

Functional FcεRI engagement by a second secretory IgE isoform detected in humans

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We have recently reported that besides the most abundant form ϵ_{S1} , there exists another human secretory ϵ H chain isoform, ϵ_{S2} , resulting from alternative splicing in the ϵ CH4 exon. Using a specific antibody targeted to the ϵ_{S2} -specific C-terminal tailpiece, we now show that this second secretory IgE isoform (IgE-S2) is constitutively co-expressed with the classical secretory IgE-S1 by human myeloma cells. The ϵ_{S2} variant was also detected in tonsils and in the serum of three non-atopic donors, but was absent in the vast majority of sera of both atopic and non-atopic individuals tested, indicating rare serum expression. IgE-S2 is capable of binding to cells expressing FcεRI, the high-affinity receptor for IgE. Analysis of intracellular tyrosine phosphorylation signal, degranulation, and rate of receptor internalization suggest a quantitatively lower response by IgE-S2 compared to IgE-S1. The modest differences observed do not appear to overall affect the degranulation competency of IgE-S2, but suggest that the unique structure of the ϵ_{S2} tailpiece can exert an effect on the interaction with the α chain of FcεRI.

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1 Introduction

In addition to the mRNA encoding the classical membrane and secreted IgE H chains, human IgE-secreting myeloma cell lines and PBL generate, by alternative splicing of the 3' exons of the Cε locus, a complex pattern of multiple transcripts potentially encoding novel forms of membrane-bound and secreted IgE with altered C termini [1, 2]. Alternative splicing occurs between splice sites located in the last two constant region exons (ϵ CH3 and ϵ CH4) and the two membrane exons M1 and M2. Alteration of the relative amounts of different ϵ mRNA splice variants observed in disease states or following *in vitro* and *in vivo* challenges has been taken as evidence for a biological role of the modulation of ϵ isoform expression patterns [3, 4]. Part of this transcriptional activity, however, could be functionally redundant as judged from data obtained in mouse transfectoma models, which revealed that post-translational regulation at the level of Ig assembly operates and can lead to intracellular retention and degradation of certain isoforms [2]. Expression of two novel isoforms, a second shorter

membrane isoform (m_s IgE) and a second secretory isoform (IgE-S2) was nevertheless shown in mouse B cells and plasma cells, implying that some of the ϵ splicing isoforms can encode functionally distinct IgE proteins [5, 6]. To validate the mouse transfectoma models, we have investigated here whether the second secreted IgE isoform ϵ_{S2} H chain is also produced and secreted by human myeloma cells and whether it can be unambiguously detected in human tissues.

The ϵ_{S2} H chain is generated by alternative splicing from ϵ CH4 into an internal acceptor splice site located in the second membrane exon M2 [1]. The resulting protein is a secretory ϵ H chain which lacks the transmembrane domain encoded by the M1 exon and contains a terminal tailpiece of eight amino acids translated from the 3' part of the M2 exon in a reading frame different from that of the membrane IgE (Fig. 1a). This C-terminal sequence distinguishes ϵ_{S2} from the classical ϵ_{S1} secretory H chain, which is six amino acids shorter. The ϵ_{S2} -specific tailpiece contains as its most distinctive feature a cysteine residue in the last position, which is similar to μ and α H chains that contain a cysteine in the penultimate position. Unlike IgM and IgA [7, 8] the ϵ_{S2} C-terminal cysteine was shown neither to induce polymerization of IgE molecules in murine cells nor to function as an intracellular

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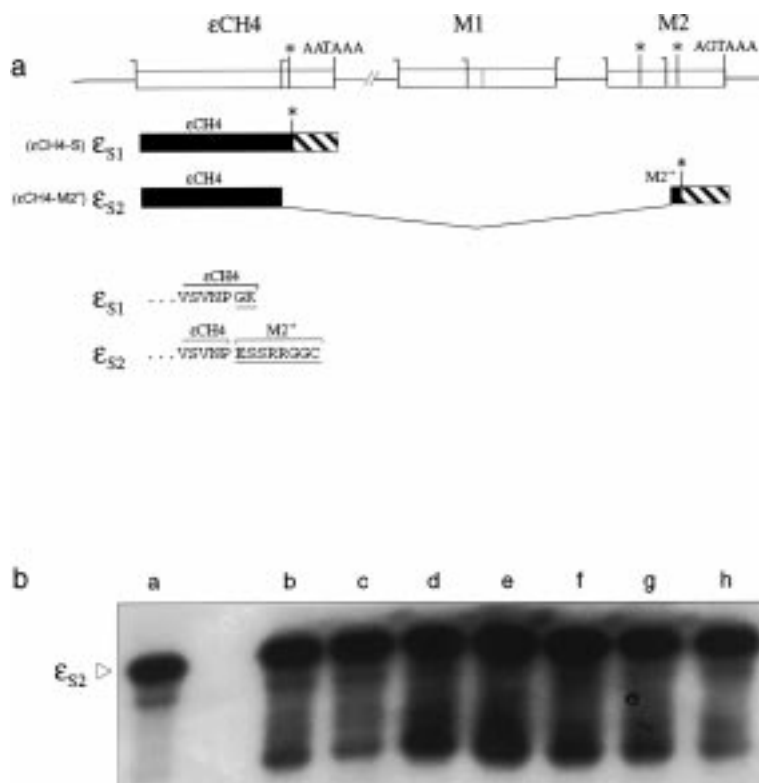


Figure 1. Structure and detection of human secretory IgE mRNA transcripts. (a) Schematic representation of alternatively spliced secretory ϵ transcripts ϵ _{S1} and ϵ _{S2}. Shadowed and hatched boxes represent coding and 3' untranslated regions, respectively. Amino acid sequences of C termini of secretory ϵ isoforms are shown underneath from C ϵ residue 542 in the ϵ CH4 domain. Asterisks indicate stop codons and underlined sequences represent isoform-specific residues. (b) RT-PCR detection of ϵ _{S2} mRNA transcripts expressed by PBL and tonsils. Lane a: positive ϵ _{S2} control of transfectoma; b–d: PBL; e–h: tonsil.

retention element for monomeric H₂L₂ complexes. Instead, the IgE-S2 isoform is secreted as efficiently as the classical IgE-S1 secretory IgE in both murine B cell and plasma cell transfectomas [5]. The C-terminal cysteine of ϵ _{S2} is involved in a stabilizing interchain disulfide bridge between the two ϵ CH4 domains, which results in a higher rate of intracellular assembly compared to IgE-S1. This additional covalent bond is also likely to confer a distinctive conformation to the C terminus of IgE-S2. To address the functional properties of the new secretory human IgE, we wanted to test whether IgE-S2 can functionally engage the high-affinity Fc ϵ receptor, Fc ϵ RI. IgE-S2-mediated intracellular phosphorylation, degranulation and receptor internalization responses on rat basophilic leukemia (RBL-2H3) cells transfected with the human α , β and γ chains of Fc ϵ RI [9] were evaluated.

2 Results

2.1 Detection of ϵ _{S2} transcripts in unstimulated human PBL and tonsils

In earlier studies it was shown that unstimulated PBL isolated from normal individuals express low levels of ϵ mRNA, mostly of the classical ϵ _{S1} secretory form [2]. Membrane exon isoforms, including the ϵ _{S2} species, can be detected following a nested reverse transcription (RT)-PCR procedure [10]. Fig. 1b shows that ϵ _{S2} transcripts could be detected in human unstimulated PBL as well as in tonsils. This indicates that both serum and tonsils are candidate tissues to look for *in vivo* expression of the IgE-S2 protein in humans.

2.2 Immunoblot analysis of IgE spontaneously secreted by human myeloma cells

We first tested if the IgE-S2 protein was detectable in the supernatant of a spontaneously IgE-secreting cell line of human myeloma origin, U266. U266 expresses a wide range of ϵ mRNA transcripts of which the classical secreted ϵ_{S1} is the predominant species [10–12]. Northern blot analysis revealed that the ϵ_{S2} transcripts are also present in significant amounts, but account only for 50–100 times less than the amount of ϵ_{S1} transcripts [10]. We wanted to investigate if the ϵ_{S2} transcripts produced by U266 cells are translated into protein and secreted in the supernatant in detectable amounts. Since the two isoforms cannot be discriminated by size, a rabbit antiserum which specifically recognizes the C-terminal tailpiece of the ϵ_{S2} heavy chain was developed [13]. Fig. 2 shows a Western blot analysis performed on total IgE purified from supernatant of unstimulated U266 cultures. An ϵ_{S2} -specific signal was clearly identifiable in IgE secreted by U266. The ϵ_{S2} specificity of the signal is shown by the reactivity of the anti- ϵ_{S2} antiserum against IgE-S2 but not against IgE-S1 controls of transfectoma origin, even under conditions of excess of IgE-S1. This excluded the possibility that the signal detected in U266 was due to weak cross-reaction with the classical ϵ_{S1} H chain, which comigrates with ϵ_{S2} at the expected size of approximately 80 kDa. The IgE nature of the ϵ_{S2} -specific signal was confirmed by removal of the first anti- ϵ_{S2} antiserum, and development with an anti- ϵ serum directed against total IgE, which detects both isoforms. By comparing the relative intensities of anti- ϵ_{S2} and anti- ϵ signals

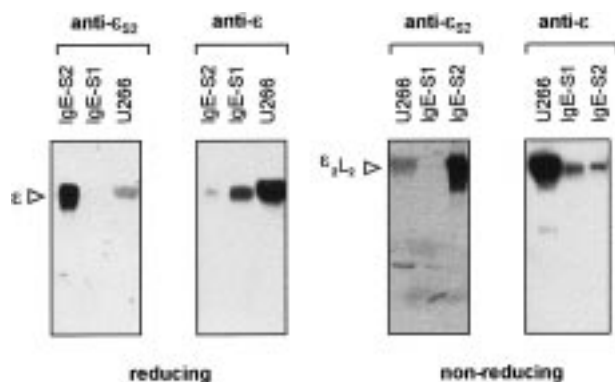


Figure 2. Expression of ϵ_{S2} protein in U266 supernatants. IgE were immunoaffinity purified as described in Sect. 4.4 and analyzed after reducing (left) and non-reducing (right) SDS/PAGE by Western blotting. Anti- ϵ_{S2} and anti- ϵ detection were performed sequentially after membrane stripping. IgE-S1 and IgE-S2 were purified from supernatants of murine transfectomas and used as controls.

in the IgE-S2 and U266 lanes, it is noticeable that the ϵ_{S2} signal accounts only for a fraction of the total IgE purified from U266 cell supernatants, which could be roughly estimated to be in the region of 1–5 %, based on the relative reactivities of the two antibodies with known amounts of purified IgE. This is in agreement with Northern analyses of the mRNA transcripts [10]. When analyzed under non-reducing conditions, the IgE of U266 origin showed a molecular mass of typical H_2L_2 Ig molecules. The absence of higher molecular mass bands demonstrates that in human myeloma the vast majority of ϵ_{S2} isoform does not associate into polymers of H_2L_2 monomers. From these data we can conclude that, similarly to what was found earlier in murine B cell and plasma cells, in human cells the terminal cysteine of ϵ_{S2} does also not appear to induce polymerization or to function as a retention signal for monomeric IgE-S2.

2.3 Detection of ϵ_{S2} in human serum and tonsils

The identification of the novel ϵ_{S2} isoform in the supernatant of IgE-secreting U266 myeloma prompted us to investigate the presence of ϵ_{S2} in human sera and tonsils, where ϵ_{S2} transcripts can be detected. Analysis of samples from both allergic and non-allergic individuals would also allow to evaluate the hypothesis of an association between ϵ_{S2} expression and atopy.

Despite the good activity of the rabbit anti- ϵ_{S2} antiserum in Western blotting (estimated to have a lower detection limit of 10–20 ng/ml; not shown), a limitation was found in the inability of the anti- ϵ_{S2} antiserum to recognize the native, non-denatured, epitope by ELISA or immunoprecipitation [13]. Therefore, detection consisted of immunoaffinity purification of IgE followed by Western blotting with anti- ϵ_{S2} . Sequential anti- ϵ H chain analysis after membrane stripping was employed to assess IgE reactivity. Serum samples analyzed were from 58 atopic, 20 non-atopic and 5 individuals affected by Schistosomiasis. Tonsils analyzed were from six individuals, four of which had proven histories of atopy. The vast majority of the tested sera proved negative for ϵ_{S2} . Most importantly, none of the atopic sera were positive for ϵ_{S2} , suggesting the absence of constitutive up-regulation of ϵ_{S2} serum levels in atopic patients. Total IgE serum levels of samples tested ranged from 30 to over 2000 IU/ml, without an apparent correlation being found between ϵ_{S2} protein detectability and total IgE serum levels. Parallel PBL mRNA analysis performed on some of the individuals whose serum has also been analyzed and found negative for the ϵ_{S2} protein revealed that transcripts encoding ϵ_{S2} were present (data not shown). This indicates that mRNA transcription of ϵ_{S2} in PBL is not always sufficient for detection of the protein in serum. The three positive

exceptions which proved positive for ϵ_{S2} were serum samples from three non-atopic individuals. The ϵ_{S2} isoform was instead more consistently expressed in tonsils. Out of six tonsils tested, four were positive for ϵ_{S2} . Three were from children with atopic histories and the fourth came from a non-atopic subject.

Fig. 3 shows a selection of ϵ_{S2} -positive samples of tonsil and serum. Under non-reducing conditions, anti- ϵ_{S2} signals (upper panels) showed the mobility of typical monomeric H_2L_2 molecules, whose IgE nature was confirmed by the coincidence with the anti- ϵ signal after membrane stripping (lower panel). A two-banded pattern was visible, with the lower band being more predominant in one of the two serum samples shown. To investigate if these difference were due to glycosylation, samples were treated with PNGaseF, which removes high mannose and complex oligosaccharides. Fig. 3b shows that after PNGaseF treatment molecular mass differences disappeared, indicating that the two bands are not due to protein degradation but instead to variations in the sugar content of the ϵ H chain. It is also noticeable that the relative abundance of ϵ_{S2} in the positive samples analyzed was higher than in U266, as judged from the relative intensities of the anti- ϵ_{S2} and total IgE signals after membrane stripping (as in Fig. 3a). As a rough estimate, based on the reactivities of the two antisera against the purified IgE-S1 and IgE-S2 controls, we calculated that in the three serum samples ϵ_{S2} accounted for 50–60 % of the total IgE, whereas in the tonsil samples it was lower, *i. e.* around 20–25 %.

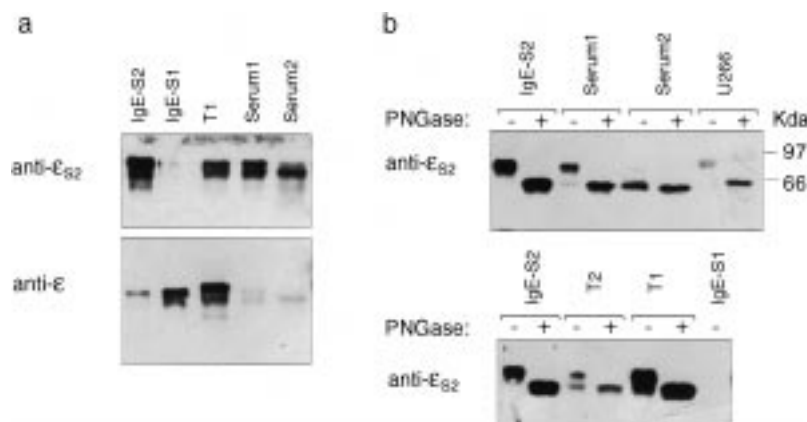


Figure 3. Expression of ϵ_{S2} protein in human sera and tonsils. Immunoblot analyses with anti- ϵ_{S2} and anti- ϵ , as indicated, were carried out sequentially following SDS-PAGE of purified IgE under non-reducing (a) or reducing (b) conditions. Two serum and two tonsil (T1, T2) samples are shown.

2.4 IgE-S2 binding to Fc ϵ RI-positive mast cells

Interaction of secretory IgE with the high-affinity receptor on mast cells is central to the effector function of allergic mediators released in the presence of multivalent antigen. Binding of monomeric IgE-S2 to two different sources of Fc ϵ RI-positive cells was evaluated by indirect immunofluorescence and flow cytometry (Fig. 4). IgE-S1 and IgE-S2 tested were of transfectoma origin and consisted of chimeric mouse/human ϵ -chain constructs with anti-NIP specificity [5]. Fig. 4a shows positive staining of human KU812, an Fc ϵ RI-expressing cell line with a basophilic and mast cell-like phenotype [14]. The degree of binding of both IgE-S1 and IgE-S2 was comparable albeit modest. This is most likely due to the low density of Fc ϵ RI molecules on KU812 [5]. Higher levels of staining were seen on rat basophilic leukemia RBL-SX38 cells expressing the human $\alpha\beta\gamma 2$ receptor (Fig. 4b) [9]. The absence of interaction with the endogenous rat receptor was confirmed in untransfected RBL-2H3 (not shown). Overall we can conclude that monomeric IgE-S2 can bind to Fc ϵ RI-positive cells and is therefore a natural ligand of the high-affinity receptor for IgE.

2.5 IgE-S2-mediated functional responses of mast cells

Cross-linking by multivalent antigen of Fc ϵ RI-bound IgE ligands on the surface of mast cells stimulates receptor aggregation and a signal transduction cascade which initiates a rapid tyrosine phosphorylation of many tyrosine kinase substrate proteins [16]. Fig. 4c shows the kinetics of total protein tyrosine phosphorylation in RBL-SX38 cells following Fc ϵ RI aggregation by IgE-S1 and

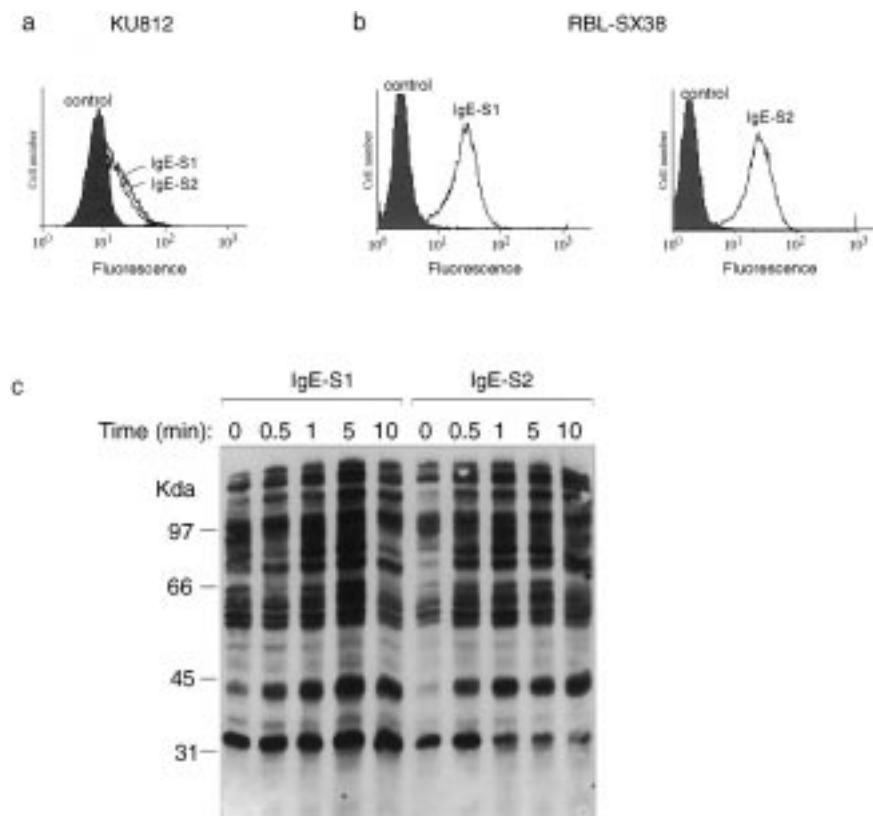


Figure 4. IgE-S1 and IgE-S2 interaction with the high-affinity receptor for IgE. (a, b) Cell surface analysis of IgE-S1 and IgE-S2 binding on FcεRI-positive KU812 (a) or RBL-SX38 (b) cells. Cells were stained with FITC-conjugated rabbit anti-ε after preincubation with IgE or medium (control). (c) Kinetics of IgE-S1- and IgE-S2-induced activation of FcεRI-mediated signaling. RBL-SX38 cells were sensitized with IgE-S1 or IgE-S2 and bound IgE were cross-linked with NIP-BSA for various times as indicated. An anti-phosphotyrosine immunoblot of cellular extracts separated on SDS-PAGE is shown.

IgE-S2 and polyvalent antigen (NIP-BSA). Phosphorylation of the same set of substrate proteins was induced by both isoforms with a total response marginally weaker for IgE-S2. Assays were performed in saturating and equimolar amounts of monomeric IgE and the effect was visible with different IgE preparations, with FcεRI-binding activity tested by cytofluorimetry on RBL-SX38 cells.

The ability to mediate mast cell degranulation was next compared by two independent assays of serotonin and histamine release, in the presence of titrated amounts of multivalent antigen (Fig. 5a, b). The results unambiguously identify IgE-S2 as competent at inducing degranulation of RBL-SX38 cells. Both histamine and serotonin release also indicate that IgE-S2 induced a weaker degranulation response with an overall differences not exceeding 15–30 % with respect to IgE-S1.

In addition to release of allergic mediators, FcεRI aggregation also results in internalization of FcεRI-IgE com-

plexes [17]. In RBL-2H3 cells, it has been shown that about 50 % of receptor-bound IgE undergo rapid internalization within 10 min after receptor cross-linking [18]. Internalization of IgE-S1 and IgE-S2 receptor complexes on RBL-SX38 was estimated by flow cytometry [19, 20], after cross-linking with NIP-BSA at 37 °C (Fig. 5c). Mean fluorescence values estimated an internalization of about 40–50 % after 20 min of antigen cross-linking, with IgE-S2 showing a slower rate compared to IgE-S1.

3 Discussion

While mRNA transcript analyses have documented a variety of alternative splicing ε isoforms potentially encoding novel human IgE isoforms bearing distinct C termini, evidences of their translation and secretion in human cells and human fluids *in vivo* have been difficult to obtain, for the lack of quality isotype-specific antibody reagents. Thanks to an antiserum with specific activity

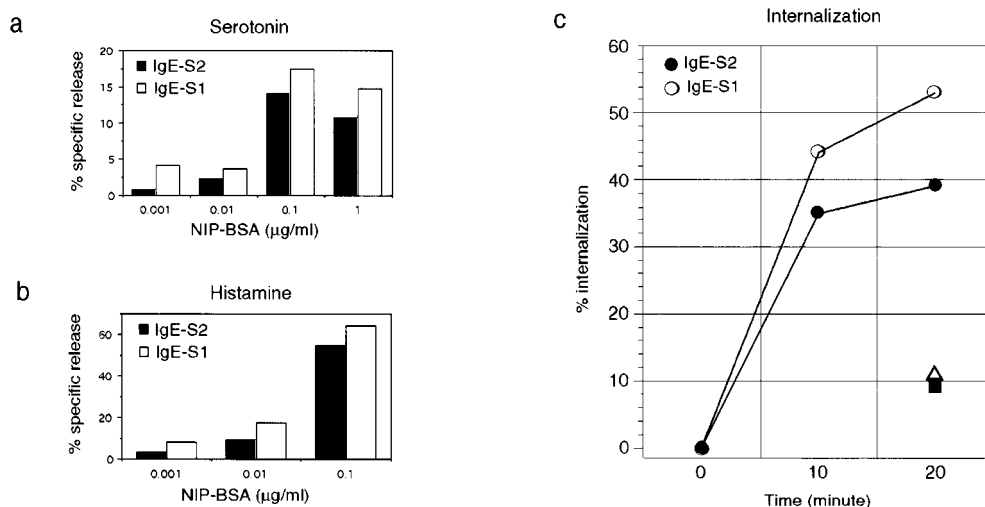


Figure 5. Comparison of ϵ_{S1} - and ϵ_{S2} -mediated degranulation (a, b) and internalization (c) functions. Degranulation was measured by release of [^{3} H]serotonin (a) or histamine (b). Results of two independent experiments are shown. Specific release was calculated from means of duplicate values after subtraction of the spontaneous release. (c) Cells were stained with FITC-conjugated anti- ϵ and internalization is represented as the difference between the fluorescence values before and after incubation at 37 °C for the indicated time periods, and expressed as percentage. The data shown are from one representative experiment. Control values of IgE-S1-sensitized cells without NIP-BSA triggering (Δ) or with triggering at 4 °C ($\blacksquare$) are shown at 20-min time points.

against the C-terminal tailpiece of the ϵ_{S2} H chain [13], we have identified a novel human secretory IgE that differs in structure and expression from the classical secreted IgE isoform. IgE-S2 is rarely detected in serum, appears more frequent in tonsils, and is constitutively expressed in the human myeloma cell line U266. The expression of ϵ_{S2} in myeloma cells as the less abundant of the two secreted isoforms suggests an independent regulation of expression, which is in agreement with the relative abundance of mRNA transcripts detected in Northern blots. As noticed earlier [10], this could in part be attributed to the use by ϵ_{S2} of the rather inefficient membrane polyadenylation site which is distinct from the polyadenylation site used by the classical secretory isoform ϵ_{S1} (see Fig. 1a). The membrane polyadenylation site contains in fact a non-canonical AGTAAA sequence which is thought to have only 30 % of the activity of the classical AATAAA [21] and could therefore contribute to less efficient transcription in U266 of IgE membrane transcripts, including ϵ_{S2} transcripts. In sera, however, even though very few samples were found positive for this isoform, the relative amount of ϵ_{S2} was surprisingly high. Distinct regulation of the two isoforms *in vivo* was further suggested by the apparent lack of correlation between total IgE serum titers and ϵ_{S2} protein detection. In fact, not only ϵ_{S2} -negative sera with high total IgE serum levels were found, but also the samples which were positive for the ϵ_{S2} isoform had only average IgE serum levels. This

suggests that serum expression of one isoform can be regulated independently of the other. Moreover, of one of the individuals whose serum proved positive for ϵ_{S2} only one out of three samples, taken at approximately 10-day intervals, was positive. While it is difficult to assess the generality of this observation, this may underlie a more common transient expression mode, and is consistent with the rapid turnover of serum IgE, whose average half-life is approximately 2.5 days. This is supported by evidence of short-lived and dose-dependent up-regulation of ϵ_{S2} mRNA transcripts in nasal airways after *in vivo* challenge with diesel exhaust particles [3] as well as by time-dependent *in vitro* regulation of ϵ transcripts by IL-6 and IL-10 [4]. The absence of ϵ_{S2} in the serum of the atopic subjects tested indicates that there is no association between ϵ_{S2} up-regulation in serum and atopy. However, given the overall rarity of ϵ_{S2} expression in human serum, the present findings do not rule out the possibility that this human isoform may play a role in allergic responses, with a pattern of expression that could be rather local and transient in time.

Visualized under non-reducing conditions, the ϵ_{S2} molecules always consisted of conventional IgE monomers composed of two H chains and two L chains. Therefore, unlike in IgM and IgA [7, 8], the unique C-terminal cysteine of ϵ_{S2} does neither function as a polymerization signal nor as an intracellular retention signal in human lympho-

cytes, in agreement with earlier data obtained from expression of ϵ_{S2} in murine transfectomas [5]. The hypothesis that ϵ_{S1} and ϵ_{S2} might have the capacity, if co-expressed in the same cell, to assemble into an hetero-hybrid H_2L_2 molecule in which one chain would consist of ϵ_{S1} and the other of ϵ_{S2} was also investigated (R. Lorenzi and S. Anand, unpublished data). While IgE-S1/S2 heteroduplexes could be secreted by a doubly transfected mouse myeloma, they never accounted for the majority of the detected species, even under the conditions tested, where the assembly of heteroduplexes was favored by the vast excess of one H chain (ϵ_{S1}), with respect to the other (ϵ_{S2}). The kinetics of ϵ_{S2} homoduplex assembly appear therefore to favor the formation of homoduplex to heteroduplex complexes. These data are in agreement with the faster rate of intracellular assembly of ϵ_{S2} , compared to ϵ_{S1} . It also justified our use of IgE-S2 homodimers for assessment of the functional interaction with Fc ϵ RI.

Positive staining by indirect fluorescence of Fc ϵ RI-positive cells confirmed the novel IgE-S2 as a natural ligand of Fc ϵ RI. This was anticipated in view of the fact that the contact sites of interaction with the receptor have been mapped to the ϵ CH3 constant region domain of the IgE molecule [22–24], which is identical in ϵ_{S1} and ϵ_{S2} . On the other hand, an indirect effect of stabilization of the active binding conformation has been shown for both the distal domains ϵ CH2 and ϵ CH4 [22, 23, 25, 26]. Moreover, altered IgE conformations induced by heat denaturation [27], domain deletion [23], or antigen binding [28] have been reported to affect the interaction between IgE and Fc ϵ RI. The C-terminal cysteine-containing tailpiece of ϵ_{S2} is attributed a stabilizing role on the formation of H_2L_2 molecules, as judged by the higher rate of intracellular assembly of IgE-S2 compared to IgE-S1 molecules [5]. We found here that the unique tailpiece of ϵ_{S2} does not impair the early effector functions of degranulation and internalization following engagement of the mast cell receptor. Interestingly, cysteine 328, which also stabilizes the dimer, but in the more upstream ϵ CH2- ϵ CH3 linker region, has been shown to have a stronger activity on histamine release upon receptor engagement, compared to a construct lacking such residue [26]. In our case, a milder response was noticed with IgE-S2 compared to IgE-S1, but the differences observed averaged 15–30 %, and therefore are not likely to significantly affect the degranulation competency of IgE-S2. Nevertheless, this weaker agonistic effect appears concomitantly with a measurable difference in dissociation rate upon direct interaction with the alpha chain of the receptor (R. Lorenzi et al., manuscript in preparation), and it could have an important effect on the later release of *de novo* formed mediators by mast cells. In fact, in addition to a rapid degranulation of pre-

formed mediators, Fc ϵ RI aggregation is also known to activate, upon prolonged antigen stimulation, gene expression and *de novo* synthesis of a number of cytokines including IL-3, IL-5, GM-CSF, IL-6, TNF and IL-4 [29, 30]. The release of these *de novo* synthesized mediators usually peaks at 3–6 h after receptor aggregation and requires sustained stimulations of the Fc ϵ RI. We are currently investigating whether the modest difference between IgE-S2 and IgE-S1 observed on degranulation is amplified on later mast cell responses, and whether ligand-induced effects upon Fc ϵ RI engagement can play a significant physiological role in the modulation of cytokine microenvironments *in vivo*.

4 Materials and methods

4.1 Cell lines

The human IgE-secreting U266 cell line and the human basophilic cell line KU812 (a kind gift of Dr. W. M. Becker, Borstel, Germany) were maintained in RPMI 1640 medium with 20 % FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin. The mouse J558L transfectomas expressing the mouse/human C ϵ_{S1} and C ϵ_{S2} constructs with anti-NIP specificity have been described previously [5] and were maintained in DMEM, 10 % FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin supplemented with 400 μ g/ml G418 (Gibco-BRL). The RBL-2H3-derived cell line RBL-SX-38, which expresses the α , β , and γ subunits of the human Fc ϵ RI IgE receptor, has been described before [9] and was maintained in Eagle's minimal essential (EMEM) medium, 16 % FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin supplemented with 700 μ g/ml G418. Untransfected RBL-2H3 cells were maintained under the same conditions except for the absence of G418.

4.2 RT-PCR analysis of ϵ_{S2} transcripts

Mononuclear cells from peripheral blood and tonsils were obtained by fractionation on Ficoll/Hypaque gradients. Total RNA was isolated using the guanidinium thiocyanate procedure [31]. A nested RT-PCR procedure was performed as described before [10].

4.3 Clinical samples

Atopic sera (a generous gift of Dr. S. Sidoli, DIBIT, Milan) were from atopic individuals with histories of atopic bronchial asthma or allergic rhinitis, and had been tested positive in skin prick tests to a variety of allergenic mixes. Non-atopic sera were from normal healthy donors with no known allergic disease and normal IgE serum levels (<100 IU/ml). Sera from patients affected by Schistosomiasis were a gift of Dr. H. Haas, Borstel, Germany. Serum samples were diluted 1:1

(v/v) in PBS prior to IgE purification. Tonsils were obtained from the children's hospital "Burlo Garofalo" in Trieste. Tissues were dissociated in PBS supplemented with 0.1 mg/ml streptomycin. The cellular and particulate fractions were sedimented by centrifugation and the supernatant kept for analysis.

4.4 IgE purification and immunoblot analysis

IgE were purified by affinity chromatography with goat anti-human IgE (ϵ chains) (Kirkegaard & Perry Labs, Gaithersburg, MD) conjugated to Sepharose (Pharmacia LKB, Uppsala, Sweden). Bound IgE were eluted from the column with 0.1 M glycine-HCl, pH 2.5, and immediately neutralized with 1 M Tris, pH 7.5. IgE were separated on SDS-PAGE and electroblotted on polyvinylidene difluoride (PVDF) membrane (Amersham Intl., Amersham, GB). Membranes were incubated with a 1:1000 dilution of rabbit anti- ϵ_{S2} serum (generated against ϵ_{S2} peptide VNPESSRRGG [13]) or with rabbit anti-human IgE (ϵ chain) (Dako, Glostrup, Denmark), followed by horseradish peroxidase-coupled goat anti-rabbit Ig (Dako). Bound Ab were visualized by enhanced chemiluminescence (ECL; Amersham Intl.).

4.5 Flow cytometric analysis

Fc ϵ RI-positive KU812 or RBL-SX38 cells were incubated for 1 h at room temperature with supernatants of IgE-S1 and IgE-S2 J558L transfectomas and surface expression of Fc ϵ RI-bound IgE was examined by flow cytometry on a FACSCalibur (Becton Dickinson Immunocytometry Sys., Mountain View, CA) using FITC-conjugated rabbit anti-human IgE (Dako).

4.6 Tyrosine phosphorylation assay

Adherent RBL-SX38 cells were trypsinized and incubated either with medium or with saturating amounts of anti-NIP IgE-S1 or IgE-S2 (5 μ g/ml/ 10^6 cells) for 1 h at room temperature. Cells were then washed, resuspended in 0.5 ml EMEM and triggered with 100 μ g/ml NIP₁₈-BSA (a kind gift of Dr. C. Faccioli, DIBIT, Milan) at 37 °C. After washing in ice-cold PBS containing 1 mM sodium orthovanadate, cells were lysed with 100 μ l of Triton X-100 lysis buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10 % glycerol, 1 % Triton X-100, 2 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitors PMSF (1 mM), aprotinin (10 μ g/ml), and leupeptin (10 μ g/ml) (all from Sigma Chemical Co., St. Louis, MO). After incubation on ice, supernatants were cleared by centrifugation at 4 °C and separated on 10 % SDS-PAGE. After electroblotting, PVDF membranes were incubated with anti-phosphotyrosine mAb PY20 (Transduction Laboratories, Lexington, KY) followed by horseradish peroxidase-coupled anti-mouse Ig Ab (Dako), and detected by ECL.

4.7 Degranulation assays

For serotonin release assays, cells (2×10^5) were suspended in medium without G418 containing 0.5 μ Ci/ml [^{3} H]serotonin (DuPont NEN, Boston, MA) and seeded into 24-well plates. After overnight incubation the medium was aspirated and cells were sensitized with medium alone or with medium containing 5 μ g/ml IgE-S1 or IgE-S2 for 1 h at 37 °C. Cells were then washed twice with sodium PIPES buffer containing 20 mM sodium PIPES, 100 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂ and 0.1 % BSA, and resuspended in 200 μ l sodium PIPES. Duplicate wells were next incubated for 30 min at 37 °C with or without addition of NIP-BSA. Radioactivity in the supernatant was calculated by liquid scintillation counting. For each well total cell-associated counts were determined after 15 min lysis at room temperature by addition of 1 % Triton X-100 in PBS. Serotonin release was calculated from the ratio between released and the sum of released and retained (cell-associated) counts. To calculate % specific release, each value was multiplied by 100 and the value for spontaneous release determined from duplicate wells which had not been sensitized with IgE was subtracted.

For the histamine release assay, cells were processed as above with the omission of radioactive serotonin addition. Lysis to calculate residual cell-retained histamine consisted of two freeze-thaw cycles in water. Histamine was measured by an enzyme immunoassay kit (Immunotech, Marseille, France) following the manufacturer's instructions. Specific release was calculated as described above.

4.8 Internalization assay

Internalization was estimated by flow cytometry as previously described [19, 20]. RBL cells were sensitized with IgE for 1 h at room temperature and stained with FITC-conjugated rabbit anti-human IgE (Dako) at 4 °C for 30 min in PBS/1 % FCS. After washing, all samples except the zero time points (no internalization) were resuspended in pre-warmed medium and incubated with NIP-BSA at 37 °C. The reaction was stopped by adding ice-cold PBS/FCS containing 0.1 % sodium azide. The fluorescence was then analyzed by flow cytometry. The internalization index was calculated from residual mean fluorescence values, and expressed as percentage of initial (zero time point) fluorescence.

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5 References

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