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Immunology

Biological properties of a recombinant human immunoglobulin ε-chain fragment

(IgE/human basophilic granulocytes/affinity for IgE receptors/histamine release)

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ABSTRACT **A recombinant human immunoglobulin ε-chain gene expression product (rFc_ε) was compared with a human E myeloma protein in the affinity for ε-chain Fc fragment receptors ($Fc_\varepsilon R$) on cultured human basophils. The association–dissociation kinetics of the rFc_ε–$Fc_\varepsilon R$ interaction are indistinguishable from that of E myeloma protein, indicating that rFc_ε and IgE have identical affinity for the receptors. The recombinant gene product sensitizes cultured basophils for anti-IgE-induced histamine release. A dose–response curve of histamine release indicates that the gene product is equally efficient in transducing the signal for degranulation as the natural IgE. Both the rFc_ε and IgE lost the affinity for $Fc_\varepsilon R$ by heating at 56°C. Upon renaturation by passage through a solution of 6 M guanidine hydrochloride, rFc_ε recovered both the affinity for $Fc_\varepsilon R$ and the original CD spectra. On the other hand, renaturation of heat-denatured IgE largely restored optical activity above 250 nm but restored neither the affinity for $Fc_\varepsilon R$ nor the CD spectrum below 220 nm. The results suggest that either the amino acid sequence or the carbohydrate present in the myeloma protein, but not the rFc_ε, may interfere with refolding of the receptor-binding structures.**

IgE antibodies bind to Fc_ε receptors ($Fc_\varepsilon R$) on mast cells and basophils through the Fc portion of heavy ε chains, and the reaction of allergen with cell-bound IgE antibodies induces the release of a variety of pharmacologically active mediators that are responsible for the clinical manifestations of allergic disorders (1). In order to analyze the structural basis for the binding of IgE to $Fc_\varepsilon R$, we have cloned a fragment of the human myeloma (ND) ε-chain gene, encoding the second, third, and fourth domains of the constant (C) region (C 2-4) and expressed this DNA sequence in *Escherichia coli* (2, 3). Expression of human gene sequences also has been obtained by other investigators (4, 5). Recombinant Fc_ε fragments (rFc_ε) isolated from transformed cultures of *E. coli* bind to $Fc_\varepsilon R$ on cultured human basophils (3, 5). In this communication, we report characteristics of the binding between rFc_ε and $Fc_\varepsilon R$ on human basophils.

MATERIALS AND METHODS

Cultured Human Basophilic Granulocytes. Human basophils were obtained from suspension cultures of mononuclear cells of umbilical cord blood (6). Mononuclear cells were cultured for 2–3 weeks in the presence of a fraction of culture supernatants of phytohemagglutinin (PHA-P)-stimulated human T cells. The proportion of basophils in the cultures varied from 30% to 80% depending on the source of growth factors. Cells were purified by a differential centrifugation with Ficoll–Paque or Percoll (Pharmacia) (7), and basophils of >70% purity were used for the experiments. Cultured basophils contained 0.5–1.6 μg of histamine per 10^6 cells and bore $2.7 \pm 0.95 \times 10^5$ $Fc_\varepsilon R$ per cell (8).

Human E Myeloma Protein, Recombinant Fc_ε Fragments, and Anti-IgE Antibodies. Human myeloma IgE (>99% pure) was isolated from the serum of a myeloma patient (PS) as described (9). The cloning, expression, and methods for the analysis of the cloned gene products have been described in earlier publications (2, 3). Although monomeric rFc_ε could be assembled into dimeric chains with a yield of >85%, upon storage at 0°C for several weeks, we observed an increase in the number of oligomeric forms. Analysis of affinity-purified rFc_ε by polyacrylamide gel electrophoresis under nonreducing conditions showed that 62% of the total protein was in the dimeric form and 27% in the tetrameric form. The remainder of the protein represented monomer or lower molecular weight fragments.

Purified IgE and rFc_ε were labeled with $Na^{125}I$ (New England Nuclear) by using chloroamine-T (10). The proportion of ^{125}I-labeled IgE (^{125}I-IgE) and ^{125}I-labeled rFc_ε (^{125}I-rFc_ε) that bound to cultured basophils was determined by the method of Kulczycki and Metzger (11). The bindable portion of ^{125}I in the ^{125}I-rFc_ε preparation was 50%, while those in two ^{125}I-IgE preparations were 74% and 77%. The concentration of ^{125}I-IgE and ^{125}I-rFc_ε described in this paper was corrected relative to the concentration that is bindable to basophils.

A rabbit antiserum and goat antiserum specific for the Fc portion of human IgE, which contained 1.8 mg and 1.38 mg of anti-IgE antibodies per ml, respectively, have been described (7). The antisera contained the antibodies specific for both ε_1 and ε_2 determinants.

Modification of IgE and Recombinant Fc_ε Fragments. Myeloma IgE (PS) and rFc_ε were heat-denatured for 1 hr at 56°C. Aliquots of the heat-denatured proteins were unfolded in 6 M guanidine hydrochloride, and the denaturing agent was removed by stepwise dialysis against 3 M guanidine hydrochloride for 3 hr, 1.5 M guanidine hydrochloride for 3 hr, and 0.75 M guanidine hydrochloride for 3 hr, followed by overnight dialysis against phosphate-buffered saline (pH 7.4).

Binding of rFc_ε to Basophils and Determination of Association and Dissociation Kinetics. The binding of IgE molecules to cultured basophils was determined by the method of Kulczycki *et al.* (12). Detailed procedures with the cultured basophils have been described (6). To determine nonspecific binding of a ^{125}I-labeled ligand, aliquots of a suspension of basophils were incubated for 15 min with a 100-fold excess of unlabeled IgE prior to the addition of ^{125}I-labeled ligand. After subtraction of the nonspecific binding, the number of ^{125}I-IgE or ^{125}I-rFc_ε molecules specifically bound per baso-

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Abbreviations: rFc_ε, recombinant Fc ε-chain fragment; $Fc_\varepsilon R$, receptors for IgE; C, constant.

phil was calculated with assumptions that the molecular weight of human IgE is 190,000 and that the form of the rFc_ε bindable to the cells is dimeric, of which the molecular weight is 80,000.

The kinetics of association and dissociation between $Fc_\varepsilon R$ and rFc_ε fragment or IgE were determined by the method described by Kulczycki and Metzger (11). To determine the forward rate constant (k_1), 1–3 × 10^6 basophils per ml were incubated with ^{125}I-IgE or ^{125}I-rFc_ε at 2 μg/ml, and cell-bound ligand was measured during the first 300 sec of incubation. The forward rate constant (k_1) was calculated as $V_0/C_0 \times R_0$ where V_0 represents the initial rate of the binding, and C_0 and R_0 represent the initial concentration of ligand and of receptors, respectively.

To determine the dissociation rate constant (k_{-1}), basophils were preincubated for 1 hr at 37°C with either ^{125}I-IgE or ^{125}I-rFc_ε at 2 μg/ml, and the cells were resuspended in fresh RPMI 1640 medium at a final concentration of 0.5–1 × 10^6/ml. Unlabeled IgE (500 μg/ml) was added to half of the cell suspension, and an equal volume of RPMI medium was added to the remainder. Both suspensions were incubated at 37°C with rotation, and aliquots of the cell suspension from both tubes were assayed for cell-bound radioactivity at various intervals. The dissociation rate was calculated by assuming that the dissociation of a ligand from receptors is a simple first-order decay process (11). The equilibrium constant (K_A) was calculated with the equation: $K_A = k_1/k_{-1}$.

Passive Sensitization and Histamine Release. Cultured basophils were incubated with various concentrations of IgE, recombinant Fc_ε or denatured protein at 37°C for 1 hr for passive sensitization. After washing with RPMI 1640 medium, the cells were resuspended in Tyrode's solution (pH 7.4) containing 1.6 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM 2-(*N*-morpholino)ethanesulfonic acid, 5 mM Hepes, and 0.5 g of gelatin per liter. The cell suspension containing 5 × 10^4 to 10^5 basophils was incubated with various concentrations of anti-IgE antibody at 37°C for 10 min. Histamine released in the supernatants was measured by the automated technique of Siraganian (13).

Measurement of Circular Dichroism (CD) Spectra. Conformational changes in the rFc_ε (0.4 mg/ml) and IgE (1 mg/ml) proteins after heat denaturation and renaturation were examined in CD spectra (14), which were recorded by a CARY 61 spectropolarimeter. Analysis of all samples was performed in phosphate-buffered saline (pH 7.4).

RESULTS

Binding of Recombinant Fc_ε Fragments to Cultured Basophils. As a preliminary experiment, aliquots of a basophil suspension were incubated with various concentrations (0.1–10 μg/ml) of ^{125}I-rFc_ε for 90 min to determine the minimum concentration of the ligand required for the saturation of $Fc_\varepsilon R$. The number of the labeled rFc_ε molecules bound to the cells increased with the concentration of ^{125}I-rFc_ε added and reached a plateau at 1.5–2.5 μg/ml. Thus, aliquots of a basophil suspension were incubated with either ^{125}I-rFc_ε or ^{125}I-IgE at 2 μg/ml for 90 min to determine the number of the ligand molecules specifically bound to the cells. The same preparations of basophils bound similar number of molecules of either human IgE or rFc_ε, provided that freshly labeled ^{125}I-rFc_ε was used (Table 1). These results, together with the fact that the binding of ^{125}I-rFc_ε was not inhibited by IgG but was completely blocked by unlabeled IgE, indicate that the recombinant gene product and native IgE bind to the same receptors.

The kinetics of association between ^{125}I-rFc_ε or ^{125}I-IgE and the $Fc_\varepsilon R$ were determined. A representative experiment for the measurement of k_1 and k_{-1} is shown in Figs. 1 and 2, respectively. It is apparent that k_1 and k_{-1} for rFc_ε are comparable to those for IgE. The equilibrium constants (K_a) for rFc_ε and for IgE were 6.46 × 10^9 M^{-1} and 8.05 × 10^9 M^{-1}, respectively. Repeated experiments with a different cultured cell preparation gave similar results. In this experiment, k_1, k_{-1}, and K_a for rFc_ε were 3.75 × 10^5 $M^{-1}\cdot sec^{-1}$, 6.58 × 10^{-5} sec^{-1}, and 5.68 × 10^9 M^{-1}, while those for IgE were 3.22 × 10^5 $M^{-1}\cdot sec^{-1}$, 7.18 × 10^{-5} sec^{-1} and 4.48 × 10^9 M^{-1}, respectively. The results collectively indicate that the affinity of the dimeric recombinant ε-chain for $Fc_\varepsilon R$ is comparable to that of native IgE.

Table 1. Binding of ^{125}I-IgE and ^{125}I-rFc_ε to cultured basophils

Cell preparation*	Molecules bound per basophil, no. × 10^{-5}	
	^{125}I-rFc_ε†	^{125}I-IgE
A	2.82	2.92
B	2.15	2.25
C	2.12	2.16
D	2.40	3.25
Mean	2.37 ± 0.32	2.63 ± 0.54

*The experiments were carried out with four different cell preparations.
†Number of rFc_ε molecules bound per cell was calculated based on the assumption that rFc_ε was in the dimeric form.

Passive Sensitization and Histamine Release. Cultured basophils were incubated overnight with rFc_ε or IgE at 2–10 μg/ml, and the sensitized cells were challenged with various concentrations of either rabbit anti-IgE or goat anti-IgE. Basophils sensitized with either rFc_ε or IgE released histamine upon challenge with the rabbit anti-IgE, whereas unsensitized cells failed to do so (Fig. 3). Maximal histamine release from the cells sensitized with rFc_ε was 47%, whereas that from the cells sensitized with IgE was 50%. The cells sensitized with rFc_ε also released histamine upon challenge with the goat anti-IgE antibodies. An optimal concentration of anti-IgE antibodies for maximal histamine release was 0.45 μg/ml for basophils sensitized with either rFc_ε or IgE. The maximal histamine released from basophils sensitized with

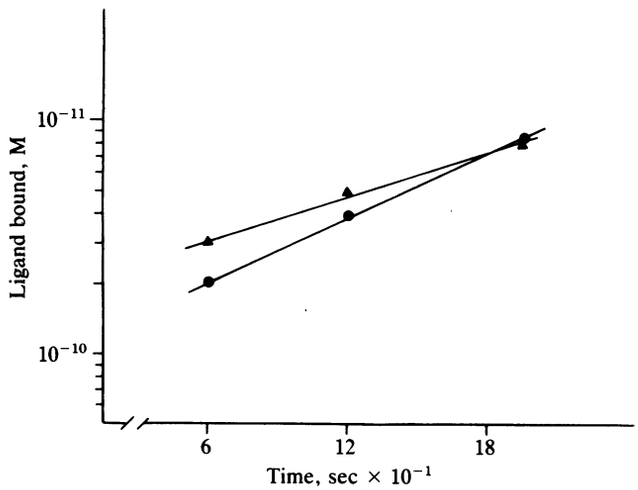

FIG. 1. Determination of the forward rate constant, k_1, for binding of IgE (k_1 = 4.87 × 10^5 $M^{-1}\cdot sec^{-1}$) and rFc_ε (k_1 = 5.32 × 10^5 $M^{-1}\cdot sec^{-1}$) molecules to cultured basophils. Aliquots of cultured cells were mixed with 2.6 μg/ml of ^{125}I-IgE (▲) or ^{125}I-rFc_ε (●), and the cell suspension was kept at 37°C with constant mixing. The reaction was stopped by adding a 200-fold excess of unlabeled IgE to the tubes at the times indicated, and the number of cell-bound molecules were determined. Each point represents the mean of duplicate samples.

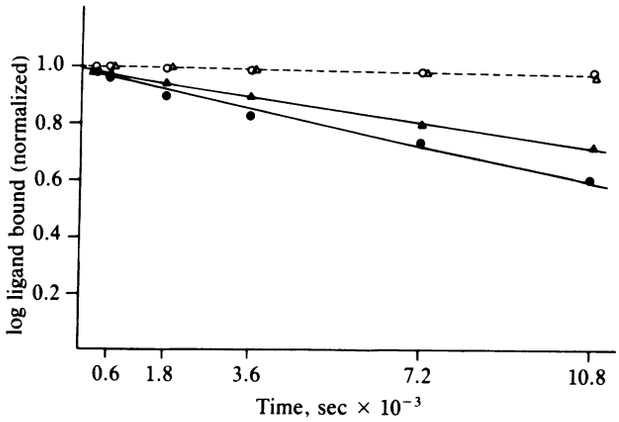

FIG. 2. Determination of the dissociation rate constant, k_{-1}, of IgE ($k_{-1} = 6.05 \times 10^{-5}$ sec^{-1}) and rFc$_\varepsilon$ ($k_{-1} = 8.23 \times 10^{-5}$ sec^{-1}) from cultured basophils. Aliquots of a cell suspension were first incubated with 2.6 μg of ^{125}I-IgE (△, ▲) or ^{125}I-rFc$_\varepsilon$ (○, ●) per ml at 37°C for 1 hr. At the initiation of the experiment (t_0), either a 200-fold excess of unlabeled IgE (——) or an equal volume of medium (------) was added to aliquots of the cell suspension. The amount of cell-bound ^{125}I-IgE or ^{125}I-rFc$_\varepsilon$ was determined at the time indicated, and the data was normalized to compensate for the difference in the concentration of cell-bound ^{125}I-labeled ligand at t_0. Each point represents the mean of duplicate measurements.

rFc$_\varepsilon$ was in the range of 43–63% in six different cell preparations.

Effects of Heat Denaturation and Renaturation of rFc$_\varepsilon$ and IgE on Their Cytotropic Activity. Possible changes in the affinity for Fc$_\varepsilon$R by heat denaturation and renaturation were assessed by the ability of modified proteins to inhibit the binding of ^{125}I-IgE to basophils. Aliquots of a basophil suspension were preincubated with various concentrations of a test sample for 15–30 min, and ^{125}I-IgE was added to the mixtures at a final concentration of 0.2 μg/ml. After incubation for 90 min, the number of cell-bound ^{125}I-IgE molecules was enumerated. Native IgE and rFc$_\varepsilon$ inhibited the binding of ^{125}I-IgE in a concentration-dependent manner (Fig. 4). Preincubation of the cells with rFc$_\varepsilon$ or IgE at 20 μg/ml inhibited the binding of ^{125}I-IgE by 87% and 90%, respectively. In contrast, the same concentration of heat-denatured rFc$_\varepsilon$ or IgE inhibited the binding of ^{125}I-IgE only by 26% and 16%. However, the cytotropic activity of the proteins was recovered after renaturation. It is apparent in Fig. 4 that the ability of the renatured rFc$_\varepsilon$ to block the ^{125}I binding was 88% of the native fragment. However, renaturation of heat-denatured IgE resulted in a less-substantial increase—i.e., only 13% recovery of blocking activity was observed (Fig. 4). The results were confirmed by two experiments of the same design.

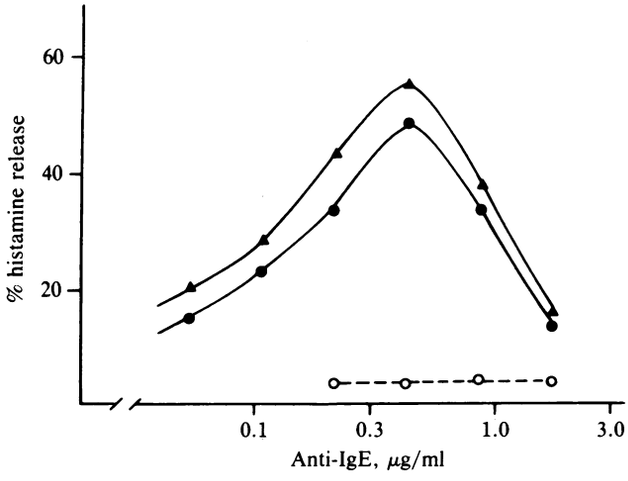

FIG. 3. Anti-IgE-induced histamine release from cultured basophils. Cells were sensitized with rFc$_\varepsilon$ (●) or IgE (▲) protein, and aliquots of the sensitized cells and unsensitized cells (○) were incubated with various concentrations of rabbit anti-IgE at 37°C for 10 min. Each point represents the mean of duplicate samples. Total histamine content in the basophils was 1.1 μg per 10^6 cells.

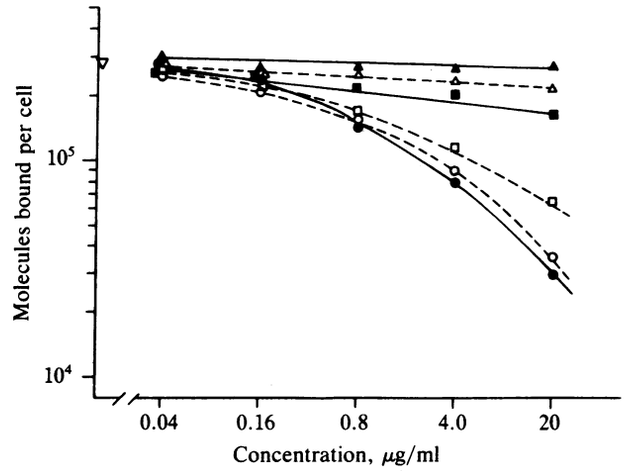

FIG. 4. Inhibition of ^{125}I-IgE binding to basophils by native IgE (●) and rFc$_\varepsilon$ (○), heat-denatured IgE (▲) or rFc$_\varepsilon$ (△), and renatured IgE (■) or rFc$_\varepsilon$ (□). Aliquots of cultured basophils were preincubated with various concentrations of one of these unlabeled molecules or medium (▽) at 37°C for 15 min prior to the addition of 0.2 μg/ml of ^{125}I-IgE. Each point represents the mean of the duplicate assays.

We also have determined the ability of the heat-denatured and renatured proteins to sensitize basophils for anti-IgE-induced histamine release. Aliquots of a suspension of cultured basophils were incubated for 1 hr with 25 μg of a test preparation per ml. The cells were washed and then challenged with an optimal concentration of rabbit anti-IgE (0.45 μg/ml). Representative results of histamine release are shown in Table 2. Failure of the denatured proteins to mediate the anti-IgE-induced histamine release was not due to loss of antigenic determinants, since the denatured proteins gave a precipitin band with the rabbit antiserum used for challenge.

Conformational changes by heat denaturation were detected by CD spectra. In both rFc$_\varepsilon$ and IgE, heating for 60 min at 56°C resulted in an increase in optical activity below 250 nm and a decrease in the activity above 250 nm (Fig. 5). The spectra of rFc$_\varepsilon$ and IgE in the far UV region are very similar, and the heating of both proteins resulted in the loss of fine structures. It was noted that the CD spectrum of the renatured rFc$_\varepsilon$ was almost the same as the spectrum of the native rFc$_\varepsilon$ fragment (Fig. 5 *Upper*). In contrast, the recovery of the CD spectrum was not complete in IgE (Fig. 5 *Lower*),

Table 2. Sensitizing ability of modified IgE or rFc$_\varepsilon$

Treatment of IgE or rFc$_\varepsilon$	Histamine release from sensitized basophils,* %	
	IgE	rFc$_\varepsilon$
Untreated	49.3 ± 5.1	48.3 ± 2.1
Heat-denatured	6.0 ± 1.0	5.7 ± 1.2
Renatured	14.7 ± 2.5	34.3 ± 1.5

*Basophils sensitized with either IgE or rFc$_\varepsilon$ were challenged with an optimal concentration of rabbit anti-IgE. Aliquots of the same cell suspension were sensitized with 25 μg/ml of either untreated or modified IgE. The percentage of histamine release is the average of three separate experiments.

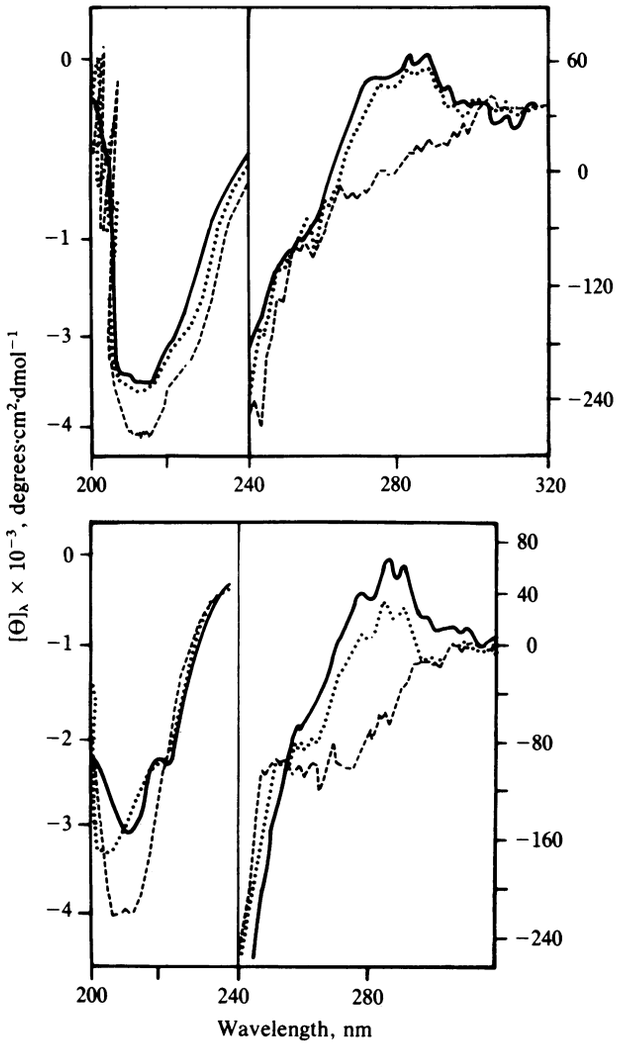

FIG. 5. (*Upper*) Comparison of the CD spectra of rFc_ε (0.4 mg/ml) (——), heat-denatured protein (------), and renatured protein (·····). (*Lower*) The CD spectrum of IgE (1 mg/ml) (——) and its denatured (------) and renatured (·····) products. Proteins were denatured by heating at 56°C for 60 min and renatured by unfolding the denatured proteins in 6 M guanidine hydrochloride followed by stepwise dialysis in decreasing concentrations of guanidine hydrochloride and finally in phosphate-buffered saline.

although above 250 nm the spectrum of renatured IgE closely resembles that of the native protein. Below 220 nm, a considerable difference was observed between the renatured IgE and native IgE.

DISCUSSION

Data presented in this paper clearly show that rFc_ε binds to the same receptors on human basophils to which the native IgE binds. The majority of the peptide in the rFc_ε preparation was in the dimeric form, although 27% of the protein in the preparation were tetramers. Since 50% of the protein in the ^{125}I-labeled rFc_ε preparation could bind to basophils, it appears that dimeric (as well as tetrameric) rFc_ε molecules bind to the receptors. Indeed, the number of rFc_ε dimer molecules bound per basophil at saturated conditions was comparable to the number of IgE molecules bound to the same cells.

The present experiments suggest that the dimeric form of rFc_ε peptide binds to $Fc_\varepsilon R$ with an affinity comparable to that of native IgE (Figs. 1 and 2). These results seem to conflict with previous observations on the lower capacity of the rFc_ε to block the binding of ^{125}I-IgE to basophils (3) or to block passive sensitization of skin and lung mast cells (15, 16). On a molar basis, blocking activities of rFc_ε were 20–30% of native IgE (PS). The discrepancies appear to be due to an assumption in the previous experiments that all peptides in a rFc_ε preparation have affinity for $Fc_\varepsilon R$. The present experiments actually show that only 50% of peptides in the preparation bind to the receptors and that these peptides and native IgE have comparable affinity for $Fc_\varepsilon R$ (Figs. 1 and 2). It is apparent that a fraction of the dimeric rFc_ε peptide assumes an inactive conformation. This is not entirely surprising because factorial analysis indicates that the 11 cysteine residues in one polypeptide chain could theoretically give rise to more than 1800 different conformers upon dimerization. In this context, it is interesting that Liu *et al.* (5) obtained a similar portion of bioactive molecules in their preparation of rFc_ε. This suggests that more than half of the rFc_ε chains will adopt an intrinsically active conformation that probably corresponds to the native structure of ε chains.

As expected from the high affinity of rFc_ε for $Fc_\varepsilon R$, the peptide could sensitize human cultured basophils for anti-IgE-induced histamine release. When basophils were saturated with either rFc_ε or IgE, an optimal concentration of anti-IgE for histamine release was comparable. These results indicate that carbohydrate in the Fc portion of ε chain does not affect the affinity of IgE molecules for $Fc_\varepsilon R$ nor the ability of the molecules for inducing histamine release.

It is well known that IgE antibodies lose their ability to sensitize basophils upon heating at 56°C (17). As expected, rFc_ε molecules also lose the affinity for $Fc_\varepsilon R$ upon heat denaturation. Inactivation of IgE by heating is associated with conformational changes as demonstrated by CD spectra (14, 18). The present experiments showed that the same principle applied to rFc_ε. Upon renaturation, rFc_ε regained its native conformation almost completely, and the restoration of the CD spectra was accompanied by a marked (>80%) recovery of biologic activity. Dorrington and Bennich (18) studied CD spectra of proteolytic fragments of native IgE and have shown that heating of a fragment corresponding to a dimer of the C_H4 domain destroyed the optical activity above 250 nm, which arises from pertubations of aromatic and disulfide transitions. In an attempt to correlate the changes in CD spectra and loss of affinity of IgE for $Fc_\varepsilon R$, they suggested a role for C_H4 domains in the binding to receptors (18). The present findings on renaturation of IgE do not support their hypothesis. When heat-denatured IgE was renatured, the optical activity above 250 nm was largely restored (Fig. 5 *Lower*). However, blocking the activity of renatured protein was <20% of the activity of native protein. The CD spectra of native and renatured IgE showed major differences just below 220 nm. It should be noted that CD spectra of native rFc_ε and renatured rFc_ε were almost identical in the region. Since the CD spectrum in this region is sensitive to changes in secondary structure, it is conceivable that the presence of oligosaccharide residues may interfere with the proper refolding of human ε chains and prevent restoration of receptor binding conformation.

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