



The novel human IgE epsilon heavy chain, epsilon tailpiece, is present in plasma as part of a covalent complex

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Abstract

Several splice variants of the secreted human epsilon heavy chain have previously been identified by reverse transcription-PCR. The heavy chain of one isoform, IgE tailpiece, differs from the originally identified IgE, IgE classic, by the replacement of the 2 carboxy-terminal amino acids by 8 novel amino acids including a carboxy-terminal cysteine residue. Recombinant human epsilon tailpiece and epsilon classic heavy chains were expressed and secreted as H₂L₂ monomers in Sp2/0 murine myeloma cells. We have investigated the in vitro function and in vivo occurrence of epsilon tailpiece heavy chains using receptor binding assays, granule release assays, flow cytometry, half-life studies, immunoprecipitation, SDS-PAGE, two-dimensional SDS-PAGE, and Western blotting. IgE tailpiece and IgE classic exhibited similar in vivo half-lives in BALB/c mice, bound the human high- and low-affinity IgE receptors with similar affinities and triggered equivalent levels of high affinity IgE receptor induced degranulation. In humans, IgE classic is present as a 190 kD circulating protein in vivo. In contrast, we found that in plasma epsilon tailpiece was primarily present as part of covalent complexes of approximately 300 and 338 kD. Dissociation of the complexes revealed that two species of epsilon tailpiece heavy chains were present therein and surprisingly, these in vivo derived epsilon tailpiece heavy chains were approximately 5 and 10 kD smaller than the recombinant expressed epsilon tailpiece or epsilon classic heavy chains. These results show that epsilon tailpiece is present in novel covalent complexes in humans. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Over 30 years ago, immunoglobulin E (IgE) was discovered to be the antibody isotype mediating immediate hypersensitivity reactions in humans (Coombs et al., 1968; Ishizaka et al., 1986). The structure of IgE was found to be typical of Ig molecules consisting of an H₂L₂ monomer comprised of two Ig heavy chains and two Ig light chains. The epsilon heavy chain consisting of four constant region domains, lacking a for-

mal hinge region, and containing 6 used N-linked carbohydrate addition sites had an apparent molecular weight of 75 kD. IgE binds three types of IgE receptors, the high-affinity IgE receptor (FcεRI), the low-affinity IgE receptor (FcεRII or CD23), and the galectin 3 (Gal 3) through contact sites within the second and third constant region domains or carbohydrate moieties (Chrétien et al., 1988; Nissim et al., 1991; Presta et al., 1994; Robertson et al., 1990). IgE plays an important role in both the afferent and efferent limbs of the immune response (Monteseirín et al., 1996; Saxon et al., 1996).

Until epsilon mRNA transcripts were investigated, only one species of human IgE was thought to exist in

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Nomenclature

IgE	immunoglobulin E	BSA	bovine serum albumin
etp	epsilon tailpiece	PBS	phosphate buffered saline
IgEtp	IgE tailpiece	TCA	trichloroacetic acid
FcεRI	high-affinity IgE receptor	TYR	Tyrod's buffer
FcεRII, CD23	low-affinity IgE receptor	PVDF	polyvinylidene fluoride
Gal3	galectin 3 (epsilon binding protein)	DNS	dansyl
C _H	constant region of heavy chain	HRP	horseradish peroxidase
HMW	high molecular weight	MFI	mean fluorescent intensity
IMDM	Iscove's Modified Dulbecco's Medium	Staph A	protein A from <i>Staphylococcus aureus</i>
CS	calf serum	rIgEtp	recombinant IgE tailpiece
mAb	monoclonal antibody		

vivo. However, several alternatively spliced human epsilon mRNAs were identified by reverse transcription-PCR (Zhang et al., 1992). The cause of these alternative splicing events seemed to be the presence of a sub-optimal splice acceptor sequence at the beginning of the first membrane exon, M1 (Zhang et al., 1992). Splicing from the splice donor sites in the heavy chain fourth constant region (C_H4) to alternative downstream splice acceptor sites yielded mRNAs that potentially encode heavy chains for four different forms of secreted IgE and two different forms of membrane bound IgE (Diaz-Sanchez et al., 1995; Peng et al., 1992; Zhang et al., 1994; Zhang et al., 1992). The mRNAs for the four secreted epsilon (ε) splice variants were present in B cells isolated from atopic and parasite-infected individuals and in normal B cells stimulated with IL-4 and anti-CD40 (Diaz-Sanchez et al., 1995).

One of the alternatively spliced RNA products encoded an epsilon heavy chain for IgE tailpiece (IgEtp), differing from the 'classic' IgE by the replacement of the 2 carboxy-terminal amino acids with 8 novel amino acids including a carboxy-terminal cysteine residue (Fig. 1). The amount of the IgEtp mRNA present appeared to correlate positively with atopy and parasitic infection (Diaz-Sanchez et al., 1995). However, the exact nature, in vivo levels, and biological activity of IgEtp protein were not elucidated.

We have found that recombinant IgE classic and IgE tailpiece, produced in the Sp2/0 cell line, had similar half-lives, biodistributions in vivo, affinities for the high- and low-affinity IgE receptors, and antigen-dependent degranulation of mast cells. However, when human IgE tailpiece in the circulation was examined, it was found to be present in a previously undescribed high molecular weight (HMW) complex. Moreover, the epsilon tailpiece (etp) heavy chains identified in vivo were found to exist in two sizes, both smaller

than the recombinant epsilon tailpiece (rεtp) heavy chain expressed in tissue culture.

2. Material and methods

2.1. Cells lines and human blood

The murine cell line Sp2/0 was grown in Iscove's Modified Dulbecco's Medium (IMDM) (Irvine Scientific, Santa Ana, CA) containing 5% bovine calf serum (CS) (Hyclone, Logan, UT). IgE producing transfectants of Sp2/0 were grown in IMDM + 5% CS containing 42 μg/ml hypoxanthine, 1 μg/ml xanthine, and 2.5 μg/ml mycophenolic acid. The rat basophilic leuke-

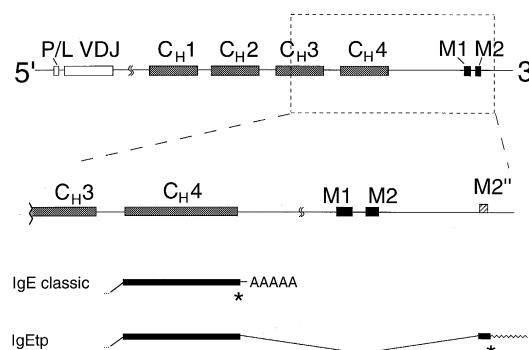


Fig. 1. Diagrammatic representation of the splicing patterns which lead to the production of two secreted isoforms of human IgE, IgE classic and IgE tailpiece. The standard exons (P/L, VDJ, C_H1, C_H2, C_H3, C_H4, M1, M2) are depicted in solid patterns, while the novel exon (M2'') is shown in crosshatches. IgEtp includes C_H1 through C_H4 and the M2'' exon. IgE classic includes exons C_H1 through C_H4. P/L is the promoter/leader sequence. Asterisks indicate positions of stop codons. The wavy line 3' of the IgEtp stop codon indicates a region where several splicing events occur which do not affect the protein product. "AAAAA" indicates the polyadenylation site of IgE classic which results in the removal of the downstream sequences from the mature message of IgE classic.

mia cell line 4RBL48, transfected with the alpha subunit of the human high-affinity IgE receptor (kindly provided by Dr. J.P. Kinet, Harvard Medical School, Boston, MA) was grown in IMDM containing 10% fetal calf serum (Hyclone, Logan, UT) and 1.2 mg/ml G418. The Chinese Hamster Ovary cell line CHO 3D10 expressing the extracellular region of the human FcεRI alpha subunit fused to the transmembrane and intracellular regions of the human IL-2 receptor p55 subunit (kindly provided by Dr. J.P. Kinet, Harvard Medical School, Boston, MA) was grown in IMDM containing 10% fetal calf serum and 10^{-7} M methotrexate. CHO7, expressing human CD23 (kindly provided by Dr. M. Sarfati, Montreal, Canada), was grown in RPMI containing 10% CS. Human plasma collected from highly allergic individuals with total IgE levels of >2000 IU/ml was purchased from Plasma Lab, Everett, WA. In addition, human serum and plasma were collected from volunteers at UCLA. Pooled serum from non-allergic individuals was purchased from BioWhittaker, Walkersville, MD. The murine monoclonal antibody 367 (anti-εtp mAb) used to detect IgEtp protein was made against a peptide antigen containing the novel 8 amino acids of human ε tailpiece (produced in collaboration with Dr. Roger Lindberg, Abbot Diagnostics, Abbot Park, IL).

2.2. Purification of recombinant IgE classic and IgEtp

Sp2/0 cells, stably transfected with expression constructs encoding either IgE classic or IgEtp, were expanded in IMDM containing 2% alpha calf serum or 1–2% Fetal Clone 1 (Hyclone, Logan, UT) and 2.5% Glutamax-1 (GibcoBRL, Grand Island, NY). IgE isoforms were purified from supernatant through their dansyl (DNS) antigen specificity on a DNS-Sepharose column as described previously (Shin and Morrison, 1989) or by affinity chromatography using rabbit polyclonal anti-ε chain Ab (ICN, Costa Mesa, CA) or murine anti-ε chain mAb (Macy et al., 1988) and acid elution.

2.3. ^{125} Iodine-labeling of purified proteins

5 μg purified of IgE was labeled with 200 μCi ^{125} Iodine (ICN, Costa Mesa, CA) using an Iodobead kit (Pierce, Rockford, IL) according to the manufacturer's instructions. The labeled IgE protein was separated from free ^{125} I on a 10 ml Sephadex G-50 Medium column previously blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.5. Fractions were tested for trichloroacetic acid (TCA)-precipitable radioactivity.

2.4. Affinity of IgE isoforms for FcεRI

CHO 3D10 cells (2×10^6 cells/ml) were washed and resuspended in Tyrode's Buffer +1% BSA (TYR/BSA). Fifty ng ^{125} I-labeled IgE combined with graded amounts of unlabeled IgE (10–30 fold excess) of the same isoform (i.e. homologous competitor) in a total volume of 250 μl was added to 250 μl 3D10 cells and binding was allowed to proceed for 90–120 min at room temperature. To determine nonspecific binding, 3D10 cells were preincubated with 25 μg IgE PS myeloma protein prior to the addition of 50 ng ^{125} I-labeled recombinant IgE. Cells were pelleted by centrifugation and the cell pellet washed in 500 μl ice-cold TYR/BSA. The washed cell pellet (bound counts) and 200 μl of the supernatant (unbound counts) were counted in a Beckman Gamma 5500 gamma counter. Data were analyzed by Scatchard plot.

2.5. Antigen-mediated mast cell degranulation

Mast cell granule release assays were performed as described (Tanaka et al., 1991) using 4RBL48 cells expressing a transfected human FcεRI alpha subunit and endogenous rat FcεRI α, β, and γ subunits. Approximately 10^6 4RBL48 cells were incubated for 1 h at 37°C in the presence of 0.75 μg of dansyl-specific IgE and varying concentrations (0.25 ng to 2.5 μg) of DNS-BSA. Release was then assessed by measurement of the β-hexosaminidase present in the cell lysate vs. supernatant fractions (Tanaka et al., 1991).

2.6. Flow-cytometric analysis of CD23 binding

Binding to CD23 was assessed using CHO7 cells expressing human CD23. 1 μg of purified IgE classic, IgEtp, or IgG₁ was combined with rabbit anti-human Fab and the mixtures were incubated overnight at 4°C in PBS + 1% BSA in a total volume of 50 μl. These preformed complexes (or a 50 μl solution containing only anti-human Fab) were added to wells containing 3×10^5 CHO7 cells and the samples were incubated on ice for 4–5 h. Following washes to remove unbound complexes, cells were incubated (30 min on ice) with FITC-conjugated F(ab')₂ of donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 in PBS + 2% CS. Samples were then analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), gating out dead cells and debris. Data were analyzed using C30/FACScan Research software and Kolmogorov–Smirnov statistics.

2.7. In vivo half-life

The 4–8 week old female BALB/c mice were injected

intraperitoneally with 200 μ l containing 10^6 cpm of ^{125} I-labeled IgE isoforms in PBS. Three mice were injected for each protein studied. Mice were given 0.1 mg/ml KI in their drinking water for at least four days prior to the experiment. Whole body radioactivity was determined at various timepoints using a Model LS-5A gamma counter (Wm. B. Johnson and Associates, Montville, NJ) with a sample holder large enough to accommodate an entire live mouse. Half-life values were obtained using only those data points at which > 15% of the initial injected dose remained.

2.8. Immunoprecipitation

Human IgE from plasma or serum was precipitated using murine anti-human IgE mAb CIA-E-7.12 (anti- ϵ mAb)(Macy et al., 1988) or polyclonal goat anti-human IgE (goat anti- ϵ Ab) (Sigma, St. Louis, MO) conjugated to CNBr-activated Sepharose 4B as described by the manufacturer. In 1.5 ml microfuge tubes, 200 μ l of human plasma or serum was incubated with 200 μ l of antibody linked Sepharose slurry overnight at 4°C. Immune complexes were then washed and the proteins of interest were eluted by boiling in SDS-PAGE sample buffer as described previously (Lyczak et al., 1996). Denatured, non-reduced samples were analyzed on 4% SDS-PAGE. Samples reduced by incubation with 1% β -mercaptoethanol at 37°C for 30 min were analyzed on 12.5% SDS-PAGE.

2.9. Two-dimensional SDS-PAGE

Immunoprecipitated samples performed in duplicate were first electrophoresed on 4% polyacrylamide gels under non-reducing conditions. The lanes of interest were excised and incubated at 37°C for 30 min in 5% dithiothreitol in dH₂O to break all disulfide bonds. Each gel slice was then placed flush against the surface of a 12.5% polyacrylamide gel (prepared using a two-dimensional gel comb) with more rapidly migrating proteins at the right and separated by size in a second dimension as described previously (Shin and Morrison, 1989). Recombinant IgEtp reduced with β -mercaptoethanol was run as a molecular weight marker in the single well on the left of the gel.

2.10. Western blot

IgE proteins separated by SDS-PAGE gels were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore, Bedford, MA) in a wet transfer apparatus at 1 A for 1 h at 4°C in 50 mM Tris, 40 mM glycine, and 4% methanol. After membranes were blocked with 3% BSA in PBS/tween (PBS + 0.02% Tween20) for 1 h at room temperature with shaking, the membranes were probed with a primary

antibody diluted 1:1000 in 3% BSA in PBS/tween overnight at 4°C with shaking. The anti- ϵ tp mAb was used to detect ϵ tp protein. This antibody bound to recombinant ϵ tp protein but failed to bind to ϵ classic. A goat anti- ϵ Ab (Sigma, St. Louis, MO) was used to detect all ϵ isoforms. After the membranes were washed with PBS/tween, the primary antibodies were detected using horseradish peroxidase (HRP) conjugated to sheep anti-mouse Ig (1:20,000) (Amersham, Piscataway, NJ) or rabbit anti-goat HRP (1:25,000) (Sigma, St. Louis, MO) and visualized by chemiluminescence detection (ECL, Pierce, Rockford, IL) for different lengths of time ranging from 1 s to 5 min.

2.11. Relative molecular mass

Along with the immunoprecipitated ϵ tp complexes, proteins of known molecular mass were run on 4% SDS-PAGE. The proteins were stained with Coomassie blue dye and their relative mobilities were calculated as the distance traveled for each protein compared to the total distance traveled by the dye front on the resolving gel layer. The relative electrophoretic mobility values (Rfs) were plotted versus molecular mass on a semi-log plot and the resulting curve was used to calculate the molecular weight of HMW ϵ tp complexes that were run on the same gel and detected by anti- ϵ tp mAb Western blotting.

2.12. Quantitative IgE ELISA

Immulon 2 ELISA plates were coated overnight at 4°C with 50 μ l of goat anti-human IgE (Sigma, St. Louis, MO) at 5 μ g/ml in carbonate buffer, pH 9.6. Plates were washed with PBS four times and blocked for 30 min at room temperature with 100 μ l 3% BSA and 0.02% sodium azide in PBS. Plates were washed and incubated overnight at 4°C with 50 μ l of human IgE standards of known concentration as well as dilutions of plasma and serum samples. All samples were diluted with 1% BSA in PBS and 0.02% sodium azide. Plates were then washed, incubated at room temperature with alkaline phosphatase conjugated goat anti-human IgE for 1 h, washed and developed for 30 min with 1 mg/ml *p*-nitrophenyl phosphate substrate (Sigma 104, Sigma, St. Louis, MO) dissolved in diethanolamine buffer, pH 9.8. The concentration of human IgE in plasma and serum samples was extrapolated from a plot of the IgE standard concentration versus optical density at 410 nm.

3. Results

3.1. Binding of IgE isoforms to FcεRI

We have previously described the alternative splicing pattern of the epsilon gene (shown in Fig. 1) and the expression of recombinant IgE tailpiece and IgE classic in the murine myeloma cell line Sp2/0 (Lyczak et al., 1996). Both recombinant IgE isoforms were approximately 190 kD on non-reduced SDS-PAGE. To function in the effector phase of immediate hypersensitivity reactions, IgE must bind to the high-affinity IgE receptor on mast cells and basophils. Both recombinant IgEtp and IgE classic showed similar rates of association with FcεRI with maximum binding being achieved between 60–90 min (data not shown). IgEtp

and IgE classic showed equivalent affinities for FcεRI with K_d values of $3 \text{ to } 4.7 \times 10^{-9} \text{ M}$ and $2.4 \text{ to } 4.7 \times 10^5$ receptors per cell as determined by Scatchard analysis (Fig. 2A).

3.2. Recombinant IgE isoforms mediate IgE-dependent, antigen-dependent granule release by the mast cell line 4RBL48

The rat cell line 4RBL48, expressing the human FcεRI alpha chain, was used to compare the ability of IgE classic and IgEtp to mediate mast cell degranulation. When increasing concentrations of DNS-BSA (32:1 substitution ratio) were added to 4RBL48 cells that had been incubated with dansyl-specific IgE classic or IgEtp, the two isoforms were equivalent in their

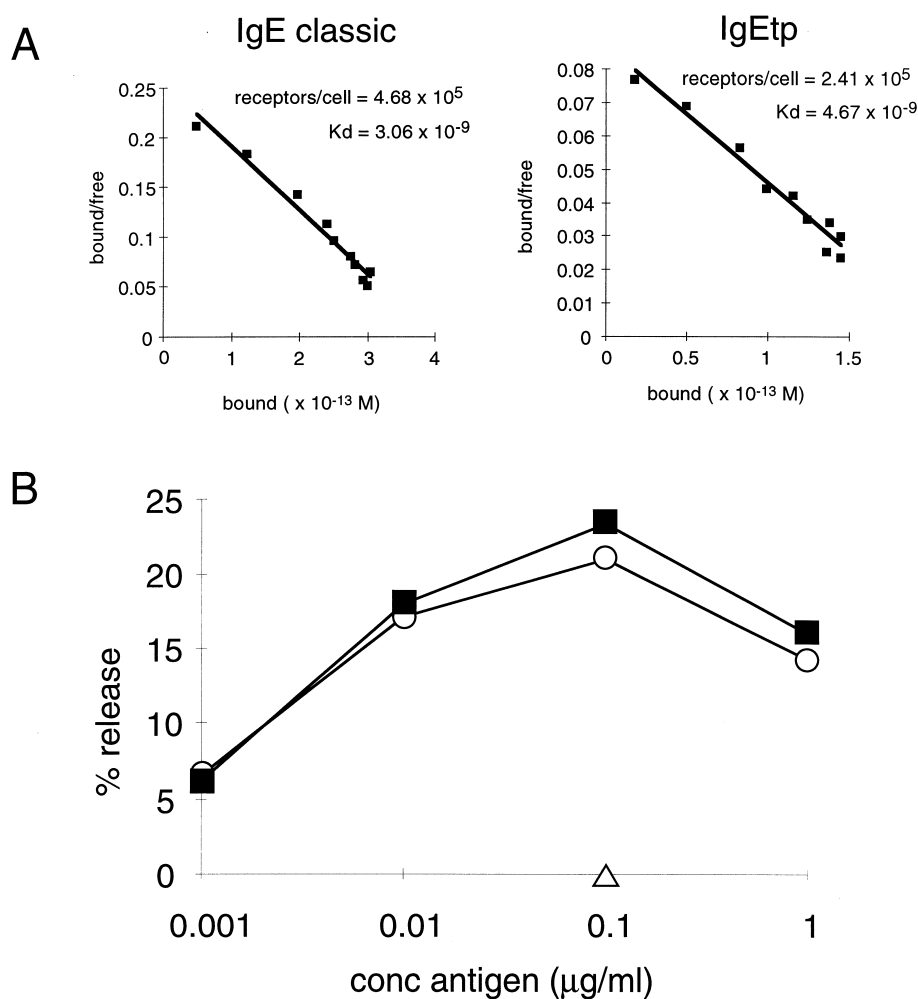


Fig. 2. IgE isoforms bind to FcεRI and trigger degranulation of 4RBL48 cells. (A) Scatchard analysis of IgE isoform binding shows receptor per cell and K_d values close to the expected values of 5×10^5 receptors per cell, and K_d = 10^{-10} . Values were determined by measuring the amount of ¹²⁵Iodine-labeled IgE isoforms bound to CHO 3D10 cells expressing human FcεRI alpha in the presence of graded amounts of unlabeled homologous competitor. (B) Comparison of 4RBL48 cell degranulation mediated by IgE classic and IgEtp. 4RBL48 cells seeded into 24-well plates were stimulated 8 h later with 32:1 DNS-BSA and with either IgE classic (open circles) or IgEtp (solid squares). Percent granule release was then determined by assaying cell supernatants and lysates for the granule component β-hexosaminidase (see Materials and methods). Maximum release was seen at 0.1 μg/DNS-BSA per ml with the magnitude similar for the two isoforms at all antigen concentrations tested. The triangle symbol indicates the level of granule release from 4RBL48 cells stimulated with 32:1 DNS-BSA in the absence of IgE.

ability to mediate degranulation as assayed by the release of β -hexosaminidase with a maximum granule release of 20–25% (Fig. 2B). Lower levels of degranulation were observed when using DNS-BSA substitution ratios of 19:1 and 6:1. No degranulation was seen when unconjugated BSA was substituted for DNS-BSA (data not shown).

3.3. Binding of IgE isoforms to *FcεRII* (CD23)

The low-affinity receptor for IgE, CD23, expressed by B cells, monocytes and eosinophils has been shown to bind IgE with a dissociation constant of 10^{-6} to 10^{-7} M (Dierks et al., 1993). Since this affinity was below the limit of detection by flow cytometry, we formed immune complexes of IgE with rabbit anti-human Fab before adding it to CD23-bearing cells. Recombinant IgEtp or IgE classic was complexed to rabbit anti-human Fab (at a ratio of 3 anti-Fab per IgE) by overnight incubation at 4°C and then added to human CD23 expressing CHO7 cells. Binding of immune complexes to CHO7 cells was then measured by flow cytometry using FITC-labeled anti-rabbit Ig. The data are presented as the mean fluorescent intensity (MFI) of the bound reagent (Table 1). Immune complexes containing IgE classic and IgEtp had similar MFI values which were more than 20 times higher than the MFI observed using either the Fab reagent alone or anti-Fab immune complexes with recombinant IgG₁. When IgEtp immune complexes were formed for only 4 h at room temperature, the MFI values were fifteen-fold higher than those obtained using IgG₁ immune complexes formed under the same conditions (data not shown).

3.4. In vivo half-life of recombinant IgE isoforms

The in vivo half-life of purified recombinant IgE

classic and IgEtp was determined following intraperitoneal injection of ^{125} I-labeled IgE isoforms into 4–8 week old female BALB/c mice. Both proteins cleared rapidly from the animals with half-lives of approximately 3.5 h (Fig. 3). These half-life values were less than the half-life of human IgE in humans (two days) but were consistent with the previous findings that the half-life of human IgE in mice mimics the half-life of murine IgE in mice (Saxon et al., 1991; Stratford and Dennis, 1994).

3.5. Epsilon tailpiece is present as a high molecular weight complex in human plasma

Previously, we had observed that some ϵ tp was present in vivo at a higher molecular weight than that predicted for single IgEtp molecules (unpublished observation). To further examine the in vivo form of ϵ tp, ϵ was immunoprecipitated from human plasma using murine anti- ϵ mAb conjugated to Sepharose 4B. The immunoprecipitate was separated by SDS-PAGE and immunoblotted with anti- ϵ tp mAb. The anti- ϵ tp mAb detected a doublet (Fig. 4A, lane 4) which was much larger than recombinant IgE tailpiece (Fig. 4A, lane 2). The anti- ϵ tp mAb was specific for ϵ tp heavy chain and did not recognize ϵ classic heavy chain (Fig. 4A, compare lanes 1–2). There was no indication of monomeric IgEtp present in these immunoprecipitates. The high molecular weight ϵ tp was also immunoprecipitated using a goat anti- ϵ Ab conjugated to Sepharose (Fig. 4A, lane 5) as well as by 3 additional IgE specific mAbs bound to protein A from *Staphylococcus aureus* (Staph A) (data not shown). Immunoprecipitation of the HMW ϵ tp complexes by multiple epsilon specific reagents from different sources supported the conclusion that the heavy chains within the complexes were structurally related to epsilon classic heavy chains. The complex was not precipitated by BSA-

Table 1
Flow-cytometric measurement of anti-Fab/IgE immune complexes binding to CD23^c

Complex added to CHO7 cells	MFI ^a
Rabbit anti-fab alone ^b	33
Rabbit anti-fab + Recombinant IgG ₁	50
Rabbit anti-fab + Recombinant IgE classic	1127
Rabbit anti-fab + Recombinant IgEtp	1224

^a Binding of immune complexes to CD23 is measured as Mean Fluorescent Intensity (MFI).

^b Rabbit anti-fab alone was used to define the background fluorescent intensity.

^c Polyclonal rabbit anti-human fab and purified IgE isoforms were mixed at a molar ratio of 3:1 and allowed to complex by incubating overnight at 4°C. The cell-bound immune complexes were detected with a FITC-conjugated reagent specific for rabbit IgG.

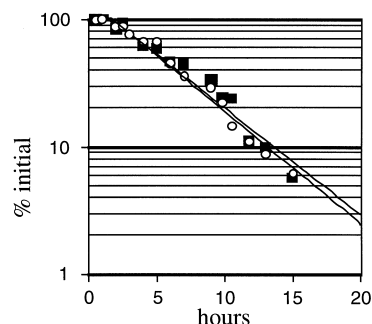


Fig. 3. In vivo half-life of IgE isoforms. IgE isoforms were labeled with ^{125} I and injected intraperitoneally into female BALB/c mice. Whole body radioactivity was then determined at subsequent time points. Data are presented as the average of triplicate animals on a semi-log graph. Open circles represent IgE classic and solid squares represent IgEtp.

Sepharose (Fig. 4A, lane 3) or by Staph A-rabbit anti-mouse Ab complexes lacking epsilon-specific mAbs (data not shown). The bands at the bottom of the gel in lanes 1, 2 and 4 represented the murine anti- ϵ mAb which leached off the Sepharose during boiling.

3.6. Molecular mass determination of HMW ϵ tp complexes

The HMW ϵ tp complexes were approximately 300 and 338 kD in size and recombinant IgEtp was approximately 170 kD as calculated from the relative electrophoretic mobility on a 4% SDS polyacrylamide

gel compared to proteins of known size (Fig. 4B). Thus, the ϵ tp complexes detected in vivo appear to be almost twice the molecular mass of recombinant IgEtp monomers.

3.7. HMW ϵ tp complexes are detected in human plasma but not serum

When human blood was collected as either serum or plasma from the same individual and analyzed by immunoprecipitation, HMW complexes containing ϵ tp were only detected in plasma samples (Fig. 5, compare lanes 1–2 and 3–4). Murine anti- ϵ mAb leached from

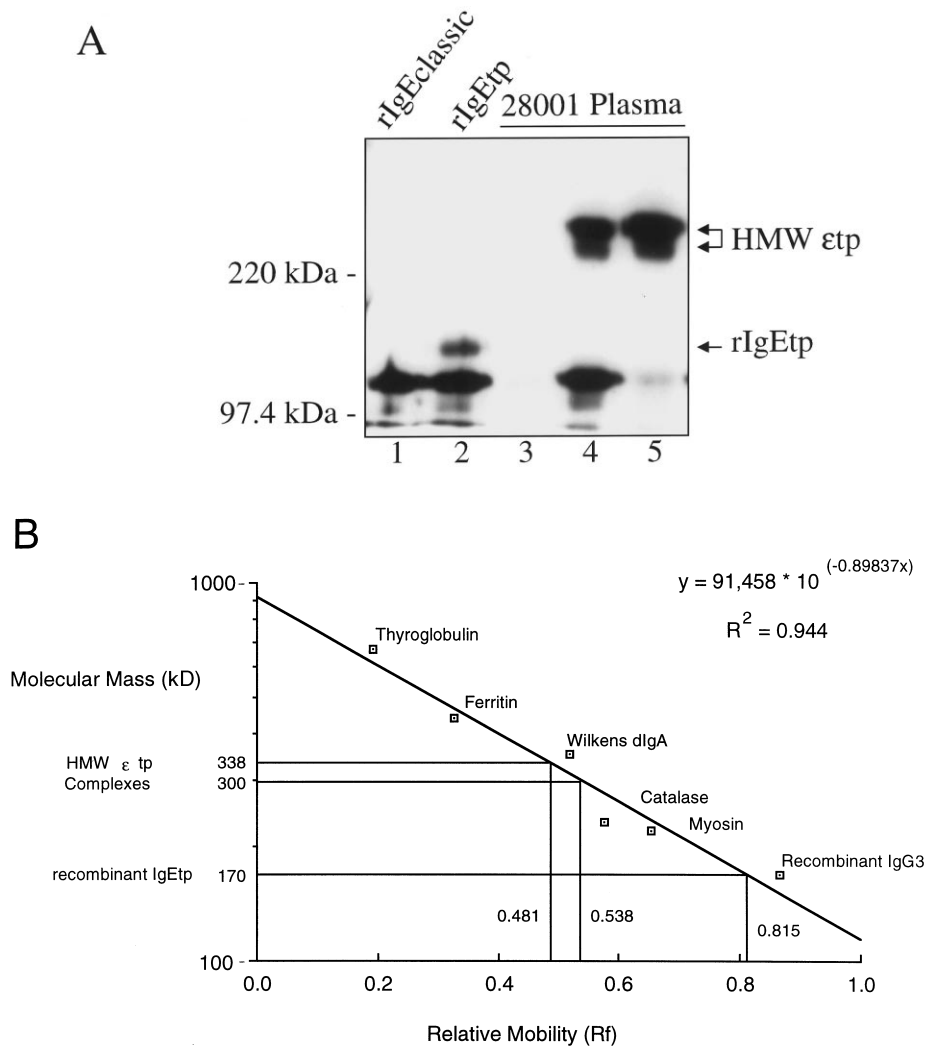


Fig. 4. Panel A. Immunoprecipitation and Western blot detection of high molecular weight species from human plasma containing ϵ tp. Proteins were precipitated from tissue culture supernatants (lanes 1 and 2) or human plasma with murine monoclonal anti- ϵ mAb-Sepharose (lanes 1, 2, and 4), BSA-Sepharose (lane 3) or goat polyclonal anti- ϵ -Sepharose (lane 5), separated on 4% SDS-PAGE gels under non-reducing conditions, and detected by anti- ϵ tp mAb Western blot. The anti- ϵ tp mAb specifically recognized immunoprecipitated recombinant (r) human IgE tailpiece (lane 2) but not IgE classic (lane 1). The sheep anti-mouse HRP reacted with the murine precipitating antibody which leached off the Sepharose and is seen as the lowest band in lanes 1, 2, and 5. The position of the molecular weight markers is indicated on the left. Panel B. Determination of the molecular mass of the ϵ tp complexes based on relative mobility using 4% SDS-PAGE. Relative mobility (Rf) was plotted against molecular mass (kD) on a semi-log graph. The best-fit curve and correlation factor are indicated in the top right corner. The molecular masses of both HMW ϵ tp complexes and recombinant IgEtp were approximated and are shown to the left of the molecular mass axis.

the Sepharose was present in all the lanes, including lane 5 in which neither plasma nor serum was added to the Sepharose.

3.8. The amount of HMW ϵ tp complexes present does not correlate with circulating IgE levels

Plasma samples from blood donors with differing levels of total IgE were examined for ϵ tp content. The high molecular weight ϵ tailpiece complex was found to differ in abundance and size among individuals (Fig. 6A). In several individuals, there were two distinct bands of higher molecular weight (Fig. 6A, lanes 3, 4, 6, and 8). Plasma from the individual shown in lane 5 had only one distinct band that was smaller. One individual did not have detectable HMW ϵ tp complexes (Fig. 6A, lane 9). HMW ϵ tp complexes were not detected in pooled serum as expected (Fig. 6A, lane 7). There was no correlation between the total IgE level and the presence of HMW ϵ tp complexes indicating that the complexes exist in both severely allergic and normal individuals (compare Fig. 6A and D). When samples were reduced with β -mercaptoethanol and analyzed by blotting with anti- ϵ tp, two or three bands were observed (Fig. 6B). The smallest 50 kD band present in all samples represented the secondary Western blotting reagent (sheep anti-mouse-HRP) reacting with the murine anti- ϵ mAb used for immunoprecipitation. The two larger bands of approximately 65 and 70 kD which were specifically recognized by the anti- ϵ tp mAb represented ϵ tp heavy chains (Fig. 6B, lanes 3–9). Interestingly, both ϵ tp bands observed in vivo were smaller than the 75 kD

recombinant ϵ tp heavy chains (Fig. 6B, lane 2). The 65 kD ϵ tp heavy chain was present only in samples in which the HMW ϵ tp complex was observed in the non-reduced samples (compare Fig. 6A and B). In contrast, the 70 kD ϵ tp heavy chain was detected in all samples including lanes 7 and 9 in which HMW complexes were not detected (Fig. 6A). This suggests that either HMW complexes were present at a level below detection or that the ϵ tp heavy chain was present in these individuals in a form not detected on the non-reducing gel.

When polyclonal goat anti- ϵ Ab was used to detect both ϵ classic and ϵ tailpiece, three or four bands were seen (Fig. 6C). The ϵ classic heavy chain was the largest polypeptide and was similar in size to the 75 kD recombinant ϵ classic seen in lane 1. ϵ tp heavy chain bands appeared as two smaller polypeptides migrating at approximately 70 kD and 65 kD. The smallest band represented the murine precipitating antibody.

3.9. The presence of the epsilon heavy chains in the HMW complex demonstrated by two-dimensional gel electrophoresis

To directly demonstrate that epsilon heavy chains were present in the HMW complex, HMW ϵ tp complexes immunoprecipitated with goat anti- ϵ mAb from an individual with a high serum IgE level (>2000 IU/ml) were analyzed by two-dimensional gel electrophoresis. Following separation in the first dimension under non-reducing conditions, the samples were reduced and analyzed in the second dimension using anti- ϵ tp mAb (Fig. 7A) or anti- ϵ mAb (Fig. 7B). Reduced recombinant IgEtp run in a lane adjacent to the two-dimensional analysis served as both a molecular weight marker and as a positive Western blot control. Anti- ϵ tp mAb recognized the recombinant ϵ tp in the control lane and three heavy chain bands derived from the HMW complexes, each of which was smaller than the recombinant ϵ tp heavy chain (Fig. 7A). The band at the far right may represent monomeric IgEtp or cross-reactivity of the secondary sheep anti-mouse HRP antibody with the goat anti- ϵ mAb immunoprecipitating antibody (Fig. 7A). The IgE specific monoclonal antibody (anti- ϵ mAb) detected the recombinant IgE tailpiece heavy chain control, two smaller heavy chain bands derived from the HMW complexes, a 75 kD ϵ classic heavy chains derived from IgE classic monomers, and an unidentified band (Fig. 7B). It is important to note that due to an inability to reblot, the two gels shown in Fig. 7 were not identical and may differ in resolution. Nevertheless, these blots demonstrate that polypeptides present in the HMW complex were recognized by both tailpiece and epsilon chain specific mAbs consistent with the previous results. Although equal volumes of recombinant ϵ tp were loaded onto

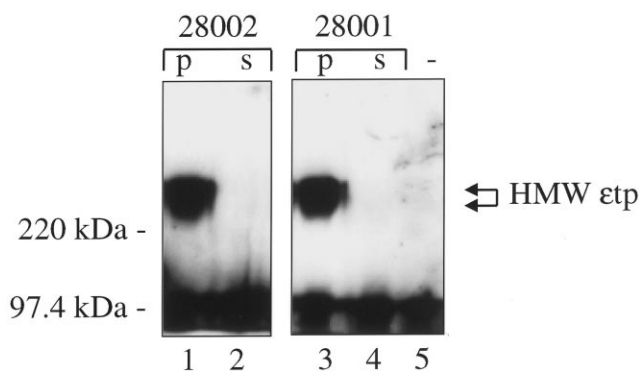


Fig. 5. Detection of HMW ϵ tp complexes in human plasma but not in serum. IgE was isolated from plasma (p) (lanes 1, 3) and serum (s) (lanes 2, 4) of individuals 28002 and 28001 by immunoprecipitation with anti- ϵ mAb-Sepharose. Anti- ϵ mAb Sepharose without addition of serum or plasma is shown in lane 5. Proteins were separated by size on 4% SDS-PAGE and detected using the anti- ϵ tp mAb. A band representing HMW ϵ tp complexes was only seen in the plasma samples (lanes 1 and 3). The position of the molecular weight markers is indicated on the left. The arrow on the right indicates the position of high molecular weight IgEtp complexes (HMW ϵ tp).

the gels, the detection of this protein is much weaker in the ϵ specific Western blot. This difference in apparent sensitivity may indicate that the anti- ϵ tp mAb produced against the 8aa peptide had a higher affinity for denatured ϵ tp than the mAb made against native epsilon chains (anti- ϵ mAb).

4. Discussion

Previous studies have examined the function of IgE purified from serum (Riske et al., 1991) or recombinant IgE produced from an expression system capable of producing multiple isoforms of IgE (Nissim et al., 1991). These studies were unable to distinguish unique characteristics associated with specific IgE isoforms. Our generation of myeloma cell lines genetically engineered to produce single IgE isoforms (Lyczak et al., 1996) has allowed us to functionally characterize individual isoforms of IgE. In addition, a monoclonal antibody specific for IgE tailpiece made it possible to identify and characterize this isoform.

Our current studies suggest that recombinant IgE tailpiece and IgE classic produced by transfectants of the murine myeloma Sp2/0 have similar properties. Both isoforms were rapidly cleared when injected into BALB/c mice. The calculated half-life, which was shorter than the half-life observed for IgE in humans, agrees with the half-life observed for purified human myeloma IgE in mice and appears to reflect the intrinsic half-life of IgE in mice (Stratford and Dennis, 1994). IgE classic and IgEtp bound equally well to Fc ϵ RI with a $K_d = 3 - 5 \times 10^{-9}$ M, an affinity similar to the $K_d = 10^{-10}$ M previously reported for human myeloma IgE (Ishizaka et al., 1986). IgE classic and IgEtp bound to Fc ϵ RI were functionally equivalent as shown by their equivalent ability to lead to mast cell degranulation. Similarly, using flow cytometric analysis, both IgE classic and IgEtp showed similar binding to the cell associated human CD23.

In contrast to recombinant ϵ tp produced in Sp2/0 which was present as a monomeric Ig of 190 kD (Lyczak et al., 1996) and monomeric IgEtp detected in some sera by Lorenzi et al. (Lorenzi et al., 1999), we

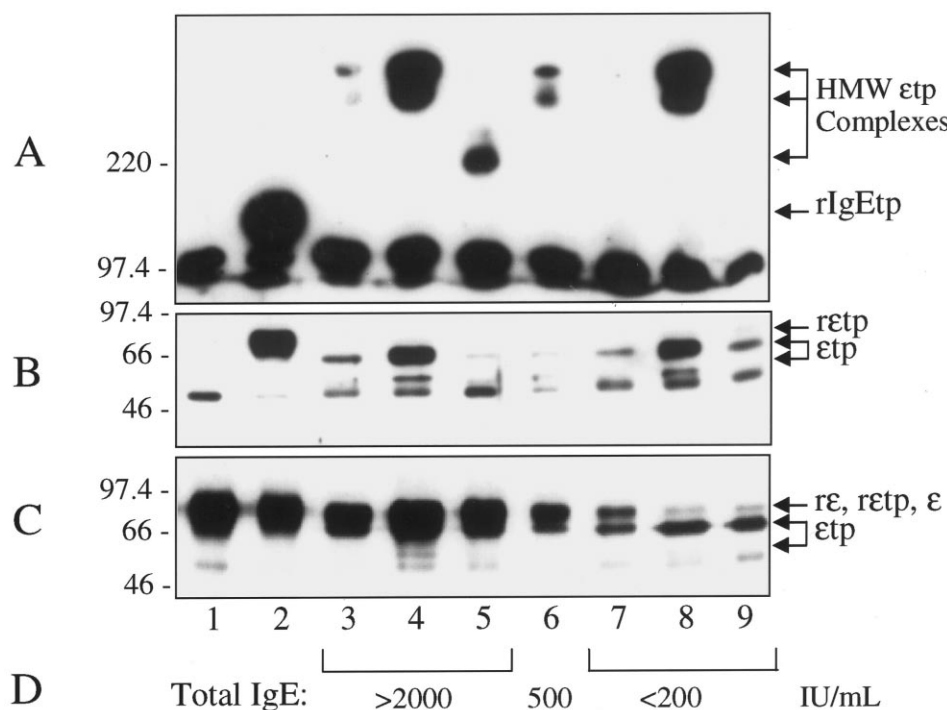


Fig. 6. Demonstration and characterization of ϵ tp protein in multiple individuals using immunoprecipitation, SDS-PAGE, and Western blot analysis. Epsilon-specific proteins were immunoprecipitated with anti- ϵ mAb-Sepharose from human plasma from six individuals (lanes 3–6, 8 and 9), pooled serum (lane 7), or culture supernatant containing rIgE (lane 1) or rIgEtp (lane 2). In panel A, samples were run non-reduced on 4% SDS-PAGE and detected by Western blot using anti- ϵ tp mAb to show the presence of HMW ϵ tp complexes in multiple individuals (lanes 3–6, 8). In panels B and C, reduced samples were run in duplicate on 12.5% SDS-PAGE and Western blotted with anti- ϵ tp mAb (panel B) and goat anti- ϵ Ab (panel C). The smallest band seen in every lane is the mouse IgG used in the immunoprecipitation. The position of the molecular weight markers run on each gel is indicated to the left. The level of total IgE present in the samples is shown in Panel D. Total IgE was quantitated by an IgE specific ELISA using an IgE standard of known concentration. IgE levels are reported as IU/ml where one international unit (IU) is equal to 2.3 ng IgE.

found that human plasma had 300 and 338 kD complexes containing ϵ tp. The presence of this HMW complex under denaturing conditions suggests that human ϵ tp isolated from plasma is part of a covalent complex. At the present time, we do not know if the HMW complex contains only an IgEtp dimer or if it is a complex of ϵ tp with other plasma proteins. In addition, the HMW ϵ tp complexes appeared as much larger native complexes between 500–600 kD when fractionated by FPLC on a Superose 6 column under non-denaturing conditions (data not shown). The difference between the size of the complex resolved by SDS-PAGE and FPLC may be due to differences in the folding and conformation of the HMW ϵ tp complexes in their native and denatured states. Alternatively, the size discrepancy could reflect non-covalent interactions with additional plasma proteins.

The carboxy-terminal cysteine on the end of the eight amino acid tail of ϵ tp is positioned similarly to the penultimate cysteine found on IgA and IgM tail-pieces. The cysteine residues on IgA and IgM are utilized during polymer formation of these isotypes (Atkin et al., 1996; Sitia et al., 1990). Nevertheless, we have not detected IgEtp polymers when recombinant ϵ tp is produced in tissue culture. However, *in vivo* multimerization may be facilitated by human serum proteins that form covalent interactions with IgEtp. For example, *in vivo* derived soluble CD21 forms complexes with CD23 and cleavage fragments of C3. These

complexes can contain IgE (Frémeaux-Bacchi et al., 1998) and may well include ϵ tp. Furthermore, the HMW ϵ tp complex was readily detected in plasma compared to serum in the same individual suggesting that ϵ tp heavy chains may form complexes with proteins such as fibrin and the serine proteases involved in blood clotting. The removal of the HMW ϵ tp complex upon clotting is consistent with the report that ϵ tp was detected in serum from only a small percentage of the individuals examined (Lorenzi et al., 1999). Those individuals may have exceptionally high levels of the ϵ tp heavy chain such that detectable levels of the ϵ tp heavy chains remain following clot formation. The associated serine proteases could play a role in the degradation of the HMW ϵ tp complexes and ϵ tp heavy chains following clotting.

Given the presence of multiple HMW ϵ tp complexes of varying size among individuals, it is possible that different complexes can be assembled. Altered assembly of identical or non-identical subunits could cause this change in size. Several <30 kD protein bands were present in the HMW IgE complex when two-dimensional gels were silver stained (data not shown). Presumably, at least one of these bands was an antibody light chain while the others could be unidentified subunits of the complex.

The ϵ tp heavy chains present *in vivo* were smaller in size than recombinant ϵ heavy chains. This difference in heavy chain size could be explained by

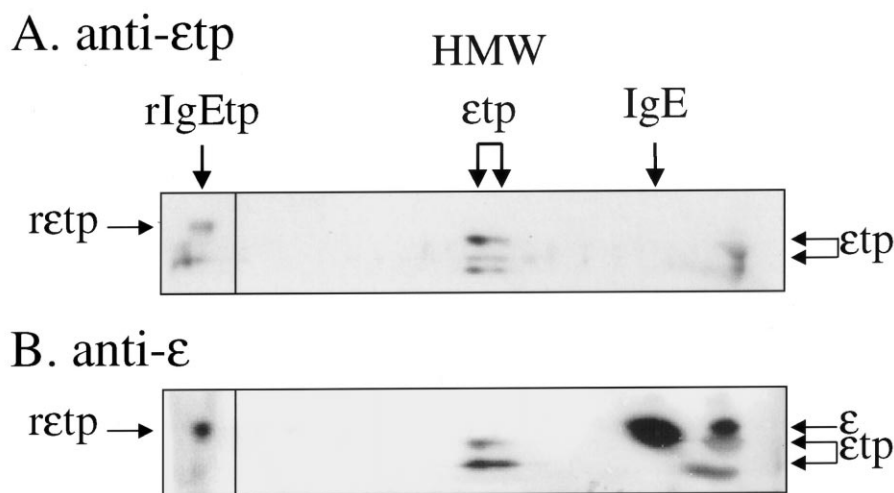


Fig. 7. Two dimensional gel analysis of HMW ϵ tp complexes isolated from human plasma. Complexes were immunoprecipitated from plasma using goat anti- ϵ -Sepharose prior to two-dimensional analysis. Along the horizontal axis, HMW ϵ tp complexes have been separated from monomeric IgE classic and the precipitating antibodies under non-reducing conditions on 4% SDS-PAGE. On the vertical axis, these covalent complexes were subsequently reduced and separated on 12.5% SDS-PAGE in a second dimension. In panel A, ϵ tp heavy chains derived from HMW ϵ tp complexes were detected by Western blotting using anti- ϵ tp mAbs (double arrow). In panel B, both ϵ tp heavy chains derived from HMW ϵ tp complexes and ϵ classic heavy chains derived from monomeric IgE were detected using anti- ϵ mAbs. On the far left, reduced recombinant IgEtp (rIgEtp) was run in the second dimension only to show the difference in size between ϵ tp and r ϵ tp heavy chains and to show the specificity of the Western blotting reagents. The denatured, non-reduced high molecular weight ϵ tp complexes (HMW ϵ tp) and IgE classic are labeled above the blots to designate the apparent location in the first dimension. The ϵ tp, ϵ classic (ϵ), and recombinant ϵ tp (r ϵ tp) heavy chains are labeled on the sides.

differences in post-translational modifications of the proteins, protein cleavage, or alternative mRNA processing. One possible post-translational modification which could account for this discrepancy is differential utilization of the N-linked glycosylation sites. Data presented by Lorenzi et al. (1999), showing that serum and recombinant etp heavy chains are the same size following PNGase F treatment, supports this idea. However, our preliminary data using PNGase F to remove N-linked sugars suggested that the difference between etp heavy chain sizes from human plasma and in vitro produced recombinant etp could not be solely attributed to differences in N-linked carbohydrate addition (data not shown). Protein cleavage of etp in vivo is another possible explanation for the size difference, although the etp heavy chain present in vivo was recognized by the anti-etp mAb indicating that the carboxy terminus remains intact. In addition, alternative splicing within the ϵ constant region could produce smaller than predicted etp heavy chains as seen in human plasma. Indeed, we have observed that other recombinant IgE isoforms expressed in Sp2/0 cells yielded several truncated proteins with large deletions within the epsilon constant region presumably due to altered mRNA splicing (data not shown).

We have found that the total IgE levels of an individual do not directly correlate with either the presence of HMW etp complexes, or the amount of etp heavy chains (Fig. 6). We previously observed that mRNA levels of the IgE isoforms were related to differentiation and disease with allergic and parasite infected individuals having relatively higher levels of mRNA for IgEtp than non-affected individuals (Diaz-Sanchez et al., 1995). It will be necessary to measure the expression of the various epsilon mRNAs and proteins in the same individual at the same time to determine the relationship between mRNA and protein levels.

It is also interesting that we could detect the larger 70 kD IgEtp heavy chain but not the smaller 65 kD heavy chain in serum and plasma samples in which we could not detect the HMW IgEtp complexes. This observation suggests a specific role for the 65 kD heavy chain in HMW complex formation. The assembly form of the 70 kD etp heavy chain is unknown in those samples lacking the HMW complex. It is possible that the 70 kD etp heavy chain is present as an H₂L₂ molecule or other form that could not be detected with the available reagents or is masked by crossreactivity with the immunoprecipitation reagent.

Although recombinant IgE tailpiece and IgE classic display very similar properties, native IgE tailpiece and classic exist in human plasma in very different forms.

IgE classic appears to circulate through the blood as a H₂L₂ monomer, whereas etp is found covalently associated in higher molecular weight complexes. Moreover, the etp heavy chain isolated from blood differs in size from that produced in tissue culture. The finding of unique molecular forms of etp in circulation suggests that it might play a novel role in the immune system; the challenge is to identify that role.

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