

ANTI-IMMUNOGLOBULIN-INDUCED HISTAMINE SECRETION
BY RAT PERITONEAL MAST CELLS
STUDIED BY IMMUNOFERRITIN ELECTRON MICROSCOPY*

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Most differentiated cells are influenced by a variety of chemical signals which bind to specific surface receptors. How such ligand-membrane interactions are transduced into intracellular signals which modify cell behavior is a central biological question. The degranulation and consequent release of histamine from mast cells when antigen or anti-immunoglobulin (Ig) antibody binds to cytophilic antibody (mainly IgE) bound to Fc receptors on the mast cell surface (reviewed in ref. 1), provides an unusually accessible model for studying this transducing function of the plasma membrane. Among the attractions of the peritoneal mast cell system are the relative ease of preparing purified single cell suspensions (2), the fact that degranulation follows within seconds of ligand binding (3), and that one can use labeled anti-Ig antibodies or antigen to follow the interaction of ligand with the relevant transducing membrane receptors during the signaling process. Blood basophilic leukocytes, which also release histamine following antigen or anti-IgE binding (1), as yet cannot be easily purified and require many minutes for maximal degranulation (4).

It has been demonstrated that for antibodies (5) or antigens (6-8) to induce degranulation of mast cells or basophils, they must be bi- or multivalent, indicating that the ligand must cross-link the IgE molecules to be an effective signal. The cross-linking could be necessary to induce a conformational change in the IgE molecules or the Fc receptors (or other associated molecules) to which they are attached, or could reflect a requirement for clustering of the receptors in the plane of the membrane, or both. Using fluorescein and ferritin-labeled ligands, Becker et al. (9) were able to show that extensive clustering or capping of IgE was not required for stimulation of human blood basophils.

In this study, we have used ferritin-labeled monovalent and divalent anti-Ig antibodies to study the ultrastructural distribution and induced redistribution of Ig receptors on rat mast cells at the same time as measuring histamine release. Our findings indicate that while divalent anti-Ig is required for histamine release and receptor redistribution, the maximum size of cluster that could be necessary for signaling is less than 10 Ig molecules.

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Materials and Methods

Cell Preparation. Male rats from a closed, random bred colony of the Lister hooded strain were sensitized by an intraperitoneal injection of 0.25 ml of a suspension of pertussis vaccine BP (Burroughs Wellcome, London) (8×10^8 organisms/ml) in a solution of ovalbumin in saline (50 mg/ml). 15–30 days later a cell suspension, containing 2–5% of mast cells, was obtained by peritoneal lavage with isotonic saline containing heparin (25 IU/ml).

Mast cells were purified to better than 80% by centrifugation through a discontinuous density gradient of human serum albumin (HSA).¹ The HSA (Kabi, A. B., Sweden) was dissolved in distilled water and desalted and concentrated by exhaustive filtration on a 76 mm PM10 filter in an Amicon pressure filtration apparatus (Amicon Corp., Scientific Sys. Div., Lexington, Mass.), cleared by centrifugation (50,000 *g* for 20 min), freeze-dried, and redissolved in distilled water at 40 g/100 ml. The solution was adjusted to 300 mOsm by adding concentrated NaCl solution under control by freezing-point osmometry, and the solution stored at -20°C in 2-ml lots. Up to 8 ml of a cell suspension containing the peritoneal washings of up to six rats were layered over an HSA gradient (1 ml of 40 g/100 ml and 1 ml of 30 g/100 ml) in cellulose nitrate tubes (Beckman 331370, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and centrifuged at 180 *g* for 5 min. The tube was pierced and the lower albumin layer, containing the mast cells, was collected and taken up in about 20 ml of Ca^{2+} -free Tyrode's solution (NaCl, 137 mM; KCl, 2.7 mM NaH_2PO_4 , 0.4 mM; MgCl_2 , 1.0 mM; glucose, 5.6 mM NaHCO_3 , 12 mM). They were washed once by centrifugation and resuspended at $2\text{--}5 \times 10^6$ cells/ml in Ca^{2+} -free Tyrode's solution.

Antibodies. Sheep antirat-Ig (S anti-RIg) was prepared by immunizing a sheep with 2 mg of rat IgG (prepared by fractionation on DEAE-cellulose) in Freund's complete adjuvant at multiple subcutaneous sites twice in 2 wk and bleeding 4 wk after the second immunization. The S anti-RIg serum was fractionated on DEAE cellulose after precipitation with 40% saturated ammonium sulphate. The fraction which was not adsorbed to DEAE cellulose in 0.02 M phosphate buffer at pH 7.3 was dialyzed against phosphate-buffered saline and used in these experiments. 25% of this protein could be adsorbed by rat IgG which was covalently linked to Sepharose, and the protein which did not adsorb was inactive in releasing histamine from sensitized mast cells (Fig. 1). Monovalent Fab fragments of the S anti-RIg (Fab anti-RIg) were prepared as previously described (10), by digestion with papain and separation on Sephadex G-100. Before conjugation with ferritin, the Fab anti-RIg was passed over G-100 twice and centrifuged for 1 h at 200,000 *g* to remove aggregates. Rabbit antiferritin antibody (R anti-FT) was raised by repeated subcutaneous injections of 2 ml of horse spleen ferritin (Koch-Light) (1 mg/ml), emulsified with an equal volume of Freund's complete adjuvant.

Ferritin Conjugates. S anti-RIg antibodies and their Fab fragments were conjugated to ferritin (Calbiochem, San Diego, Calif.) with glutaraldehyde as previously described (11, 12). The concentration of ferritin in the conjugates was 27 mg/ml for the S anti-RIg and 12 mg/ml for the Fab anti-RIg-FT (measuring optical density at 440 nm and correcting the optical density of the Ig at this wavelength), giving Ig concentrations of 5–6 mg/ml and 0.5–1 mg/ml respectively, assuming the ferritin and Ig were present in equimolar ratios in the conjugates. The conjugates were used at a final concentration of 600 μg S anti-RIg and 250 μg Fab anti-RIg/ml.

Histamine Release and Assay. Purified mast cells were incubated without Ca^{2+} , in the presence of the appropriate anti-Ig reagent for 30 sec or 30 min at 37°C , after which they were sampled for a further 10-min incubation with and without Ca^{2+} (1.8 mM). The cells were then sedimented and the supernate assayed for histamine by the enzyme-radioisotopic (double label) microassay of Snyder and Taylor (13). The histamine assay depends on the enzyme-catalyzed transfer of the ^{14}C -methyl group of L-[methyl- ^{14}C]-S-adenosyl methionine to the histamine, to which a trace of [2,5- ^3H]histamine has been added. The concentration of histamine in the sample is proportional to the ratio $^{14}\text{C}/^3\text{H}$ in the product (1-methyl-histamine), which is isolated from the reactants by extraction into chloroform. The concentration of histamine in the samples, and the fraction of histamine released from the cells was calculated by the use of a simple computer programme. In experiments where immunoferritin electron microscopy was done on the same cells, these were sampled at 30 sec or 30

¹ Abbreviations used in this paper: Fab anti-RIg, monovalent Fab fragments of sheep antirat immunoglobulin antibody; FT, ferritin; HSA, human sheep albumin; R anti-FT, rabbit antiferritin antibody; S anti-RIg, sheep antirat Ig antibody.

min, and fixed by the addition of 6 ml of cold Ca^{2+} -free glutaraldehyde (3% in 0.1 M Na cacodylate buffer pH 7.3) and processed as detailed below.

Immunoferritin Electron Microscopy. Cells were fixed in 3% glutaraldehyde for 30 min at 0°C and 90 min at room temperature, washed, postfixed in 2% Na cacodylate-buffered osmium tetroxide, washed, resuspended in 0.5% aqueous uranyl acetate, washed and resuspended in two drops of warm (47°C) 2% agar, centrifuged in the warm and then cooled. The agar-embedded cell pellet was cut into 1–2 mm³ pieces, which were dehydrated in ethanol followed by propylene oxide, and embedded in araldite. Thin sections ($\sim 700 \text{ \AA}$) were sometimes stained with lead citrate followed by uranyl acetate and examined with an AEI-6EMB electron microscope at 60 kV. Quantitative analysis of the ferritin binding and clustering was carried out by examining enlarged prints of 10 randomly selected cells from each specimen, sectioned close to their greatest diameter, under a dissecting microscope. In this way, a band, approximating to 0.4% of the cell surface, was observed. Ig-FT micules were counted as "singlets" when the distance separating them from their neighbors exceeded the diameter of a single ferritin molecule (80–100 \AA in these experiments).

Results

Requirement for Divalency of Anti-RIg for Triggering of Histamine Secretion. Dose-response curves for the calcium-dependent release of histamine induced by ferritin-conjugated and unconjugated S anti-RIg reagents are shown in Figs. 1 and 2. Compared to unconjugated S anti-RIg, the S anti-RIg-FT showed reduced activity, indicating some inactivation of the antibody during the conjugation procedure. As has been reported for histamine release in basophils (9, 14), monovalent Fab anti-RIg induced some histamine secretion, but only at very high concentrations, requiring approximately 20 times the concentration of S anti-RIg to induce comparable secretion. In view of the tendency for Fab

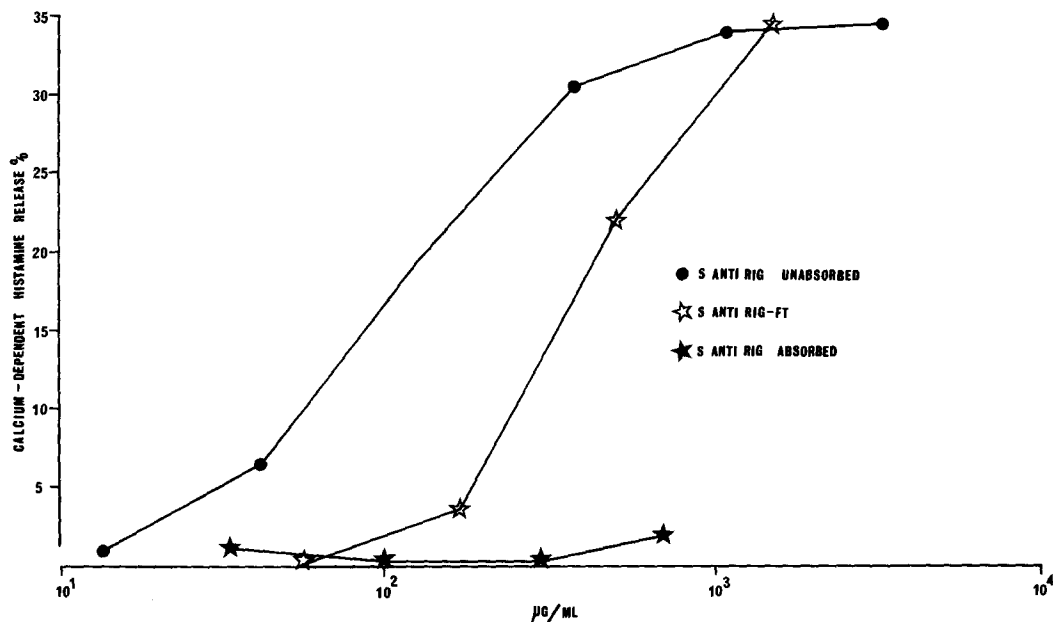


FIG 1. Dose-response curves for divalent S anti-RIg. Ca^{2+} -dependent ligand-induced histamine release is expressed as percent of the total cell histamine content. Calcium-independent release was in the range 1–6% and was not significantly different from spontaneous release. Absorption of S anti-RIg was with rat IgG linked to Sepharose.

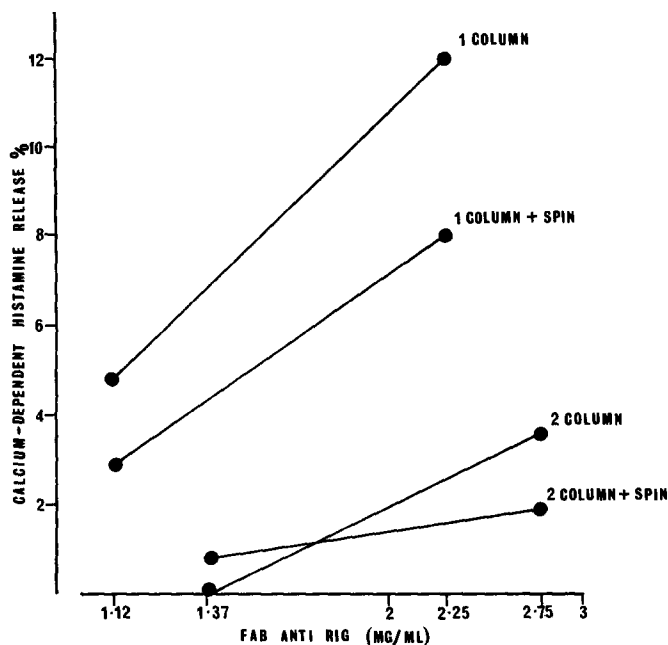


Fig. 2. Dose-response curves for monovalent Fab anti-RIg, showing progressive reduction in histamine release as a consequence of treatments designed to remove aggregates.

fragments to aggregate, the Fab preparation was re-run over G-100 Sephadex and centrifuged at 200,000 g for 60 min before testing and conjugating to ferritin. As seen in Fig. 2, this procedure eliminated the histamine-releasing activity of the Fab anti-RIg.

In a single sample treated with divalent S anti-RIg in the presence of Ca^{2+} it could be seen that some cells were not visibly altered (Fig. 3) while others had almost completely degranulated (Fig. 5). Most cells in such samples were partially degranulated (Fig. 4). Even when cells were treated with S anti-RIg for much longer periods (up to 10 min), most cells were only partially degranulated.

Failure of Fab Anti-RIg Triggering is Not Explained by Reduced Binding. In Table I, the numbers of molecules of S anti-RIg-FT and Fab anti-RIg-FT bound to mast cell sections after 30 sec and 30 min are tabulated together with the fraction of histamine released and the extent of degranulation seen in the same experiment. It can be seen that the failure of Fab anti-RIg-FT to induce secretion is not related to decreased ligand binding. Thus, whereas an average of 72 divalent molecules bound per cell section at 30 sec were sufficient to produce marked degranulation and histamine secretion, 86 monovalent molecules bound at 30 sec had no effect.

Distribution of Monovalent and Divalent Anti-RIg-FT on Mast Cell Surface. The distribution of the ferritin conjugates on the surface of mast cells is tabulated in Table I. When Fab anti-RIg-FT was used, 90% of the bound ferritin was distributed as single molecules, 6-8% as doublets, 1% as triplets and 0.1% as groups of four (rarely five) and no pinocytosis was seen. This distribution was unchanged at 30 min, in spite of a much greater degree of binding (Fig. 6). This

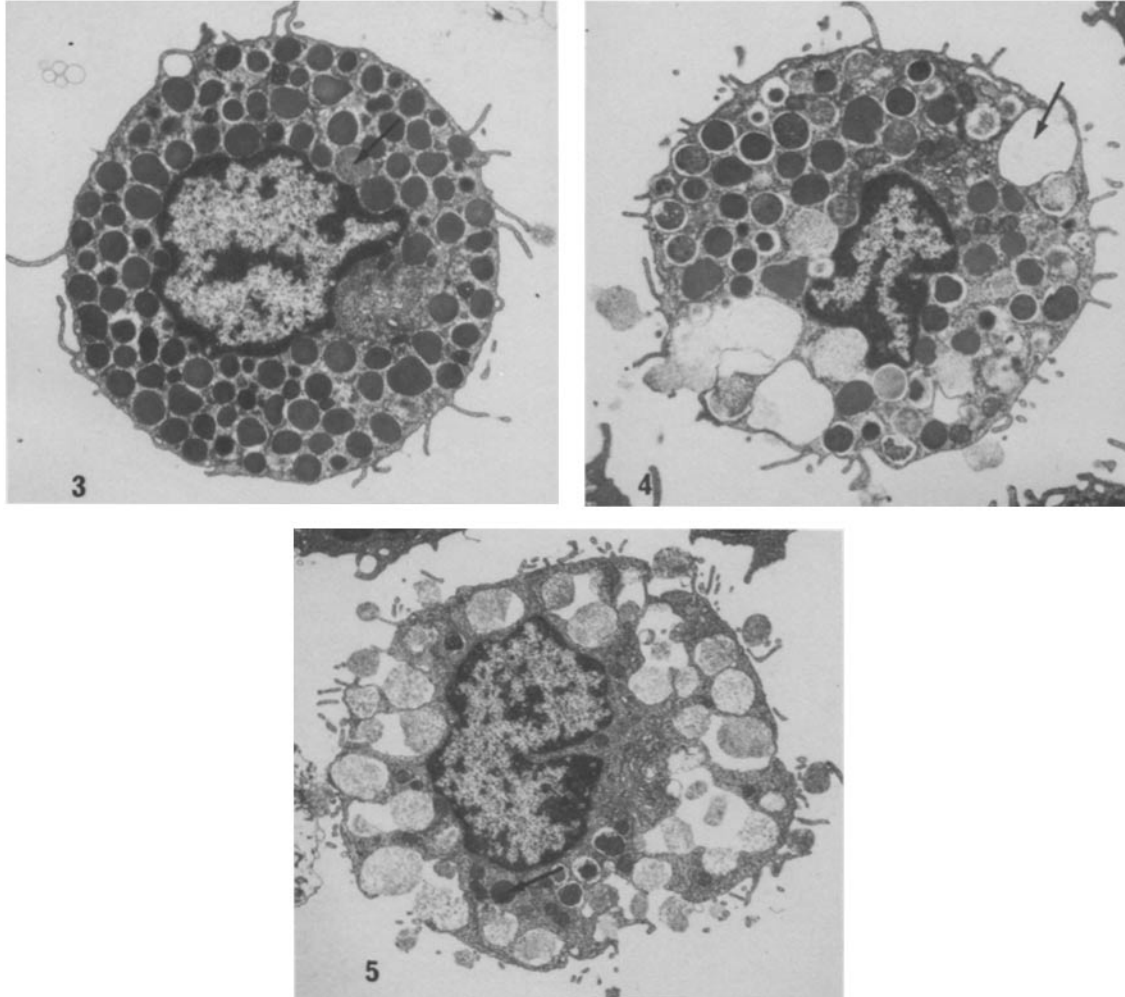


FIG. 3. Mast cell treated with S anti-RIg-FT in the presence of 1.8 mM Ca^{2+} for 0.5 min at 37°C. Only one granule (arrow) appears to have released its histamine. Lead-stained section. $\times 6,250$.

FIG. 4. Partially degranulated mast cell from the same sample as Fig. 3. Whereas some of the granules have released their histamine (arrow), many have not. Lead-stained section. $\times 7,500$.

FIG. 5. Mast cell from the same sample as Figs. 3 and 4 with only one intact granule (arrow). Although the cell is almost fully degranulated the rest of the cell organelles (Golgi, mitochondria) appear intact. Lead-stained section. $\times 6,250$.

suggests that the surface receptors [mainly IgE, see below) are randomly distributed, that up to four, or at most five, molecules of Fab anti-RIg-FT can bind to a single Ig molecule (15)], and that the Fab anti-RIg-FT cannot redistribute the surface receptors in the plane of the membrane. These results are in agreement with observations previously reported for basophils (9, 14, 16) and lymphocytes (10, 12).

30 seconds after addition of divalent S anti-RIg-FT, a time when maximal

TABLE I
Distribution of Monovalent and Divalent Anti-Rlg-FT and Induced Degranulation and Histamine Release

Ligand	Ca ²⁺ present	Time	FT molecules per cell section*	Distribution of FT molecules in clusters of:						Pino-cytosis	Granules released*	Histamine secretion
				One	Two	Three	Four	Five	Six			
250 µg Fab anti-Rlg-FT/ml		<i>min</i>		%	%	%	%	%	%	%	%	
Fab anti-Rlg-FT	0	0.5	86 ± 5	92 ± 6	6 ± 1	0.7 ± 0.3	0.1	0	0	0	5 ± 2	2.8
Fab anti-Rlg-FT	+	0.5	—	—	—	—	—	—	—	—	—	1.9
Fab anti-Rlg-FT	0	30.0	292 ± 13	90 ± 4	8 ± 0.7	1 ± 0.2	0.2 ± 0.1	0.1	0	0	4 ± 1	7.8
Fab anti-Rlg-FT	+	30.0	—	—	—	—	—	—	—	—	—	6.4
600 µgS anti-Rlg-FT/ml				%	%	%	%	%	%	%	%	
S anti-Rlg-FT	0	0.5	133 ± 13	75 ± 8	13 ± 1	7 ± 0.7	2 ± 0.6	0.7 ± 0.4	0.6 ± 0.1†	0	1 ± 0.6	3.0
S anti-Rlg-FT	+	0.5	72 ± 7	74 ± 8	19 ± 1	4 ± 0.5	1 ± 0.5	0.8 ± 0.2	0	0	35 ± 8	27.0
S anti-Rlg-FT	0	30.0	327 ± 32	60 ± 7	20 ± 2	11 ± 1	5 ± 0.6	1 ± 0.3	0.3 ± 0.6§	+	7 ± 2	—

* Expressed as mean ± sd of 10 different cells.

† On 10 cells, one cluster of eight, nine clusters of seven FT molecules were seen.

§ On 10 cells, 67 clusters of >6 (7-18) FT molecules were seen.

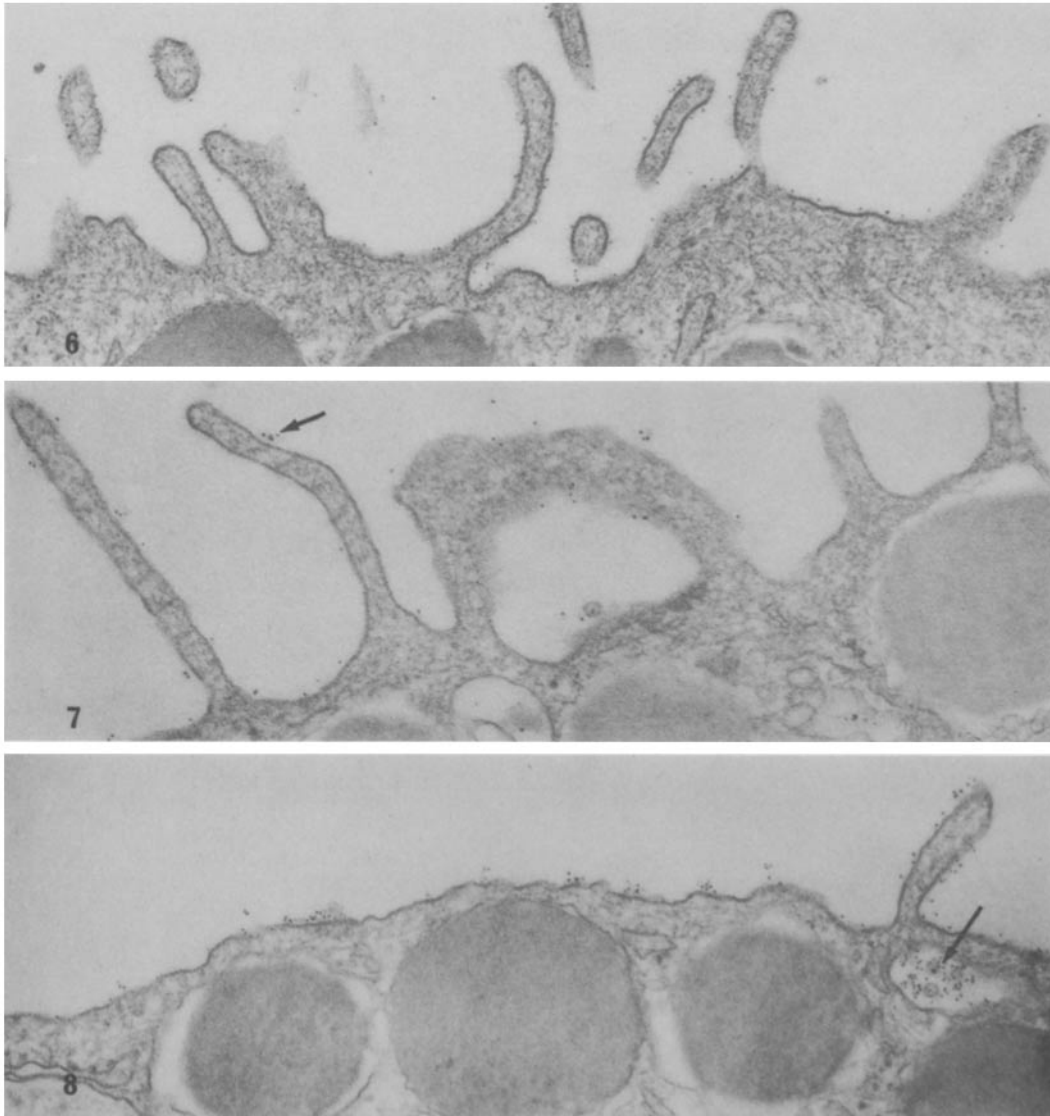


FIG. 6. Mast cell treated with Fab anti-R1g-FT for 30 min at 37°C. Most of the FT molecules are singlets with the occasional doublet and triplet. Section stained with uranyl acetate. $\times 58,000$.

FIG. 7. Mast cell treated with S anti-R1g-FT for 0.5 min at 37°C in the absence of Ca^{2+} . Although most of the FT molecules are singlets, doublets or triplets, one cluster of five molecules is seen (arrow). No pinocytosis is seen. Unstained section. $\times 66,000$.

FIG. 8. Mast cell treated with S anti-R1g-FT in the absence of Ca^{2+} for 30 min at 37°C. Note the clusters of FT molecules (up to eight in one cluster shown here), and the presence of a pinocytotic vesicle (arrow). Section stained with uranyl acetate. $\times 60,000$.

histamine release should be occurring (2), 75% of the ferritin was still seen as single molecules, about 15% as doublets, 5% as triplets, 1% as quadruplets and there were a few clusters of five or more ferritin molecules (Table I, Fig. 7). In 10 cells surveyed, the largest cluster contained eight ferritin molecules, and only one such cluster was seen, while there were nine clusters of seven ferritin molecules. After 30 min at 37°C, 60% of the ferritin molecules were still present as singlets, with an increased fraction and size of clusters, and some pinocytosis (Table I, Fig. 8). After 4 h at 37°C (Fig. 9) much more impressive patching and pinocytosis was seen (but this was still less than the extent of patching seen on lymphocytes after a few minutes (12)) and no capping was ever observed. The failure of the mast cells to cap after 4 h at 37°C was confirmed by examining over 500 cells by immunofluorescence, using S anti-RIg-fluorescein. Massive clustering and pinocytosis could be induced if mast cells were labeled first with S anti-RIg-FT for 30 min at 37°C followed by anti-FT (1/100) for a further 15 min at 37°C (Fig. 10); but even here, capping was not seen. These results suggest that divalent anti-RIg-FT minimally redistributes mast cell Ig into clusters after 30 sec, and

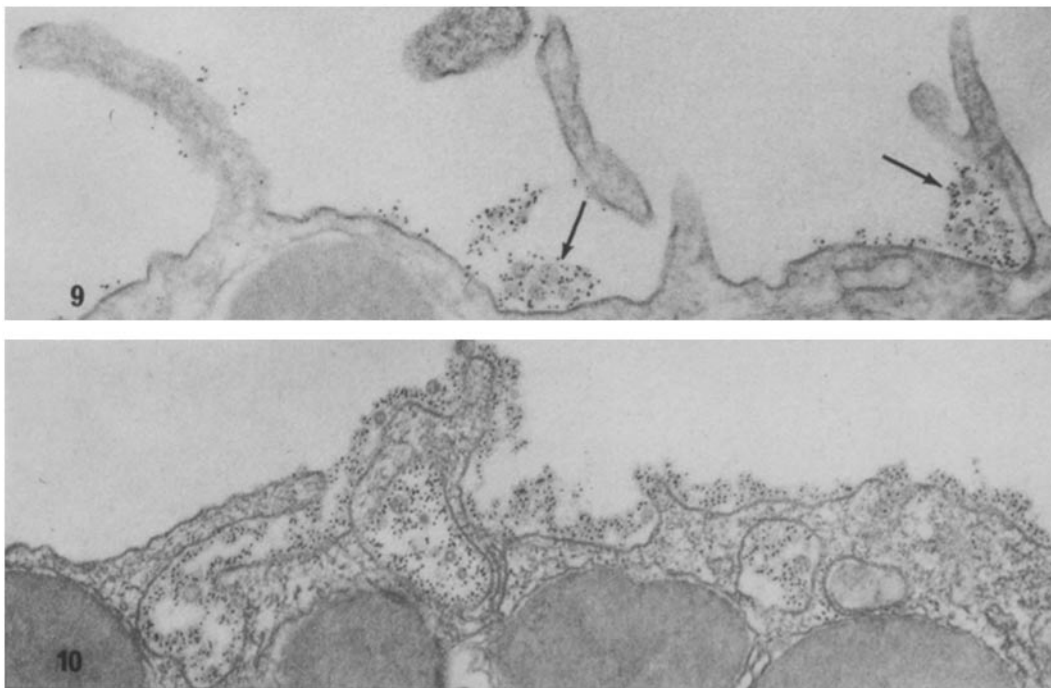


FIG. 9. Mast cell treated with S anti-RIg-FT for 4 h at 37°C in the absence of Ca^{2+} . FT molecules are collected in patches of varying size separated by areas of unlabeled membrane. Most of the clustered FT molecules appear to be associated with shed Ig-carrying membrane vesicles (arrows). Pinocytosis was present in this cell although it is not shown here. Unstained section. $\times 66,000$.

FIG. 10. Mast cell treated with S anti-RIg-FT for 30 min at 37°C followed by R anti-FT for 15 min. All of the FT molecules are clustered in large patches separated by extensive areas of unlabeled membrane. Large scale pinocytosis has occurred. Section stained with uranyl acetate. $\times 57,000$.

does not induce pinocytosis, and that the maximum cluster size required to trigger degranulation is less than 10 Ig molecules. Although clustering and pinocytosis increase slowly with time, and can be markedly increased by a second layer of cross-linking ligand (antiferritin), capping does not occur.

No consistent effect of calcium on the extent or distribution of the binding of divalent conjugates was noted in these experiments. Moreover, no obvious relationship could be discerned between the amount of binding and the extent and distribution of clustering on a cell, and the number and location of released granules.

Discussion

Our results confirm previous observations made on human (5, 9) and guinea pig (14) basophil leukocytes, and monkey lung mast cells (17), namely that divalent anti-Ig is required for calcium-dependent degranulation and histamine release. As reported by others (9), while monovalent Fab fragments release histamine if used in high concentration, this effect is progressively abolished by maneuvers aimed at removing aggregated material. The tendency for Fab fragments to aggregate may well explain some of the surprising effects that monovalent antibodies have been reported to have, such as inducing antigenic modulation (18) and pinocytosis in lymphocytes (12). Our experiments (Table I) provide formal proof that the failure of Fab anti-RIg to induce histamine secretion is not due to insufficiency of binding caused by the reduced avidity of the ligand. They are consistent with the notion that the requirement for ligand multivalency reflects the need for receptor cross-linking. Recently, Magro and Alexander (7), and Siraganian et al. (8) have provided elegant evidence that intermolecular, rather than intramolecular cross-linking is required, since divalent antigens (having two different haptens) are able to induce histamine secretion from sensitized mast cells. If we assume that Fc receptors for IgE on mast cells are monovalent, then it appears probable that it is the formation of discrete groups of IgE molecules, cross-linked in a linear fashion without complex lattice formation, which generates the initial transducing signal.

It has been demonstrated that when multivalent ligands cross-link macromolecules on the surface of various types of intact cells they can redistribute them in the plane of the membrane, inducing the passive clustering by the macromolecules into patches, followed in some cases by an active movement of the patches to one pole of the cell forming a cap (10-12). In addition, pinocytosis of the complexes is frequently seen (10, 11). Our experiments make it clear that pinocytosis and capping are not required for mast cell activation by anti-Ig and indicate that if clustering is required, less than 10 Ig molecules need be aggregated per cluster. Previously, Becker et al. (9) showed that extensive patching and capping were not required for the activation of human basophils, but they were unable to put an upper limit on the size of the clusters that could be involved. It is interesting that in our experiments, the S anti-RIg-F_T was a relatively poor cross-linking agent for cell surface Ig, and induced only moderate patching and pinocytosis after 30 min at 37°C. The addition of anti-F_T antibody increased the clustering and pinocytosis extensively, making it clear that hindered mobility of the Ig molecules is not the explanation for the ineffective

patching induced by the S anti-RIg-FT. In contrast to basophils (9, 14, 16) and lymphocytes (10, 11, 12), anti-Ig-FT did not induce capping on mast cells even after 4 h at 37°C.

Thus, it is still unclear whether the crucial change in the Ig molecules (and the Fc receptors to which they are attached) induced by the cross-linking ligand is the clustering of less than 10 Ig molecules, or a conformational change in these molecules, or both. Moreover, it is not known whether the cross-linking of two adjacent Ig molecules would be a sufficient signal and whether the cross-linked molecules need come into close proximity with each other to activate the cell. The recent report of Siraganian et al. (8), provides suggestive indirect evidence that cross-linking two adjacent Ig molecules is the optimal signal and our results are consistent with this possibility. In view of the increasing evidence that the entry of Ca^{2+} into the mast cell is the "second message" in ligand induced histamine secretion (19-21) it seems likely that the cross-linking of two or several IgE molecules could be responsible for the opening of " Ca^{2+} gates" in the membrane, allowing Ca^{2+} to enter the cell.

As has been shown for the activation of mast cells by the polyamine, 48/80 (22), histamine secretion correlates reasonably well with the degree of degranulation visualized ultrastructurally when mast cells are released with anti-Ig. In addition, our results show that degranulation is not an all-or-none event at the level of the single cell. The fact that it is almost always incomplete, even after a prolonged incubation with ligand, could be due to the presence of granules of varying maturity within each cell, and/or a homeostatic mechanism which shuts off the process.

Although the polyspecific S anti-RIg used in these studies would be expected to react with any class of Ig on the mast cells, it is likely that the binding observed was mainly to IgE. Although IgGa may also be cytophilic for rat mast cells, its affinity for mast cell Fc receptors is low compared to IgE and it probably would have dissociated during the purification and washing of the cells (23). The conclusions drawn from our experiments would be the same even if this were not the case.

Summary

We have used ferritin-conjugated divalent and monovalent anti-Ig antibodies to study simultaneously, histamine secretion and the ultrastructural distribution and redistribution of Ig receptors on rat peritoneal mast cells. We conclude that (a) divalent anti-Ig is required for both receptor redistribution and for calcium-dependent degranulation and histamine release, (b) divalent anti-Ig induces patching and pinocytosis but not capping of Ig molecules, (c) neither capping nor pinocytosis are required for triggering and if clustering is necessary, then less than 10 Ig molecules are required per cluster, and (d) degranulation (and histamine release) is not an all or none response of the mast cell.

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