Implanted IgE–$Fc_\epsilon R$ Complexes Elicit IgE-Mediated Activation of RBL-2H3 Cells†

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ABSTRACT: The high-affinity receptor for IgE ($Fc_\epsilon R$) is the cellular trigger of the antigen-induced activation of mast cells and basophils. To examine the functional integrity of $Fc_\epsilon R$, we have adopted a protein implantation procedure whereby the purified receptor complex was coreconstituted with Sendai virus envelopes. The latter promoted fusion of the hybrid vesicles with recipient cells such as rat basophilic leukemia, RBL-2H3, thus serving as a vehicle for the receptor. The implanted $Fc_\epsilon R$ was complexed with ^{125}I-labeled mouse IgE (anti-DNP) to permit receptor quantification as well as specific triggering by $DNP_{20}BSA$. Implantation in the presence of unlabeled rat IgE, which blocked the native receptors on the recipient RBL-2H3 cells, resulted in incorporation of up to 15 ng of receptor-bound IgE/10^6 cells. This was roughly equivalent in amount to 10–20% of the native receptors on such cells. The exocytosis which was triggered in the recipient cells by reagents that specifically recognized the implanted IgE reached between 15 and 50% of the maximal response. Various treatments that interfered with the activities of the viral envelopes reduced both receptor incorporation (3–5-fold) and cell degranulation (3–10-fold). These included separation of the receptor from the reconstituted envelopes, addition of serum to the incubation mixture (to inhibit vesicle–cell binding), and trypsinization of the virus (to inhibit vesicle–cell fusion). Poly(ethylene glycol) 8000 (4%) enhanced both the incorporation of the receptor and its functional responses. These treatments distinguished between real incorporation of IgE–$Fc_\epsilon R$ complexes and other mechanisms of ^{125}I-IgE association with the recipient cells. The results indicate that the amount of IgE that was exchanged between the implanted and native receptors was minor. This conclusion was confirmed by use of an unstable variant of RBL-2H3 which expressed 10-fold less receptor on the cell surface. ^{45}Ca uptake was also elicited by implanted receptors. Sendai envelope treated cells show a reduced uptake when triggered via their native receptors, as compared to that of untreated cells. However, ~80% of this control response was attained by aggregation of implanted IgE–$Fc_\epsilon R$ complexes. We thus conclude that under the employed conditions the purified receptor appears to be intact with respect to the obligatory functions necessary for the exocytotic process.

The high-affinity receptor ($Fc_\epsilon R$)[1] for immunoglobulin E (IgE), which is located on the surface of normal or transformed mast cells and basophils, initiates upon aggregation the biochemical events which culminate in degranulation (Foreman, 1980; Ishizaka, 1980; Gomperts & Fewtrell, 1985). This receptor is comprised of three nonidentical subunits, α (50 kDa), β (32 kDa), and γ_2 (20 kDa). The latter is a disulfide-linked dimer of ~9-kDa polypeptides (Metzger et al., 1986). Various biochemical perturbations occur in response to $Fc_\epsilon R$ aggregation; however, identification of the intrinsic function(s) of this receptor has not been fully accomplished as yet. Activation of calcium-specific channels has been suggested as an intrinsic function of $Fc_\epsilon R$. This was based on studies with intact cells (Beaven et al., 1984; Mohr & Fewtrell, 1987a,b; Ran & Rivnay, 1988) and on reconstitution experiments in which receptor aggregation elicited calcium-specific channel opening in the planar bilayer containing $Fc_\epsilon R$ and the channel protein (Mazurek et al., 1986; Corcia et al., 1986, 1988). Other activities may be intrinsic for the receptor in addition to the latter (Metzger et al., 1986). For instance, various receptors were shown to activate a polyphosphoinositide-specific phospholipase C in a process that involves a guanine nucleotide binding protein [G protein (Berridge & Irvine, 1984)]. A similar activity was also elicited in response to IgE–receptor aggregation on mast cells (Cockroft, 1981; Gomperts & Fewtrell, 1985) or on the transformed cell line RBL-2H3 (Beaven et al., 1984; Maeyama et al., 1986; Pribluda & Metzger, 1987; Meyer et al., 1988), but the inherence of this activity to the $Fc_\epsilon R$ complex has not yet been established rigorously (e.g., by reconstitution experiments).

The identification of receptor-activated (potentially intrinsic) functions, combined with the availability of the purified, structurally intact $Fc_\epsilon R$ (Rivnay et al., 1984), renders it possible now to initiate studies designed to elucidate structure–function relationships. One way of performing such an analysis is to reincorporate the receptor back into RBL-2H3 cells and aggregate it in situ. This should permit examination of the functional status of untreated or chemically modified $Fc_\epsilon R$ with respect to any biochemical event which it induces in the intact cell, including the ultimate degranulation. The in situ analysis is also advantageous in providing the receptor

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[1] Abbreviations: bveq, resin bed volume equivalent; B-IgE, biotin-derivatized IgE; BA-IgE, benzenearsonylated IgE; DNP, dinitrophenol; $DNP_{20}BSA$, bovine serum albumin derivatized with 20 mol of DNP/mol of albumin; ER-RSVE, IgE–$Fc_\epsilon R$ complex coreconstituted with RSVE; $Fc_\epsilon R$, high-affinity receptor for IgE; IgE_m, mouse IgE; IgE_r, rat IgE; MBC, maximal binding capacity equivalence; PEG, poly(ethylene glycol); RBL-2H3, rat basophilic leukemia cell line 2H3; RSVE, reconstituted Sendai virus envelopes; SVP, Sendai virus envelope protein.

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with its natural membrane milieu.

In this study, we have used this approach to demonstrate the functional integrity of the purified $Fc_\epsilon R$. We have implanted the purified receptor in sufficient quantities to trigger upon aggregation both ^{45}Ca influx into the recipient cells and the functional pathways that lead to degranulation.

Materials and Methods

Reagents. [^{3}H]Serotonin as 5-hydroxy[1,2-^{3}H(N)]tryptamine binoxalate (15–30 Ci/mmol), was purchased from New England Nuclear (FRG). $^{45}CaCl_2$ was obtained from Amersham International (Amersham, U.K.). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT), trypsin, phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin, pepstatin, and ethidium bromide were purchased from Sigma (St. Louis, MO). Poly(ethylene glycol) 8000 was obtained from Serva (Heidelberg, FRG). Cyanogen bromide activated Sepharose was obtained from Pharmacia. Fluorescein isothiocyanate was from Miles-Yeda (Israel). General laboratory reagents were all of analytical grade and were purchased from Sigma (St. Louis, MO).

Proteins. Monoclonal mouse anti-dinitrophenyl-IgE (IgE_m) was purified from ascitic fluid of hybridoma HI-DNP-ϵ-26.82 as previously described (Liu et al., 1980). Rat IgE (IgE_r) and purified rabbit anti-IgE_r antibodies were a gift from Dr. H. Metzger (NIH). IgE_m was iodinated by the chloramine T method (Hunter & Greenwood, 1962). Conjugation of IgE_m with azobenzenearsonate groups (BA-IgE_m) was done according to Kanellopoulos et al. (1979). Conjugation of the IgE_m to biotin (B-IgE_m) was performed as follows: ^{125}I-IgE (2 mg at a specific activity of 2×10^4 cpm/μg and 1 mg/mL of borate-buffered saline) was mixed with 200 μL of 1 M $NaHCO_3$ and then reacted with freshly dissolved biotin *N*-hydroxysuccinimide ester (Calbiochem, La Jolla, CA, 4.8 μL of a 25 mM solution in dimethylformamide). After 2 h at room temperature, the reacted IgE was dialyzed vs 400 volumes of Tris buffer (125 mM NaCl, 5 mM KCl, 15 mM Tris, pH 7.4). Fluoresceinated IgE (F-IgE_m) was prepared as described (Holborow & Johnson, 1967).

Cells. Rat basophilic leukemia cells (RBL-2H3) (Barsumian et al., 1981) and sublines thereof were used in this study. Cells were maintained as previously described (Ran & Rivnay, 1988). In degranulation experiments with implanted or nonfused control cells, 1 μCi/mL [^{3}H]serotonin was added to the culture 1 day before the assay. The native receptors of these cells were saturated by adding 1 μg/mL IgE_r (recipient cells) or IgE_m (cells to be used as positive control). For $^{45}Ca^{2+}$ uptake experiments, the pretreatments were similar yet without [^{3}H]serotonin. Other IgE combinations were used (see Experimental Design).

Variants of RBL-2H3. Parental RBL-2H3 cells were exposed to the mutagen ethidium bromide (1.5 μg/mL) in serum-free medium for 3 h at 37 °C and cultured for 10 days. This treatment and the culturing were repeated once again. Cells were then harvested, reacted with F-IgE_m (overnight at 37 °C), and then washed, suspended to $\sim 10^6$/mL of phosphate-buffered saline (PBS), and analyzed by a fluorescence-activated cell sorter (FACS, Becton Dickinson Ltd.). The fluorescence intensity histogram obtained from treated cells was typically broader in distribution than that of parental cells. Fluorescence histograms were all gated by size at a window derived from unlabeled parental cells. Sorting was performed on two fractions of cells: those with very low F-IgE_m fluorescence (lower 3%) and those with high F-IgE binding (80–100%). Both groups were cloned by the limiting dilution technique, and the clones were tested for ^{125}I-IgE binding and [^{3}H]serotonin secretion. Two clones were used during the implantation studies: (1) a normal binder and very high responder (80–120 ng of IgE/10^6 cells; 65–85% net [^{3}H]serotonin release) designated R20; (2) a low binder and a normal responder (7–15 ng of IgE/10^6 cells; 45–60% net serotonin release) designated P5. The latter retained the low IgE binding at least 3 months after the clone had been isolated. However, within the following 2–3 months a gradual increase in binding and decrease in responsiveness were observed, suggesting that it is an unstable variant. R20 has been stable in properties over 6 months.

IgE–$Fc_\epsilon R$ Purification. IgE–$Fc_\epsilon R$ purification was performed by a modified version of an earlier protocol (Rivnay et al., 1984). RBL-2H3 cells were grown in 0.5-, 1-, or 2-L spinner bottles until cells reached (1.5–1.8) $\times 10^6$ cells/mL in density. Cells were washed twice in Tris buffer and saturated with IgE (^{125}I-IgE_m, BA-^{125}I-IgE_m, or B-^{125}I-IgE_m, 0.5 μg/10^6 cells at 37 °C). The washed cells were solubilized by the zwitterionic detergent CHAPS (15 min at 4 °C and ρ = 2.65) as described (Rivnay & Metzger, 1982; Rivnay et al., 1984). PMSF (1 mM), aprotinin (25 μg/mL), pepstatin (3 μg/mL), and leupeptin (1 μg/mL) were present to inhibit proteolysis. The 100000*g* (1 h) supernatant (spun at 4 °C) was recovered and incubated with Sepharose beads coupled to (dinitrophenyl)lysine (1–2 h at 4 °C with shaking and at a ratio of 2 μL of wet resin/μg of IgE). The resin was washed batchwise in the cold with 20 bveq of Tris buffer containing 2 mM EDTA, 2 mM rat liver phospholipids, and 10 mM CHAPS (10/2 buffer, 1 h), then with 50 bveq of 2 mM EDTA and 2 mM CHAPS in Tris buffer (1 h), and finally with 20 bveq of 10/2 buffer overnight at 4 °C. Immediately prior to elution, the column was washed vigorously with 2 mM CHAPS in Tris buffer and eluted with 3 bveq of the same buffer containing 10 mM DNP-glycine at 10-, 30-, and 60-min intervals. The eluate was concentrated to 0.5–1 mL in a Centricon-10 microconcentrator (Amicon Mass.), counted to quantify ^{125}I-IgE, and immediately mixed with freshly solubilized Sendai virus envelope proteins (SVP, see below).

Reconstitution of IgE–$Fc_\epsilon R$–Sendai Envelope Hybrid Vesicles (ER–RSVE). The purification of Sendai virus, its solubilization by CHAPS, and the hemolysis assay were described earlier (Ran et al., 1988). Several batches of CHAPS were functionally more deleterious for the dissolved SVP. This problem was solved by reducing the micellar detergent: phospholipid ratio from 10 to 5, at the expense of a lower yield. Reconstitution of Sendai virus envelopes alone (RSVE) or its coreconstitution with IgE–receptor complexes (ER–RSVE) was achieved by dialyzing the appropriate dissolved protein preparations in Tris buffer (500 volumes, three changes) in a 6 mm diameter bag. RSVE or ER–RSVE were pelleted (2 h, 100000*g*, at 4 °C) and resuspended in 200–300 μL of the same buffer. Protein concentration was determined by the Bradford assay (Bradford, 1976), and ER–RSVE were recounted in the γ-counter (Gammamatic, Kontron, Switzerland) to quantify the incorporated IgE–receptor complexes. Every preparation of reconstituted envelopes was tested before the implantation studies for its fusogenic capacity by the hemolysis assay (Ran et al., 1988). The initial ratio of IgE (as IgE–$Fc_\epsilon R$) to solubilized SVP was chosen with the following considerations: (1) The minimal amount of receptor reported as sufficient to activate RBL-2H3 cells to nearly maximal response is $\sim$5 ng/10^6 cells (Fewtrell & Metzger, 1980). (2) The maximum amount of RSVE tolerated by RBL-2H3 cells without any damage (lysis) is $\sim$25 ng/2 $\times 10^6$ cells (Ran et al., 1988). (3) The average efficiency of implantation ranged

Table I: IgE and Agonist Combinations Employed in Implantation Experiments

$Fc_\epsilon R$ bearing the IgE	I	II	III
purified $Fc_\epsilon R$ (implanted)			
IgE	IgE_m	$BA\text{-}IgE_m$	$B\text{-}IgE_m$
trigger	$DNP_{20}BSA$, anti-IgE_m[a]	anti-BA	avidin + anti-avidin
native $Fc_\epsilon R$ on recipient cells (blocked)			
IgE	IgE_r	IgE_m	IgE_m
trigger	anti-IgE_r	anti-IgE_m	anti-IgE_m

[a] Anti-IgE_m must be adsorbed on immobilized IgE_r to become specific for the implanted IgE.

between 2 and 6% of the added hybrid vesicles. Therefore, 12.5 μg of Sendai virus envelope proteins (SVP) needs to be associated with 100–250 ng of IgE (bound to $Fc_\epsilon R$). We have used 200 ng/12.5 μg of SVP, i.e., a weight ratio of IgE:SVP = ~1:60. To compensate for the differential losses in the preparatory steps, the conditions chosen for the initial reconstitution mixtures were 8–10 ng of IgE–receptors/0.8 mg of SVP (1:80–1:100). Variations in the *final* IgE:SVP ratio in the range of 1:30–1:60 were observed.

Implantation. A total of 2×10^6 RBL cells that had been saturated with IgE_r (see Cells) were washed twice and incubated in 200 μL of PBS with 25 μg (unless otherwise indicated) of RSVE or ER–RSVE (20 min, 4 °C). This is referred to as step I, in which the vesicles attached to the cell surface. Vesicle–cell fusion was initiated by transferring the tubes (polystyrene, Falcon) to a 37 °C shaker bath for 30 min (step II). Cells were then washed twice with MEM tissue culture medium containing 16% fetal calf serum (FCS, GIBCO) and plated in a 24-well cluster at 10^6 cells $well^{-1}$ mL^{-1} for an additional 4 h (recovery, step III). Only live undamaged cells adhered to the well bottom during this incubation, and these were used for functional experiments. Unless mentioned, no apparent difference in cell density among wells could be detected. Due to limiting quantities of ER–RSVE, most treatments were not done with replicates; however, each finding was reproduced at least twice.

Enhancement of Fusion by PEG 8000. PEG 8000 was dissolved in PBS at a concentration of 40% (w/w). This stock solution was diluted whenever desired to the indicated final concentrations (usually 4%). PEG was only present during steps I and II.

Serotonin Release (Degranulation). Culture monolayers of implanted or control cells were washed three times with Tyrode's solution (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 10 mM Hepes/Na^+, pH 7.2) to remove leaked [^{3}H]serotonin, unbound IgE, and cell debris from the recovery step. Secretion was initiated by adding $DNP_{20}BSA$ (0.5–1 μg/mL final concentration). After 30 min at 37 °C, the reaction was terminated by EDTA (5 mM final). The total [^{3}H]serotonin content was measured in two wells disrupted by Triton X-100. Aliquots of the supernatants or lysates were counted for ^{3}H radioactivity (Kontron, Betamatic, Switzerland), and the release was expressed as a percentage (above the spontaneous release) of total [^{3}H]serotonin incorporated.

^{45}Ca Uptake. ^{45}Ca uptake was done as described earlier (Ran & Rivnay, 1988).

Experimental Design. Receptor preparations were always bound to IgE which could be recognized by specific reagents. A different type of IgE was used for blocking native receptors during the implantation protocol. Table I depicts three of the tested combinations that were employed. These combinations not only enabled a specific activation of implanted receptors but also provided a means to obtain a positive control for the maximal response of the very same RSVE-treated cells when triggered via the blocking IgE.

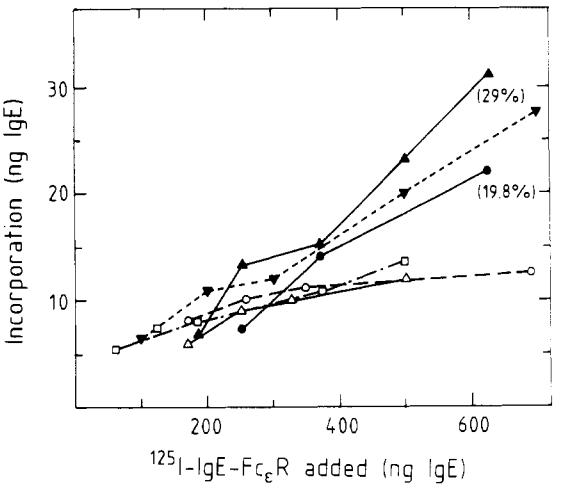

FIGURE 1: Incorporation of ^{125}I-IgE–$Fc_\epsilon R$ complexes into RBL-2H3. Hybrid vesicles were prepared as described under Materials and Methods, and their ^{125}I-IgE and total protein were determined. RBL-2H3 cells were incubated with 5–25 μg of ER–RSVE according to the routine protocol, and the radioactivity at the end of step III was determined. Concentrations over 30 μg/plate were lytic. Each symbol and line pattern represents a separate experiment with a separate vesicle preparation. Maximal IgE binding and the IgE:SVP ratio in these experiments were as follows: (△) 107 ng/plate, 1:29, (○) 105 ng/plate, 1:30; (▲) 108 ng/plate, 1:40; (●) 111 ng/plate, 1:40; (▼) 107 ng/plate, 1:55; (□) 85 ng/plate, 1:37. Numbers in parentheses indicate the quantitative relationship to the native receptors.

Results

Receptor Incorporation. To assess the extent of IgE–$Fc_\epsilon R$ incorporation into RBL-2H3 cells, hybrid envelopes containing ^{125}I-IgE–$Fc_\epsilon R$ were mixed at increasing concentrations with the cells and processed for fusion. Following the recovery step, incorporation was quantified by the cell-associated radioactivity. The recovery was generally inversely related to the concentration when expressed as percentage of the added amount, particularly at low vesicle concentration [see also Ran et al. (1988)]. In the range between 10 and 25 μg of Sendai envelope protein (SVP)/tube, the percentage of incorporated IgE remained almost constant (2–5%). Thus, more IgE became incorporated when the initial concentrations were raised (Figure 1). Hence, while the lower concentration seemed preferable for economizing with regard to both SVP and IgE–$Fc_\epsilon R$ complexes, the amounts incorporated under these conditions were far too low to be useful in further functional studies. Figure 1 depicts the dose–incorporation curve of six experiments. This curve shows that hybrid vesicle (ER–RSVE) preparations with an IgE to RSVP weight ratio of 1:60 or 1:40 linearly introduce IgE–receptors into RBL-2H3 cells. At sublytic doses of such vesicles, an equivalent of 10–30% of the native receptors became cell associated. Preparations with higher IgE to SVP ratios (~1:30–1:20) were apparently less efficiently incorporated. Since the maximal binding capacity of the these cells is 80–130 ng of IgE/10^6 cells (the range observed in our laboratory over the last few years) and

Table II: Antigen Stimulation of RBL-2H3 Cells through Native or Implanted Receptors

protocol	IgE on cells[a]	treatment[a]	IgE_m incorporated[b] ng	IgE_m incorporated[b] % MBC	trigger (1 μg/mL)	[^{3}H]serotonin release % of contained	[^{3}H]serotonin release net[c]
1	IgE_r	E_m*R-RSVE	nd	nd	anti-IgE_r	49.5 ± 1.70	42.8 (7)
2	IgE_r	E_m*R-RSVE	6.0	5.6	$DNP_{20}BSA$	7.26 ± 0.07	0.0 (8)
3	IgE_r	E_m*R-RSVE	9.34	8.72	$DNP_{20}BSA$	12.3 ± 0.55	2.5 (8)
4	IgE_r	E_m*R-RSVE	10.1	9.43	$DNP_{20}BSA$	24.3 ± 1.15	14.5 (8)
5	IgE_r	E_m*R-RSVE	12	11.2	$DNP_{20}BSA$	38.2 ± 1.65	24.4 (8)
6	IgE_r				anti-IgE_r	61.8 ± 3.65	55.1 (7)
7	IgE_r					6.7 ± 0.06	
8	IgE_r				$DNP_{20}BSA$	9.8 ± 0.35	
9	IgE_m				anti-IgE_m	46.7 ± 2.3	40.2 (11)
10	IgE_m				$DNP_{20}BSA$	42.5 ± 1.44	36 (11)
11	IgE_m					6.5 ± 0.05	

[a] See nomenclature under Materials and Methods. E_m* denotes radioactive IgE_m. [b] Extent of implantation was assessed by radioactivity of ^{125}I-IgE_m. The specific activity in this experiment was 10^5 cpm/μg. MBC = maximal IgE binding capacity (107 ng/10^6 cells). nd = not determined, treatment as in protocol 5. [c] Number in parentheses indicates the protocol of the subtracted background.

Table III: Capacity of Implanted Receptors To Mediate Degranulation

protocol	treatments: IgE on cells	treatments: fusion[a]	IgE incorporated, % MBC[b]	trigger[c]	[^{3}H]serotonin release: % of contained	[^{3}H]serotonin release: net[d]	[^{3}H]serotonin release: % of control
1	IgE_m	RSVE		+	1.79 ± 0.1	0.84 (5)	
2	IgE_m	BA-E_m*R-RSVE	14.6	+	10.7 ± 0.9	9.75 (5)	26.3
3	IgE_m	RSVE + BA-E_m*	2.7	+	2.09 ± 0.1	1.14 (5)	3.07
4	IgE_m	RSVE + BA-E_m*R	nd	+	1.07 ± 0.15	0.12 (5)	0.32
5	IgE_m	RSVE		−	0.95 ± 0.04		
6	BA-IgE_m			+	38.7 ± 0.30	37.1 (7)	"100"
7	BA-IgE_m			−	1.64 ± 0.46		

[a] 25 μg of ER-RSVE and 0.4 μg of IgE in protocols 2–4. [b] MBC = maximal IgE binding capacity. [c] 50 μL of antiserum against BA-hemoglobin. This serum still stimulated the same extent of degranulation in protocol 6 at a 1:3 dilution. [d] Numbers in parentheses indicate the protocol of the substrated background.

since variably 5–20% of native receptor occupancy is sufficient to elicit full degranulation [Fewtrell and Metzger (1980) and our unpublished observations], proper implantation of 4–26 ng of receptor-bound IgE/10^6 cells should be sufficient to trigger maximal degranulation. Such amounts were indeed incorporated by 10–25 μg of ER-RSVE/tube; hence, additional experiments were performed to further characterize the mode of ^{125}I-IgE incorporation and the functional responsiveness of the implanted receptor.

Functional Analysis: Degranulation. Increased incorporation of IgE-Fc_ϵR complexes was associated with increased degranulation. Cells implanted with 12 ng of IgE (Table II, protocol 5) responded approximately 65% of the nontreated, fully saturated control cells (Table II, protocol 10). In addition, the complete activation of the *recipient* cells via their own *native* receptors (Table II, protocol 1) indicates that the cells have not been damaged by the fusion process. If approximately half of the reconstituted ^{125}I-IgE is properly oriented (right side out), then the cells in protocol 5 acquired ~6 ng of IgE accessible for aggregation on the cell surface. The net release triggered by these implanted receptors (24.4%) comprised ~60% of the full degranulation, when related to the average of controls in protocols 1, 9, and 10. Since 6 ng of bound IgE is within the linear segment of the response–occupancy curve, one can extrapolate and anticipate full response at about 10 ng (equivalent to 9–10% of full occupancy). This value is in good agreement with published values (Fewtrell & Metzger, 1980) and therefore indicates that no severe denaturation occurs to the purified protein complex under the employed purification protocol. Note, however, that precise quantitative calculations are made difficult by the variability in the positive controls which in turn depended on the nature of the agonist, the stoichiometry between the available IgE and the added agonist, and the status of the control cell culture (treated vs untreated).

The functional capacity of implanted receptors was examined with several agonists (see Table I). Anti-BA and BA-^{125}I-IgE_m were employed in the following experiment to evaluate the amount of IgE associated with truly fused complexes. An essential control in which *receptor-free* envelopes were fused with the cells was examined first (Table III, protocols 1 and 5). These cells contained comparable amounts of [^{3}H]serotonin as untreated cells (not shown), and their secretion in the presence or absence of anti-benzenearsonate (a triggering antibody) was minimal like that of the untriggered control (Table III, protocol 7). These results corroborated our earlier finding that RSVE at concentrations of less than 30 μg/2 × 10^6 cells did not reduce cell viability, nor did it induce leakiness. In contrast to these controls, RBL-2H3 cells which were treated with *receptor-containing* envelopes have incorporated an amount of receptor equivalent to 14.6% of the native receptor contents and released 10.7% of their contained [^{3}H]serotonin (Table III, protocol 2). When corrected for the spontaneous release by cells treated with the Sendai vehicle only, this response was found to comprise 26.3% of that of control cells saturated with BA-IgE. Extrapolations such as those made above suggested that these implanted cells would have responded maximally at ~27% occupancy (higher than in the previous experiment) and might indicate a partial damage in this receptor preparation. Most experiments extrapolated to 10–20% of the maximal occupancy.

Functional examinations of the implanted receptor were done with awareness of the possibility that exocytosis may be triggered via antigen-specific IgE molecules which in the course of the process had dissociated from the liposomal complexes (ER-RSVE) and then bound to the native receptors on the recipient cell surface (exchanged IgE). This was evaluated with the following approaches: (1) modulation of the binding of RSVE to the cell membrane; (2) modulation of the fusion between vesicles and the cell membrane; (3) modulation of

Table IV: Effect of Serum on ^{125}I-IgE Incorporation and Antigen-Induced Degranulation

test	treatment: steps I + II[a] ER-RSVE[a]	steps I + II[a] serum[b]	step III[c] ER-RSVE	step III[c] serum	incorporated ^{125}I-IgE (ng)[d]	percent [^{3}H]serotonin release[e] -Ag	+Ag	net
(1) control fusion	+	-	-	-	4.1	9.8	20.1	10.3
(2) exchange during steps I + II only	+	+	-	-	1.8	5.2	8.6	3.4
(3) exchange during step III only	-	-	+	+	1.6	6.6	8.7	2.1

[a] 285 ng of receptor-bound ^{125}I-IgE coreconstituted with 15 μg of SVP. No poly(ethylene glycol) was added. [b] 16% FCS in culture medium. [c] The recovery step in this experiment was 3 h. [d] Nanograms of IgE incorporated per confluent well. Maximal occupancy of the R20 variant in this experiment was 76 ng of IgE/confluent well. [e] Ag = antigen stimulation. Total [^{3}H]serotonin in the three cultures was 19420 ± 780 (means ± SD).

native receptor pool available for the exchanged IgE. These experiments are detailed below.

(1) Reduced Binding. This approach for evaluating the exchange was based on exposing the cells to $Fc_\epsilon R$-bound IgE yet under conditions that reduce the binding of $Fc_\epsilon R$ to the cell surface. Such conditions were created by adding RSVE and IgE–receptors together but in a protocol whereby the two components have *not* been *co*reconstituted (i.e., mixed after the dialysis). This control (Table III, protocol 4) shows a reduced IgE incorporation and degranulation. A variation of this control was performed with *free* IgE (Table III, protocol 3) under otherwise identical conditions. This control protocol permitted the maximal potential contribution of exchanged IgE since in the absence of the bound receptor dissociation ceased to be a rate-limiting step for the exchange. Nevertheless, it showed considerably less IgE incorporation and cell degranulation than those found in protocol 2.

A different experiment within this approach was performed with intact ER–RSVE (receptors coreconstituted with the envelope proteins), but in the presence or absence of *serum* at various stages of the implantation process. In a routine fusion protocol, in the absence of serum (Table IV, test 1) the cells incorporated 4.1 ng of IgE, which mediated the release of 10.3% of the stored tritiated serotonin above basal release. In the presence of serum during steps I + II of the protocol, incorporation was reduced to 1.8 ng of IgE, and the respective response decreased 3-fold (Table IV, test 2). Comparably low binding and response were obtained when vesicles and serum were coincubated during the recovery step only (Table IV, test 3). However, in the routine protocol, only 10% of the ^{125}I-IgE input remain associated with the cells at that step, so that the major portion of exchanged IgE originates in the initial dose of vesicles to which the cells are exposed at steps I + II.

(2) Enhanced or Reduced Fusion. Another approach to differentiate between exchanged IgE and true implantation was to raise or reduce the portion of truly fused complexes within a comparable population of cell-attached vesicles. Figure 2 depicts the incorporation of ^{125}I-IgE and the degranulation response following fusion in the presence of increased concentrations of poly(ethylene glycol) 8000 (PEG 8000), which is known to affect the hydration of the lipid bilayer and thus to enhance the fusion step, but not the binding (D. Hoekstra, personal communication). One such experiment was performed with the parental RBL-2H3 with a 3-h recovery step while the other was performed with the R20 variant and with a 4-h recovery. Despite the lower antigen-induced response and the elevated spontaneous release observed in the control group (0% PEG) of the former experiment, both experiments clearly show that PEG 8000 potentiated the final incorporation and the response to antigen, although the initial input (and hence the extent of exchange) was the *same*.

Inhibition of fusion with yet no effect on vesicle binding to the cell surface was obtained with ER–RSVE prepared from trypsin-treated virus particles (Hosaka & Shimizu, 1977).

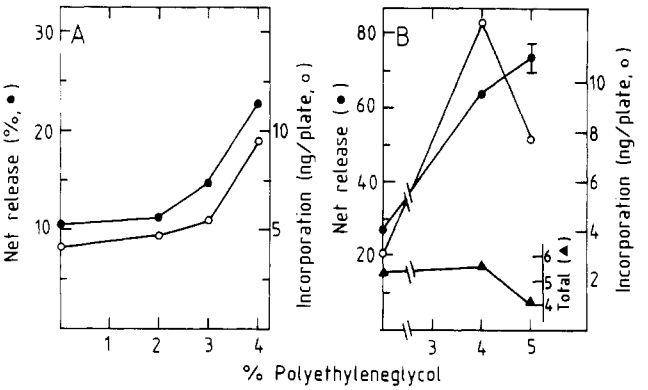

FIGURE 2: Effects of poly(ethylene glycol) 8000 on IgE implantation. Fusion protocols were carried out with PEG added at steps I + II to different final concentrations. (A) Parental RBL-2H3 cells. Step III was shortened to 3 h only. The untreated control cells in this experiment, which were saturated with IgE_m (81 ng/well) and triggered by 1 μg/mL $DNP_{20}BSA$, released 59.2% above the basal release (6.1 ± 1.8%). [^{3}H]Serotonin content in all dishes was 18340 ± 290 cpm/plate. (B) R20 variant. Step III was 4 h. The control cells in this experiment were saturated by 76.3 ng of IgE/plate and released 77.6% above basal release (2.9 ± 0.5%). "Total" in the inset indicates total [^{3}H]serotonin contents per plate. This curve suggests that the apparent decrease in incorporation with 5% PEG is due to loss of cells in this plate.

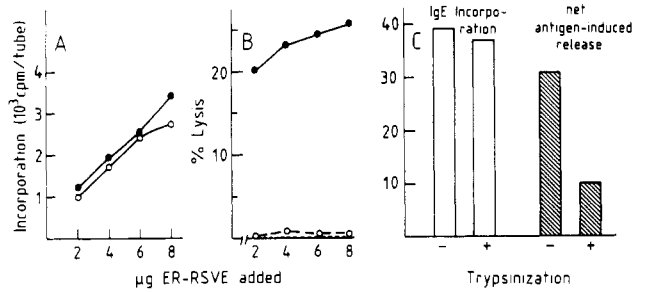

FIGURE 3: Effects of trypsinization on IgE implantation. Viral envelope proteins from trypsinized (50 μg/5 mg of virus, 30 min at 37 °C) or control Sendai virus were extracted, and similar quantities of protein were coreconstituted with ^{125}I-IgE–$Fc_\epsilon R$ as described under Materials and Methods. Vesicles were comprised of 14.0 and 18.8 ng of IgE/μg of viral protein in the two preparations, respectively. (A) ^{125}I-IgE bound to red blood cells, pelleted at 10K rpm (microfuge) after hemolysis. (B) Hemolysis. Complete lysis yielded 5.25 OD_{540}/tube. (●) Vesicles made of untreated viral proteins (ER–RSVE); (○) vesicles made of trypsinized viral proteins (ER–$RSVE_t$); (dashed line) hemolysis by receptor-free trypsinized viral envelopes ($RSVE_t$). (C) ^{125}I-IgE incorporation to and degranulation of RBL-2H3 cells reacted with 23 μg of either preparation. Total [^{3}H]serotonin in the degranulation experiment was 5300 and 5700 cpm/plate, and the spontaneous release was 2.7% and 2.6% for the control and treated preparations, respectively. (Open bars) ng of ^{125}I-IgE incorporated/plate; (hatched bars) net release, % above spontaneous release (same ordinate for both variables).

Figure 3 depicts the binding (panel A) and fusogenic capacity (panel B) of such vesicles (ER–$RSVE_t$) with human erythrocytes as target cells. ER–$RSVE_t$, like the receptor-free

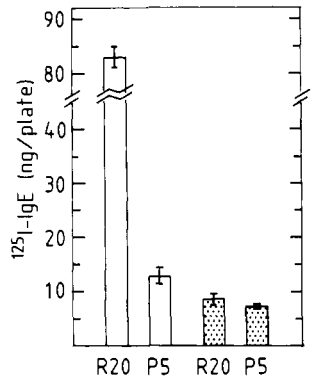

FIGURE 4: IgE binding or $Fc_\epsilon R$-bound IgE incorporation into high- and low-receptor variants of RBL-2H3. (Open bars) $^{125}IgE_m$ (1 μg) was incubated with 3×10^6 R20 or P5 variant cells for 60 min at 37 °C. Cells were washed and plated (10^6 cells mL^{-1} $well^{-1}$) for 4 h. (Stippled bars) ^{125}I-ER–RSVE containing 600 ng of IgE_m were incubated with 3×10^6 cells according to the routine fusion protocol (+4% PEG 8000 in steps I + II). Plating after step II was done in triplicate. After 4 h cells of all the four groups were washed twice and then lysed in water, and aliquots were counted for γ radioactivity.

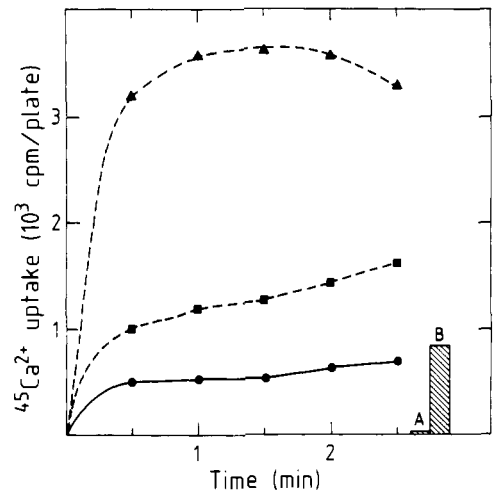

FIGURE 5: Net antigen-induced $^{45}Ca^{2+}$ uptake in untreated and implanted (fused) R20 variant cells. (Dashed line) Untreated control cells: (▲) saturated with IgE_m (76.3 ng/10^6 cells), (■) partially saturated (18.5 ng of IgE_m/10^6 cells). (Solid line) Cells that had incorporated 9.9 ng of IgE_m by fusion. Histograms: (A) Cells processed for implantation with FCS present during steps I + II (as in Table IV, line 2) and triggered by antigen, t = 2.5 min; (B) same cells as solid line, triggered by anti-IgE_r (via blocking IgE), t = 2.5 min. Basal uptake of the implanted cells was 800–1200 cpm between 0.5 and 2.5 min and between 1200 and 1600 cpm/plate for the untreated controls. Note that R20 cells are faster responding in $^{45}Ca^{2+}$ uptake than the parental cells (Ran & Rivnay, 1988), reaching a plateau already within 30 s.

treated envelopes ($RSVE_t$), cause minimal lysis compared to intact (nontrypsinized) ER–RSVE. In contrast, the association of ^{125}I-IgE–receptor to the erythrocytes was comparable for both types of envelopes (Figure 3A), suggesting that the adsorption capacity was undamaged. Likewise, despite comparable adsorption of both types of vesicles to the RBL-2H3 cells, the net antigen-induced exocytosis in cells that were reacted with the treated envelopes was 3-fold lower than that of the respective control (Figure 3, panel C). These results demonstrate again that the extent of cell activation due to exchange is in the order of one-third (or less) of the attained signal.

(3) Manipulation of Native $Fc_\epsilon R$ on Acceptor Cells. Obviously the best tool to examine activation by implanted receptor would be a receptor-deficient subline of RBL-2H3 cells. We [like others (Barsumian et al., 1981; Stracke et al., 1987)] have invested efforts in this direction, yet such a variant has not been isolated. However, we have isolated a variant (P5) that bound 8–14 ng of IgE/10^6 cells (~10% of the normal RBL-2H3 binding). ^{125}I-IgE incorporation to this variant was compared to that of the R20 variant cells, following an incubation with ER–RSVE under identical conditions (Figure 4). Cells of the P5 variant bound in this experiment 7-fold less IgE when incubated with free IgE at saturating concentrations (open bars); however, the incorporation by fusion was similar (stippled bars). Incorporation by P5 cells was ~82% of that of R20, indicating that IgE *exchange* in R20 is in the order of ~20%.

Exchange could also be reduced by raising the blocking IgE concentration present during the implantation. This was not pursued any further since at concentrations higher than 0.2 μg/mL (20% excess over the saturating rat IgE that was bound to the recipient cells overnight in the basic protocol) rat IgE also displaced the mouse (anti-DNP) IgE_m from the ER–RSVE-associated receptors and from the truly implanted receptors. Collectively, these results indicate that exchanged IgE cannot account for the functional responsiveness of the implanted $Fc_\epsilon R$.

Functional Analysis: $^{45}Ca^{2+}$ Uptake. Figure 5 shows the net $^{45}Ca^{2+}$ uptake in control, nonimplanted cells at two different extents of native receptor occupancies (100% and 24%, dashed lines) and in cells implanted with 10.4 ng of IgE (11% MBC, solid line). Unlike the results on degranulation, the positive control of vesicle-treated cells (triggered by anti-IgE_r, histogram B) in this experiment and two others (not shown) was variably lower than the untreated control cells (triangles), indicating that functions which involve ionic movements might be more sensitive to the Sendai envelope treatment even when recovery (step III) had been prolonged to 6 h as in this experiment. Incorporated receptors mediated 83% of the $^{45}Ca^{2+}$ uptake observed in the *internal* positive control, and only 4% of this could be triggered by the negative control incubated with serum (histogram A).

Discussion

Sendai virus envelopes have been used extensively to graft membrane components from one cell type to another (Loyter & Volsky, 1982). Only in very few cases were those components purified protein (Cabantchik et al., 1980; Mazurek et al., 1983; Doyle et al., 1979), and none of which was a labile multimer. Moreover, in no previous case did the implanted species have to be noncovalently associated to any ligand during the implantation. This paper dealt with Sendai envelope mediated implantation of the *purified* tetrameric receptor for IgE ($Fc_\epsilon R$), which dissociates irreversibly in lipid-free detergent solutions. The protocols commonly employed to purify $Fc_\epsilon R$ are based on the tightly (but noncovalently) bound ligand, IgE, and its affinity to various matrices. However, once bound, IgE cannot be removed quickly without exposure of the protein to extreme conditions [e.g., pH 3, 60 s (Kulczycki & Metzger, 1974)] which destroy the receptor integrity (Kinet et al., 1985) and the RBL-2H3 cell responsiveness. Whereas the limitation of the pure receptor stability had already been overcome in earlier studies (Rivnay et al., 1984; Rivnay & Metzger, 1982), the presence of IgE on the purified receptor raised a new difficulty, namely, RBL cells to be employed as recipients express on their surface the same receptors so that ligand exchange could result in occupancy of native receptors by IgE from the implanted complex. The following approaches were attempted or considered in relation to this problem: (1) Implantation of $Fc_\epsilon R$ into receptor-negative non-RBL cells which employ Ca^{2+} influx or PI turnover as signaling was considered

to be an unlikely approach to examine $Fc_\epsilon R$ functional integrity at this stage, since no identity has yet been established between the effector protein(s) activated by $Fc_\epsilon R$ of RBL-2H3 and those of any receptor system in other cell types. (2) We have attempted to induce an $Fc_\epsilon R$-negative variant of RBL-2H3 by ultraviolet irradiation or by the mutagen ethidium bromide. Selections by treatment with antibodies to the IgE-binding subunit of $Fc_\epsilon R$ (α) and complement, by passage through IgE-specific affinity columns or by fluorescent IgE-mediated sorting in the cell sorter, were applied. Neither of those treatments and selections alone nor any of several combinations thereof have provided $Fc_\epsilon R$-free cells after cloning. Experiments with other useful variants which were obtained during this effort (R20 and P5) were detailed. (3) Another approach to eliminate the problem of IgE exchange involved covalent attachment of a small hapten to the implanted receptor while still on the donor cells followed by the purification and implantation protocols. This approach should have enabled *direct* receptor aggregation without the requirement for IgE as a mediator. Along this line we have modified the receptor with biotin or DNP through the side chains of lysine (by *N*-hydroxysuccinimide esters), cysteine (by a maleimide), or sugars (by hydrazides following two different protocols of oxidation). Using ^{125}I-avidin in Western blot analyses confirmed that the α subunit of $Fc_\epsilon R$ had been modified (in the case of biotin). Nevertheless, none of these attempts yielded $Fc_\epsilon R$ preparations that could be triggered directly via the biotin (i.e. by anti-biotin antibodies, by avidin, or by avidin + anti-avidin antibodies) or via the DNP (by anti-DNP antibodies) following implantation. In the absence of the above solutions we pursued this study on the available RBL-2H3 cells but considered very critically the constraint imposed by the presence of native $Fc_\epsilon R$ on the recipient cell surface. We have examined the incorporation under a variety of conditions that have been known to interfere with vehicle function. In addition, we have induced and examined an unstable RBL-2H3 variant that expressed one-tenth of the parental receptor density. The experimental and control groups were in all the cases exposed to identical amounts of receptor-bound ^{125}I-IgE (in solution or adsorbed to the cell surface) and hence to a comparable extent of IgE translocation. Yet, since the examined treatments clearly modified the incorporation and the associated responsiveness and since the low-receptor variant showed comparable incorporation to control cells, we concluded the following: (a) The radioactive IgE on the recipient cell is primarily bound to the implanted rather than the native receptors (namely, exchange accounts for a minor fraction of the cell-associated IgE). (b) A large fraction of the implanted receptors are properly oriented (right side out). This was also qualitatively corroborated with ^{125}I-avidin binding to biotinylated viral envelopes after the fusion and recovery steps (not shown). (c) The implanted receptors in most preparations are functionally intact and can become engaged with the appropriate effector molecules for signal transduction following aggregation.

Due to features inherent to the $Fc_\epsilon R$ system, like the complex relationship between specific lattice formation and receptor activation, these conclusions are only semiquantitative in nature. Nonetheless, we have been able to demonstrate that the implanted receptors are functionally intact so that both the overall degranulation and individual signaling events like Ca^{2+} uptake could be studied.

These findings provide some information on the arrangement of the signaling components in the plasma membrane. First, these data indicate that in the untriggered cells the signaling effector molecules are not permanently engaged with a portion of the native receptors, and hence, both implanted and native receptor complexes have comparable probability for such an engagement to take place upon aggregation. Second, since effectors are accessible to implanted receptors, one can likely exclude unique laterally separated lipid phases as being the residence for the effector molecules. Such domains have been implied recently (Yechiel & Edidin, 1987) and may act as barriers to the engagement unless fusion and insertion occurred within the same domain.

The demonstration that $Fc_\epsilon R$ is functionally intact is a logical prerequisite for various reconstitution experiments and corroborated in situ the earlier results on the Ca^{2+} channel opening obtained in the reconstituted membranes in vitro. The combination of active $Fc_\epsilon R$ and the well-documented use of Sendai virus envelopes for implantation makes it possible now to examine functions which at present cannot be reconstituted. This combination also enables examination of $Fc_\epsilon R$ activity following chemical or enzymatic modification without reservations concerning the effect of the modifier on other proteins involved in the signaling. The modifying treatment can be applied under conditions that permit modifications of otherwise inaccessible parts of $Fc_\epsilon R$ so that topological studies can be designed.

Current methodologies in molecular biology enable modifications at the level of gene structure so that similar questions on the relationship of structure and function may be asked, provided that native and modified receptors can be discriminated. Amino acid replacement(s) or deletion(s) produce different structural modifications than those produced upon chemical modifications. Hence, $Fc_\epsilon R$ implantation may complement genetic approaches in studying structure–function relationship.

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Conformational Changes of Cytochromes P-450$_{cam}$ and P-450$_{lin}$ Induced by High Pressure†

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ABSTRACT: Absorbance and fluorescence spectra of bacterial cytochrome P-450$_{cam}$ and cytochrome P-450$_{lin}$ have been studied as a function of pressure. These pressure-induced spectral perturbations fall into two categories, which are interpreted as resulting from denaturation domains and are discussed in terms of protein structural dynamics. The results presented herein support a view that these two bacterial cytochromes have large structural differences and suggest a picture in which the gellike cortex of each protein may play an essential role in stability and function.

High pressure has been used to induce conformational changes in several heme proteins (Ogunmola et al., 1977), and this perturbation has been previously applied to the bacterial cytochrome P-450$_{cam}$ (Hui Bon Hoa et al., 1982; Fisher et al., 1985), a monooxygenase that catalyzes the hydroxylation of camphor. Like many other heme proteins, the ferric form of this cytochrome is characterized by a spin state influenced by various parameters (Sligar, 1976; Lange et al., 1979) and also by a transition from the native form to a nonnative form termed cytochrome P-420 (Yu et al., 1974). Both of these processes can be induced by increasing pressure and can be readily "ressolved" in time (Marden et al., 1982, 1986).

The dominant fluorophores in proteins are tryptophan residues attached to well-defined sites along the polypeptide backbone. Fluorescence yield, lifetime, and emission maxima are often very sensitive to the local environment and hence can sometimes serve as a good probe of the structure and dynamics of local polypeptide chain fluctuations. In heme proteins, the fluorescence emission of tryptophan residues overlaps the absorption band of the heme, and there can be efficient nonradioactive energy transfer between these groups. This energy transfer serves to reduce the fluorescence yield and excited-state lifetime and can reveal subtle changes in distance, defined to a first approximation via the theory of Forster (1959).

The three-dimensional structure of P-450$_{cam}$ (Poulos et al., 1985) revealed the presence of five tryptophan residues at amino acid positions 42, 55, 63, 374, and 406. The closest distance to the heme is that of Try-42 (17.5 Å) and the longest that for Try-406 (31.3 Å). The distance of the three other

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