Small Oligomers of Immunoglobulin E (IgE) Cause Large-scale Clustering of IgE Receptors on the Surface of Rat Basophilic Leukemia Cells

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ABSTRACT We examined the distribution of small oligomers of IgE bound to rat basophilic leukemia cells using fluorescence microscopy. The oligomers were seen to cluster into visible patches on the cell surface at 4°C; at higher temperatures internalization also was observed. In contrast, cells labeled with IgE monomers remained predominantly ring-stained. Evidence is provided that the observed clustering of IgE oligomers is a cell-induced phenomenon, and the possible significance of this clustering is discussed in the context of the oligomer-triggered degranulation of rat basophilic leukemia cells.

The cross-linking of cell surface receptors for IgE triggers the degranulation of mast cells, basophils, and the tumor analog, rat basophilic leukemia (RBL)[1] cells. This cross-linking can be achieved by antireceptor antibodies (1, 2), oligomers of IgE (3, 4) or by reagents such as anti-IgE antibodies or multivalent antigen that cross-link receptor-bound IgE. Small oligomers of IgE suffice to trigger degranulation (3–5), and these have been considered to be the unit elements of the extensive aggregation induced by external cross-linkers such as multivalent antigen. Studies with fluorescently labeled monomeric immunoglobulin E (IgE) have shown that the receptor for IgE is univalent, partially mobile, and diffusely distributed on the cell surface (6, 7). This diffuse distribution is observed microscopically as a smooth, fluorescent ring stain and large-scale cross-linking of the IgE receptor complexes by anti-IgE or multivalent antigen transforms the smooth ring stain into a punctate distribution of fluorescence (6; unpublished observations). The binding of small oligomers of IgE might be expected to produce only small oligomeric clusters of receptors. However, other cell membrane-associated components could redistribute these small oligomeric clusters into larger aggregates of possible relevance in the context of the triggering signal for degranulation.

As a means of exploring the nature of the oligomer induced stimulation of RBL cells, the distribution of cell-associated, fluorescently labeled oligomers was studied as a function of time and temperature. The experiments reported here were motivated in part by the fluorescence and electron microscopic investigations of the redistribution of cell surface receptors on fibroblasts as a consequence of ligand binding (8). These include receptors for insulin, epidermal growth factor, α_2-macroglobulin, and low density lipoprotein. In many cases, it appears that diffusely distributed receptors bind ligand and the ligand-receptor complexes are then trapped in coated pits and subsequently internalized. Ligand-induced endocytosis via uncoated surface invaginations also has been reported (9, 10). The focus of many of these studies has been the endocytosis of ligand-receptor complexes, the lysosomal processing of these complexes, and receptor recycling. The clustering of ligand-receptor complexes, however, is of great interest in the context of the transduction and regulation of biological signals at cell surfaces. In this paper we describe and discuss the cell-induced clustering of small oligomers of IgE on RBL cells.

MATERIALS AND METHODS

Materials: Tetramethyl rhodamine isothiocyanate was obtained from Research Organics Inc. (Cleveland, OH), fluorescein isothiocyanate isomer I was from Molecular Probes Inc (Junction City, OR) and [5-1,2-^{3}H(N)]hydroxytryptamine binoxalate ([^{3}H]-5HT) was from New England Nuclear (Boston, MA). The bivalent affinity cross-linker *bis*-2,4-dinitrophenyl pimelic ester (BDPE) (11) was a gift from Dr. Paul Plotz (National Institutes of Health). The murine monoclonal anti-2,4-dinitrophenyl (DNP) IgE was obtained and purified as previously described (12, 13). ^{125}I-IgE was prepared by the chloramine T method (14).

Cells: RBL cells (subline 2H3) were maintained in stationary culture (15, 16) and used 4–6 d after passage. Cells typically were grown adherent to 75-cm^2 flasks and treatment for 5–10 min with trypsin-EDTA (Microbiological

[1] *Abbreviations used in this paper:* IgE, immunoglobulin E; RBL, rat basophilic leukemia; [^{3}H]-5HT, [5-1,2-^{3}H(N)]hydroxytryptamine binoxalate; BDPE, *bis*-2,4-dinitrophenyl pimelic ester; DNP, 2,4-dinitrophenyl; RI and RII, rhodamine conjugates of BDPE-reacted IgE monomers (I) and dimers (II).

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Associates, Walkersville, MD) released them into suspension. Trypsin activity was quenched by the addition of supplemented medium (Eagle's minimum essential medium with Earle's balanced salt solution, 10% new-born calf serum, 20 mM HEPES, pH 7.4) and the harvested cells were washed by centrifugation and resuspension.

If the cells were to be used for a [^{3}H]-5HT release assay, they were resuspended at a concentration of ~2.2 × 10^6/ml in a buffered salt solution (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 0.1% gelatin, 20 mM HEPES, pH 7.4). If the cells were to be saturated with IgE for microscopy, they were resuspended at a concentration of 10^7/ml in supplemented medium.

IgE Binding: The receptors on RBL cells typically were saturated with IgE in the course of two successive incubations at 4°C. Each step involved the addition of a one- to twofold molar excess of bindable IgE monomer units, followed by a 1.5-h period during which the cells were rocked on a Labquake rocker (Labindustries, Berkeley, CA). At the end of the second incubation, the cells were washed by centrifugation and resuspension at 4°C in the buffered salt solution. The cells were finally resuspended in the same buffer at a concentration of 10^7/ml. By this procedure, the cells first became available for microscopic observation ~4 h after the first incubation was begun. To observe the evolution of the distribution of cell-associated IgE, we continued to rock the washed cell suspensions at 4°C, ambient temperature, or 37°C, and periodically removed aliquots as indicated.

[^{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 above and aliquots of cells were added to wells in a U-bottom microtiter plate containing dilutions of the IgE derivatives to be tested. The plate was incubated at 37°C for 1.25 h and harvested using the Skatron Supernatant Collection System (Flow Laboratories, McLean, VA). A Beckman LS 250 counter (Beckman Instruments, Inc., Palo Alto, CA) was used to determine ^{3}H activity. The assay procedure is described in greater detail elsewhere (17).

Rabbit Anti-IgE: Rabbit anti-mouse IgE was produced and purified as previously described for rabbit anti-rat IgE (18), except that the affinity adsorbant was mouse IgE coupled to Biogel A5M (Bio-rad Laboratories, Richmond, CA) and anti-IgE was eluted with 3.5 M $MgCl_2$. Rabbit anti-mouse IgE was labeled with rhodamine as described below. Any residual free dye was removed by layering 250 μl aliquots onto a 2.5-ml centrifuge column (19) of Sephadex G-50 Fine equilibrated with borate-buffered saline, pH 8, and collecting the effluent after centrifugation at 200 *g* for 2 min.

Fixing: Cells were fixed at ambient temperature. 50 μl of a cell suspension (10^7 cells/ml) was dropped onto a 22 × 30 mm #2 coverslip placed in a petri dish, and the cells were allowed to adhere for 15 min before being exposed to 3.7% formaldehyde for 1 h. The cells were then washed and 75 μl of a solution containing ~0.3 mg/ml rhodamine-labeled, rabbit anti-IgE, and ~1.5 mg/ml bovine serum albumin was added. After 10 min, the cells were washed again and the coverslip was inverted and placed on a glass slide for microscopic observation.

Microscopy/Photography: A Leitz Ortholux II fluorescence microscope equipped with I2 (excitation band pass filter 450–490 nm, emission barrier filter 515 nm) and N2 (ex. 530–560 nm, em. 580 nm) cubes was used with a Leitz 50X water immersion fluorescence objective (NA = 1.00). Micrograph exposures were made on Kodak Tri-X film, which was then push-processed in Kodak D19 to ASA 1600. Prints were made on high contrast paper.

IgE Oligomers: Mouse IgE to which a trace amount of ^{125}I-IgE had been added, was cross-linked with BDPE by the following procedure: a freshly prepared solution of BDPE in dimethyl formamide was added to a solution of IgE (~6 mg/ml) in borate-buffered saline (0.16 M NaCl, 0.2 M borate, pH 8) such that the BDPE:antibody molar ratio was 5:1 and the dimethyl formamide concentration in the reaction mixture was about <5% by volume. The reaction was carried out for 30 min in the dark at ambient temperature (22°C) and quenched with a 1,000-fold molar excess of glycine. The mixture was dialyzed against borate-buffered saline overnight; the cross-linking procedure was then repeated and the mixture was again dialyzed. Under these conditions, BDPE has been shown to selectively cross-link anti-DNP antibodies via residues near their combining sites (11).

The oligomers were separated according to size by gel filtration chromatography on a 2 cm^2 × 100 cm column of Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) with a flow rate of 0.1 ml/min. The eluting buffer was Na^+/K^+ HEPES (135 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4). 2 ml fractions were collected after separately collecting the first 45 ml of eluent. Protein concentrations were determined from ^{125}I activity using a Beckman Gamma 4000 radiation spectrophotometer (Beckman Instruments, Inc.). The column was separately calibrated with gel filtration standards from Bio-rad Laboratories (molecular weight range 1,350–670,000). Based on this calibration, monomeric IgE runs at a molecular weight of ~235,000. Fractions were analyzed by SDS PAGE. Slab gels containing 5% (wt/vol) acrylamide were prepared by the method of Laemmli (20).

Several fractions corresponding to monomeric IgE on the calibrated column were pooled into one sample which ran as a single band on SDS PAGE gels. This sample is referred to as I. Other contiguous column fractions corresponding to small oligomers were pooled, refractionated, and analyzed by SDS PAGE. Of these, fractions containing predominantly dimeric IgE (undetectable monomer; dimer: larger oligomer about >1:0.04 by densitometer scans of SDS PAGE gels) were pooled. This preparation is referred to as II.

Fluorescence Conjugation: Rhodamine and fluorescein derivatives of IgE were prepared with tetramethyl rhodamine isothiocyanate and fluorescein isothiocyanate, respectively, using a dialysis method described elsewhere (21). IgE concentrations were determined by the method of Lowry et al. (22) using a correction factor of 0.8 in order to obtain results consistent with the extinction coefficient ($\epsilon_{280\,nm}^{0.1\%}$ = 1.62) reported for unmodified IgE (12); the molecular weight of IgE was assumed to be 184,000 (12). Absorption spectra were used to obtain approximate concentrations of bound fluorophore: fluorescein-IgE spectra were matched to the data of Mercola et al. (23) and rhodamine-IgE spectra were matched to the data of Selwyn and Steinfeld (24).

Rhodamine conjugates of BDPE-reacted IgE monomers (I) and dimers (II) are designated RI and RII, respectively. The average degree of conjugation in the rhodamine-labeled preparations was rhodamine: IgE monomer unit = 4.5:1 for RI and 3:1 for RII. The corresponding values for the fluorescein preparations were 9.5:1 and 6:1, respectively. A sample of BDPE-reacted dimeric IgE (II) that was labeled with fluorescein, was further modified with ^{125}I. 65% of these iodinated, fluorescein-labeled IgE dimers were capable of binding specifically to RBL cells.

RI and RII were fractionated by gel filtration chromatography as above. Rhodamine fluorescence was used to obtain the column elution profiles and unfractionated RI and RII were used to calibrate the fluorescence in terms of IgE concentration. The calibration procedure is complicated by the tendency of rhodamine moieties to stack dimerically to a nonfluorescent form (24). By matching the absorption spectra of the various eluted fractions to the data of Selwyn and Steinfeld (24), we found that for any given fraction the ratio of unstacked:stacked rhodamine differed by at most a factor of 2 from the average. Thus protein concentrations based on rhodamine fluorescence are in error by at most a factor of 2 assuming that the concentration of bound rhodamine is the same in all fractions. This error in protein concentration does not significantly affect the interpretation of the results presented here.

Characterization of Fluorescently Labeled, BDPE Cross-linked IgE Preparations: The IgE preparations were examined for structural composition. Initially the rhodamine and fluorescein derivatives of I and II had the same SDS PAGE patterns as I and II (see above), showing that the labeling procedures did not perturb covalent associations of the BDPE-reacted IgE. However, the cross-linking became partially reversed to a similar degree for all of the labeled and unlabeled samples of II during storage 4°C (10–2,000 μg IgE/ml in Na^+/K^+ HEPES) such that approximately half of the IgE changed from covalent dimers to monomers as shown by SDS PAGE analysis. The degradation took place within a month of the initial fractionation of the BDPE-reacted IgE and did not progress noticeably beyond that period.

The partial reversal of the covalent BDPE cross-linking in RII was counteracted by limited noncovalent aggregation. The tendency of the rhodamine-labeled preparations to undergo this self aggregation was revealed by fractionating RI and RII by gel filtration (Fig. 1, *a* and *c*). Molecular weight standards showed that IgE oligomers of pentamer size and larger would appear in the void volume of the column used and as seen for both fractionation profiles, RI (Fig. 1 *a*) and RII (Fig. 1 *c*), most of the fluorescence corresponds to aggregates of tetramer size and smaller. Both profiles are similar, although in comparison with RI, RII is somewhat shifted to higher molecular weights. The stability of the aggregation states contained in RI and RII was tested by assaying the ability of different fractions to trigger [^{3}H]-5HT release several weeks after the fractions were collected. As shown in Fig. 1, *b* and *d*, fractions containing predominantly tetramers induced significantly more release than those corresponding to smaller aggregates and, when tested together, tetramer fractions from RI and RII displayed identical abilities to trigger release (data not shown). The same trends were observed when release was potentiated with 30% D_2O (data not shown) (4). The differences observed between fractions are consistent with previous observations of the dependence of triggering ability on oligomer size (4). These results indicate that the noncovalent self-aggregation seen in RI and RII is limited and stable in solution and that extensive further aggregation of this type does not occur on the cell surface under the conditions of the release assay.

Somewhat different results were obtained from an analysis of a preparation of BDPE-reacted dimers and larger oligomers of IgE labeled with fluorescein. Fractionation of this fluorescein-labeled preparation on the same gel filtration column used for the rhodamine derivatives displayed a clearly separated IgE monomer peak reflecting partial reversal of the BDPE cross-linking. Also, there

appeared to be no shifting of the column profile to molecular weights larger than those present in the preparation before fluorescence modification (data not shown). Thus, fluorescein derivatives display little tendency to undergo noncovalent aggregation. Similar to the rhodamine derivatives fractionated fluorescein derivatives of higher molecular weight induced greater [^{3}H]-5HT release than fractions corresponding to lower molecular weights; fractions from the monomer peak caused no release (data not shown).

RESULTS

In preliminary experiments, BDPE cross-linked dimers of IgE were labeled with rhodamine and incubated with cells. Microscopic observation showed that under certain conditions, the pattern of cell-associated fluorescence changed progressively from a smooth ring stain to discrete patches. These IgE preparations were further characterized by SDS PAGE analysis and gel filtration chromatography (see Materials and Methods), and selected fractions were used in the experiments described below.

For microscopy, the cells were incubated with fluorescently labeled IgE preparations at 4°C as described in Materials and Methods and were available for initial microscopic observation ~4 h after the incubation was begun. In the experiments described, all the time periods given begin after this initial treatment. Different patterns of cell-associated fluorescence were observed. Cells with visible patches were distinguished by the characteristic punctuated appearance of their periphery (Fig. 2, *b* and *c*). The patches were typically quite large (a few micrometers when viewed edge on at the cell boundary) although finer punctate distributions (Fig. 2*c*, cell at bottom right) could also be seen. The large patches appear to be qualitatively different from the small numerous patches seen when fluorescently labeled, receptor-bound IgE monomers

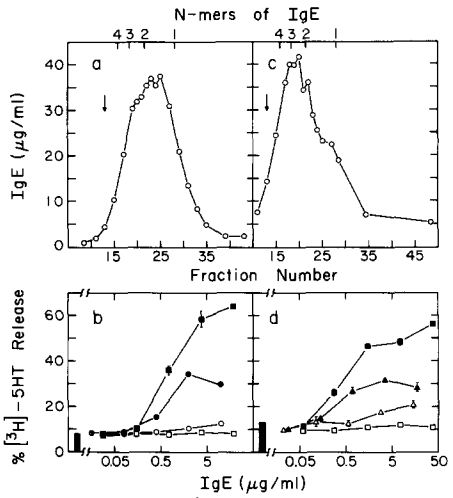

FIGURE 1 Analysis of rhodamine conjugates RI and RII of BDPE-reacted IgE monomers (I) and dimers (II). In the *upper* panels are shown elution profiles obtained by fractionation of (*a*) RI and (*c*) RII with gel filtration chromatography; the void volume is indicated by the arrows and the calibration shown of the number (*N*) of IgE monomer units that are oligomerically associated is based on molecular weight standards and the migration of IgE monomers. The *lower* panels show the [^{3}H]-5HT release from RBL cells elicited by various fractions taken from the elution profiles (*b*) RI, fractions 17 (●) and 29 (○) and (*d*) RII, fractions 17 (▲) and 23 (△). Also included are control samples showing release caused by unlabeled BDPE-reacted monomers (□) and these monomers in the presence of multivalent antigen, DNP_{16}-bovine gamma globulin (■). Spontaneous release with no IgE bound to the RBL cells is shown by the shaded bars. Each point represents the mean of duplicates with the range shown by the bars.

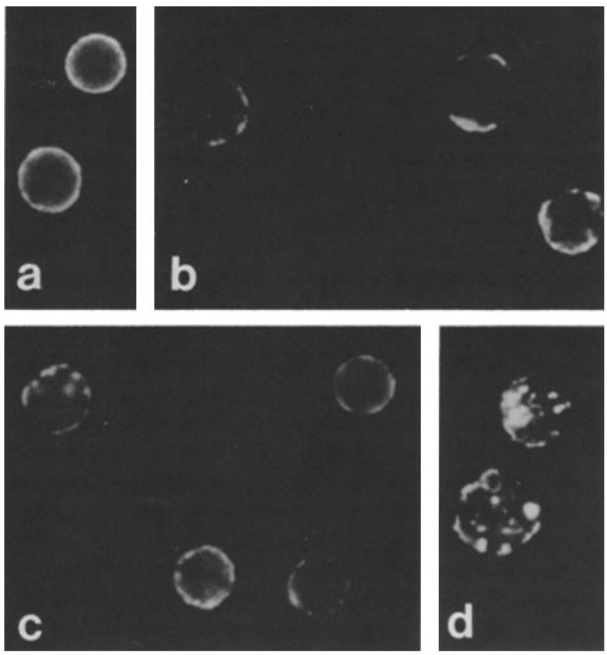

FIGURE 2 Fluorescence micrographs of RBL cells that had been labeled with tetramers of IgE (fraction 17, Fig. 1*a*) in the course of a 4-h period at 4°C. Time periods given begin after the labeling procedure. (*a*) Ring-stained cells at 0 h. (*b*) Patched cells after 2 h at 4°C. (*c*) Patched cells after 1 h at 22°C (the cell at top left shows some internalized fluorescence). (*d*) Cells displaying dramatic internalization after 7.5 h at 22°C. × 500.

are aggregated with external cross-linkers such as anti-IgE or multivalent antigen (not shown). The presence of round fluorescent spots within the boundary of the cell when that boundary was in focus was interpreted as internalized fluorescence (Fig. 2*d*). No capping was seen, although instances of surface fluorescence localized to opposite poles of a cell were observed (Fig. 2*b*, cell at top right). Evidence that the observed patching is largely confined to the cell surface is provided below.

Fig. 2 shows fluorescence micrographs of cells maintained at 4°C or ambient temperature (22°C) after incubation with a fraction corresponding to tetramers of IgE obtained from the fractionation of RI (fraction 17, Fig. 1*a*). The time-course of the evolution of the fluorescence patterns shown in Fig. 2 is included in Fig. 3, *b* and *d*. At the outset ~40% of the cells were ring stained and ~40% were patched, the remaining 20% being too dim to assess accurately. This pattern evolved at 4°C to one in which the great majority of cells were patched, the fraction of ring stained cells diminishing to <10% after 5 h (Figs. 2*b* and 3*b*). No internalization was seen over these extended periods of time at 4°C.

When the cells were placed at ambient temperature after incubation with rhodamine-labeled tetramers at 4°C, the pattern of events was different. Appreciable internalization (~40% of the cells) was seen after 3 h and the fraction of ring stained and patched cells each dropped to about <30% over this time (Fig. 3*d*; representative micrographs are shown in Fig. 2, *c* and *d*).

In distinct contrast to the behavior of cells incubated with rhodamine-labeled tetramers, cells incubated with lower molecular weight fractions containing primarily IgE monomers (e.g. fraction 30, Fig. 1*a*) were predominantly ring stained when first observed with <20% of the cells displaying a

patched appearance. This pattern remained roughly constant over extended periods at 4°C (Fig. 3*a*). At ambient temperature, some internalization was apparent but most of the cells remained ring stained (Fig. 3*c*).

Table I summarizes the results of further experiments at 4°C with fractions from the RI and RII column profiles (Fig. 1 *a* and *c*) as well as with the unfractionated preparations. Also shown in Table I is the effect of incubating the cells for 30 min at 37°C after they had been at 4°C for 5 h. Under these circumstances, cells saturated with rhodamine-labeled oligomers (Table I, A2, A3, and C1) displayed substantially more internalization than cells saturated with fractions containing primarily monomers (Table I, C2).

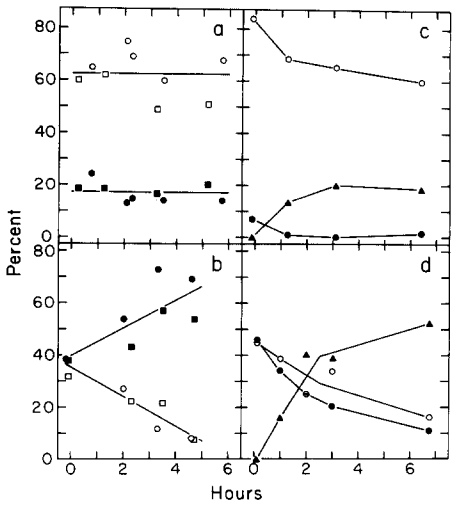

FIGURE 3 Time course of the evolution of cell-associated fluorescence under different conditions. (*a*) Cells were labeled with monomer fractions 29 (○, ●) and 30 (□, ■) from RI (see Fig. 1*a*) and incubated at 4°C. (*b*) Cells were labeled with tetramer fractions 17 (○, ●) from RI or 17 (□, ■) from RII (see Fig. 1*c*) and incubated at 4°C. (*c*) Cells were labeled with monomer fraction 30 from RI and incubated at 22°C. (*d*) Cells were labeled with tetramer fraction 17 from RI and incubated at 22°C. The percentages of ring-stained cells (○, □), patched cells (●, ■), and cells with internalized fluorescence (▲) were calculated after scoring 100–200 cells over many fields in the microscope. On the average, ~20% of the cells counted were too dim to assess accurately. Time periods given begin after the 4 h, 4°C labeling procedure (see legend to Fig. 2).

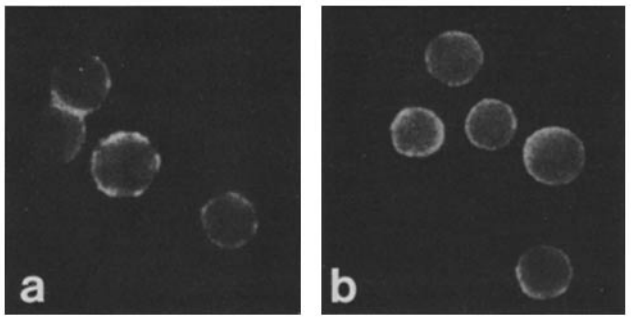

FIGURE 4 Distribution of unlabeled BDPE-reacted IgE (a) tetramers and (*b*) monomers on RBL cells. Cells were labeled with IgE preparations at 4°C for 4 h, and then maintained at 22°C for 1 h before being fixed with 3.7% formaldehyde and stained with rhodamine-labeled rabbit anti-IgE. Cells were scored as follows: monomer sample, 90% ring stained, 10% patched; tetramer sample, 50% ring stained, 50% patched. × 500.

Fractionated fluorescein-labeled IgE preparations behaved comparably to the rhodamine-labeled preparations on cells at 4°C (see Fig. 6*a*), ambient temperature and 37°C, with the notable exception that internalization was less pronounced (Table I; compare C1, C3). That this was not due to an inability to visualize internalized fluorescein fluorescence was shown by inducing internalization with multivalent antigen and observing the characteristic round fluorescent spots within the cell boundary.

To verify that the clustering of fluorescently labeled tetramers was not an artifact of the fluorescence modification, cells were incubated with unlabeled monomers and oligomers of IgE. Cell surface distributions of these unlabeled samples were visualized by using rhodamine-labeled rabbit anti-IgE antibody after fixing cells adherent to coverslips. Like the fluorescently labeled preparations, unlabeled oligomers were seen to cluster into patches (Fig. 4*a*) in contrast to unlabeled monomers (Fig. 4*b*).

Experiments also were carried out to investigate whether the BDPE modification caused the IgE preparations to undergo self-association on the cell surface. The possibility that

TABLE I

*Distribution of Cell-associated IgE**

		0 h, 4°C				+5 h, 4°C				+0.5 h, 37°C			
Experiment	Sample	R	P	I	D	R	P	I	D	R	P	I	D
A 1	R-IgE‡		—			78	17	0	5		—		
2	Unfractionated RII	57	26	0	17	16	67	0	17	2	22	71	5
3	Unfractionated RII§	60	20	0	20	31	56	0	13	0	21	62	17
B 1	Unfractionated RII	62	24	0	14	9	81	0	10	7	20	64	9
C 1	Tetramer‖(RI)	42	45	0	13	9	68	0	23	12	23	42	23
2	Monomer¶(RI)	60	19	0	21	51	20	0	29	66	0	18	16
3	Tetramer**(F)	39	48	0	13	25	59	0	16	25	40	15	20
4	Monomer**(F)	67	14	0	19	63	19	0	18	73	3	0	24
D 1	Tetramer‖(RI)	39	39	0	22	8	69	0	23		—		
2	Tetramer‖(RII)	32	38	0	30	8	54	0	39		—		

* Cells had receptors saturated with IgE preparations during a 4-h period at 4°C (see Materials and Methods); the indicated times and conditions proceeding from left to right *followed* this initial treatment. The percentages of total cells scored are classified under the following heading: *R*, ring stained; *P*, patched; *I*, internalized; *D*, dim/not determined. For all samples shown, $D = 18 \pm 7$ (mean ± SD).

‡ Monomers of IgE from a non-BDPE-reacted preparation, labeled with rhodamine and centrifuged before use to remove aggregates.

§ RII purified by passage over an anti-DNP affinity column.

‖ Fraction 17, Fig. 1 a or c as indicated.

¶ Fraction 30, Fig. 1 a.

** Fractions 16 (tetramer) and 28 (monomer) from the fractionation of fluorescein-labelled oligomers.

exposed, residual DNP groups on BDPE molecules interact with free IgE combining sites thereby causing self association, was ruled out for the following reasons. A sample of RII passed over an anti-DNP affinity column (see Materials and Methods) showed patterns of cell-associated fluorescence comparable to those obtained with untreated RII (Table I compare A2 and A3) and triggered [^{3}H]-5HT release equally well (not shown). Also, DNP-lysine (0.25 and 2.5 mM) did not disrupt patches of RII on the cell surface, although 0.1 mM DNP-lysine caused the dispersal of DNP_{16}-bovine gamma globulin induced patches of fluorescently labeled IgE monomers (not shown).

Further evidence that the clustering of oligomers on the cell surface is not caused by self-aggregation comes from observations made on cells with attached membrane blisters (blebs). These structures are depleted of filamentous F-actin (25), but contain highly mobile membrane proteins (25, 26), including the receptor for IgE (14). In all observed instances of cell-attached blebs, the blebs were ring-stained although generally not as bright as the cells. Importantly, ring-stained blebs were seen attached to clearly patched cells (Fig. 5). This is a significant observation, because if the patching was caused by self-aggregation, then it would also be expected to occur on blebs.

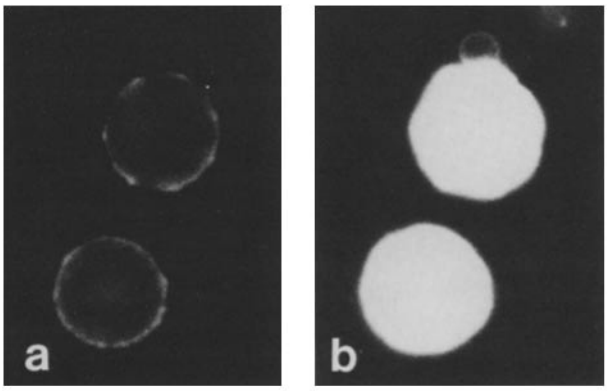

FIGURE 5 Cell labeled with unfractionated RII has patched fluorescence but a protruding membrane bleb is ring stained. (*a* and *b*) Different printing exposures of the same photographic negative. (*a*) Distinctly patched cells; the bleb attached to the top cell is too faint to be seen. (*b*) Ring-stained bleb attached to the top cell; the exposure obscures the cell-surface patches visible in *a*. By eye, patched cells with attached ring-stained blebs are easily observed simultaneously. × 625.

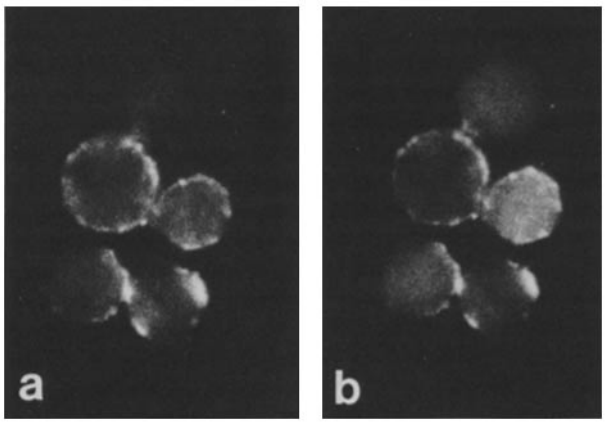

FIGURE 6 Cells with patched fluorescein-labeled IgE tetramers were fixed with 3.7% formaldehyde and then stained with rhodamine-labeled rabbit anti-IgE antibody. (*a*) Fluorescein fluorescence (Leitz I2 filter cube). (*b*) Rhodamine fluorescence (Leitz N2 filter cube). × 625.

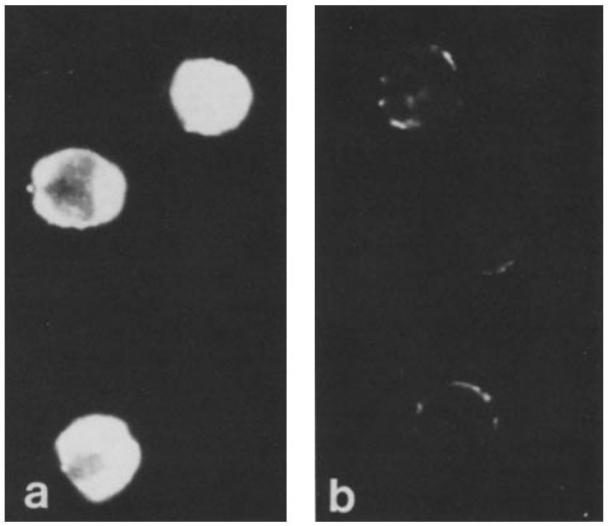

FIGURE 7 Cells having patched IgE tetramers (RI, fraction 17; see Fig. 1*a*) that were taken from the same sample and subsequently were (*b*) or were not (*a*) treated with a glycine buffer (pH 3) to dissociate surface receptor-bound IgE (27). *a* and *b* were photographed and printed with identical exposure times. *a* has the same photographic negative as Fig. 2*b* (rotated through 90°) where it is printed with a longer exposure. × 500.

Observed patches were shown to be largely confined to the surface of the cell by two distinct methods. When cells with patched, fluorescein-labeled tetramers were fixed and stained with rhodamine-labeled rabbit anti-IgE antibody, the pattern of fluorescein fluorescence (Fig. 6*a*) was seen to correspond exactly to the pattern of the rhodamine fluorescence (Fig. 6*b*). The procedure whereby receptor-bound IgE can be dissociated from the cell surface by resuspension of the cells in a pH 3 buffer (27) was also employed. When patched cells were exposed to this treatment, it was possible to dissociate the majority of the cell-associated fluorescence. This can be seen in the considerably lower fluorescence associated with the treated cells in Fig. 7*b* compared to untreated, patched cells in Fig. 7*a*. In contrast, internalized fluorescence (e.g., Fig. 2*d*) was not seen to diminish noticeably after the same treatment (data not shown).

DISCUSSION

The experiments presented here demonstrate that small oligomers of IgE bound to univalent receptors for IgE on RBL cells cluster into large patches under the apparent control of cellular components. The fraction of patched cells increases with time at 4°C (Fig. 3*b*) and under these conditions no internalization is seen. Internalization is observed, however, at higher temperatures (Fig. 3*d*, Table I) and this is consistent with the observations of Isersky et al. (28). The lack of characteristic internalization at 4°C and the accessibility of the patches to external agents (e.g., antibody, Figs. 4 and 6; low pH treatment, Fig. 7) provides strong evidence that the clustering observed occurs on the cell surface.

Evidence that the observed clustering of oligomers is a cell-induced phenomenon and not merely an artifact of the methods used is provided by the following experiments. (*a*) The possibility that BDPE oligomers self-associate on the cell surface via free, unreacted haptenic groups of the cross-linker

was eliminated since DNP-lysine did not disrupt patches and a preparation of oligomers passed over an anti-DNP affinity column clustered similarly to the untreated preparation (Table I, A2 and A3). Also, Segal and colleagues have investigated the binding (29) and internalization (30) of BDPE-cross-linked IgG (anti-DNP) oligomers on P388D$_1$ cells and have shown that oligomers made in this way do not self-associate on the cell surface. (*b*) Patching was observed on cells incubated with rhodamine-labeled oligomers (Fig. 2, *b* and *c*), fluorescein-labeled oligomers (Fig. 6*a*) as well as with unlabeled oligomers (Fig. 4*a*). Experiments carried out in parallel with column fractions containing mostly monomers (BDPE-reacted) revealed predominantly ring stained cells (Figs. 3, *a* and *c*, and 4*b*). Thus the phenomenon of oligomer clustering is independent of the method of visualization. (*c*) No patches were seen on fluorescent blebs attached to distinctly patched cells (Fig. 5). This was true of both rhodamine and fluorescein-labeled oligomers and may reflect the involvement of cytoskeletal architecture in cell-induced oligomer clustering (26).

The clearly resolved patches discerned on the cell surface correspond to very large scale aggregation of receptors. These patches are much larger than the small clusters expected for individual receptor-bound IgE oligomers which, given the high surface density of the receptors ($\sim 10^3\ \mu m^{-2}$; see reference 31), would be impossible to resolve in the light microscope. On the basis of estimates of the number of patches per cell (30–300) and the number of receptors (3×10^5), the patches correspond to clusters of 1,000–10,000 receptors. Since the oligomer preparations used apparently do not undergo self-aggregation on the cell surface (Fig. 1, *b* and *d*; Fig. 5), the large scale aggregation observed appears to be controlled by the cell, possibly mediated by cytoskeletal attachment of receptors.

Fractions corresponding to tetramers of IgE were used in the experiments described here in order to increase the percentage of multivalently bound species on the cell surface. For example, knowing that 65% of a dimer preparation is capable of binding to cells and assuming a statistical distribution (4), ~60% of cell bound tetramers are estimated to be multivalently bound, whereas only ~25% of bound dimers are multivalently bound. Also, in preparing cells for microscopic observation, multivalent binding of oligomers was maximized by performing two sequential incubations of cells with a concentration of oligomers in small molar excess of cell surface receptors (see Materials and Methods). The clustering of cell-bound IgE oligomers that is readily observed at 4°C under the conditions described here was not detected by autoradiography in a previous study (28) that documented cellular internalization at 37°C of dimethyl suberimidate-cross-linked dimers and trimers of rat IgE that were trace labeled with ^{125}I. In addition to the different method of visualization used, possible explanations for this apparent disagreement in observations include differences in the size of the oligomers and the degree of multivalent binding and differences in the structures of the oligomers resulting from the particular species of IgE used and the location of the chemical cross-links. The difference in temperature may also be important if rapid internalization occurring at 37°C prevents the accumulation of large, resolvable clusters at the cell surface (see Fig. 3, *b* vs *d*, and Table I, 4°C vs. 37°C). In their study, Isersky et al. (28) did observe some co-internalization of unoccupied receptors with IgE oligomers, and more recent studies indicate that receptor-bound monomeric IgE is co-internalized with oligomeric IgE (K. Furuichi and C. Isersky, personal communication). These results suggest that some type of cell-induced clustering of receptors occurs before or during the internalization process. Previous observations of co-capping of unoccupied receptors with IgE-receptor complexes bridged by anti-IgE on human basophils (32) are also consistent with the observations of nonexternally cross-linked receptor clustering described here.

The cell-induced redistribution of IgE oligomers may be related to the triggering signal for degranulation. The formation of visible patches involving large scale aggregation of oligomer-receptor complexes is presumably preceded by microclustering, and this could be the state required for triggering. Such an increase in the local density of receptors above a critical value has been proposed as a possible signaling event on theoretical grounds (33) and McConnell and colleagues (34, 35) have provided some experimental evidence to support this hypothesis by showing that RBL cells with receptor-bound IgE can be triggered to degranulate by lipid vesicles or monolayers containing laterally mobile monovalent haptens. Cross-linking of receptors by IgE and multivalent antigen or anti-IgE antibodies, or by antireceptor antibodies, also enhances local receptor density, but such large-scale external induction of receptor clustering would probably obscure the cell-mediated clustering process revealed in the experiments described here. Excessive cross-linking of receptors by anti-IgE to yield visible patches and caps is known to be accompanied by inhibition of degranulation (36), but whether this phenomenon occurs for the large-scale clustering stimulated by small oligomers of IgE has not yet been determined. The nature of the clusters that are constrained by external cross-linkers may be quite different from those directed by cellular components, and the cell-induced mechanism of clustering may discriminate between dimers and higher oligomers of IgE in such a way as to result in the previously shown differences in the magnitude of the triggered response (4). However, a direct relationship between cell-mediated receptor clustering and degranulation has yet to be demonstrated, so the possibility remains that clustering is merely a prelude to internalization and that triggering is accomplished by individual oligomeric units of receptors that are diffusely distributed on the cell surface.

Further studies will be necessary to determine more precisely the significance of the cell-induced clustering of small oligomers described here. Investigation of the kinetics of microcluster formation and of possible structural alterations in the receptor and its interaction with other cellular components will be of interest in this regard.

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