sephacryl S-300 gel filtration chromatography of rhodamine conjugates of IgE_M (○) and IgE_R (▽). An elution profile for ^{125}I-IgE_M is shown for comparison (□). The arrows indicate molecular weight standards: *A*, protein aggregates (void volume); *B*, bovine thyroglobulin (670,000), *C*, bovine gamma-globulin (150,000); and *D*, chicken ovalbumin (44,000). IgE_M fractions used for FPR measurements (Fig. 3, *d–f*) are indicated by filled circles.

oligomers trigger degranulation (Fig. 4*a*) and can be internalized by RBL cells (not shown). These phenomena are not observed with R-IgE$_R$.

Other Fluorescent IgE Preparations

Several experiments were carried out with a TRITC-labeled IgE oligomer preparation (RIII) analogous to preparations characterized in earlier work (Menon et al., 1984). In brief, oligomers of IgE were prepared using the bivalent affinity cross-linker bis-2,4-DNP pimelic ester, purified by Sephacryl S-300 gel filtration chromatography, and fractions containing primarily dimeric IgE (~5% trimer impurity) were pooled and labeled with TRITC as described above. The rhodamine labeling induced limited, non-covalent aggregation to oligomers of hexamer size and less. This preparation was used without further fractionation.

Monomeric TRITC-labeled IgE$_M$ was prepared by vortexing TRITC (65 μl, 10 mg/ml in dimethyl formamide) quickly and gently with IgE$_M$ (5 ml, 2 mg/ml in borate-buffered saline, pH 8) and incubating at ambient temperature for 4 h. Free dye was removed by layering 500-μl aliquots onto 2.5-ml centrifuge columns (Penefsky, 1977) of Sephadex G-25 (equilibrated with the buffer, pH 8, and packed by centrifugation for 2 min at 200 *g*) and collecting the effluent after centrifugation at 200 *g* for 2 min. When Sephacryl S-300 chromatography of this preparation was carried out 2 yr after the modification the elution profile was similar to that shown for monomeric R-IgE$_R$ in Fig. 1, and peak fractions were used in all experiments described here. The degree of fluorophore conjugation was rhodamine/IgE monomer, 1.6:1.

Diffusion Measurements

The FPR technique has been described elsewhere (Webb et al., 1981; Bloom and Webb, 1983) and is summarized here. TRITC-labeled IgE was used for the experiments described, and rhodamine fluorescence was excited by the 514-nm line of an argon ion laser. The laser beam was first shaped into a Gaussian intensity profile by a spatial filter then split into monitor and bleach beams by multiple reflection within a quartz optical flat. Bleaching pulses in the 5–15:ms range were achieved by staggering the opening and closing times of two independent shutters in the path of the bleach beam. The bleach and monitor beams were recombined by a second matched optical flat with an anti-reflectance coating to reduce the monitor intensity to 1/1,000 that of the bleach beam. The combined beam was then focused through the vertical illuminator of a Zeiss Universal microscope and onto the cell sample via a dichroic mirror and a 100× oil immersion objective (numerical aperture, 1.3). Fluorescence was passed through a barrier filter and monitored by a photomultiplier tube with photon counting electronics. The e^{-2} radius of the illuminated spot was measured by a convolution scan method (Schneider and Webb, 1981) and was 0.55–0.65 μm. The monitor beam intensity was reduced by stationary neutral density filters introduced into the beam path: a 0.02–0.04-mW beam was used to focus the spot on a cell surface and was then rapidly attenuated to 1–9 μW for the photobleaching experiment. Bleaching of fluorescence while focusing was estimated to be <10%. Monitor beam intensities used for the actual FPR measurement caused no detectable bleaching. FPR curves of fluorescence intensity versus time before and after a bleaching pulse were stored, averaged, and analyzed by computer. Fluorescence recovery was monitored for 20–40 s and, unless stated otherwise, all measurements were made at ambient temperature. Most of the results reported here are based on FPR curves accumulated from five cells (one bleach/cell) and averaged. The five-cell averaged recovery curve was fitted according to Axelrod et al. (1976) to give R, the percent recovery (an indication of the mobile fraction of the fluorescently labeled species) and D, the diffusion coefficient. The shapes of the recovery curves were characteristic of diffusion processes: no indication of flow was seen except in the form of occasional "glitches" in diffusive recovery. Unless otherwise stated the data are presented as histograms, each block representing a five-cell average. Summarized data are given as mean $R(\bar{R}) \pm$ SEM, mean $D(\bar{D})D$, or $\overline{\log(D/D_0)} \pm$ SEM, where $D_0 = 10^{-10}$ cm^{2}s^{-1}.

Other features of these FPR experiments were as follows. Localized heating of the cell surface under the photobleaching conditions used should be less than a few tenths of 1°C (Axelrod, 1977). No systematic differences were detected in measurements made on round cells versus flattened cells. Nonspecific fluorescence (determined after cells were sensitized with TRITC-labeled IgE in the presence of excess unlabeled IgE) was similar to cell autofluorescence and <10% of the signal from labeled cells. Repeated bleaching of a given spot on a cell resulted in a systematic increase in the value of R as expected (Schlessinger et al., 1976*a*). Measurements at different spots on a given cell showed D and R values sufficiently disparate to account for much of the spread in the data over many cells. This contrasts with the observations of Edidin and Wei (1982) on the diffusion of H-2 antigens on mouse fibroblasts where it was found that the cell-to-cell variation in H-2 diffusion was greater than the diffusion measured at several sites on a single cell.

Results

Oligomer Clustering

The oligomeric IgE–induced large scale clustering of receptors on RBL cells that we described previously (Menon et al., 1984) and that occurs under conditions of low temperature with prolonged observation periods is probably indicative of an interaction between small receptor clusters and other cellular components that occurs long before the clusters grow large enough to be resolved by light microscopy. To gain insight into these interactions, the effects on large scale aggregation of various reagents and conditions were tested. The presence of 3.3 mg/ml rat IgG, which blocks the weak binding of IgE$_R$ to Fcγ receptors (Segal et al., 1981), did not affect oligomer (RIII) patching at 4°C relative to untreated control samples, indicating that only Fcε receptors were involved. Similarly, patching at 4°C was unaffected in the presence of 0.1 μM cytochalasin D, a microfilament disrupting drug, or in BSS prepared without Ca^{2+} and with 1 mM EGTA. The rate of oligomeric IgE patching at ambient temperature could be followed if the cells were maintained in BSS containing 10 mM sodium azide and 10 mM 2-deoxy-D-glucose, a combination of reagents found to inhibit internalization of oligomers (unpublished observations). This rate was found to be generally somewhat slower than the rate observed at 4°C with cells in the same buffer. (The patching rate at 4°C was about the same with or without azide and deoxyglucose.) This result suggests that the large-scale clustering is not diffusion limited, since the diffusion of monomeric IgE-receptor complexes at 22°, $\bar{D} = 3 \times 10^{-10}$ cm^{2}s^{-1} ($\overline{\log(D/D_0)} = 0.47 \pm 0.05$; Fig. 3*b*), is two- to threefold faster than at lower temperatures, e.g., $\bar{D}(10°C) = 1.3 \times 10^{-10}$ cm^{2}s^{-1}, ($\overline{\log(D/D_0)} = 0.12 \pm 0.10$, data not shown). Cells incubated at ambient temperature (with azide and deoxyglucose) and subsequently placed at 4°C appeared to patch faster than at ambient temperature. Cold treatment is known to cause depolymerization of microtubules (Dustin, 1978), and these results may reflect some control of the large-scale clustering by this class of cytoskeletal filaments.

Large-scale Clustering and Degranulation

The extent of large-scale, oligomer-induced clustering seen at 4°C was found not to affect the magnitude or the kinetics of degranulation assayed by measuring [^{3}H]5HT release after warming the cells to 37°C. Fig. 2*a* compares the rates of degranulation for cells exposed to oligomeric IgE$_M$ (RIII) for 4 h at 4°C then warmed to 37°C and for cells incubated for 4 h at 4°C without IgE then exposed to RIII immediately after warming to 37°C. The normalized curves superpose exactly, both showing a half-maximal release time of 15 min. The nearly identical rates seen with these two samples suggest that oligomer binding and clustering are not rate limiting in the triggering process. In Fig. 2*b*, cells incubated with RIII for increasing time at 4°C were sampled, warmed to 37°C, and assayed for [^{3}H]5HT release at the half-maximal time of 15 min (□, ◇). The kinetics of degranulation clearly remain unchanged with extended incubation at 4°C, although microscopically the percentage of cells displaying large patches of fluorescence increases to ~80% over the 4°C-incubation period examined. As expected, cells pretreated with monomeric IgE at 4°C (Fig. 2, ○) showed very little release after warming to 37°C unless a multivalent antigen (DNP$_{16}$ bovine gamma-

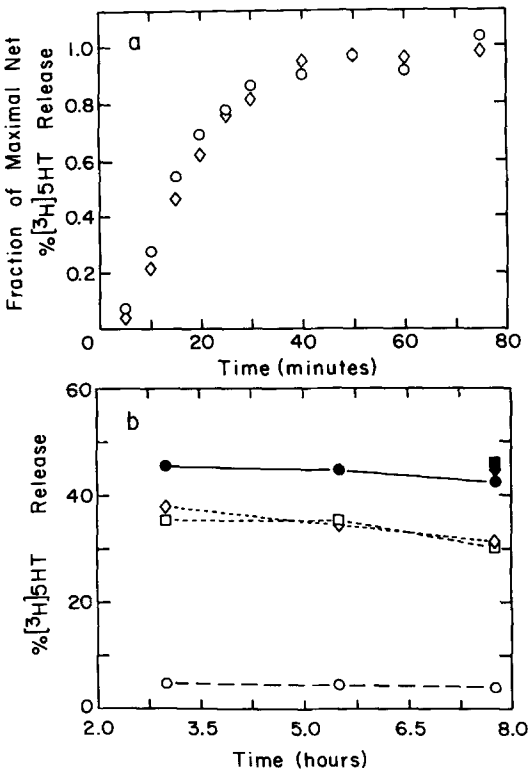

Figure 2. (*a*) Time course of [^{3}H]5HT secretion for cells incubated with RIII oligomers (see Materials and Methods) for 4 h at 4°C, then washed and placed into a 37°C bath at time = 0 min. ○, as compared with cells incubated for 4 h at 4°C, washed, then placed in a 37°C bath and exposed to RIII oligomers (5 μg/ml) at time = 0 min (◇). Maximal net percent [^{3}H]5HT release was 30% (○) and 25% (◇). (*b*) Cells were incubated with RIII oligomers (□, ◇) or monomeric IgE_M (○) at 4°C. At each time point, an aliquot of cells was removed, washed, then incubated at 37°C for 15 min to assay secretion kinetics. A multivalent antigen, DNP_{16} bovine gamma-globulin (0.75 μg/ml), was added to some aliquots (monomeric IgE_M-cells [●], RIII oligomers-cells (■, ♦) at the beginning of the 37°C incubation. Cell concentration was 5×10^6/ml.

globulin) was added (●). Maximal secretion, which occurs after 60 min at 37°C (Fig. 2*a*), was also unchanged, as demonstrated in another experiment (data not shown) in which cells incubated with oligomeric IgE_M at 4°C for various times up to 6 h were assayed for degranulation. Thus it appears that large receptor clusters formed at 4°C neither stimulate nor inhibit the magnitude and rate of oligomer-induced degranulation. In Fig. 2*b* it is of interest to note that despite the occurrence of considerable oligomer-induced patching, it was possible to enhance secretion with the addition of DNP_{16} bovine gamma-globulin (■, ♦). This was an enhancement of maximal secretion and not of its rate because the kinetics of antigen-induced secretion and oligomer-induced secretion are identical (data not shown).

Lateral Mobility of Receptors

The large scale clustering described above indicates selective interactions between oligomerized receptors and membrane-associated or cytoskeletal structures that do not occur with monomeric IgE-receptor complexes. These interactions must be initiated at the level at which the number of receptors cross-linked together is less than or equal to the number of Fc segments in the IgE oligomer and at which they are not detectable by fluorescence microscopy. We have employed FPR to monitor potential constraints or receptor mobility that occur at these initial stages. In prelimnary experiments we compared the diffusion of receptor-bound RIII oligomers and R-IgE_M monomers on the cell surface. Sensitized cells maintained at 4°C (oligomers) or ambient temperature (monomers) were sampled, and FPR measurements were made at ambient temperature within 10 min, during which time internalization was found to be negligible. Histograms of the diffusion coefficient (D) and percent recovery (mobile fraction, R) values for R-IgE_M monomers accumulated from multiple samples in several different experiments are shown in Fig. 3*b*. The mean values calculated from these data are $\overline{D} = 3 \times 10^{-10}$ cm^{2}s^{-1} and $\overline{R} = 84\%$, in agreement with previous measurements of monomer diffusion (Wolf et al., 1980). D in the RIII oligomer experiments (Fig. 3*a*) was similar to that obtained for monomers (Fig. 3*b*), but R for the oligomers was substantially lower than for monomers. Thus it appears that with bound RIII oligomers only a small fraction of receptors is free to diffuse but that these receptors diffuse with a D similar to that of monomeric IgE-receptors. Since the affinity of a single IgE molecule for its receptor is sufficient to bind oligomeric IgE to the cell (Metzger, 1978), the fact that some cell-bound RIII oligomers are not multivalently bound probably accounts for the fraction that is mobile. The fraction

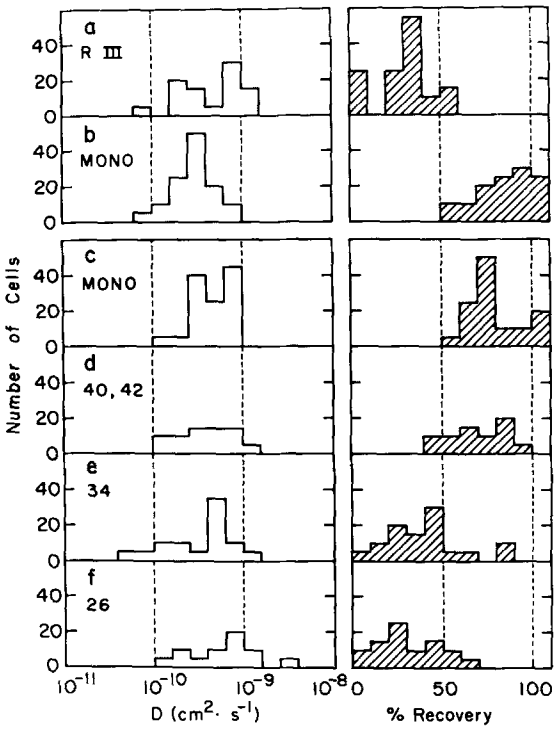

Figure 3. Histograms of diffusion coefficient (D) and percent recovery (R) values from FPR measurements on cells labeled with various IgE_M preparations as indicated. Five FPR measurements made on five different cells were averaged and numerous such averages were used to construct the histograms. Fraction numbers (*d–f*) refer to fractions indicated by filled circles in the gel filtration profile for rhodamine-labeled IgE_M shown in Fig. 1. MONO refers to monomeric R-IgE_M, and RIII refers to rhodamine-labeled, *bis*-DNP pimelic ester-reacted IgE_M oligomers (see Materials and Methods). In *a* and *c–f* cells were sensitized and maintained at ambient temperature. FPR measurements were made at ambient temperature.

classified as immobile by the FPR analysis has $D \leq 5 \times 10^{-12}$ cm^2s^{-1}.

For a more detailed study of oligomer diffusion we tested R-IgE_M oligomer fractions from the gel filtration profile shown in Fig. 1. As described above, cells sensitized with these fractions were maintained at 4°C, and samples were withdrawn for FPR measurements. Histograms of these measurements from a number of different experiments are shown in Fig. 3, *d–f*. Cells sensitized with R-IgE_M monomers and maintained under identical conditions were used as a comparison in each experiment (Fig. 3*c*). Several observations can be made from the results shown in Fig. 3 and related experiments:

(*a*) Comparison of Fig. 3, *b* and *c* shows that the diffusion of monomeric IgE-receptors on cells maintained at 4°C (Fig. 3*c*; $\overline{D} = 5.1 \times 10^{-10}$ cm^2s^{-1}; $\overline{\log(D/D_0)} = 0.71 \pm 0.04$; $\overline{R} = 77 \pm 3\%$) is different from those on cells maintained at ambient temperature (Fig. 3*b*; $\overline{D} = 3 \times 10^{-10}$ cm^2s^{-1}; $\overline{\log(D/D_0)} = 0.47 \pm 0.05$; $R = 84 \pm 3\%$) although FPR measurements were made at ambient temperature in both cases. Thus for monomeric IgE-receptors, pretreatment of cells in the cold appears to shift D to significantly higher values without obviously affecting R.

(*b*) Measurements on successive fractions (40-26), which correspond to oligomer size increasing very roughly from monomers to hexamers, show generally constant distributions for D (Fig. 3, *d–f*), similar to the monomer distribution (Fig. 3*c*). However, the R distributions shift markedly from a mean of 70% for the smaller oligomer fractions 40 and 42 to a mean of 30% for the larger oligomer fraction 26 (Fig. 3, *d–f*). These results are summarized in Fig. 4, *b* and *c* together with the measured ability of these fractions to trigger degranulation relative to IgE and multivalent antigen (Fig. 4*a*). Although maximal degranulation requires that only a fraction of receptors be aggregated whereas R reflects an average property of the entire population, a comparison of Fig. 4, *a* and *b* suggests that the triggering ability of these oligomers is directly related to their capacity to immobilize receptors.

(*c*) As observed by fluorescence microscopy (data not shown), the oligomer fractions used in the experiments in Fig. 3, *d–f* did not undergo the large-scale clustering described for RIII oligomers above and for other preparations elsewhere (Menon et al., 1984). Some patching could be seen with the fraction 26 oligomers, but cells sensitized with the other fractions, e.g., 34, were invariable ring-stained, even with extended (8 h) incubation at 4°C. This difference in the ability to form visible patches by the two different preparations may reflect either a larger average size for the RIII oligomers or a qualitatively different structure due to the bis-DNP pimelic ester cross-links. Thus immobilization of oligomerized receptors occurs even in the absence of detectable large-scale clustering. Furthermore, no systematic decrease in R with time was observed in FPR measurements involving either the oligomer fractions or RIII (data not shown). Immobilization of oligomerized receptors therefore occurs by the time the cells are first available for observation, and experiments described in the accompanying paper indicate that the immobilization occurs quite rapidly (≤2 min) upon receptor cross-linking.

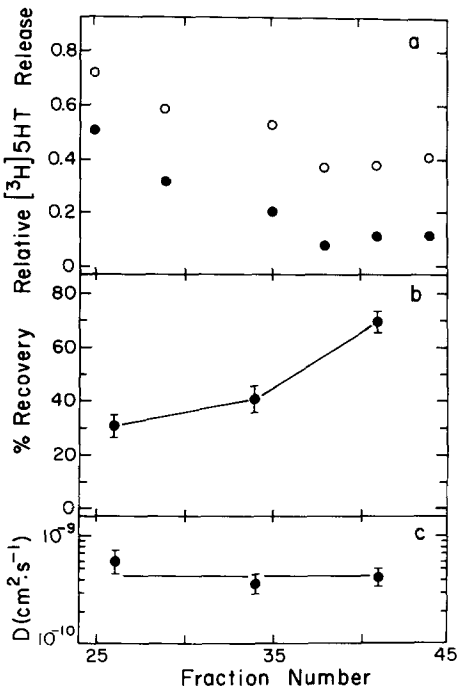

Figure 4. (*a*) [^{3}H]5HT release induced by 4 μg/ml (○) or 0.4 μg/ml (●) of various oligomer fractions from the gel filtration chromatography of rhodamine-labeled IgE_M shown in Fig. 1. The data are expressed relative to release induced by unlabeled, monomeric IgE_M + DNP_{16} bovine gamma-globulin. *b* and *c* show mean percent (R) and diffusion coefficient (D) values for fractions from the same preparation. These are indicated by filled circles in Fig. 1 and correspond to the histograms in Fig. 3, *d–f*. Bars represent SEM.

Discussion

Our results indicate that the oligomerization of IgE receptors by binding of oligomeric IgE triggers a constraining interaction between these receptors and other cellular components that does not occur with monomeric IgE-receptor complexes. The earliest manifestation of this interaction that we have observed is the immobilization (reduction in R) of receptor oligomers detectable by FPR measurements (Fig. 3). After extended times large aggregates may accrue from the immobile, oligomerized receptors and appear as patches under the fluorescence microscope (Menon et al., 1984). It is the initial interaction at the level of small receptor oligomers that appears to be fundamental to oligomer-triggered degranulation because (*a*) larger IgE oligomers cause smaller R and have greater degranulation activity (Fig. 4), (*b*) FPR measurements reveal immobilization of receptor oligomers whether or not subsequent clustering is extensive enough to be visible, and (*c*) the degranulation activity of patched cells is neither stimulated nor inhibited as compared with that of cells with receptors clustered to a smaller degree (Fig. 2).

Large-scale clustering of IgE receptor by small oligomers of IgE was our first indication of an interaction between the receptors and other cellular structures (Menon et al., 1984). We also noted that clustering did not occur on blebs, which are spherical protrusions of cell membrane that can be induced to form on RBL cells (Holowka and Baird, 1983*a*; Menon et al., 1984). Since blebs appear to be depleted of filamentous F-actin (Barak and Webb, 1982) and to contain highly mobile membrane proteins (Barak and Webb, 1982; Tank et al., 1982), these findings suggest a correlation between the absence of oligomer clustering and the absence of an intact cytoskeleton on blebs. The suggestion of cytoskeletal

involvement with receptor oligomers on normal RBL cell plasma membranes came from experiments in which we used the F-actin-specific fluorescent probe nitrobenzoxadiazole phallacidin (Barak et al., 1980) to investigate the distribution of actin in patched cells and found that in 40% of cells with clustered RIII oligomers the nitrobenzoxadiazole-phallacidin stain co-localized with the rhodamine-labeled oligomer patches (unpublished results), implying that actin can become associated with the large-scale clusters.

Our best evidence that an interaction between oligomerized receptors and other cellular components occurs is that there are striking effects on receptor diffusion as measured by FPR. We found the diffusion of IgE-receptor monomers to be typical of many membrane proteins with $D \sim 10^{-10}$ cm^{2}s^{-1} and $R \sim 85\%$. This result agrees with earlier FPR measurements (Wolf et al., 1980) and also with an electrodiffusion study using postfield relaxation to estimate D (McCloskey et al., 1984). The production of small oligomers of receptors upon the binding of IgE oligomers results in a reduction in D of more than two orders of magnitude and is observed as a low percent recovery. Thus, the mobile fraction for receptor oligomers (Fig. 3, *e* and *f*) is considerably smaller than that for receptor monomers (Fig. 3, *b* and *c*) and is nonzero presumably because of insufficient or incomplete oligomerization. In support of this interpretation, the diffusion coefficient for these residual mobile receptors (Fig. 3, *d–f*) is similar to that for receptor monomers (Fig. 3*c*). If the sole effect of binding IgE oligomers were to cross-link together small numbers of receptors to form a larger diffusing entity, then hydrodynamic models of diffusion in a lipid bilayer would predict a very weak (logarithmic) dependence of D on molecular size and not the substantial reduction that is observed (Saffman and Delbrück, 1975; Hughes et al., 1981; Vaz et al., 1982; Criado et al., 1982; Peters and Cherry, 1982).

In the accompanying paper (Menon et al., 1985) we describe studies that test the generality of receptor-cluster immobilization and its correlation with cell triggering by producing small receptor clusters by another method. In particular we have used a monoclonal anti-IgE antibody that has allowed receptor dimers to be distinguished from higher oligomers. We have also used multivalent antigen to investigate the rate and reversibility of the immobilization process. On the basis of all of the results several possible explanations for the observed immobilization are then considered in terms of the interaction of receptor clusters with other cellular components.

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