

Short communication

Sequence-specific antibodies against human IgE isoforms
induced by an epitope display system

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Abstract

Background: Unlike other immunoglobulin isotypes, the human C ϵ gene generates by alternative splicing two types of secretory and two types of membrane ϵ chains. The two secreted ϵ heavy chains, ϵ_{S1} and ϵ_{S2} , differ only in the sequence of the last eight C-terminal amino acids, being ϵ_{S2} six amino acids longer. The two types of membrane isoforms differ in the extracellular membrane proximal domain, with the longer variant, ϵ_{mL} , containing 52 extra amino acids which are absent in the shorter ϵ_{mS} isoform. **Objectives:** We wished to produce quality antibody reagents that specifically detect epitopes that are ϵ isoform-specific. **Study design:** Short sequences of seven or ten amino acids were chosen as target epitopes and expressed as part of the highly immunogenic loops of deletion variants of engineered Flock House Virus capsid protein RNA2. Chimeric proteins were expressed in *E. coli*, and used to immunize rabbits. Antisera were screened by immunoblotting of purified IgE isoforms expressed by murine transfectomas. **Results:** Chimeric proteins expressing ϵ isoform-specific epitopes proved to be strong immunogens in vivo and induced highly specific rabbit antisera. Two antisera so obtained recognize specifically the IgE-S2 isoform. A third one recognizes the long membrane variant m_L IgE and a fourth one detects an epitope specific to m_S IgE. **Conclusion:** Here we describe a simplified and efficient protocol of immunization which does not require peptide synthesis and conjugation to carrier protein. Our results show that short peptides of unknown immunogenicity, when genetically introduced into the modified Flock House Virus epitope display system, successfully induced IgE isoform-specific polyclonal antisera in rabbits. These are valuable tools to specifically identify secretory and membrane isoforms of human IgE, and the method is potentially applicable to other variant isoforms or mutants of a given protein. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The human ϵ gene produces by alternative splicing of the 3' exons a complex pattern of multiple mRNA transcripts that is not paralleled in other immunoglobulin types [1]. In addition to the classical membrane and secretory human IgEs, two other splicing variants have been shown to be correctly assembled and expressed in murine B cells and plasma cells and encode putative membrane and secretory isoforms with distinct properties [2,3] (R. Lorenzi, unpublished). The splicing variants are generated through usage of alternative splicing donor and acceptor sites in the CH4 and the two membrane exons M1 and M2 [1,4]. The pattern of alternative splicing and the carboxyl-terminal sequence of the four ϵ heavy chains is shown in Fig. 1. The ϵ_{S2} is six amino acids longer than the classical secretory heavy chain ϵ_{S1} (ϵ CH4-S) and contains a C-terminal tailpiece with eight different amino acids of which the last one is a cysteine involved in an interchain disulfide bridge [2]. The classical membrane variant ϵ_{mL} (ϵ CH4-M1'-M2) contains 52 amino acids which are absent in the shorter ϵ_{mS} (ϵ CH4-M1-M2) isoform.

Since the two secretory isoforms differ only in eight amino acids and cannot be discriminated by size, we wanted to develop an antibody reagent that specifically recognizes the carboxyl-terminal tailpiece of the ϵ_{S2} heavy chain. Previous attempts to develop an ϵ_{S2} -specific antibody response in rabbits with a peptide containing the C-terminal ϵ_{S2} sequence were not successful (Batista and Burrone, unpublished). To enhance immunoreactivity, the Flock House Virus (FHV) epitope display system carrying ϵ_{S2} -specific epitope sequences was chosen as alternative in vivo immunogen. The same approach was employed for ϵ_{mL} - and ϵ_{mS} -specific epitopes.

The *RNA2* gene of the FHV genome encodes a capsid protein whose X-ray structure is known and contains five highly immunogenic loops [5]. Deletion mutants have been engineered each containing a deleted loop suitable to insert short foreign peptides [6]. Each deletion variant differs in the position of the modified loop and this

allows for presentation of foreign epitopes in multiple internal sites which confer a different conformation for a chosen target peptide. Hybrid FHV proteins carrying a small immunoreactive epitope of HIV-1 and expressed as recombinant baculoviruses in insect cells have been shown to induce specific in vivo responses in guinea pigs [7].

Here we present a simplified protocol of bacterial expression of the recombinant FHV-*RNA2*-encoded protein, which led to the production of epitope-specific rabbit antisera to human ϵ isoforms ϵ_{S2} , ϵ_{mL} and ϵ_{mS} .

2. Materials and methods

2.1. Choice of target epitopes

The ϵ_{S2} - and ϵ_{mS} -specific epitopes overlap sequences from CH4 and the CH4-M2' and CH4-M1 exons, respectively (Fig. 1b) [4]. The C-terminal cysteine in ϵ_{S2} was excluded from the chosen target epitope to avoid possible disulfide bond formation. The ϵ_{mL} target epitope localizes in the M1' ϵ_{mL} -specific coding region. Two target epitopes (ϵ_{S2} and ϵ_{mL}) are ten amino acids long, whereas the epitope for ϵ_{mS} is seven amino acids long as shown in Fig. 1b.

2.2. Recombinant plasmid construction and protein expression of chimeric FHV protein

The two FHV-*RNA2* gene deletion variants L2 and L3 are in pET36 expression vector and contain an engineered *Bsu36I* restriction site for insertion of foreign epitopes, as previously described [6]. Inserts encoding the desired peptide target were generated by annealing a pair of complementary oligonucleotides containing *Bsu36I* cohesive ends and cloned into positions L2 or L3. Chimeric immunogens so generated (s2-L2, s2-L3, ms-L2, ms-L3, mL-L2, mL-L3) were expressed in BL21(DE3) *E. coli* and purified from bacterial lysates by fractionation on 10% SDS-PAGE under reducing conditions, followed by electroelution as described [8].

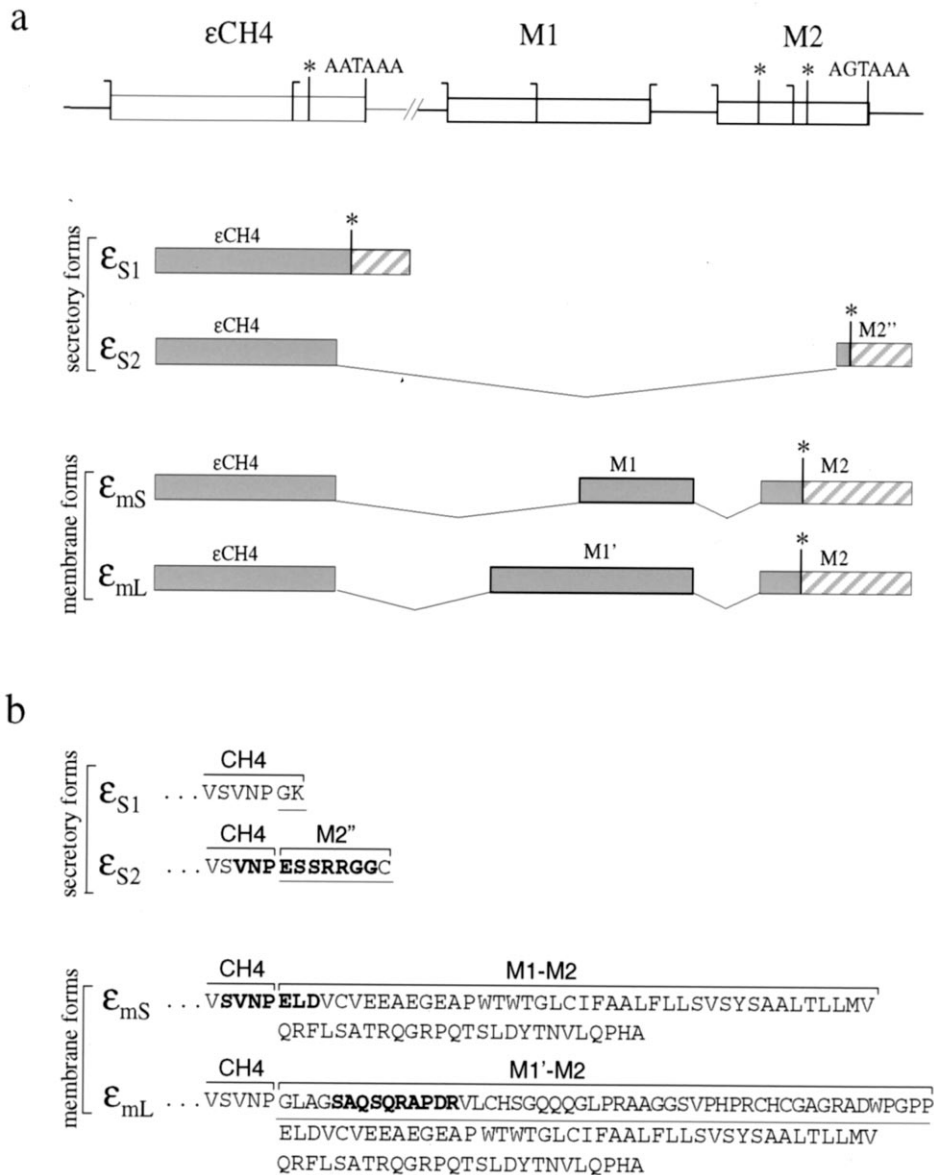


Fig. 1. Schematic representation of IgE heavy chain isoforms. (a) Diagram of the 3' part of the $C\epsilon$ gene and pattern of alternative splicing that generates secretory and membrane ϵ transcripts. Shaded and striped boxes represent, respectively, coding and 3' untranslated regions. (b) Amino acid sequences of carboxyl termini of secretory and membrane ϵ isoforms are shown from $C\epsilon$ residue 422 in the CH4 domain. Isoform-specific sequences are underlined and their exon origins indicated above them. The three chosen target epitopes inserted in the FHV capsid protein to generate recombinant isoform-specific immunogens are shown in bold typing.

2.3. Animals inoculation

Rabbits were immunized with approximately 100 μ g of recombinant immunogens with com-

plete Freund's adjuvant for the first immunization (day 0) or incomplete Freund's adjuvant for booster injections at days 14, 28 and 42 and bled 50 days after the first inoculation.

2.4. Cell lines and immunoassays

Immunoreactivity and specificity of rabbit antisera were tested by Western immunoblotting and flow cytometry. Supernatants and cell lysates of previously described J558L and WEHI231 transfectomas [2,3] were the source of isoform-specific IgEs. IgEs were purified by affinity chromatography with goat immunoglobulins anti-human IgE (ϵ chains) (Kirkegaard & Perry Labs, Gaithersburg, MD) conjugated to Sepharose (Pharmacia LKB, Uppsala, Sweden). IgEs were separated on 10% SDS–PAGE under reducing conditions and blotted on PVDF membrane (Amersham, Buckinghamshire, UK). Membranes were incubated with 1:100 dilution of immunized rabbit sera or with rabbit anti-human IgE (ϵ chain) (Dako, Glostrup, Denmark), followed by horseradish peroxidase-coupled goat anti-rabbit immunoglobulins (Dako). Bound antibodies were visualized by enhanced chemiluminescence detection system (Amersham).

2.5. Flow cytometry

Expression of surface IgE was examined by flow cytometry on a FACSCalibur (Becton-Dickinson Immunocytometry Sys., Mountain View, CA) using FITC-conjugated rabbit anti-human IgE (Dako) or antisera from immunized rabbits and FITC-conjugated goat anti-rabbit IgG (Dako).

3. Results and discussion

Sequences specific to ϵ_{S2} , ϵ_{mL} and ϵ_{mS} isoforms were expressed into positions L2 or L3 of the FHV-*RNA2* gene. All six chimeric proteins were expressed in *E. coli* and used to immunize rabbits.

Antisera reactivity and specificity were assessed for their ability to recognize by Western blot analysis IgE isoforms bearing the immunizing epitope. All antisera had anti-FHV capsid protein activity (data not shown). Fig. 2a shows the reactivity of antisera obtained with the s2-L3 and s2-L2 immunogens against transfectoma-derived IgE-S1 and IgE-S2 [2] separated under reducing

conditions on SDS–PAGE. Both anti- ϵ_{S2} sera detect the IgE-S2 isoform and there is no cross-reaction with the classical secretory IgE-S1 isoform. This indicates that the seven-amino acid long ϵ_{S2} -specific sequence contained within the chosen epitope elicited a specific immune-response in rabbits when inserted at both L2 and L3 positions of the FHV capsid protein. Reactivity of a rabbit serum to total human ϵ -chain is shown for comparison of the relative signal and of the amount of

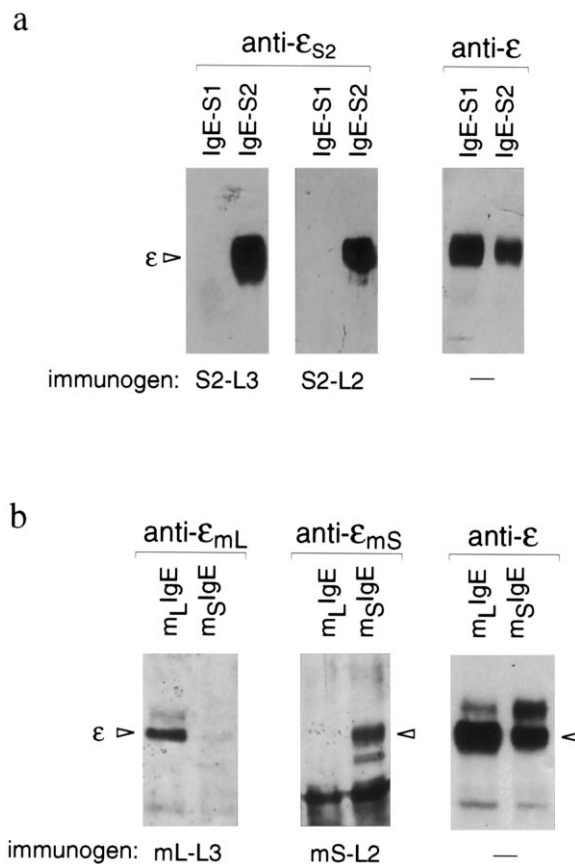


Fig. 2. Western immunoblot reactivity and specificity of anti- ϵ isoform antisera. IgE molecules were separated on 10% SDS–PAGE under reducing conditions and membranes incubated with the immunized rabbit antisera followed by detection with HRP-labelled anti-rabbit immunoglobulins by ECL. Anti- ϵ signals are detected with a rabbit polyclonal anti-human IgE serum. (a) Reactivity of antisera obtained with s2-L3 and s2-L2 immunogens to secretory isoforms IgE-S1 and IgE-S2. (b) Reactivity of antisera obtained with the mL-L3 and mS-L2 immunogens to membrane isoforms m_L IgE and m_S IgE. The position of the ϵ heavy chain is indicated with an arrowhead.

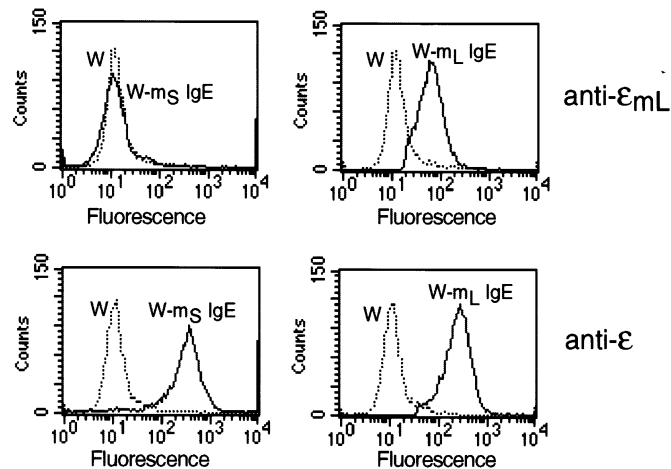


Fig. 3. Surface detection of m_L IgE with anti- ϵ_{mL} by fluorescent flow cytometry. Staining of m_S IgE and m_L IgE on the surface of WEHI transfectomas (W- m_S IgE, W- m_L IgE) by the antiserum obtained with immunogen m_L-L3 (upper panels) is shown compared with total anti- ϵ fluorescence (lower panels). Dotted histograms are control WEHI (W) untransfected cells.

IgE loaded. The combined use of anti- ϵ_{s2} and anti- ϵ also allows to conveniently identify both secretory molecules, also when heterogeneous samples which contains both species are analyzed. In particular, we found that sequential immunoblotting with anti- ϵ_{s2} followed by anti- ϵ detection after a standard protocol of membrane stripping is an adequate procedure for detection of ϵ_{s1} . This compensates for the lack of an anti- ϵ_{s1} specific reagent. No attempts were made to raise antibodies against this isoform with the FHV system.

Fig. 2b shows reactivity and specificity of antisera obtained with immunogens m_L-L3 and m_S-L2. Both sera specifically recognize the respective immunizing epitope present in the ϵ chains derived from cellular lysates of IgE transfectoma expressing the m_L IgE or the m_S IgE isoform [3]. Differently from responses against the ϵ_{s2} epitope, however, the reactivity against both ϵ_{mL} and ϵ_{mS} epitopes was not indifferent to the position in which the foreign epitope was inserted in the FHV capsid protein. In fact, the ϵ_{mL} -specific epitope elicited an *in vivo* response only when inserted at position L3 but not when inserted at position L2 (not shown). The opposite occurred for the ϵ_{mS} -specific target epitope.

This confirms the efficacy of the epitope display system to rely on multiple insertion positions in order to enhance the chance of a positive response against a chosen epitope.

The ability of rabbit antisera to detect the chosen epitopes in their native conformation was also investigated. Fig. 3 shows staining of m_L IgE positive cells with the anti- ϵ_{mL} antiserum by flow cytometry. No cross-reaction with m_S IgE expressing cells is observed.

Instead, the anti- ϵ_{mS} antiserum does not recognize the ϵ_{mS} epitope on the native protein expressed on the cell surface, as judged by the lack of reactivity in flow cytometry. One reason for this could be the lesser accessibility of the ϵ_{mS} native epitope compared to ϵ_{mL} . The ϵ_{mS} epitope is in fact more proximal to the membrane surface and may be masked by non-covalent association with other protein components of the B cell receptor [3]. This is, however, not supported by the observation that immunoprecipitation after cellular lysis and membrane solubilization does not result in recognition. It is possible that the lack of reactivity is due to the intrinsic conformation of the region at the junction between the ϵ CH4 and the membrane exons. The anti- ϵ_{s2} target epitope also localizes in

this region. It is noteworthy that both anti- ϵ_{S2} antisera were equally incapable to detect the ϵ_{S2} epitope in ELISA capture assays, as well as in immunoprecipitation assays. Since the denatured IgE-S2 is recognized in Western blotting in both reducing and non-reducing conditions, this suggests that, similarly to the ϵ_{mS} -target epitope on m_S IgE, the C-terminal tailpiece of ϵ_{S2} is also structurally inaccessible in its native conformation.

The differences in molecular weight between membrane and secretory molecules aided by the lack of cross-recognition of anti- ϵ_{S2} and anti- ϵ_{mS} and anti- ϵ_{mL} antisera for their non-specific targets makes this set of reagents here described a useful tool for assessing the distribution and role of the different ϵ isoform in humans.

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