Structural Mapping of IgE–FcεRI, an Immunoreceptor Complex

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The immune response system operates, usually successfully, to prevent an animal from being taken over by foreign invaders such as bacteria, viruses, and nonself cells. In the midst of this complex array of mechanisms involving cellular and soluble components, there are a few processes that appear to do more harm than good. One example, familiar to many human sufferers, is the allergic response that is mediated by the antibody immunoglobulin E (IgE) and its cell surface receptors. Similar to the more common IgG and other classes of antibodies, IgE is a tripartite structure with ligand-binding sites located at the ends of its two Fab segments and effector-binding region located within its Fc segment (see Figure 1).[1,2] The effector for IgE is FcεRI, a high-affinity receptor located on the surface of mast cells and basophils. When IgE–FcεRI complexes are cross-linked by multivalent ligands (antigens), such as proteins from pollen or cat dander in allergic individuals, the cells are activated and undergo degranulation. The result of this process is the secretion of histamine and other chemical mediators of allergic reactions. Value has been ascribed to IgE for its role in battling parasitic infections by mobilizing mediators of inflammation.[3] In modern developed countries, however, parasites are not a major problem, and allergic reactions are at best unpleasant and sometimes fatal.

Beyond medical interest in the treatment of allergies, research on the IgE–FcεRI system has broader importance because this system has many structural and functional features common to other types of immunoreceptors. For example, the T cell receptor for an antigen plays a central role in most kinds of immunological responses including cell-mediated killing of virally infected cells and stimulation of B cells to become antibody-producing cells. The T cell receptor, IgE–FcεRI, and many other immunologically important proteins are members of the Ig superfamily.[4] Similar in operation to IgE receptors, T cell receptors must be aggregated to cause cell activation; physiological activation of a T cell involves antigens that are presented multivalently on the surface of an apposing cell. IgE–FcεRI receptors are the immunoreceptors most extensively studied by biochemical and biophysical methods for a variety of reasons including the relative simplicity of the system and the experimental components available. These studies provide general information about the operation of immunoreceptors.

Our laboratory investigates the molecular properties of IgE–FcεRI complexes and how they can be cross-linked by antigens to initiate signal transduction and thus stimulate a cellular response. This Account describes our studies on the structure of receptor-bound IgE. A wealth of X-ray crystallographic data has provided a detailed view of the Ig domain structure, the Fab and Fc segments, and the antigen-binding sites.[1,2,5] The structure determined for rigid mutant ("hinge-deleted") IgG showed the axes of the Fab and Fc segments lying roughly in the same plane[6] which led to the common view that Ig's are Y- or T-shaped. Recently, an intact IgG was successfully crystallized, and its Fab and Fc segments were found to be displaced

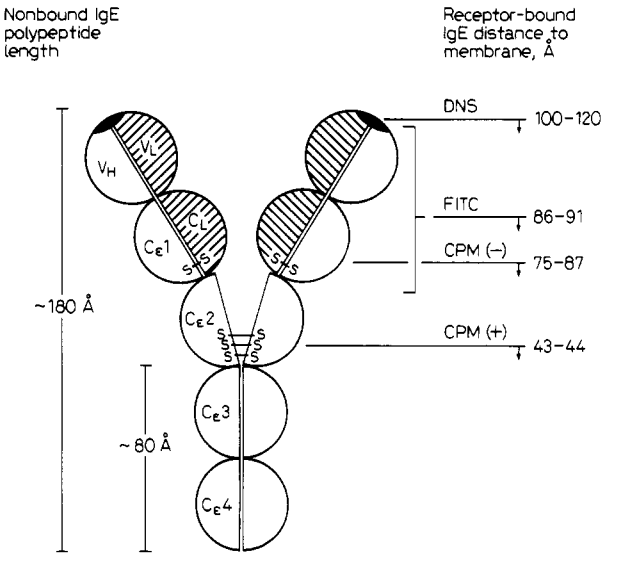

Figure 1. Energy-transfer distances between sites on receptor-bound IgE and the membrane surface (right side) are compared to dimensions of the IgE polypeptides (left side). V_L and V_H represent the variable domains of the light (L) and heavy (ϵ) polypeptide chains, respectively; C_L, $C_\epsilon 1$, $C_\epsilon 2$, $C_\epsilon 3$, and $C_\epsilon 4$ represent the constant domains of the light and heavy chains; S–S indicates the location of interchain disulfide bonds. The Fab segments include the V_LV_H–$C_LC_\epsilon 1$ domains, and the Fc segment contains the $(C_\epsilon 2)_2$–$(C_\epsilon 3)_2$–$(C_\epsilon 4)_2$ domains. The dimensions of IgE assume the length of a single domain to be 40 Å (on the basis of X-ray crystallographic data for IgG_1) and the angle between the Fab segments to be 90°; angles of 180° to 0° yield full-length values of 140–200 Å, respectively. Energy-transfer distances are from labeled samples and measurements summarized in the text and Table I. Reprinted with permission from ref 48. Copyright 1989 Karger.

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asymmetrically, a feature that was interpreted as being indicative of Ig flexibility.[7] No crystal structures have been determined for IgE, although a model for the three-dimensional structure of IgE–Fc has been developed with the coordinates derived from homologous portions of IgG.[8] Other types of structural studies, including fluorescence resonance energy transfer, fluorescence anisotropy, and small-angle X-ray scattering, have been carried out for several different classes of Ig, and these provide valuable complementary information about overall Ig structure and flexibility.[2]

Because of the complexities of the experimental systems, very little three-dimensional structural information is available for any cell surface bound immunoreceptor. Yet this is the environment in which the receptors operate biologically, such that complete understanding, possibly leading to more rational medical intervention, requires that these more complex systems be approached. For example, detailed information about the IgE–FcεRI interaction could lead to the design of a drug that could inhibit this interaction and thereby be useful in the treatment of allergies. Of basic and medical interest is the possibility that the structural properties influence the way in which immunoreceptor complexes are clustered into a cell-activating configuration.

Structural Mapping with Fluorescence. The method best suited to an investigation of the three-dimensional structural arrangement of proteins on cell membranes is fluorescence resonance energy transfer. This spectroscopic method requires the selective placement of donor and acceptor probes and measures distances between them. The structural determination is limited by the sites labeled, and consequently the level of resolution is rather low compared to the information obtained with X-ray crystallographic and NMR methods. However, unlike these other methods, energy transfer can be applied to highly heterogeneous biological systems in which the structure of interest is present at very small (nanomolar) concentrations.

In addition to chemical reactivity for specific placement, the energy-transfer probes must have a matched set of electronic energy levels such that the fluorescence spectrum of the donor overlaps the absorption spectrum of the acceptor. Energy transfer can then be observed as the quenching of the donor fluorescence (i.e., reduction of quantum yield, Q) in the presence of the acceptor. Experimental comparison of donor fluorescence in the absence (Q_D) and presence (Q_{DA}) of the acceptor yields an "efficiency of energy transfer" (E), and this is related to the distance (R) between these two probes according to the Förster theory.[9]

$$E = 1 - Q_{DA}/Q_D \quad (1)$$

$$E = \frac{(R_0/R)^6}{1 + (R_0/R)^6} \quad (2)$$

$$R_0 = (9.79 \times 10^3)(J\kappa^2 Q_D n^{-4})^{1/6} \quad (3)$$

R_0 is the critical transfer distance between the donor and acceptor corresponding to $E = 0.5$. This reference value can be determined independently for the particular pair of donor and acceptor probes in a particular medium according to eq 3; J is the spectral overlap integral; κ^2 describes the relative orientations of the donor and acceptor transition dipoles; n is the refractive index of the medium through which the energy transfer occurs. An assessment of uncertainties associated with the calculation of R_0 has been discussed previously;[10] small errors in R_0 have relatively little effect on the calculation of R because of the sixth root dependence. Useful R_0 values are in the range 20–55 Å, allowing measurement of energy-transfer distances R up to about 100 Å. The simple energy-transfer relationship described in eq 2 for a single donor–acceptor pair can be extended to more complicated cases of multiple donors and acceptors in particular geometric arrangements.[10]

Thus energy-transfer measurements provided the means to initiate a structural investigation of cell surface IgE–FcεRI, seeking at first information about the conformation and orientation of the bound IgE.[11] Our experiments have been carried out with a rat mucosal mast cell line, RBL-2H3, which expresses a relatively large number of IgE receptors, ~200 000 FcεRI's per cell. Maintained in a tissue culture, these cells have no bound IgE, but added rodent IgE binds with a very high affinity ($K \geq 10^{10}$ M^{-1}) and essential irreversibility ($k \leq 10^{-5}$ sec^{-1}) on the time scale of the experiments.[12] With these cells, monoclonal IgE's of different specificities can be employed, and these can be labeled selectively with spectroscopic probes prior to binding. Our studies have also taken advantage of a set of "switch-mutant" antibodies with the same antigen-binding sites specific for the fluorophore [5-(*N*,*N*'-dimethylamino)naphthalen-1-yl]sulfonyl (dansyl) but different heavy-chain constant regions corresponding to different Ig isotypes. These were first generated by selecting somatic variants in hybridoma cells lines,[13] and genetic engineering was subsequently used to create further variations.[14]

Our first set of experiments (summarized in Figure 1 and Table I) measured distances between donors located in various regions on IgE and amphipathic acceptor probes located in the plane of the cell membrane. Fluorescence measurements with whole cells can be technically difficult because internal components contribute a high background of autofluorescence and the cells also tend to absorb the amphipathic probes into intracellular membranes. There-

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Table I. Summary of Distances (L) between Donor-Labeled Sites on Receptor-Bound IgE and Acceptors Located at the Membrane Surface[a]

donor	location	acceptor	R_0 (Å)	L (Å)[b]	ref
dansyl–lys	antigen-binding sites	HAE, ORB	54, 57	100–120	17
FITC	Fab	HAE, ORB	53, 53	86–91	16
CPM(–)	Fab (Cε1 domain)	HAF, $DiOC_6$	51, 56	75–87	17
FITC	B5 or B5 $F(ab')_2$ bound to Fab (Cε1)	HAE	55, 58	78–87	18
FITC	A2 Fab′ bound to Fc	HAE	46	54	18
CPM(+)	Cε2 domain	HAF, $DiOC_{10}$	47, 52	43–44	16

[a] Table adapted from ref 18. Abbreviations: lys, lysine; FITC, fluoresceinyl 5-isothiocyanate; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide; HAE, 5-(hexadecanoylamino)eosin; ORB, octadecylrhodamine B; $DiOC_6$, 3,3′-dihexyloxacarbocyanine; $DiOC_{10}$, 3,3′-didecyloxacarbocyanine. [b] The distance range shown includes variation arising from use of more than one acceptor in different experiments. The values determined should be considered as "distances of closest approach" between the donor-labeled site on IgE and acceptors at the membrane surface, and this may be somewhat greater than the vertical distance, depending on the unknown width of the receptor at the membrane surface.

fore we employ cell surface membranes prepared by chemically-induced vesiculation of the RBL cells; these vesicles contain no internal membranes and are generally large (several micrometer diameter), spherical, and right-side-out.[15] Thus we are able to carry out measurements on IgE receptor complexes located on their native cellular membranes but without many of the complications present with the whole cell.

As depicted in Figure 1 we can place a donor probe, CPM, in the Cε2 domain of IgE by reduction and alkylation of the disulfides located there (CPM(+)).[16] For another sample, we found that CPM modifies a site in Cε2 in the absence of reducing agents (CPM(–)).[17] Another sample was labeled with fluorecein isothiocyanate (FITC), and although we expected random labeling over the whole IgE, we found that this probe predominately reacts within the Fab segments.[16] The selective covalent modification of these probe locations can be confirmed with a variety of evidence including identification of fluorescent proteolytic fragments using polyacrylamide gel electrophoresis. For another distance measurement, we used the anti-dansyl IgE to put a donor probe in the antigen-binding site.[17] The acceptor probes, including for example 5-(*N*-hexadecanoylamino)eosin, are hydrophobic molecules with a charged chromophoric head group; this amphipathic combination causes the probe to partition into the membrane with the chromophore remaining on the membrane surface.[15] Donor fluorescence plotted as a function of acceptor surface density can be fitted with a model parameterized by the distance of a donor point above a uniform plane of acceptors.[11]

The energy-transfer distances between these different donor-labeled sites on IgE and the membrane surface (Figure 1, right side) are compared to the dimensions of a planar IgE as inferred from crystallographic measurements of Ig domains[1] (Figure 1, left side). This comparison shows that the disulfide bond in the Cε2 domains are a distance from the membrane (~45 Å) that is less than the length of two Ig domains (~80 Å), and the distances from other sites in the Fab segments increase in the direction of the antigen-binding sites. The picture that began to emerge from this first set of measurements was that receptor-bound IgE is bent such that the Fab segments point away from the membrane surface while the Fc segment is closer, but its long axis is oriented at some nonperpendicular angle to the plane of the membrane.[17]

Further distance measurements to test this model employed monoclonal anti-IgE antibodies (of the IgG class): B5 antibody is specific for the Fab segments (Cε1 domains), and A2 antibody is specific for the Fc segment.[18] As exploited in many laboratories, antibodies and their Fab fragments are highly valuable research tools as specific tags in complex systems. As probes for energy-transfer measurements of distances ≤100 Å, their bulky size is problematic. However, this uncertainty can be addressed with Fab fragments that are labeled with fluorophores, randomly over their length or selectively at their N-terminal or C-terminal ends.[11] Thus comparative measurements of energy transfer between such donors bound to a membrane protein and amphipathic acceptor probes located at the surface of the membrane bilayer can yield information about the orientation of the bound Fab as well as provide a distance to the site on the protein where the antibody binds. As listed in Table I, the distance-to-membrane measurements made with these anti-IgE probes are consistent with the other distances measured and thereby support the bent IgE model. The set of distances together provides confidence in the individual measurements.

Region of Interaction between IgE and FcεRI. Since the initial demonstration that the Fc segment of IgE binds to FcεRI[19] there has been a steady effort to localize more precisely the region of interaction. Proteolytic digestion was used to show that a site near the Cε2–Cε3 interface is protected when rat IgE binds to FcεRI on RBL cells,[20] and studies with Fc fragments prepared from genetic manipulation of human IgE[21] also indicated the involvement of this region in receptor binding. We addressed this question by genetically engineering a set of chimeric Ig's from a combination of two of the switch-mutant anti-dansyl Ig's. In particular, one or both of the two C-terminal domains in mouse IgE were exchanged with the homologous C-terminal domains in human IgG_1 (IgG, subclass 1). Thus we were able to prepare intact Ig molecules in which Cε3 was exchanged with Cγ2 and/or Cε4 was exchanged with Cγ3.[22]

The purified chimeric IgE/IgG molecules were tested for their ability to bind to FcεRI on RBL cells and to trigger antigen-mediated cellular degranulation.[23] The results were unequivocal that IgE with Cε4 replaced by Cγ3 behaves identically to wild-type IgE, whereas neither IgG nor any of the other chimeric IgE/IgG molecules had any activity with the RBL cells. These data are consistent with the current view that residues distributed throughout the Cε3 domains interact di-

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Table II. Head-to-Tail Distances for IgE and IgG_1[a]

sample	E[b]	R (Å)[b]	ρ (Å)[c]	hw (Å)[c]	R_ρ[d]
IgE	0.20	71	75	24	72
IgE–FcεRI	0.22	69	71	12	70
IgG_1	0.15	75	100	89	77
IgG_1–FcγRI	≤0.08	>85	nd[e]	nd	nd

[a] Table abridged from ref 28. For all samples, the donor was fluorescein conjugated to cysteines located at the C-terminus of the Fc segment and the acceptor was 1-*N*-eosinthiocarbamyl-5-*N*-dansylcadaverine located in the antigen-binding sites at the N-termini of the two Fab segments. For this energy-transfer pair, R_0 = 50 Å.[27] Calculated head-to-tail distances calculated shown here assume that the two N-termini are equidistant from the C-terminus.[29] [b] Average transfer efficiencies (E) and distances (R) for IgE and IgG_1 taken from energy-transfer experiments of refs 27 and 28, respectively. [c] Values taken from experiments in which the R_0 was varied by addition of I^- in order to assess the distribution of head-to-tail distances.[28] ρ is the most probable distance of an assumed Gaussian distribution of distances between donors and acceptors; hw is the full width at half-maximum of this distribution. [d] R_ρ represents a weighted average of distance values calculated with eq 2 (adjusted for two acceptors) from the Gaussian distribution of head-to-tail distances (based on varied R_0) for comparison with the average distance values R obtained from energy-transfer measurements with constant R_0.[28] [e] Not determined.

rectly with FcεRI.[24] The Cε2 domains appear uninvolved in receptor binding, and Cε4 may be required only to maintain the dimerized state of Cε3.[25]

Measurement of Head-to-Tail Distances for IgE and IgG. The anti-dansyl IgG_1 has a derivative in which a serine residue near the C-terminal end in Cγ3 has been mutagenized to cysteine.[26] Genetic exchange of this domain for Cε4 in IgE provided a chimeric IgE that is identical to wild-type IgE in its binding and cell activation properties but contains sulfhydryls at the C-terminus that can be selectively labeled with fluorescent probes. Thus we could measure the energy-transfer distance between fluorescein donors at this site and eosin–dansyl acceptor probes located in the two antigen-binding sites.[27] Because the donor-labeled sites and acceptor-labeled sites are identical in all cases, we can directly compare these head-to-tail distances for IgE and IgG_1 both in solution and bound to their respective Fc receptors on cell membranes (Table II).[28]

For IgE bound to FcεRI, the average head-to-tail distance[29] was found to be 69 Å, consistent with our previous data which indicated a bent IgE (Figure 2). The results further showed that IgE in solution appears to be similarly bent with an average head-to-tail distance of 71 Å. Thus it appears that the common view of a planar Y-shaped Ig structure is not accurate for receptor-bound IgE or for nonbound IgE. Independent support for a compact IgE comes from sedimentation

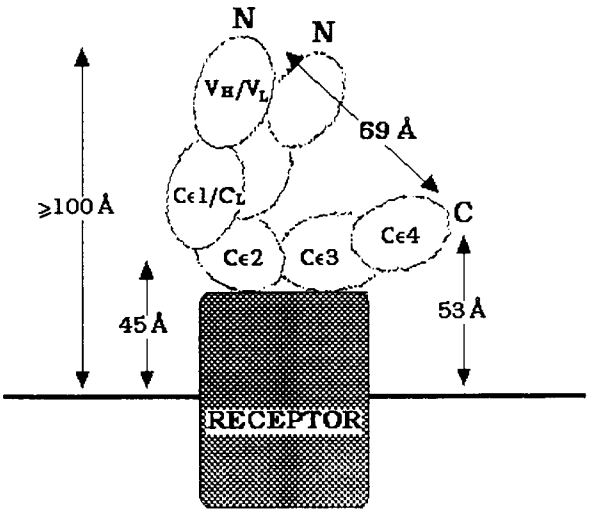

Figure 2. Model of IgE bound to a receptor on the cell membrane. The amino-terminal heads and the carboxy-terminal tail are indicated as N and C, respectively. The solid horizontal line represents the outer surface of the cell membrane, and FcεRI is represented as a box. Distances are from measurements summarized in Tables I and II. Reprinted with permission from ref 27. Copyright 1991 American Chemical Society.

and small-angle X-ray scattering measurements for IgE in solution.[30]

The average head-to-tail distance determined for IgG_1 in solution (75 Å; Table II) also suggests an average bent structure as this distance for a planar shape is estimated to be 120–170 Å for IgG (covering the range of T to Y shapes). The longer distance for IgG_1 bound to FcγRI receptors on U937 cell membranes (>85Å) indicates that the average IgG_1 conformation is different after binding.

Flexibilities of IgE and IgG_1. Like other structural measurements, fluorescence resonance energy-transfer distance measurements represent an average over the distribution of structures present in the sample. As indicated in eq 2, the distances assessed by energy transfer are weighted according to R^{-6} such that the average distance determined is shorter than an arithmetic average of actual distances. Consideration of a distance distribution is particularly important in evaluating Ig structures because these proteins are known to have some flexibility between domains, such as in the hinge region joining the Fab and Fc segments.[2] The flexibility characteristic of a class of antibodies probably plays some role in its immunological function. This property has been investigated with fluorescence anisotropy measurements. Previous studies compared the segmental flexibilities of switch-mutant IgE and several different subclasses of IgG that all had identical antigen-binding sites specific for the dansyl fluorophore.[13] This work revealed that IgE is more rigid than all of the IgG molecules tested. Subsequently our group examined the same anti-dansyl IgE and found that IgE in solution has some segmental flexibility that decreases but is still significant after binding to FcεRI on RBL cell membranes.[31,32]

To assess the distribution of head-to-tail distances corresponding to Ig flexibility, we took advantage of an

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(29) The head-to-tail distances calculated assume the two N-termini are equidistant from the C-terminus. It is possible that the structural conformations available to IgE or IgG_1 place the C-terminus closer to one of the two N-termini (e.g., coplanar sideways bending of a Y shape). This would not make a large difference in the distance extracted from the energy-transfer measurements. As an extreme comparison, if R corresponds to two acceptors being equally spaced from the donor and R' corresponds to only one acceptor being close to the donor, then for the same efficiency of energy transfer $R' = R \times 2^{1/6} = 1.12R$.
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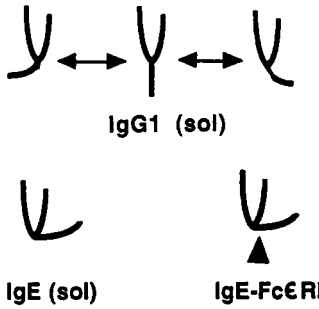

Figure 3. Structures and flexibilities are compared for IgE and IgG_1 with a schematic model shown in perspective; the Fc C-terminal end is drawn to be equidistant from the two Fab N-terminal ends. IgE in solution (bottom left) and IgE bound to FcεRI on the cell membrane (bottom right) appear to be similarly bent and relatively rigid; the arrowhead indicates the probable region of receptor interaction for IgE–FcεRI. IgG_1 in solution (top) shows much greater flexibility than IgE. The distributions of distances represented by these models are consistent with the view that IgE is asymmetrically bent on one side, whereas IgG_1 flexes in more than one direction. Reprinted with permission from ref 28. Copyright 1992 American Chemical Society.

energy-transfer method that systematically alters the R_0 for the same donor–acceptor pair and thus modulates the probe sensitivity over a range of distances.[33] This was accomplished by the addition of variable amounts of iodide which collisionally quenches the fluorescein fluorescence, thereby reducing its quantum yield and consequently the R_0 for the fluorescein–eosin donor–acceptor pair (see eq 3). In this manner, energy transfer between the C-terminal fluorescein and eosin–dansyl in the two antigen-binding sites was measured as a function of R_0. The efficiencies observed can be fitted with Gaussian distributions of probability *vs* distance that are characterized by the most probable (average) distance (ρ) and the width of the distance distribution at half maximal probability (hw).[28]

The best fits from this independent analysis of IgE in solution, IgE bound to FcεRI receptors on membranes, and IgG1 in solution (Table II) are consistent with the average bent structures indicated in the earlier measurements, and they also provide new information about relative conformation flexibilities of these Ig. The distributions obtained provide the following view (Figures 2 and 3): IgE in solution is a bent molecule such that the distance between the ends of the Fab segments and the end of the Fc segment is, on average, 75 Å (ρ); a limited amount of flexibility allows this distance to vary in the range of roughly 63–87 Å (hw = 24 Å). IgE bound to FcεRI receptors on RBL cell membranes appears to be slightly more bent with somewhat less flexibility having an average end-to-end distance of 71 Å within a rough range of 65–77 Å. IgG_1 in solution shows much greater flexibility than IgE such that the corresponding distribution of distances is much broader, ranging roughly over 57–143 Å. Comparison of the structural dimensions of these Ig's with their apparent end-to-end distance ranges suggests that the Fc of IgE is bent to one side, whereas the Fc of IgG_1 can bend in more than one direction (Figure 3). As shown by the comparison of R to R_ρ in Table II, the weighted average of the distribution of head-to-tail distances determined by this method agrees quite well with the average distances determined in the earlier measurements.

A three-dimensional model of the IgE–Fc segment has been constructed from its known amino acid sequence, identification of its disulfide bonds, and structural homologies of Cε3 and Cε4 with Cγ2 and Cγ3 of IgG_1–Fc for which the crystal structure has been determined.[8] The eight-residue stretch bridging the Cε2 and Cε3 domains was modeled as an extended region of the polypeptide chain. Although this IgE segment could be considered comparable to the hinge of IgG, our structural measurements suggest that its structure is bent and rather inflexible. A plausible basis for the difference in IgG and IgE flexibilities is the conformational freedom allowed by glycine and the fact that IgG_1 contains two adjacent glycines in its hinge segment compared to the single glycine in the corresponding IgE segment.[34] Site-specific mutagenesis and energy minimization techniques should allow determination of whether the amino acids present within this hingelike segment or in the adjacent domains (Cε2–Cε3) stabilize a bent structure for IgE.

Measurement of the Distances between Antigen-Binding Sites. Bivalency of antibodies is a critical feature that affords tighter binding in the presence of multiple antigenic sites and also enables the formation of cross-linked immune complexes containing multiple antigens and Ig's. Such immune complexes can trigger cellular activation via cell surface Fc receptors, and they can also activate the components of the cytolytic complement system in the blood. Parameters of interest in considering the ability of antibodies to bind bivalently are the flexibility of the Fab segments (as described above) and the distance between the antigen-binding sites.[35] We have used energy-transfer distance measurements to compare members of switch-mutant antibodies with identical antigen-binding sites specific for dansyl, using fluorescein and eosin (both linked to dansyl) as donors and acceptors, respectively.[36]

The average interbinding site distances determined for mouse IgE, mouse IgG_1, and human IgG_1 are shown in Table III. These distance values are consistent with other physical parameters including the mean rotational correlation times as determined from fluorescence anisotropy measurements (a lower number indicates faster internal motion of the Fab segments)[14] and the number of amino acids in the upper hinge, which is considered to be a major source of Fab flexibility. The results support the suggestion that a longer hinge allows greater flexibility such that the binding sites can get closer together. Hence, shorter energy-transfer distances are observed for the more flexible IgG than for the more rigid IgE which appears more constrained with a larger average angle between the Fab segments.

Distances between Cross-Linked IgE–FcεRI Complexes on the Cell Membranes. Although aggregation of cell surface receptors is essential in most immunological cellular responses, very little is known about the features of aggregation that are critical for the activation

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Table III. Energy-Transfer Distances between Immunoglobulin Fab Binding Sites Compared to Other Structural Properties[a]

immunoglobulin	E^b	R (Å)[c]	angle (deg)[d]	$\langle\phi\rangle$ (ns)[e]	upper hinge[f]
mouse IgE	0.03	≥96	>70	124	0
mouse IgG_1	0.09	83	60	81	6
human IgG_1	0.08	84	60	60	10

[a] Table abridged from ref 36. [b] Average transfer efficiency from four or five measurements; standard deviation of ≤0.02. [c] Average distance assumes a binomial distribution of the fluorescein and eosin probes. The R_0 for this donor–acceptor pair is 56 Å. [d] Angle between Fab segments assuming the distance between binding sites is given by R and length of Fab is 80 Å. [e] Average rotational correlation time determined from measurement of fluorescence anisotropy of dansyl probes located in antigen-binding sites. Values were taken from ref 14. [f] Number of amino acids in the upper hinge region that contribute to the separation of the Fab segments and probably provide the major structural basis for Fab flexibility.[2,14]

mechanism. Structural properties that correlate with biological functions can provide valuable insight into this problem. In the case of FcεRI activation of mast cells, it is clear that massive receptor aggregation is not necessary: less than 10% of the IgE–FcεRI complexes cross-linked as trimers or larger can trigger a maximal response.[37] What is probably involved in the initiation of signal transduction is specific interactions of small aggregates of IgE–FcεRI complexes with other cellular components, and effective coupling may depend on certain structural features such as receptor–receptor contact or receptor–receptor orientation.

Our laboratory investigated the possible requirement for physical contact between cross-linked receptors by preparing oligomers of avidin that were cross-linked by bis-biotin and capped on either end with dinitrophenyl (DNP)-biotin. Thus we had bivalent ligands ranging from 40 to 240 Å in length corresponding to DNA–$(avidin)_n$–DNP with n = 1–6, respectively.[38] These were tested for their ability to trigger degranulation from RBL cells sensitized with monoclonal IgE specific for DNP, and we found that the avidin hexamer (n = 6) stimulates a strong response (similar to n = 2–5 and better than n = 1). Considering the dimensions of IgE–FcεRI, these results indicate that it is not necessary for the directly cross-linked IgE–FcεRI to come into physical contact for the initiation of signal transduction.[38] However, because a small amount of external cross-linking can cause larger scale receptor coalescence,[39] it remains possible that physical associations occur between receptors that are not directly cross-linked to each other. It is also possible that membrane proteins associated with FcεRI come into contact to produce a signal.

Some evidence for the importance of receptor–receptor orientation has been provided by a study with several different monoclonal antibodies specific for FcεRI which cause degranulation of RBL cells (in the absence of IgE). Particularly interesting are the data indicating that the magnitude of the response does not correlate with the antibodies' ability to bind and cross-link. Hence the results suggest that the orientation in which the receptors are brought together by the different antibodies is an important factor.[40]

We have obtained some information about the orientation for IgE–FcεRI complexes from ligand-binding, degranulation, and energy-transfer studies. Binding studies with anti-DNP IgE and the symmetric bivalent ligand, (DNP–caproyl–tyrosine)$_2$–cystine ((DCT)$_2$–cys), have revealed that this ligand preferentially cross-links the IgE–FcεRI into cyclic dimers (containing two IgE and two (DCT)$_2$–cys) on the cell surface.[41] Furthermore, these cyclic dimers do not effectively stimulate degranulation even when they are cross-linked together by a monoclonal anti-IgE to a higher aggregation state.[42] Thus the orientation corresponding to the IgE–FcεRI cyclized in this manner appears to be incompetent for stimulating a cellular response.

We can use energy transfer to assess orientation as exemplified by measurements made with fluorescently modified anti-dansyl IgE derivatives and didansylcadaverine[43] which appear to have binding and triggering properties similar to those of anti-DNP IgE and (DCT)$_2$–cys. The anti-dansyl IgE was labeled with fluorescein donors either by reducing and alkylating the disulfide bonds in the hinge region ($C_\epsilon 2$) or by modification of the genetically introduced sulfhydryls at the C-terminal tail. A different sample of IgE was labeled with eosin acceptors at the C-terminal tail. A mixture of the donor-labeled IgE and acceptor-labeled IgE was bound to FcεRI on RBL cells and then cross-linked with didansylcadaverine such that most of the IgE–FcεRI complexes were expected to be in the form of cyclic dimers.

The sample containing the tail-to-tail donor–acceptor pair yielded an efficiency of 0.10. Assuming a binomial distribution in the combination of donor-labeled and acceptor-labeled IgE's, this efficiency corresponds to an average distance of 70 Å between the C-termini for IgE's cross-linked into cyclic dimers. The hinge-to-tail donor–acceptor pair yielded an energy-transfer efficiency <0.03 corresponding to an average distance >100 Å. Compared to didansylcadaverine in these experiments was a monoclonal antibody specific for the C-terminal domain of the IgE derivative. This antibody effectively activates IgE-sensitized cells, indicating the formation of competently cross-linked IgE–FcεRI. With this antibody, the tail-to-tail donor–acceptor pair yielded an energy-transfer efficiency of <0.05 corresponding to an average distance >100 Å.[43] Taken together, these results indicate that not all configurations of cross-linked IgE–FcεRI are capable of interacting with other cellular components necessary to initiate signal transduction. In particular, an orientation that brings the IgE tails in proximity with the hinge regions further apart (Figure 4) appears to be incompetent to activate a cellular response.

Concluding Remarks. The structural measurements described in this Account yield a self-consistent picture that IgE bound to its cell surface receptor FcεRI has a bent and rather rigid conformation and that IgE in solution is structurally similar. This constrained, asymmetrical conformation stands in contrast to IgG_1, which appears to have substantially more segmental

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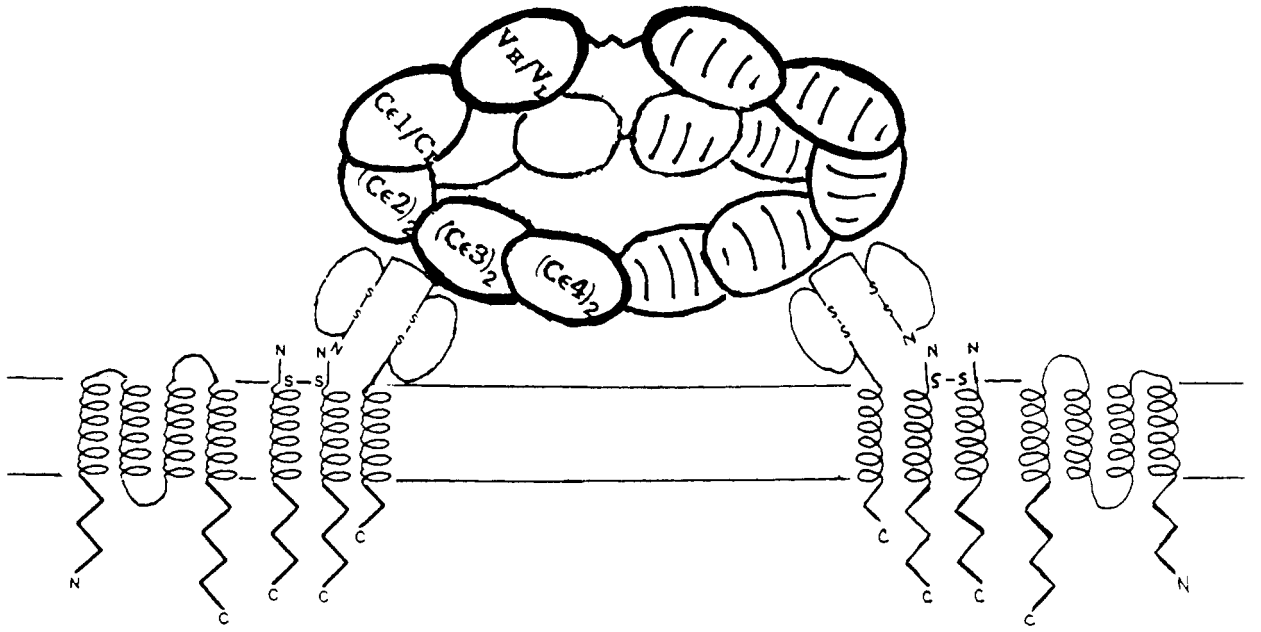

Figure 4. One possible structural configuration for two IgE–FcεRI complexes that have been cyclically dimerized by a pair of bivalent ligands into a form that is incapable of activating a cellular response. The energy-transfer results indicate that the distance between labeled sites in the C-terminal tails is ~70 Å, whereas the distances between these sites in the tail of one IgE and labeled sites in the hinge of the apposing IgE is >100 Å. The disposition of the subunits of FcεRI with respect to the membrane, as predicted by gene sequencing,[49] is shown. The region of the IgE–FcεRI interaction is within Cε3 of IgE (as discussed in text) and in the α subunit of FcεRI.[50] Flexibility in the α subunit has been indicated in measurements of phosphorescence anisotropy[51] and fluorescence anisotropy.[43]

flexibility, although it may assume a more constrained conformation when it binds to cell surface receptors.[2,28]

Significant insight into biological processes can be derived from this level of structural information. For example, the flexibility of an antibody's Fab segments is advantageous for cross-linking by antigens, and the restricted flexibility of IgE–FcεRI probably limits its ability to be cross-linked on the cell surface by a multivalent antigen. Because an immune response to a particular antigen is likely to include the generation of several classes of antibodies specific for that antigen, there may be competition between these different classes that activate different effector functions. Thus the relative abilities of IgE and IgG on cells and in solution to bind an antigen may play some role in determining whether an allergic response or some other type of immune response occurs.

The influence of receptor structure on function may also be exerted during receptor aggregation leading to cell activation such that misalignment leads to an ineffective aggregate. For example, bound IgE may play some role in orienting the FcεRI subunits for optimal interaction with signal-transducing components in the membrane or cytoplasm. Thus IgE–FcεRI complexes that are forced into tail-to-tail cyclic dimers by short bivalent ligands may be incompetent because the proper interactions cannot occur. Alternatively, some orientations of the cross-linked receptors may be more susceptible to enzymatic modification (e.g., phosphorylation) that leads to receptor desensitization.[44] More detailed information about the structural arrangements of cross-linked receptors that are either competent or incompetent for activating cells will be valuable for understanding the molecular interactions occurring during the initiation of signal transduction.

Fluorescence resonance energy transfer is clearly a powerful method for extracting three-dimensional structural information in complex systems such as cell-bound receptors that are far beyond the reaches of current X-ray crystallography and NMR techniques. The methods we have described here can be extended further to more demanding situations. For example, detection of fluorescence changes occurring in small populations of cells within a heterogeneous mixture can be achieved with flow cytometry,[45] and quantitative microscopy[46] can examine single cells. The major challenge remains specific placement of donor and acceptor probes. Specific antibodies and their Fab fragments are generally applicable tools for this purpose, and genetically engineered single-chain antibodies that consist of the antigen-binding site with minimal additional protein[47] will be particularly valuable for improved distance resolution.

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