

Immunological Intervention Strategies in Allergy

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Regulation of the expression of distinct human secreted IgE proteins produced by alternative RNA splicing

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Production of a family of ϵ mRNAs and IgE proteins by human B-cells

IgE was identified as the immunoglobulin that mediates immediate allergic reactions some 30 years ago [1]. Although functional data provided indirect evidence for possible heterogeneity of IgE, proof of this heterogeneity and its molecular basis have only been recently elucidated. It is now clear that humans, in contrast with rodents, produce a family of secreted and membrane-bound IgE proteins, the production of which is under developmental and disease-related control. The challenge will be to use this new information to modify the manifestations of IgE-mediated diseases.

Immunoglobulins generally exist in two forms, membrane-bound and secreted, depending on alternative splicing and polyadenylation patterns of primary RNA transcripts. Humans have only one functional ϵ gene [2], so that after the switch to the human ϵ gene, it was expected that it would encode a single membrane-bound and a single secreted IgE protein [3]. However, as the result of alternative ϵ mRNA splicing, human IgE is far more complex, comprising four secreted and two membrane-bound proteins [4–7].

Production of a family of IgE proteins by alternative 3' RNA splicing (Figure 1)

Splicing of CH ϵ 4 to two splicing acceptor sites in the second membrane exon, M2, results in

mRNA for two novel secreted IgE isoforms. The absence of the conserved hydrophobic transmembrane region encoded by M1' or (M1) makes them secreted proteins. One mRNA species, designated CH4-M2', results from splicing of CH4 directly to the normal M2 exon splice acceptor. This encodes the ϵ 'grandé' heavy chain which carries 136 amino acids from the M2' exon and is thus 134 amino acids larger than the classic secreted ϵ chain. A second species of ϵ mRNA, designated CH4-M2'', is generated by splicing of CH4 to a novel splice acceptor in the terminal portion of the M2' exon. This CH4-M2'' mRNA has a one-nucleotide reading frame shift compared with the CH4-M2' form and encodes a protein (IgE tailpiece or IgE tp protein) containing eight unique amino acids and a terminal cysteine compared with classic secreted ϵ .

To determine whether CH4-M2'- and CH4-M2''-mRNA-encoded IgE proteins exist *in vivo*, polyclonal and monoclonal antibodies (mAbs) were generated against them. When the M2' antiserum was used to probe human polyclonal IgE, it reacted with a single high-molecular-mass band representing IgE grandé. Detection of the CH4-M2''-encoded IgE protein (IgE tailpiece) used mAbs against its C-terminal eight amino acids. On Western blots, the M2'' mAbs detected IgE tailpiece among total serum IgE from atopic persons who had very high serum IgE, with levels differing dramatically among subjects.

The altered C-terminal domain of IgE grandé may conceivably interfere with Fc ϵ RI α binding by disturbing the interchain CHR-CH4 interactions that maintain the necessary

Abbreviations used: mAb, monoclonal antibody; IL, interleukin; DEP, diesel exhaust particle.

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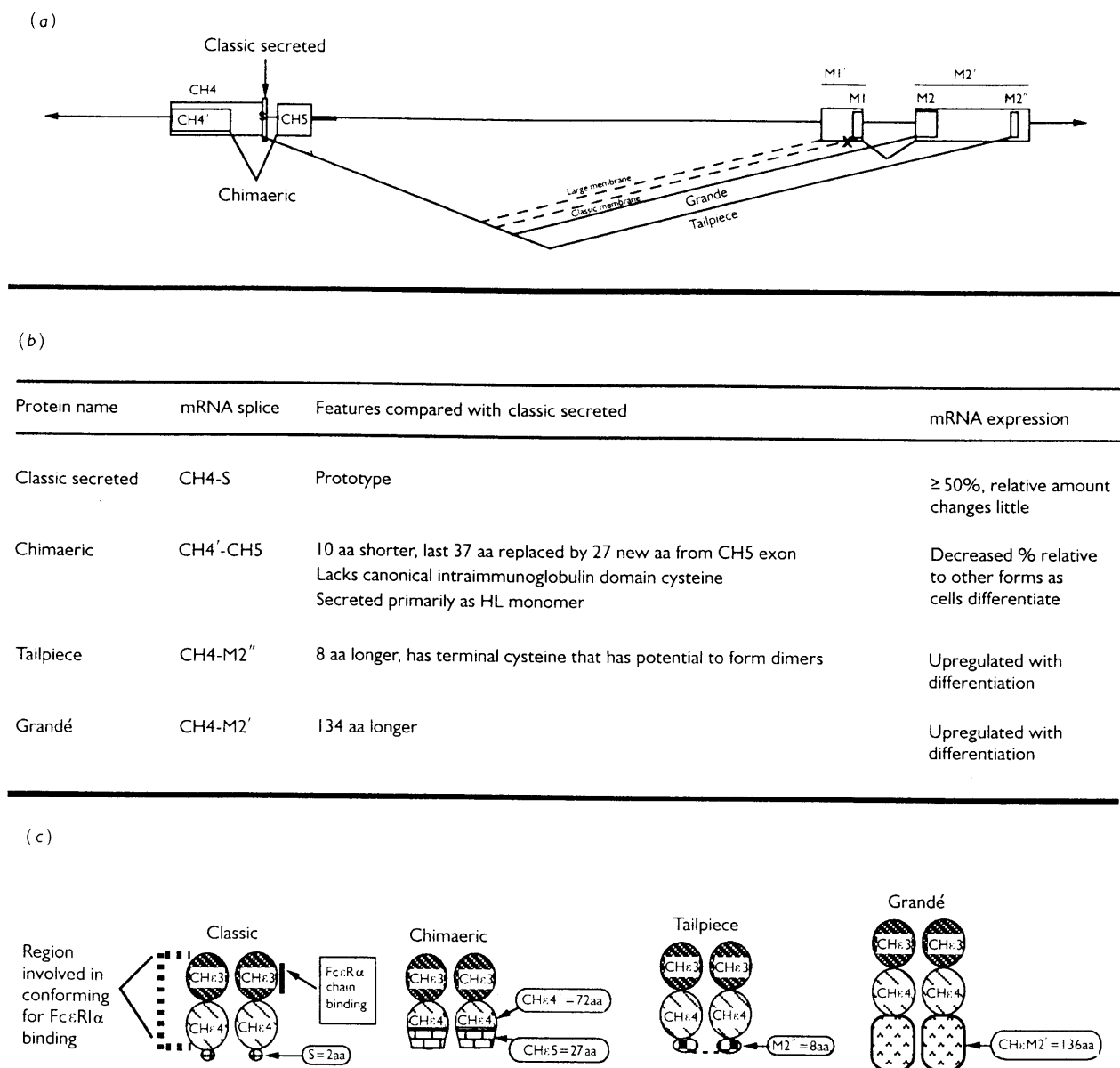
[CH2-CH3-CH4]₂ structure or by directly blocking access to the CH3-binding site for FcεRI or FcεRII. IgE tailpiece contains a C-terminal cysteine. C-Terminal cysteine residues in immunoglobulin heavy chains are usually associated with the J-chain, which is involved in formation of dimers or polymers of IgM and IgA1. Immunoglobulin isotypes that exist solely as

monomers all lack this cysteine. If dimer or polymer forms of IgE tailpiece exist, they may be expected to differ biologically from the classic secreted IgE or other monomeric IgE isoforms. We have not observed multimeric forms of IgE tailpiece *in vitro* [8], nor have Batista et al. [9]. However, we know that different cell lines process IgE isoforms differently [8,9], and the

Figure 1

A guide to the human ε splicing variants

(a) Sequence representing the 3' portion of the primary RNA transcript from the single functional human ε gene is shown from the last constant region domain (CH4) to the end of the M2' exon. RNA splices that generate mRNA for secreted IgE proteins are shown as solid lines below the gene schematic, and splices that generate mRNA for membrane IgE isoforms are shown as dashed lines and are so named. (b) Characteristics of the alternatively spliced secreted human IgE isoforms and their mRNA are listed. aa, amino acid. (c) Cartoon of the C-terminal protein sequence (CH3 onward) of the four human secreted IgE proteins. The region involved in the conformation for FcεRIα binding, and the region of the specific contact peptides is shown in the left hand diagram for classic IgE. The size and positional differences between the secreted forms are illustrated.



potential for formation of multimeric forms of IgE tailpiece needs to be specifically determined *in vivo*. Indeed, Western blots of immunoprecipitated IgE from high-IgE sera show bands at the appropriate size for dimers and trimers of IgE tailpiece when allowed to react with IgE tailpiece mAb.

We also defined a fourth secreted form of IgE generated by splicing to a previously unknown cryptic exon, CH5, containing 106 nucleotides, located in the intron between the CH4 and M1' exons (Figure 1). The ϵ mRNAs using the CH5 exon splice to it from a donor site inside the CH4 exon (CH4'). All the CH4'-CH5 mRNAs encode the same ϵ -protein isoform (IgE chimaeric or IgE ψ CH4), as translation stops at a termination codon in CH5. IgE chimaeric is 10 amino acids shorter than classic secreted ϵ , the C-terminal 37 amino acids of the CH4 being replaced with the 27 amino acids encoded by CH5. This creates a hybrid CH4'/CH5 domain that has a cysteine residue in a position different from the conserved cysteine that forms the immunoglobulin interchain domain disulphide bond. The domain with an altered cysteine position is predicted to affect immunoglobulin domain folding.

Two species of mRNAs encode human membrane-bound IgE. One species called CH ϵ 4-M1'-M2 is generated by splicing from CH4 to a novel splicing acceptor site 156 nucleotides upstream of the M1 exon, to produce a membrane-bound form of IgE with 52 additional amino acids in the extracellular portion between CH ϵ 4 and the M1 exon. Semiquantitative PCR by Peng et al. [4] and ourselves revealed that this CH ϵ 4-M1'-M2 is the dominant form of mRNA for membrane-bound IgE. This unique IgE protein can be detected in the surface of IgE-secreting cell lines ([4]; A. Saxon, D. Diaz-Sanchez and K. Xhang, unpublished work) by using specific antiserum against the extracellular 52-amino acid domain. The mRNA for 'typical' membrane IgE, CH4 spliced to the acceptor site of M1 exon (CH4-M1-M2), is very rare. This failure to generate 'typical' membrane IgE is apparently the result of humans having gained an AG dinucleotide in the polypyrimidine tract upstream of the M1 splice acceptor; the occurrence of this dinucleotide prevents normal use of the splice acceptor immediately downstream.

The extracellular region of the large membrane IgE contains a proline-rich domain that is unique with regard to its size and structure

among murine and human membrane immunoglobulins. Such an extracellular domain may play an important role in the signal transduction via membrane IgE by binding to specific ligand(s). Potentially, this unique extracellular region could be targeted to eliminate the membrane IgE-bearing IgE B-cells, i.e. cells that are destined to become IgE-secreting B-cells, while not interacting with B-cells that have passively acquired IgE through either high- or low-affinity receptors for IgE.

Expression of the novel human secreted IgEs as genetically engineered proteins

The low levels of IgE present even in high-IgE sera and the similar molecular size of several of the isoforms argued against many approaches to purification of the individual isoforms. Expression vectors were developed to produce genetically engineered secreted ϵ -isoforms [8], and four human IgE isoforms, 'classic' (CH4-S), 'grandé' (CH4-M2'), 'tailpiece' (IgE tailpiece protein) (CH4-M2'') and 'chimaeric' (IgE ψ CH4 protein) (CH4'-CH5), were expressed as human anti-dansyl antibodies. The variable expression of these isoforms in specific cell lines [8,9] gave clear evidence that the synthesis, assembly, glycosylation and degradation of immunoglobulin proteins vary *in vitro* and that what actually occurs in humans at the protein level will need to be determined *in vivo*.

IgE classic, IgE grandé and IgE tailpiece were each secreted as fully assembled H₂L₂ molecules (where H represents heavy chain and L light chain). IgE chimaeric was secreted primarily as HL monomers, which migrated as a broad band of 75–90 kDa under non-reducing conditions. The failure to produce H₂L₂ molecules was also confirmed by pulse-chase experiments. The diffuse migration of IgE chimaeric was due to heterogeneous glycosylation, as it was not observed when cells were cultured in tunicamycin. The incomplete assembly of IgE chimaeric is not surprising. H₂ pairing is initially stabilized by non-covalent interactions between the heavy-chain C-terminal domains, and the C-terminal portion of CH ϵ 4 is replaced by a sequence from CH5 in IgE chimaeric, making it likely that the normal immunoglobulin domain structure is not present in the terminal portion of IgE chimaeric.

It is not surprising that individual members of the human secreted IgE family differ in their ability to mediate IgE functions; although the

contact residues for Fc ϵ RI and Fc ϵ RII are present in all the IgE isoforms, the complete CH4 domain is critical in constraining the three-dimensional doughnut shape of the paired CH3-CH4 IgE regions that provide for Fc binding of IgE [10]. IgE chimaeric merits special attention in this regard since it is secreted as HL or H₂L. If bound to Fc ϵ RI, it will be at a marked disadvantage in cross-linking receptors and therefore be less able to arm mast cells and basophils for antigen-triggered release. It may even inhibit such release.

Differentially regulated developmental and disease-associated expression of IgE isoform mRNAs

We also discovered that some stimuli that regulate IgE production also change the nature of the IgE produced, i.e. alter the relative amount of the different ϵ mRNAs [11]. This provides a mechanism for altering the qualitative IgE response. While interleukin (IL)-10 increased total ϵ mRNA approx. 12-fold, it also had a marked differential effect on ϵ mRNAs *in vitro*. Production of the grandé splice variant increased 46-fold in cultures with IL-10 whereas amounts of IgE tailpiece, classic and chimaeric splice variant mRNA increased less than twofold. Accordingly, the ratio of grandé to classic increased from 12:1 to 60:1. The amount of IgE grandé in supernatants of IL-10-treated cells increased approx. 50-fold whereas total IgE increased only threefold, thereby showing a marked proportional increase in IgE grandé [7].

Similarly, polyaromatic hydrocarbons extracted from diesel exhaust, which enhance IgE production *in vitro*, led to a similar but not identical shift in IgE isoforms, the most striking change being increased mRNA for grandé and a far smaller increase in the levels of tailpiece [12]. Addition of IL-2, IL-13 or interferon γ did not alter the relative ratios of the ϵ mRNA variants. IL-6, while increasing total IgE and total ϵ mRNA, did not change the ϵ mRNA splicing pattern.

Circulating B-cells that spontaneously produce IgE were used to determine the pattern of ϵ mRNAs *in vivo*. Such B-cells showed a pattern analogous to that seen with IL-10 *in vitro*. Grandé mRNA was markedly increased and chimaeric mRNA was barely discernible in the B-cells of allergic patients and was often completely absent from the B-cells of parasite-infected patients. In contrast, immature B-cells

of immunodeficiency patients did not exhibit a 'normal' pattern of ϵ mRNA observed with IL-10 [13].

We also determined the *in vivo* pattern of ϵ mRNAs expressed after *in vivo* nasal challenge. Challenges were performed with diesel exhaust particles (DEPs), ragweed antigen or both. DEP challenge resulted in an approx. fivefold increase in lavage IgE protein, no increase in ragweed-specific IgE and a clear change in the relative levels of the ϵ mRNAs in the lavage cells (a minimal increase in chimaeric mRNA and an approx. 28–80-fold increase in the other ϵ mRNAs) [14]. Ragweed challenge resulted in a similar pattern and an approx. fivefold increase in ragweed-specific IgE. Notably, DEP plus ragweed challenge gave an approx. 50-fold increase in ϵ mRNAs for the secreted isoforms other than chimaeric, which became relatively less abundant than before challenge. Topical steroids administered 1 week before ragweed challenge only partially blocked the rise in ragweed-specific IgE, and a 'less mature' pattern of ϵ mRNAs was observed, mRNA for IgE chimaeric comprising a greater proportion.

Summary

The use of splice sites for human ϵ mRNAs is tightly regulated, as the potential number of splice products far exceeds that actually produced. Our studies show that use of the ϵ alternative splices is regulated by a limited number of stimuli, and the relative production of the ϵ mRNA variants follows a developmental profile. In addition, we have found disease-related changes in the pattern of ϵ mRNA variants encoding distinctive IgE isoforms. Analysis of the biological activity of expressed recombinant IgE proteins and measurement of their levels in disease conditions will directly answer questions as to the biological relevance of the changes observed in ϵ mRNA splicing. This information will then be able to be used for rational therapeutic design interventions in IgE-mediated disorders.

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Structure-based design of peptides that inhibit IgE binding to its high-affinity receptor FcεRI

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Introduction

The interaction between IgE and its high-affinity receptor, FcεRI, is a critical step in the development of an allergic reaction, and is therefore a major focus of attention for the development of strategies to intervene at the molecular level to control allergy [1]. Although our understanding of the three-dimensional structures of the two molecules and of the nature of the complex is far from complete, sufficient is now known for a structure-based approach to be undertaken. The design of an inhibitor of this interaction, which has an association constant of the order of 10^{10} M⁻¹, is clearly a challenge, but several promising approaches are now being pursued, one of which we describe here.

IgE binds to FcεRI through its Fc region, which consists of the disulphide-linked dimer of the Cε2, Cε3 and Cε4 domains. Various studies involving recombinant fragments [2–4], chimaeric immunoglobulin molecules [5–8] and, most recently, site-directed mutagenesis [8] all point to Cε3 as the domain in which the site of interaction lies. FcεRI, expressed principally on mast cells and basophils, consists of four transmembrane polypeptide chains, αβγ₂, and IgE-binding activity resides in the extracellular region of the

α-chain. This consists of two immunoglobulin-like domains termed α(1) and α(2). Although α(2) alone has been shown to display significant activity, both domains are required together for the expression of near-maximal binding activity [9–11], and full binding activity may only be achieved with the intact multichain receptor. The two structures and their mode of interaction will now be considered in more detail.

Modelling and mutagenesis of IgE–Fc

The structure of the Cε3 and Cε4 domains of IgE–Fc have been modelled [12,13] on the basis of their sequence similarity to the Cγ2 and Cγ3 domains of IgE–Fc, the crystal structure of which is known [14]. Previous work had implicated the N-terminal region of the Cε3 domain in the interaction with FcεRI [1,15,16], and we therefore targeted a number of mutations to this region which were predicted to be solvent accessible in the model: D330S, P333E, P333Q, R334S and R351E (residue numbering of Dorrington and Bennich [17]). These mutations were first inserted into IgE–Fc and expressed transiently in CHO cells. The supernatants were assayed by surface plasmon resonance (SPR) using a BIAcore instrument to determine the kinetics of binding to FcεRI. Briefly, a fusion protein consisting of the soluble extracellular domains of the receptor (sFcεRIx) linked to

Abbreviation used: SPR, surface plasmon resonance.

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