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Protein and cell engineering of components of the human immunoglobulin E receptor/effector system: applications for therapy and diagnosis

Birgit A. Helm [a,*], Ian Sayers [a], Jillian Swan [b], Lucy J.C. Smyth [a], Stuart A. Cain [a], Mark Suter [c], Denise C. Machado [d], Alan C. Spivey [e] and Eduardo A. Padlan [f]

[a] *Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2UH, UK*

[b] *Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, Health and Safety Laboratory, Broad Lane, Sheffield S3 7HQ, UK*

[c] *University of Zürich, Institute for Virology, Winterthurerstr. 266a, 8057 Zürich, Switzerland*

[d] *Laboratorio de Microbiologia Molecular, Centro de Biotechnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Goncalves, 9500-Predio 43421, Campus do Vale, CEP 91501-970, Porto Alegre, Brazil*

[e] *Krebs Institute of Biomolecular Research, Department of Chemistry, The University of Sheffield, Sheffield S10 2UH, UK*

[f] *NIDDK: National Institutes of Health, Bethesda, MD 20892, USA*

Abstract. Adaptive immune responses characterised by the synthesis of antibodies of the immunoglobulin E (IgE) isotype play an important role in type I hypersensitivity disorders and parasitic infestations, diseases which have an significant socio-economic impact world-wide. This paper considers potential applications of recent advances in our understanding of the origin of isotype specific immune responses which emerged as a result of cell and protein engineering studies on components of the human IgE/receptor/effector system. Furthermore, the identification of the receptor binding regions in IgE as a result of the development of a stable assay system has important applications for the design of rational therapeutic interventions in allergy and asthma, the treatment of mast cell tumours, and the establishment of procedures for the selective isolation of cells expressing the high-affinity receptor for IgE for functional studies.

Keywords: Immunoglobulin (Ig)E, high-affinity receptor for IgE, allergy, parasitic infestations

1. Introduction

An investigation of structure/function relationships in human (h) immunoglobulin (Ig)E and its receptors together with a study of the molecular mechanisms which cause IgE-mediated hypersensitivity

*Address for correspondence: Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2UH, UK. Tel.: +44 114 222 4375; Fax: +44 114 279 5495; E-mail: B.Helm@Sheffield.AC.UK.

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reactions and stimulate immunity to parasitic infestations is very timely in view of the socio-economic impact of both diseases. In the developing world some 100 million people suffer from parasitic infestations, while in industrialised countries, the incidence of IgE-mediated allergies and asthma has more than doubled during the past 25 years [1–5].

At birth, IgE levels are either not measurable or exceedingly low. In normal adults, plasma levels of IgE rarely exceed 100 μg/l, but are elevated in allergic and parasitic disease. The sustained production of IgE antibodies in response to parasitic infestations is considered a beneficial immune defence mechanism, while the induction of IgE synthesis by a large number of seemingly diverse and inoccuous antigens is a pathological immune response which results in the development of type I hypersensitivity responses.

An outline of consequences of IgE-mediated target cell activation is shown in Fig. 1. Antibodies of the IgE isotype are usually synthesised and secreted from B lymphocytes in response to allergens or parasite proteins, although substances with suitable adjuvant activity can stimulate an IgE response to by-stander antigens [2,6]. Very little IgE is found in the circulation because IgE antibodies bind with high affinity to Fc receptors (FcεRI) found predominantly on mast cells and basophils, and with low-affinity to receptors (FcεRII) found on various inflammatory cells including macrophages and platelets. At any time, most IgE molecules are cell bound and extensively distributed on the surface of mast cells found in the mucosal lining of the eyes, lungs, skin and the intestine. These IgE sensitised cells are the major target organs in immediate hypersensitivity reactions. Following challenge with cognate antigens/allergens, they respond with the secretion of a wide spectrum of pro-inflammatory molecules, including histamine, prostaglandins, leukotrienes, proteases and chemokines. Cytokines released from IgE-activated mast cells and basophils include interleukin (IL)-4, which induces the expression of MHC class II and FcεRII molecules in target cells. These molecules also provide an up-regulatory feedback signal that stimulates IgE synthesis in B cells. In addition to causing the symptoms of the acute phase of the allergic response, they also induce, via the release of chemotactic mediators such as tumour necrosis factor (TNF) α, the recruitment of inflammatory cell sub-populations, which include eosinophils, macrophages and platelets, into the site of immediate hypersensitiviy, while IL-5 plays a key role in the activation of eosinophils. Eosinophil-mediated cytotoxicity depends on mast cell mediators and in the lungs of asthmatics and tissues invaded by parasites, eosinophils are found in close association with mast cells. Furthermore, oxygen metabolites, which are released from IgE-activated eosinophils, can induce mast cell secretion through an IgE-independent stimulus [6]. These mechanisms contribute to the clinical manifestations of the late phase of the allergic response and illustrate the importance of mediator release from mast cells and basophils in immediate and delayed hypersensitivity responses. An inhibition of the initial degranulation event should therefore be associated with wide ranging short and long term anti-inflammatory benefits [1].

Despite intensive efforts, there are no effective medications to treat allergies and asthma. Currently available therapeutic interventions are inadequate and many are associated with undesirable and often severe side effects. Similarly, attempts to develop effective vaccination schedules for the treatment of parasitic infestations have had only limited success. An improved understanding of the molecular mechanisms involved in these disease processes should assist the development of rational therapeutic interventions.

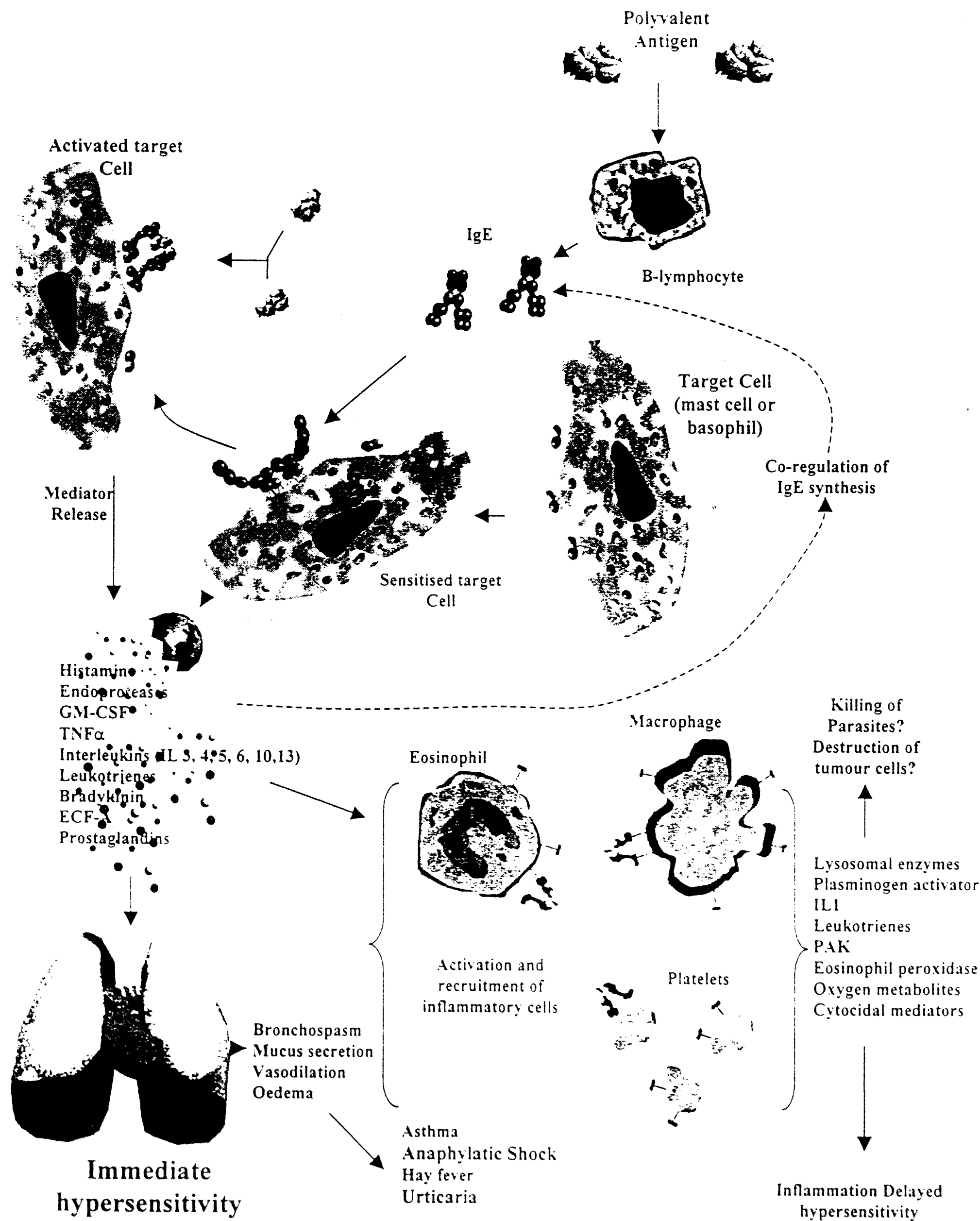

Fig. 1. IgE-mediated cell activation and its consequences in immediate and delayed hypersensitivity reactions. Following synthesis and secretion from B lymphocytes, IgE binds rapidly to high-affinity receptors. The initial interaction does not cause mediator secretion. This take place upon subsequent interaction of receptor bound IgE with cognate antigen. It initiates cell degranulation. Pharmacologically active mediators are rapidly released and these cause the clinical symptoms associated with type I hypersensitivity. In addition, chemokines secreted by IgE-activated mast cells and basophils activate and recruit inflammatory cells into tissues affected by immediate hypersensitivity responses. These cause the symptom associated with delayed hypersensitivity responses. Furthermore, via the secretion of IL-4 an up-regulatory feedback occurs on IgE synthesis by B cells. An inhibition of the initial sensitisation with IgE may therefore be associated with considerable anti-inflammatory benefits. The development of small molecules which block the initial docking of the ligand into the receptor is therefore an important goal of medicinal chemistry programs.

2. Materials and methods

2.1. *Generation of rodent cell lines expression the ligand binding domain of the high-affinity receptor for human IgE*

Rat basophilic leukaemia cell lines (RBL) expressing the human (h) α-chain of the FcεRI complex were engineered using as a host cell line a high secreting variant of the rat RBL 2H3 cell line [8], which expresses a functional receptor complex for rodent IgE. The h FcεRI α-chain gene was subcloned from pUC19 into the multiple cloning site of the vector pcDNA3 which supports constitutive expression of recombinant proteins in mammalian cells. Correct insertion was confirmed by gene sequencing. The plasmid containing the h FcεRI α-chain gene was transfected by electroporation into the RBL-2H3 cells [8] and is expressed as a functional unit with the rodent receptor on the cell surface. The generation and characterisation of the RBL 2/2/C cell line, which supports dexamethazone inducible expression of h FcεRIα, and the characterisation of IgE binding and secretory responses in native and transfected cells, has been described in earlier publications [8–11].

2.2. *Identification of the high- and low-affinity receptor binding site in h IgE*

The methodology has been described in earlier publications [1,9].

2.3. *IgE-independent activation of mast cell mediators by potential allergens*

The methodology has been described in earlier publications [10,11,19,22].

3. Results

3.1. *Strategies for the development of therapeutic interventions in allergy and asthma*

Although the ancient Egyptians already knew of sudden death as a result of bee stings, the dramatic rise in recent years in the incidence of allergic disease and IgE-mediated asthma in industrialised countries has stimulated the quest for the development of more effective rational therapeutic interventions in allergic disease [1–3].

One such approach focuses on the nature of the complementary binding site between IgE and its receptors. The binding of IgE to both types of receptors is a reversible process which sensitises, but does not induce mediator release until the ligand becomes aggregated, usually by cognate antigen, lectins or anti-IgE antibodies [1,11]. Soon after the discovery of the IgE antibody as the mediator of the allergic response, it was demonstrated that a h IgE fragment, which was prepared by papain cleavage and comprises amino acid (a.a.) residues 227–547 of the disulphide linked Cε2–4 dimer, can competitively inhibit the sensitisation of skin mast cells in passive cutaneous anaphylaxis (PCA) tests [12]. This initiated the search for progressively smaller Fcε peptides as potential blocking agents. In order to overcome the limitations imposed by proteolysis, IgE-derived peptides and chimaeric rodent/human IgE constructs were generated by chemical synthesis or recombinant DNA techniques with the aim of identifying the sequence requirements for the complementary interaction. There is still considerable discrepancy concerning the precise location of the FcεRI binding site in h IgE, although there is now a broad consensus that the binding region for both receptors is located in the Cε3 domain [1]. This disagreement can be

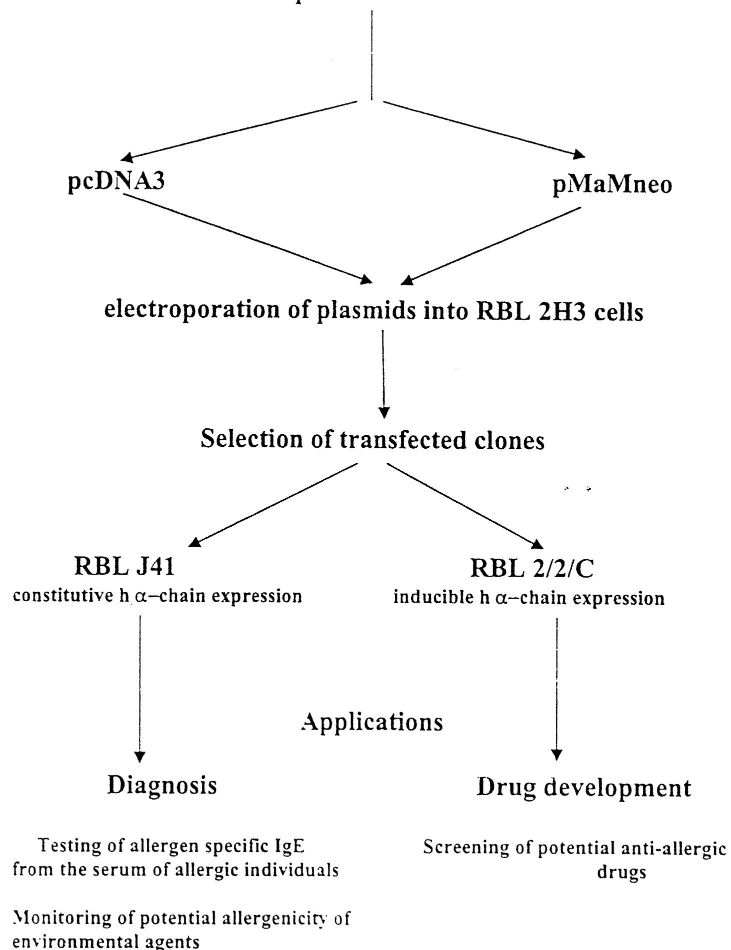

Fig. 2. Establishment of stable rodent mast cell line expressing the ligand binding domain of the human high-affinity receptor complex for IgE. The engineered cell lines support constitutive (RBL J41) and inducible expression (RBL 2/2C) of h FcεRIα. Cells were employed to map the FcεRI binding site in human IgE, using a family of overlapping IgE-derived peptides, shown in Fig. 3. Transfected cells respond to a human IgE-mediated antigenic stimulus with mediator secretion and have useful applications in diagnosis and for the screening of potential anti-allergic drugs.

attributed largely to the fact that h IgE only binds to primate or human FcεRI, and in the absence of a permanent cell line, which expresses the h FcεRI complex, nearly all data reported before the 1990s, relied on the inhibition of the PCA reaction, which produced inconsistent results (reviewed in [1]).

In order to eliminate the problems associated with this temperamental assay system, we developed well-defined *in vitro* assays where the binding of IgE to the soluble extracellular domain of h FcεRIα can be assessed [8,13,14,19]. In addition, we transfected the gene encoding the α-chain of the h FcεRI complex into RBL cells. A functional rodent FcεRI complex is expressed in these cells, which does not bind h IgE. It is made up of an α-subunit, which comprises the IgE binding site, a β-subunit, and two disulphide linked γ-chains. We chose this cell line, which represents an accepted model system for the study of mucosal mast cell function, because earlier investigations had shown that γ-chains of rodent origin can facilitate cell surface expression of h α-chain gene products in, e.g., COS7 cells [15].

Mapping of receptor binding regions in human IgE

Cε2 Cε3 Cε4

Construct	FcεRI Interaction	FcεRII Interaction
GST~	-	-
GST~226 — 547	+	+
GST~226 — 361	+	-
GST~226 — 357	+	-
GST~226 — 354	+	-
GST~226 — 353	-	-
GST~226 — 352	-	nd
GST~226 — 340	-	-
GST~226 — 329	-	-
GST~340 — 357	-	-
GST~44[illegible] — 547	-	-
GST~350 — 547	-	-
GST~345 — 547	-	-
GST~342 — 547	-	-
GST~340 — 547	+	+
GST~330 — 547	+	+
GST~326 — 547	+	+
226 — Cγ3	+	+

Receptor binding regions:

FcεRI
FcεRII

Fig. 3. Identification of the receptor binding regions in human IgE. For experimental details see [1,7].

In addition, the high sequence homology between rodent and human α-chains in the transmembrane domains suggested that the transfected h α-chain should form a functional complex with the β- and γ-subunits of the endogenous rodent receptor complex. Thus, sensitisation with h IgE should activate the host cell's signal transducing machinery in response to a h IgE-mediated antigenic stimulus. Figure 2 shows the technology employed in the engineering of RBL 2/2/C cells, which express $\sim$ 100 000 h α-chains following receptor induction with dexamethazone and that of another variant, RBL J41, which supports constitutive expression of $<$ 10 000 h α-chains per cell. Transfected cells bind h IgE and support h IgE induced mediator release. The RBL 2/2/C cell line was employed to map the FcεRI binding site in h IgE, using a family of overlapping IgE-derived peptides, expressed in *E. coli*, shown in Fig. 3.

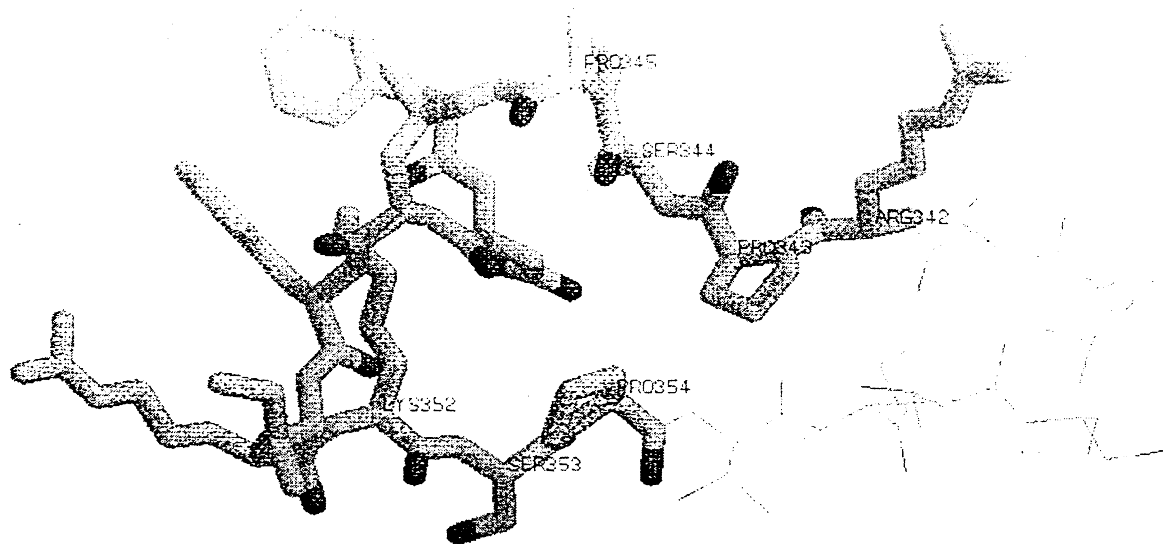

Fig. 4. Model structure of a cyclic peptide based on the A-B loop of h Cε3. The peptide blocks h IgE binding to to h FcεRIα with an affinity in the μmol range and may form the basis for the development of blocking agents which inhibit the binding of IgE to FcεRIα.

Furthermore, values for the kinetics of association and dissociation obtained from the *in vitro* assay system (BiaCore) are in excellent agreement with studies where the binding to the receptor on transfected cells is assessed [14].

3.1.1. *Identification of the FcεRI binding site in h IgE: applications for the structure based design of anti-allergic drugs*

Figure 3 shows that the sequences common to all Fcε fragments capable of recognising FcεRI comprise Pro343-Ser353 in the Cε3 domain. Further deletion from either the N- or C-terminal end is associated with a loss of receptor recognition [9]. The Pro343-Ser353 peptide blocks IgE/FcεRI binding with an IC_{50} in the mmol range [1,16]. Such low affinity is commonly observed with linear peptides and attributed mainly to the ability of the peptide to adopt a large number of conformations in aqueous solutions. There is however evidence that appropriate conformationally restrained analogues can exhibit enhanced specificity and affinity. Viewed in the context of the model structure we developed for h IgE-Fc, the Pro343-Ser353 sequence has been computed to form an exposed loop [1,9,17], and this provided the basis of the disulphide bond constrained peptide shown in Fig. 4, which blocks IgE/FcεRI interaction in a competitive manner with an IC_{50} in the μmol range. This increase in affinity suggests that this "lead" peptide may form the starting point for the development of low molecular weight anti-allergic drugs [1].

Furthermore, when this peptide was employed to raise antibodies against the FcεRI binding region in h IgE, it was found that these antibodies bind to IgE in solution, but do not recognise receptor bound IgE and inhibit the binding of IgE to FcεRI [1,13]. The structural basis of this phenomenon, which appears paradoxical in view of the fact that IgE is a homodimer and antibodies are divalent, is unknown. It has been explained in terms of a bent conformation of IgE shown in Fig. 5, where the second ε-chain becomes inaccessible to an additional copy of the receptor, or to antibodies directed against epitopes in IgE that become masked following receptor engagement. As our study shows, such IgE epitopes may have applications as immunogens in the therapy of all IgE-mediated allergies through active immunisation irrespective of the nature of the allergen [1].

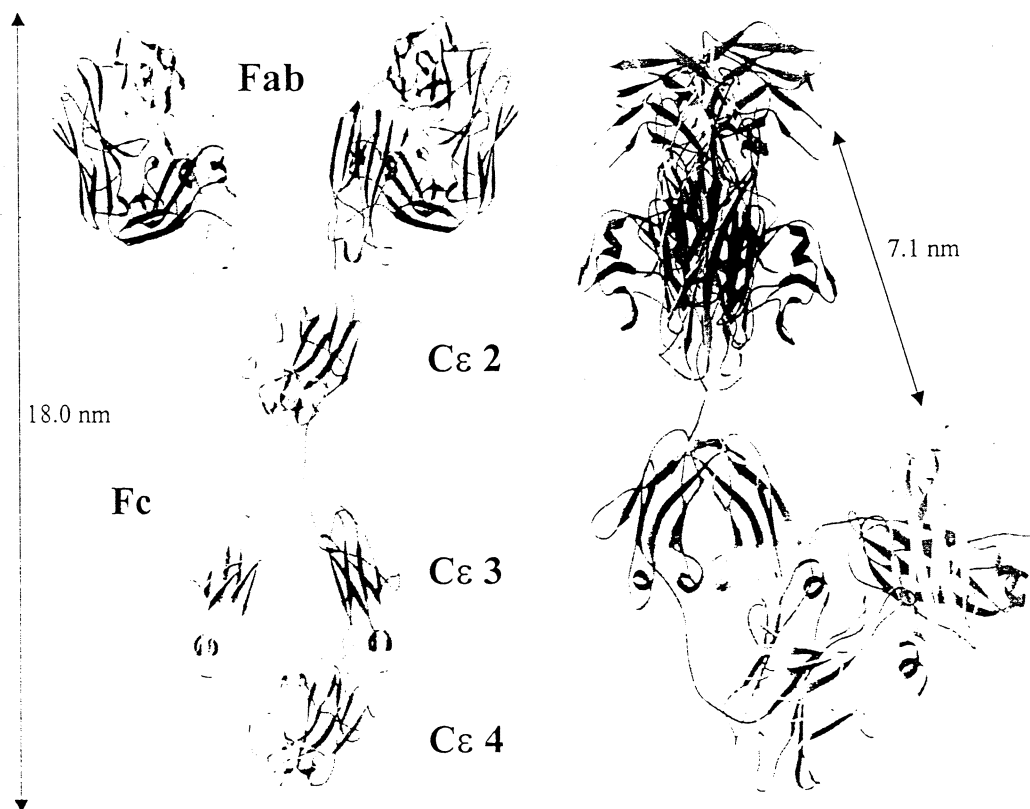

Fig. 5. Structural models of IgE in coplanar (left) and bent conformation (right). A bent conformation of the IgE explains the observation that despite bilateral symmetry, the IgE molecule binds to FcεRIα and non-anaphylactogenic antibodies in a 1 : 1 stoichiometry. It indicates that the identification of epitopes recognised by the receptor of non-anaphylactic antibodies can lead to the design of peptide immunogens for active immunisation of asthmatics and patients at risk of anaphylactic shock.

The development of such vaccines is very timely in view of the dramatic rise in IgE-mediated allergies in recent years for which there exist no obvious underlying cause. Although genetic evidence indicates the importance of hereditary factors in immunity to parasites and susceptibility to develop IgE-mediated allergies, it appears probable that environmental factors play a decisive role in the current epidemic of allergic diseases since the gene pool of the population cannot have changed sufficiently to explain the recent increase in the incidence of type I hypersensitivity responses [2]. Although some observations point to a connection between the decline of infectious diseases and the rise in allergies and asthma [2, 3], there is also compelling evidence that pollutants in air such as diesel exhaust particles, polyaromatic hydrocarbons, oxygen radicals produced by engine emissions or cigarette smoke, can have adjuvant activity and enhance IgE ongoing synthesis [22–29].

3.2. Uncovering a link between the nature of substances that activate cells of mast cell/basophil lineage and the allergic response

The exposure to several types of antigens, including pollen grains, mould spores, house dust mite and cockroach emanations, latex, fruit and nut associated substances or parasite secretions, gives rise with preference to the synthesis of antibodies of the IgE isotype in susceptible individuals. However despite a large amount of information regarding the molecular structure of many allergens and parasite proteins, no

unifying principle has been proposed that explains the nature of the isotype selection which consistently occurs in response to these diverse substances. Our own studies emerged with an unexpected alternative explanation for the selective isotype induction elicited by these antigens [10,11,18].

This became apparent when we employed the h α-chain transfected RBL cell line to study allergic sensitisation *in situ* as an alternative to the PCA test, since this procedure is associated with the inherent danger of boosting an already sensitised individual [19]. In order to assess the technology, we sensitised the cells with the serum of a bee venom sensitive individual (EMC) and, following challenge with the major bee venom phospholipase A_2 (PLA_2), we could, as expected, demonstrate mediator release. Surprisingly, however, control experiments, where non-sensitised cells had been incubated with the same concentration of antigen in the absence of the serum containing bee venom specific IgE, cells also responded with the degranulation of cellular mediators [10,11,18]. Further studies showed that only enzymatically active bee venom PLA_2, but not an inactive variant, is able to induce IgE-independent mediator release, including IL-4 from this cell line. Furthermore, only mice immunised with enzymatically active PLA_2, but not an inactive variant, produce high levels of PLA_2-specific IgE [10]. This suggested that the catalytic activity, manifested as IgE-independent mast cell secretagogue activity, determined the outcome of isotype specific immune responses.

This initial observation led to an extensive investigation into the IgE-independent activation of cells of mast cell/basophil lineage by potential allergens. Interestingly, potent hydrolytic enzymes, most of which are associated with catabolic pathways, have been isolated from nearly all sources of allergenic materials [20]. Similar proteins are secreted by parasites as part of the invasive process [10,11].

As summarised in Table 1, proteolytic and lipolytic enzymes from organisms as diverse as plants, fungi, house dust mites and schistosomes stimulate degranulation of cellular mediators from RBL cells and induce IL-4 synthesis and secretion. This IgE-independent cell activation is critically dependent on enzymatic activity since inactive forms of, e.g., bee venom PLA_2 or the house dust mite protease Der p I do not induce mediator release [10,11]. Other classes of substances which induce mediators release and stimulate cytokine synthesis are lectins, including those present in natural latex and ragweed, virus associated proteins with protease or lectin-like haemagluttanin activity [11,22], or substances like the polycationic mast cell degranulating agents including mellitin, mastoporan, substance P, and compound 48/80. These are thought to activate heterotrimeric G proteins of the Rab family, which act as regulators of membrane fusion [21]. In addition, components in car engine emissions and cigarette smoke induce mast cell mediator release [23–29]. There is evidence that the former can act as adjuvants, since stimulation of ongoing IgE synthesis has been observed following exposure to diesel exhaust particles and polyaromatic hydrocarbons [23–27] and it is interesting to note that cigarette smoke contains many components also found in diesel engine emissions [24].

Although the RBL cell line presents a cellular model system for the study of mast cell function, we extended our investigation to assess the responses of preparations containing human lung (HLMC) and skin mast cells (HSMC) and basophils to confirm the commonality of our findings. Similar observations were made, indicating that we have identified an important biological principle underlying potential allergenicity [11].

4. Discussion and conclusion

Our results show that protein and cell engineering studies on components of the h IgE receptor/effector system have important applications for the diagnosis and therapy of allergic, parasitic and possibly also viral diseases in relation to the development of allergies [1,10,19].

Table 1
Antigen-induced mast cell mediator release from RBL J41 cells in the absence of sensitisation with antigen specific IgE

	Mediators measured		
	5-HT	Histamine	IL-4
Venoms, bee/wasp (1% suspensions)	90	85	d
Bee venom PLA2 (10 μg/ml) recombinant enzymatically active	17	17	d
Bee venom PLA2 (10 μg/ml) recombinant enzymatically inactive	0	0	nd
Der p I (3 μg/ml) enzymatically active	18	19	d
Der p I (3 μg/ml) enzymatically inactive	0	0	nd
Schistosomal protease (3 μg/ml) enzymatically active	17	19	d
Apergillus protease (10 μg/ml)	21	24	d
Natural latex (1:400) *Hevea brasiliensis*	15	20	d
Condom extract (1:400) (Gossamer)	12	18	d
Hevein (1 μg/ml) *Hevea brasiliensis*	8	9	nd
Influenza virus F (5% suspensions)	5	5	nt
Respiratory syncytial virus (5% suspensions)	7	5	nt

d = detected, n.d. = not detected, n.t. = not tested. 5-HT = [^{3}H]-5-hydroytryptamine (% release). Experimental details have been described in previous publications [10,11]. The enzymatic activity of all enzymes was tested before mediator release was assayed. In the absence of a quantitative assay for rat IL-4, the cytokine was detected by Western blotting.

4.1. *Assessment of anti-allergic drugs*

The development of stable cell lines, which express the ligand binding domain of h FcεRI and which respond to a h IgE-mediated antigenic stimulus with mediator release, led to the identification of the minimum sequence requirements for the binding to both receptors. This may form the basis of for the design of anti-allergic drugs, based on the structural motif contributed by the A-B loop in the Cε3 domain of h IgE shown in Fig. 4. Both the *in vitro* assay system [14] and the FcεRIa transfected cell lines [9,27] can be employed for the screening and evaluation of potential blocking agents of IgE/receptor interaction and mast cell activation.

The engineering of a variant form of IgE ([IgE] R16) [9,13] which selctively recognises cells expressing the high-affinity receptor, but which does not bind to FcεRII/CD23 has potential therapeutic applications in the treatment of systemic mastocytomas when linked to an immunotoxin or radioactive isotope. In addition, it can be used for the selective isolation of cells expressing FcεRI for functional studies.

4.2. *Design of vaccination schedules in allergic and parasitic disease*

The demonstration that enzymatically active allergens and parasite proteins activate cells of the immune system to induce cytokine secretion has potential applications for the design of immunisation schedules in allergic and parasitic disease. Immunisation with active parasite protein and/or the use of IL-4 as an adjuvant may induce a protective immune response. Conversely, immunisation schedules employing biologically inactive allergens, anti-IL-4 antibodies, or adjuvants known to induce alternative Ig isotypes may prove effective for the treatment of allergies.

4.3. *Development of new diagnostic tests to monitor allergic sensitisation*

An *in situ* cellular assay system for the testing of allergic sensitisation offers an attractive and safe alternative to the skin prick test, which may carry the risk of boosting an already sensitised individual [19]. In addition, a considerable number of PCA tests are positive, although no allergen specific IgE can be demonstrated in the patient's serum [19]. This can problably be attributed to the IgE-independent secretagogue which we have shown to be associated with many sources of allergens and our assay system can clearly differentiate between these situations and eliminate potentially false positive results.

4.4. *Development of an assay system to predict the potential allergenicity of environmental and occupational hazards*

In addition, our observations indicate that the reason why diverse aero-allergens, environmental pollutants, parasite proteins or insect venoms induce and/or enhance IgE synthesis, is their ability to stimulate the release of cellular mediators, including IL-4. It indicates that any substance which induces the secretion of IL-4 must be considered as a potential allergen. The monitoring of air quality involves measurements of NOx gases (nitrogen dioxide, nitric oxide, and sulphur dioxide), ozone, pollen counts and particulate matter. No biological assay exists to assess the immediate and long term effects of these substances on cells of the respiratory tract. Risk assessment by means of a biological assay on cells which closely resemble the mucosal mast cells of the airways [2] is preferable to assessment of epidemiological evidence since this will facilitate pro-active rather than reactive responses when considering airborne allergens in the environment and at the workplace.

The demonstration of non-immunological mediator release from RBL and primary human mast cells and basophils indicate that common cellular responses to these substances occur in rodents and humans. This suggests that RBL cells can be employed to monitor potential allergenicity of occupational and environmental pollutants. The establishment of a cellular assay system to correlate air quality with potential allergenicity may have wide ranging applications in industry and the environment.

Acknowledgments

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