

## Synthetic IgE peptide vaccine for immunotherapy of allergy

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### Abstract

An immunotherapeutic vaccine for allergy was produced by designing IgE-based synthetic peptide immunogens and selecting them for functional immunogenicity. The vaccine targets the binding site on IgE for the high affinity receptor FcεRI, by active immunization. The peptide target site on IgE heavy chain was selected from among the amino acid sequences for the Cε2, Cε3, and Cε4 domains. These were characterised by epitope mapping studies for cross-reactivity to IgE and functional antigenicity. A peptide, modified from positions 413–435 of a loop region of Cε3 and subjected to conformational constraint, elicited anti-IgE antibodies that blocked IgE-mediated histamine release. It was immunopotentiated by linkage to a promiscuous T helper site to produce a wholly synthetic chimaeric immunogen. This immunogen was shown to induce polyclonal site-specific anti-IgE antibodies that obstruct binding to FcεRI, inhibit histamine release by IgE-sensitised basophils, inhibit passive cutaneous anaphylaxis, and do not signal degranulation. Immunized dogs experienced significant reductions in total serum IgE.

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### 1. Introduction

Immunoglobulin E (IgE) sensitises mast cells and basophils by binding to its high affinity receptor FcεRI, on the surface of those effector cells. Contact with antigen or anaphylactogenic anti-IgE antibodies causes the cross-linking of the bound IgE and the underlying FcεRI. The cross-linked receptors initiate a signal transduction cascade and rapid degranulation. The mast cells and basophils release stored histamine, followed by the synthesis and release of prostaglandins, leukotrienes, cytokines and other inflammatory mediators. These attract and activate inflammatory cells, produce the symptoms of allergy, and up-regulate the biosynthesis of IgE by B cells to promote heightened sensitivity. IgE-FcεRI interactions and the degranulation event are central to type 1 allergic reactions and to the development of atopic asthma [1–3]. The binding of IgE to the high affinity receptor has been a major target for intervention at the root level of type 1 allergic reactions [4].

The site on human IgE responsible for binding to FcεRI has been associated with the Cε2, Cε3, and Cε4 domains by

binding inhibition studies involving recombinant IgE truncations [5,6], chimaeric IgE [7], site-directed mutagenized IgE [8,9], synthetic peptides corresponding to IgE Fc domains and antibodies induced by such peptides [10–14], and mimotope peptides [9,15]. These observations pointed to a highly conformational receptor binding site that has recently been solved by resolution of the crystal structure of a human IgE-FcεRI complex. This site has contact residues scattered among four surface loops that are asymmetrically dispersed between the two Cε3 domains of an IgE dimer and conformational scaffolding provided by Cε4 and the Cε2–Cε3 junctions [16]. Antibodies can obstruct this convoluted site by direct steric effect or induced conformational change.

Certain anti-IgE monoclonal antibodies whose recognition sites map to Cε3 interfere with the high affinity receptor binding site. These do not cross-link FcεRI-bound IgE and so they do not trigger degranulation and anaphylaxis [15,17,18]. Two of these antibodies are in clinical trial, and passive immunizations have provided desensitisation for patients with allergic rhinitis and allergic asthma [19–23]. An anti-IgE approach to immunotherapy by active immunization may have even greater promise than passive immunization due to cost effectiveness and potential

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for wider application. Rats sensitised to ovalbumin experienced suppression of skin reaction to the antigen following vaccination with a bacterially-expressed recombinant antigen comprising IgE domains C $\epsilon$ 2–C $\epsilon$ 3 coupled to a carrier protein [24] or with a C $\epsilon$ 4 peptide coupled to a carrier protein [14]. However, those approaches to immunotherapy by active immunization were limited by (i) the risk that a large IgE fragment has the potential to generate anaphylactogenic antibodies, (ii) the inappropriateness of the target of the site-specific carrier-linked peptide, and (iii) low immunogenicity [25]. Here we sought to demonstrate the feasibility of an immunotherapeutic vaccine for allergy that uses wholly synthetic IgE peptide immunogens to target a site involved in Fc $\epsilon$ RI binding. Synthetic peptide immunogens were designed that generated high affinity, yet non-cross-linking (i.e. non-anaphylactogenic), site-specific anti-IgE. These antibodies blocked binding to the high affinity receptor, and were sufficiently immunogenic to evoke functional anti-dog IgE immune responses in dogs.

## 2. Materials and methods

### 2.1. Immunogen and antigen synthesis and processing

#### 2.1.1. Peptide synthesis

Peptides were synthesized on a solid-phase support using an Applied Biosystems Peptide Synthesizer Model 430A and characterised as described [26]. Peptides having combinatorial library Th were prepared by providing a mixture of the desired amino acids at the specified positions. Combinatorial immunogens were characterised by size exclusion chromatography to a specification that requires 90% of the integrated area to exceed a mass threshold limit value, and by Edman degradation for N-terminal amino acid analysis.

#### 2.1.2. Peptide modifications

Peptide-carrier protein conjugate immunogens were produced by chemical linkage of the target peptide to keyhole limpet hemocyanin (KLH) by MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinamide ester (Pierce, Rockford IL, USA). Liquid phase cyclization of peptide immunogens was accomplished by the formation of intramolecular disulphide bonds between substituent cysteines. The peptides were dissolved in water at 0.8 mg/ml, pH 3, DMSO was added to 1% (v/v) and NH<sub>4</sub>OH was used to adjust to pH 7.5. The solution was incubated at ambient temperature in air and checked daily for 3 days by calorimetric assay using Ellman's reagent until disulphide bond formation was at least 90% complete.

#### 2.1.3. Vaccine formulations

Peptide-based vaccines were water-in-oil single emulsions mixed 1:1 with Freund's complete/incomplete adjuvants or an adjuvant based on metabolizable oil, Montanide ISA 720 (Seppic, Fairfield NJ, USA).

### 2.2. *In vivo* analyses

#### 2.2.1. Animals and immunizations

Duncan-Hartley guinea pigs were immunized intramuscularly with 100  $\mu$ g of peptide immunogen or peptide-carrier conjugate on weeks 0, 3, 6, and 10. The first dose was administered with Freund's Complete Adjuvant and subsequent doses in Incomplete Freund's. Yorkshire-Landrace cross swine were immunized on a similar schedule with peptide vaccine in Montanide ISA 720. Balb/c mice were immunized subcutaneously on weeks 0, 3, and 6 with 20  $\mu$ g of peptide immunogen in Freund's Complete for initial dose and Incomplete on subsequent boosts. Non-atopic beagles were immunized intramuscularly with peptide immunogen in Montanide ISA 720, on weeks 0, 3, and 7. Investigational procedures and animal care were in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, 1996), and the guidelines of the Institutional Animal Care and Use Committees of United Biomedical Inc. and Covance Research Products Inc. (Denver PA and Kalamazoo MI, USA).

#### 2.2.2. Measurement of anti-IgE antibodies

Anti-IgE peptide titres were determined by IgE peptide ELISA and cross-reactivities to human IgE by human IgE ELISA. Peptide ELISAs for determination of anti-IgE peptide reactivity were conducted in microtitre plates coated with the target antigen site peptide without the T helper site, as described [26,27]. For determination of anti-human IgE cross-reactivity, human IgE ELISAs were conducted in microtitre plates coated in a likewise fashion with a human IgE myeloma protein (American Biosystems Inc. cat. no. A113) at 5  $\mu$ g/ml. Captured anti-peptide or anti-IgE antibodies were detected by horseradish peroxidase (HRP)-labelled anti-guinea pig IgG goat antibody or HRP-anti-swine IgG goat antibody. ELISA titres, expressed as log<sub>10</sub> of reciprocal dilution, were calculated based on linear regression analysis of the absorbances, with cutoff A<sub>492</sub> set at 0.5. This cutoff value was rigorous as the values for diluted normal guinea pig and swine control samples run with each assay were less than 0.15.

The anti-mouse IgE ELISA was as described for the anti-human IgE ELISA except that microtiter wells were coated with 1  $\mu$ g/ml of mouse IgE anti-DNP monoclonal antibody SPE7 (Sigma, St. Louis MO), and HRP-goat antibody IgG (Kirkegaard and Perry Laboratories, Gaithersburg MD) was used for detection of captured mouse IgG.

Anti-dog IgE responses to the trial immunization were determined by the peptide and IgE-based ELISAs as described above for the anti-human IgE assays, except that a hybridoma-derived dog IgE (Bethyl Laboratories, Montgomery TX, USA) was the solid-phase immunoabsorbant and bound dog IgG was detected by HRP-goat anti-dog IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

### 2.2.3. Measurement of serum IgE

**2.2.3.1. Mouse serum IgE.** IgE content of mouse sera was determined by a quantitative ELISA where mouse IgE was captured by coating the microtitre wells with 2 µg/ml of anti-mouse IgE mAb R-35–72 (Pharmingen, San Diego CA, USA), and the captured IgE was detected by HRP-sheep anti-mouse IgE (The Binding Site Inc., San Diego CA, USA).

**2.2.3.2. Canine serum IgE.** Immune dog sera were analyzed for total serum IgE, i.e. free IgE and IgE in immune complexes, by quantitative sandwich ELISA for IgE in heated sera [28]. Plates were coated with polyclonal anti-dog IgE, and captured IgE was detected by monoclonal antibodies specific for heat-stable IgE epitopes, as described [28].

### 2.3. *In vitro* analyses for functional immunogenicity

#### 2.3.1. Inhibition of histamine release assay

IgG antibodies were purified from immune serum by Protein A affinity chromatography (ImmunoPure® Immobilized Recomb® Protein A, Pierce, Rockford IL, USA) and the eluted antibodies were prepared at a standard concentration of 8 mg/ml in 25 mM PIPES buffer, 0.15 M NaCl, pH 7.2. A control antibody preparation was prepared from sera of animals of the relevant species that were immunized with an irrelevant peptide immunogen. These antibodies were used in assays that measured the reduction in IgE-mediated sensitisation of human basophils. Human basophils were prepared from the venous blood of volunteers as described [29] except that the PAGCM buffer used to suspend the cells was made up with water containing 44% D<sub>2</sub>O. The IgE used for sensitisation was human gp120-specific IgE (antibody to a peptide of the HIV glycoprotein gp120) [30] at 0.25 µg/ml. The allergen-specific IgE was preincubated with an equal volume of purified antibody at 8 mg/ml or dilution thereof, total volume 0.1 ml, for 15 min at 37 °C, prior to being added to the basophils. The antibody mixture was added to the cells and incubated for 20 min to allow for sensitisation of the cells by uncomplexed IgE (final concentration of the IgG antibody in the preincubation step was one-half the added concentration, and in the final incubation with cells, it was one-fourth the added concentration). The sensitised cells were then stimulated by addition of the allergen, gp120 peptide-ovalbumin (gp120P-OVA) [30] and the cells were incubated for 45 min. The cells were separated and the supernatant assayed for histamine content by an automated fluorimetric technique [31]. All reactions were performed in duplicate. The percentage of histamine release was calculated from the ratio of sample to total histamine after spontaneous release was subtracted from both. Results are expressed as per cent inhibition of histamine release, as determined from the ratio of histamine release by IgE preincubated with experimental antibody to histamine release by the IgE preincubated with control antibody of irrelevant specificity.

#### 2.3.2. Inhibition of IgE binding by flow cytometry

IgG antibodies from immunized and control animals were purified by protein A affinity chromatography as described above. Human basophils were purified from leukapheresis cells as described previously [32]. The basophils (>99% purity) were sensitised with gp120-specific IgE (see above). IgG antibodies at various dilutions of the stock purified antibody at 8 mg/ml were incubated with an equal volume of 1 µg/ml of gp120-specific IgE (for a total volume 0.1 ml) for 15 min at 37 °C, prior to being added to an equal volume of basophils in suspension (≈100,000 cells per condition). The antibody mixture was added to the cells and incubated for 20 min at 37 °C to allow for binding to the cells by uncomplexed IgE (final concentration of the IgG antibody in the preincubation step was one-half the added concentration, and in the final incubation with cells, it was one-fourth the added concentration). After two washes, the cells were labelled with mouse anti-idiotypic antibody (specific for the gp120-specific IgE) [30], then secondary anti-mouse-phycoerythrin (PE) conjugate. The results were read by flow cytometry with controls being isotype matched mouse IgG. An additional control included cells that had not been sensitised with anti-gp120-specific IgE but were otherwise labelled with the anti-idiotypic antibody and the anti-mouse-PE conjugate.

#### 2.3.3. Flow cytometry

Flow cytometry was performed with a Becton-Dickinson cytometer. Forward and side scatter gates were set for the basophil population and PE fluorescence is calculated from the median of the distributions obtained.

#### 2.3.4. Direct release

To determine whether the antibodies directly induced histamine release, Percoll separated leukocytes were challenged with either a standard goat polyclonal anti-IgE antibody (acting as a positive control) or with dilutions of the antibodies found to inhibit passive sensitisation. Leukocytes were challenged in PAGCM buffer for 45 min at 37 °C and supernatants harvested for analysis of histamine as described above.

### 2.4. Passive percutaneous anaphylaxis (PCA)

The 50 µl samples of diluted mouse serum from ovalbumin-sensitised mice were incubated with immune mouse sera or PBS controls for 1 h at 37 °C and then injected intradermally into the shaved backs of Sprague-Dawley retired breeder rats. After 24 h, PCA reactions were induced by intravenous injection of 1 mg of DNP-ovalbumin conjugate in 1% Evan's Blue dye. One hour later, the rats were euthanized and skinned. The DNP-Oa antigen had cross-linked receptor-bound mouse anti-Oa IgE on the rat mast cells. The cross-linking triggered degranulation, increased permeability of the Evans blue dye, and the appearance of blue zones on the underside of the rat skins proportional to the

extent of degranulation. However, wherever free IgE had been depleted by the site-specific murine anti-IgE, less was available to sensitise the rat mast cells and PCA reactions were suppressed. PCA reactions were evaluated by measuring the diameters of the blue zones on the undersides of the rat skins in two directions at right angles and taking the average.

### 3. Results

#### 3.1. Identification of the IgE target site

Sites within the C $\epsilon$ 2, C $\epsilon$ 3, and C $\epsilon$ 4 domains of human IgE were selected for synthesis as peptide immunogens based on previous observations and structural models that suggested potential effector sites [5,8,14]. These sites were also analyzed for surface-exposed loop structures deduced from models for the three-dimensional structure of IgE-Fc $\epsilon$ RI [8,16,33]. For peptides corresponding to predicted loop sites, disulphide-bonded loops were incorporated into the designs so as to stabilize the mobile peptides into conformations that resembled predicted IgE loop structures, and to maximize cross-reactivity between these designed target antigenic peptides and the native IgE molecule. Potential target antigenic sites were synthesized and made immunogenic either by chemical conjugation to KLH following solid-phase peptide synthesis, or by covalent attachment to promiscuous Th epitopes and other immunostimulatory sequences by continuous synthesis [25]. All 35 sites from IgE that were screened are shown in Fig. 1.

Candidate effector target sites were then identified by the preparation of hyperimmune sera in guinea pigs and testing of the antisera for reactivities to the target peptides and for cross-reactivities to human IgE. All peptide immunogens elicited anti-peptide responses. The kinetics of the antibody response show that anti-peptide ELISA reactivities appeared first, and for those peptides that evoked anti-IgE cross-reactivities, the cross-reactivity did not approach the levels of anti-peptide reactivity until week 8 post-initial immunization (data not shown). Antibodies from the 8 immune sera generated by the respective IgE antigens numbered in Table 1 had high cross-reactivities to human IgE. These antibodies were purified and evaluated for ability to inhibit the IgE-mediated sensitisation of human basophils by the *in vitro* assay for histamine release. Anti-peptide antibodies evoked by cyclized peptide immunogens 15b and 15c (Table 1) displayed strong cross-reactivity for IgE and most consistently displayed high inhibitory activity in the histamine release assay. This target epitope, at positions 413–435, corresponded to a segment of the C $\epsilon$ 3 domain on a predicted surface-exposed loop.

The target site on peptide immunogens 15b and 15c was modified from that of the naturally occurring IgE sequence so as to provide a constrained loop structure. A cysteine residue was added to the N-terminus side of position 413 of human IgE ([34] for human sequence positions), the cysteine at 418 was replaced by serine, a cysteine was added at C-terminus side of position 435, and a disulphide bond was formed between the terminal cysteines to produce a cyclic structure. The target site peptide by itself was non-immunogenic. Extrinsic T cell help was provided

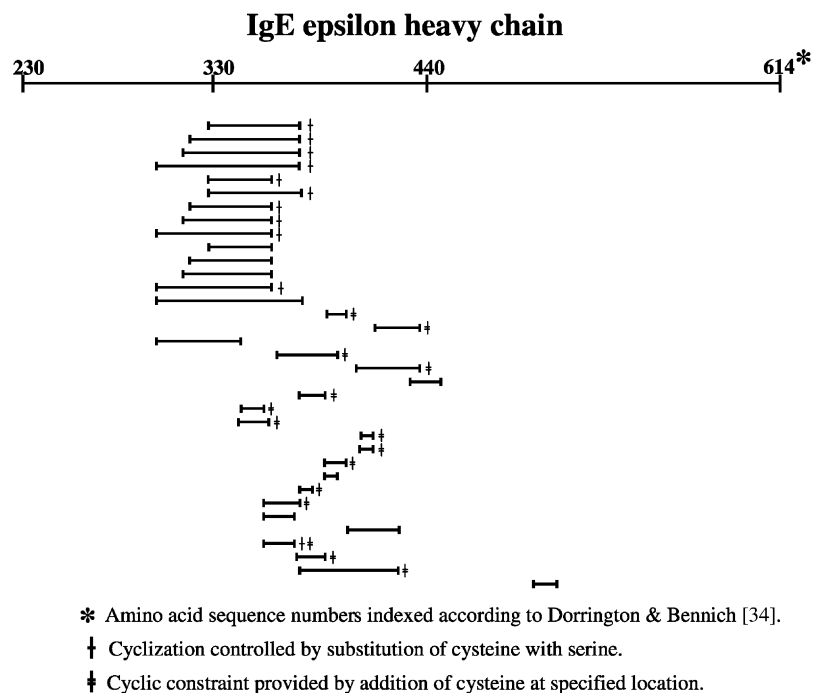


Fig. 1. Epitope mapping of functionally antigenic sites on human IgE epsilon chain.

Table 1  
Evaluation of anti-IgE antibodies for inhibition of histamine release

IgE antigen no.	IgE antigen description	Immunogenic elements attached to IgE antigen	Cross-reactivity with human IgE (log <sub>10</sub> titre)	% inhibition of histamine release <sup>c</sup> (%)
1	CH2/3 (328–376) (C <sub>358</sub> → S) <sup>a</sup>	KLH	3.66	0
2	CH2/3 (317–376) (C <sub>358</sub> → S) <sup>a</sup>	KLH	5.08	14
		UBITH <sup>®</sup> A-GG-	3.77	17 and 0
5	CH2/3 (328–362) (C <sub>358</sub> → S) <sup>a</sup>	KLH	4.40	0
6	CH2/3 (317–362) (C <sub>358</sub> → S) <sup>a</sup>	KLH	4.30	0
7	CH2/3 (313–362) (C <sub>358</sub> → S) <sup>a</sup>	KLH	3.92	6
8	CH2/3 (301–362) (C <sub>358</sub> → S) <sup>a</sup>	KLH	3.37	6
11	CH2/3 (313–356)	KLH	4.31	6
15	(C)CH3 (413–435) (C) <sup>b</sup> (C <sub>418</sub> → S) <sup>a</sup>	Syn Th(1, 2, 4)-GG	4.24	58 <sup>c</sup> and 71 <sup>d,e</sup>
		Inv-GG-Syn Th(1, 2, 4)-GG-	4.17	
20	(C)CH3 (374–382-(C)-383-385) <sup>b</sup>	HB <sub>S19–32</sub> Th-GG	3.98	0
30	CH3 (399–424)	HB <sub>S19–32</sub> Th-GG-	4.01	9 and 0
32	(C)CH3 (370–390) (C) <sup>b</sup>	HB <sub>S19–32</sub> Th-GG-	3.45	0

<sup>a</sup> (C → S) serine substituted for cysteine.

<sup>b</sup> Cyclized peptide (C) cysteine introduced into native sequence for cyclization.

<sup>c</sup> Histamine release inhibition by antibodies to peptides, purified from serum collected at week 8, except as otherwise noted.

<sup>d</sup> Results are shown for pooled anti-15b and anti-15c IgG's.

<sup>e</sup> Histamine release inhibition by antibodies to peptides, collected at week 12.

by linkage to a synthetic UBITH<sup>®</sup> epitope “Syn Th (1, 2, 4)”, a palindromic sequence derived from hepatitis B virus [25,35] or later by the artificial combinatorial T helper site derived from measles virus “UBITH<sup>®</sup>A” (shown in Fig. 2). UBITH<sup>®</sup>A has been shown to be promiscuously immunogenic across species [35]. T cell help was also imparted to a series of peptides by a T helper epitope at positions 19–33 of hepatitis B virus surface antigen [36].

### 3.2. *In vitro* characterisation of functional immunogenicity

A peptide immunogen having UBITH<sup>®</sup>A, the modified human 413–435 Cε3 target site, and an (ε-N) Lysine linker was synthesized (Fig. 2) and used to immunize a swine. This UBITH<sup>®</sup> IgE immunogen, Ap2878, was formulated with Montanide ISA 720 and 300 μg doses were administered intramuscularly to pig no. 183 on weeks 0, 3, 6, and 10. IgG was purified by protein A affinity chromatography from serum collected on week 12 and control IgG was prepared from a pig immunized with an irrelevant peptide immunogen. Purified human basophils were used to detect inhibition of sensitisation with IgE. Fluorescence flow cytometry data are compared (Fig. 3) for the antibody preparation from swine no. 183, and the control antibody, for inhibition of

binding. Fig. 4A shows a titration of the 8 mg/ml purified antibody stock used to inhibit sensitisation, showing an IC<sub>50</sub> of 1/250 (concentration in the cell suspension). The polyclonal antibody purified from swine no. 183 immune serum exhibited inhibitory activity for human IgE binding. In Fig. 4B, bar graphs are shown for the indirect assay of sensitisation of basophils by measuring subsequent antigen-driven histamine release. Measurement by flow cytometry or by histamine release yielded essentially equivalent results. For the basophils sensitised in the presence of swine no. 183 antibody, their response to goat polyclonal anti-IgE antibody was the same as for cells sensitised in the presence of control antibody, indicating that the vaccine antibodies had no non-specific effects on basophil function and did not desensitise the general IgE-mediated response (data not shown).

### 3.3. *Non-anaphylactogenicity*

The swine no. 183 antibody preparation and several other antibodies from guinea pigs that had been hyperimmunized with UBITH<sup>®</sup> human IgE immunogen (Fig. 2) were used to directly challenge human basophils. Histamine release by the vaccine-induced anti-IgE specimens did not exceed the level of spontaneous release by the basophils of the donors tested, indicating that the site-specific anti-IgE

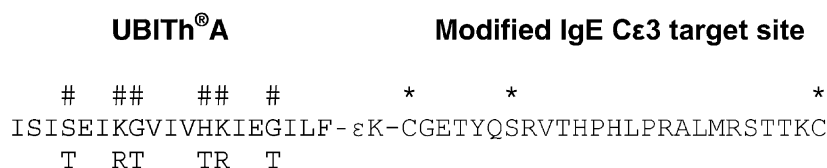


Fig. 2. UBITH<sup>®</sup>A immunogen for human IgE. T cell help is provided by the UBITH<sup>®</sup>A site shown, with combinatorial positions marked by (#). The Cε3 target antigenic site, linked to the T helper epitope through the ε-Lysine linker, has been modified from the native human sequence at the positions marked by (★).

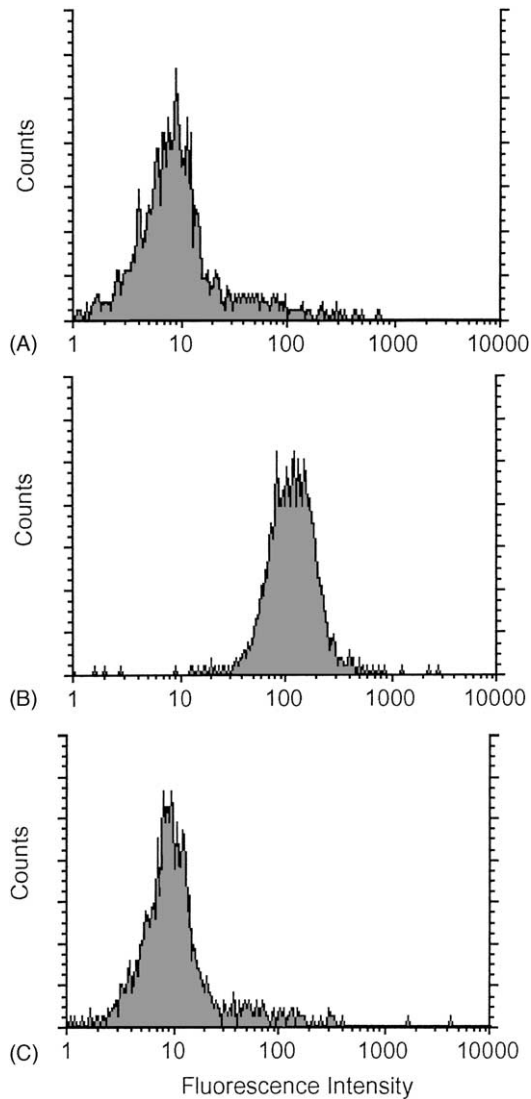


Fig. 3. Inhibition of human basophil sensitisation with vaccine-elicited anti-IgE antibodies. Purified basophils were sensitised in the presence or absence of vaccine-elicited anti-IgE antibodies and the effect of sensitisation assessed by flow cytometry. Panel A: cells not sensitised with gp120-specific IgE but labelled with the primary and secondary antibodies for flow cytometry, panel B: cells sensitised in the presence of control vaccine-elicited antibodies, and panel C: basophils sensitised in the presence of vaccine-elicited antibodies (swine no.183) at a concentration of 2 mg/ml (see text).

antibodies did not cross-link receptor-bound IgE and were non-anaphylactogenic. In particular, swine no. 183 antibody, which was characterised in Fig. 3 for inhibition of sensitisation, induced no release at a final concentration of 1:10 (of 8 mg/ml stock) from basophils obtained from four donors which otherwise released  $33 \pm 14\%$  to a positive goat polyclonal anti-IgE antibody.

#### 3.4. Functional immunogenicity by passive cutaneous anaphylaxis assay

To study the effect of immunization by a UBITH<sup>®</sup> IgE immunogen on an IgE-mediated inflammatory reaction, an

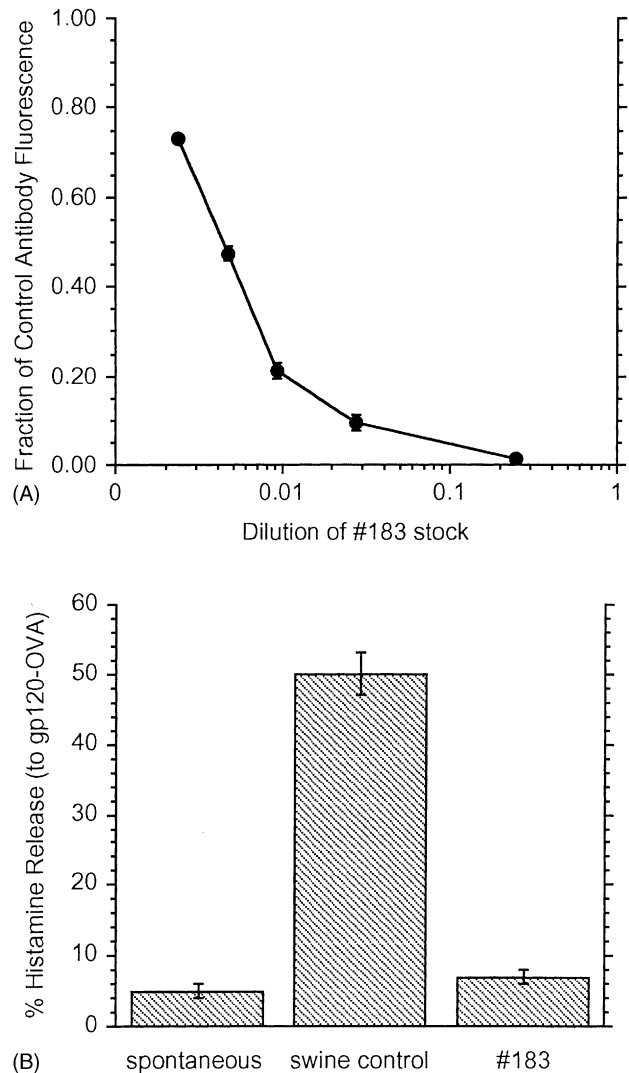


Fig. 4. Inhibition of human basophil sensitisation assessed by direct and indirect measures. Panel A shows the average of 2 experiments in which several concentrations of swine no. 183 antibody were tested for inhibition of sensitisation as detected by flow cytometry (as performed in the experiment described in Fig. 3). The data is calculated from the medians of the cytometric histograms. The data is expressed as a fraction of the median fluorescence (minus background) for cells sensitised with gp120-specific IgE in the presence of control swine antibody. The abscissa is the dilution of no. 183 as it is in the presence of cells (see Section 2). Panel B shows the results for experiments where the level of sensitisation was assessed by subsequently stimulating the basophils with specific antigen (gp120-OVA in these studies). In this example, sensitisation was tested in the presence of swine no. 183 antibody (or control Ab) at a concentration of 1:4 of the stock Ab.

antibody response was elicited by immunizing mice with a UBITH<sup>®</sup> peptide immunogen having the autologous murine IgE-C $\epsilon$ 3 target antigenic site (modified from the mouse sequence shown in Table 2). The resulting mouse antiserum was then used to suppress the passive cutaneous anaphylaxis (PCA) triggered by the cross-linking of mouse IgE bound to rat mast cells.

Table 2  
Homologous IgE Cε3 target sequences

Human ε	GETYQCRVTHPHLPRALMRSTTKTSGPR
Chimp ε	GETYQCRVTHPHLPRALVRSTTKTSGPR
Rat ε	GEYQCRVDHPHFPKPIVRSITKAPGKR
Mouse ε	GYGYQCIVDHPDFPKPIVRSITKTPGQR
Dog ε	GETYYCRVTHPHLPKDIVRSIAKAPGKR
Cat ε	GETYQCKVTHPDLPKDIVRSIAKAPGRR
Horse ε	GETYKCTVSHPDLPREVRSIAKAPGKR
Pig ε	GETYYCNVTHPDLPKPILRSISKGPGR
Goat ε	GETYYCKVSHGDLPKDIQRSISKDVGKR

Balb/c mice were immunized subcutaneously with 20 μg of the UBITH<sup>®</sup> mouse IgE peptide on weeks 0, 3, and 6. On week 8, mouse sera were collected and evaluated for cross-reactivity to IgE by the mouse IgE ELISA. Thirteen out of 20 immunized mice had cross-reactive antibodies against mouse IgE. Sera was pooled from seven mice showing ELISA titres against mouse IgE of  $\geq \log_{10} 2.3$  for use as the site-specific anti-IgE. Another group of 10 balb/c mice was sensitised to ovalbumin (Oa) (1 μg per 0.2 ml in PBS on 0.4% alum, intraperitoneal injection). IgE content of the mouse sera was measured at day 20 by the ELISA for detection of murine IgE. Out of the 10 Oa-immunized mice, 7 had appreciable IgE responses of titre  $\geq \log_{10} 1.6$ . These sera were pooled for use as the anti-Oa IgE working stock.

The anti-Oa IgE serum pool was serially diluted 1:62, 1:124 and 1:248 into PBS, for use in PCA reactions in rats. These dilutions were later further diluted by pre-incubations with equal volumes of the site-specific anti-IgE serum pool. Thus, final dilutions of the PCA reactants was 1:124, 1:248, and 1:496 for mouse IgE serum and 1:2 for mouse anti-IgE

serum. Control dilutions of IgE were prepared having only PBS as diluent. The IgE dilutions, with and without anti-IgE serum, were incubated for 1 h at 37° and 50 μl of each was taken for evaluation by PCA reaction in Sprague-Dawley rats.

The 50 μl samples of mouse IgE, pre-incubated with mouse anti-IgE serum or PBS control, were injected intradermally into the shaved back of rats in a pattern that was a set of two rows of four injections. The rows were a row of three controls of IgE diluted 1:124, 1:248, and 1:496 in PBS only, in parallel with a row of the serially diluted IgE incubated with the site-specific anti-IgE. The fourth injection of each row was PBS only, as a control for tissue trauma. The pattern was duplicated on two rats. After 24 h, PCA reactions were induced by intravenous injection of DNP-Oa conjugate in 1% Evans blue dye and results were observed 1 h later. The rats differed by their inherent sensitivities to the mouse IgE so that control and anti-IgE inhibited PCA reactions are compared independently for each rat (Fig. 5). Mouse IgE-mediated PCA reactions were inhibited in both rats by the murine antiserum with specificity for the mouse IgE target site.

### 3.5. In vivo functional immunogenicity in dogs

Non-atopic beagles were immunized with the UBITH<sup>®</sup> canine IgE peptide immunogen. This peptide has the canine IgE target antigenic sequence (modified from the dog sequence of Table 2) linked to the UBITH<sup>®</sup>A T helper site. The dogs were divided into five groups, four dogs per group. Groups 1–4 were immunized with 2000, 400, 100,

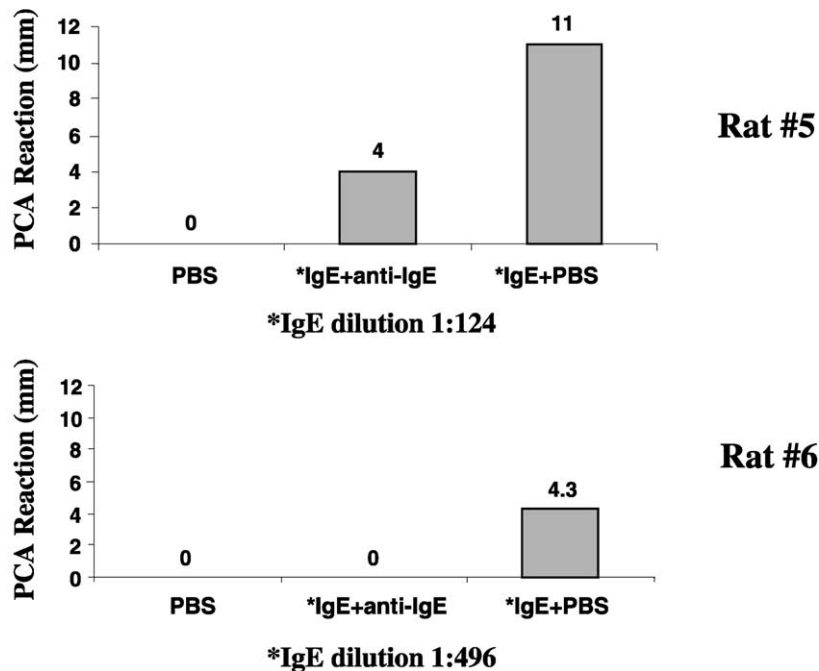


Fig. 5. Passive percutaneous anaphylaxis assays. Murine IgE pre-incubated with murine anti-IgE antibodies were used for the in vivo sensitisation of rat mast cells. Inhibition of sensitisation evaluated by extent of allergen-induced anaphylaxis.

or 25  $\mu\text{g}$ , respectively, of the UBITH<sup>®</sup> canine IgE peptide in ISA 720, at 0 and 3 weeks post-initial immunization (wpi). At 7 wpi, the dogs of groups 1–3 were boosted with 100  $\mu\text{g}$  while the group 4 dogs were given 25  $\mu\text{g}$ . A fifth group was immunized with an irrelevant UBITH<sup>®</sup> vaccine for control. Dog serum was collected and analyzed for an anti-IgE response by anti-dog IgE ELISA. All four dogs of group 3, given the 100  $\mu\text{g}$  doses on weeks 0, 3, and 7, achieved peak responses over background level that were sustained through week 9 (Fig. 6). For groups 1 and 2, three out of the four dogs of each group were responsive though less so than group 3, while the dogs of group 4, given 25  $\mu\text{g}$

doses, were only minimally responsive above the control group (data not shown). The results indicate that 100  $\mu\text{g}$  was the optimum immunogenic dose for the beagles.

The sera collected from two responsive dogs from each of groups 1–3 and a dog from control group 5 were assayed for total IgE content, including free IgE and IgE within anti-IgE/IgE immune complexes, by the quantitative assay on heated serum [28]. Serum IgE for responsive dogs from group 1 (nos. 27 and 28), group 2 (nos. 30 and 31), group 3 (nos. 32 and 33) and control dog no. 01 from group 5 are shown in Fig. 7 for weeks 0, 5 and 9. The range of initial IgE levels varied among these normal dogs, but the relative

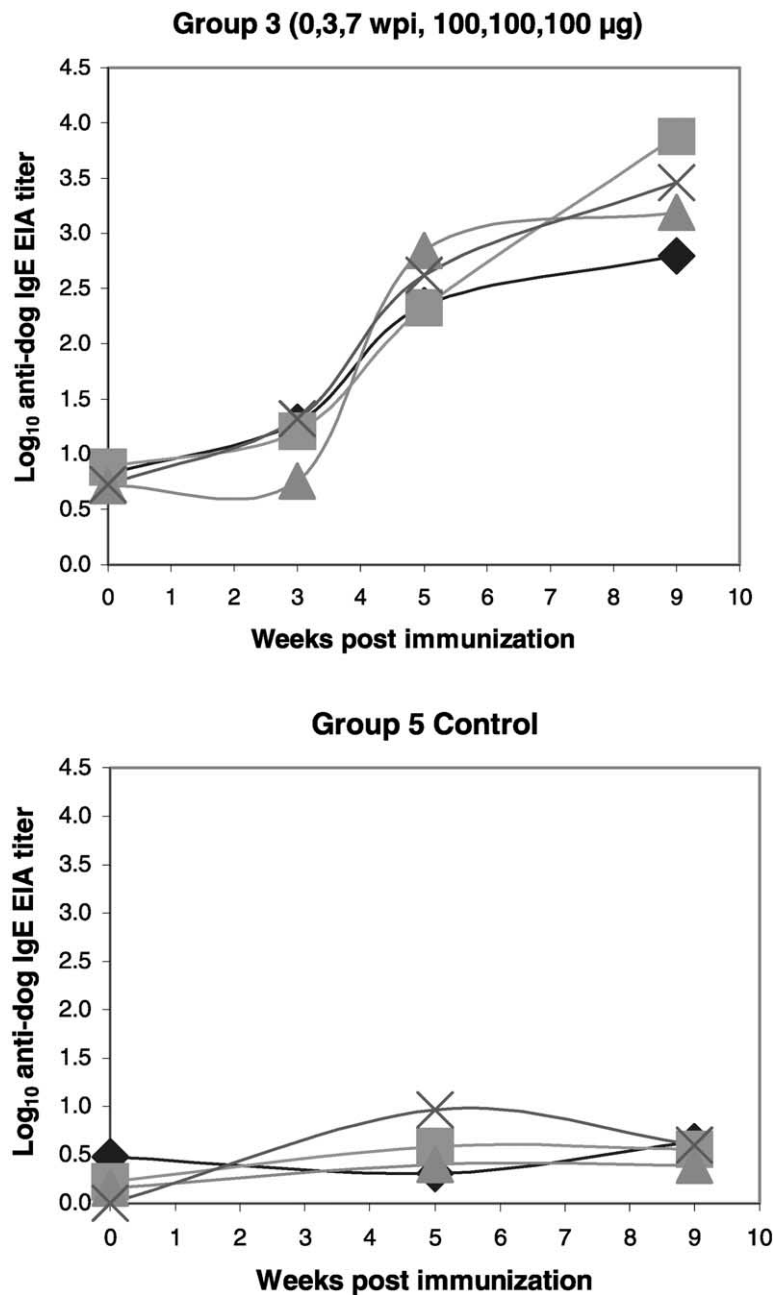


Fig. 6. Anti-IgE IgG responses in dogs to the dog IgE target immunogen.

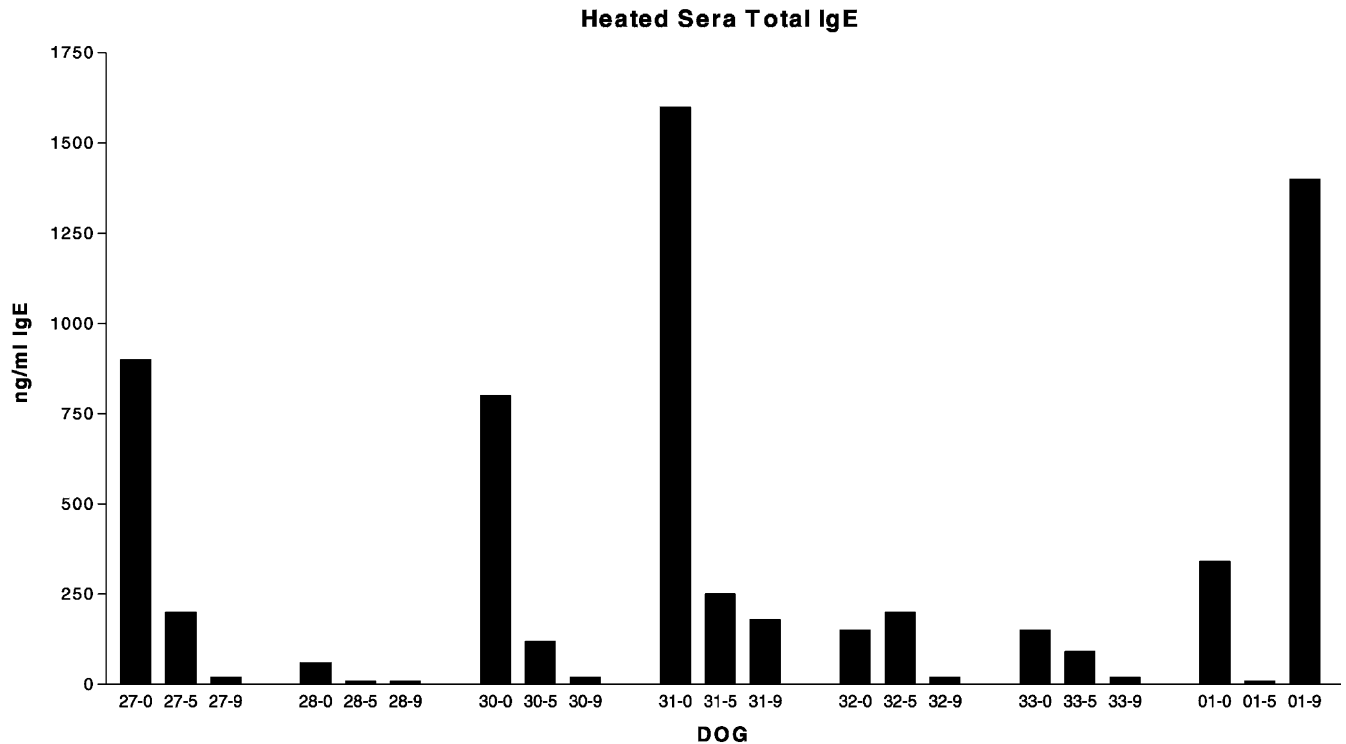


Fig. 7. Total IgE in heated dog sera, both free and complexed, was determined for dogs at weeks 0, 5, and 9 post-initial immunization. Dog nos. 27 and 28 were given initial dose 2000  $\mu$ g, nos. 30 and 31 were given 400  $\mu$ g, nos. 32 and 33, 100  $\mu$ g, and no. 01 received placebo.

levels of total IgE in circulation declined substantially during the course of the trial immunizations. Dog no. 31, with the lowest anti-IgE (reciprocal titre  $\log_{10}$  1.8) at week 9 and the highest IgE at 0 week of the group 1–3 dogs tested, had total IgE at week 9 reduced by close to 10-fold from week 0.

#### 4. Discussion

An IgE-directed immunotherapeutic approach aims to prevent initiation of the allergic cascade, and unlike classic desensitisation therapy, it is not allergen-specific. It can be effective for all IgE-mediated allergic reactions with no need to identify the offending antigen (e.g. pollens, dust mite allergen, molds). Proof-of-concept for an anti-IgE vaccine has already been established by clinical trials using passive immunization with non-anaphylactogenic anti-IgE monoclonal antibodies [19–23]. We have provided for a chemically-defined immunogen that elicits a polyclonal site-specific anti-IgE response. The IgE target antigenic site was immunopotentiated by linkage to a combinatorial T helper epitope derived from measles virus, UBITH<sup>®</sup>A. This T help was promiscuous across species, in guinea pigs, mice, swine, dogs, and elsewhere in baboons [35] and goats and cattle (to be submitted), when combined with various self and foreign B cell epitopes taken from Luteinizing Hormone Releasing Hormone (LHRH or GnRH), domain 1 of CD4, an N-terminal fragment of  $\beta$ -amyloid protein,

somatostatin, and foot-and-mouth disease virus, in addition to IgE. Tolerance to the self target IgE antigen was overcome in dogs and to a lesser extent in mice by the artificial UBITH<sup>®</sup>A T helper epitope as shown by the production of auto IgG anti-IgE antibodies. The artificial T helper epitope also provided for antibody maturation. The kinetics of the antibody response in guinea pigs and swine (data not shown) showed that by week 8, anti-IgE cross-reactivities approached the anti-peptide reactivities. We concluded that as the anti-peptide antibody response matures, higher affinity antibodies are expressed and this secondary anti-peptide response becomes cross-reactive with the target protein.

Like the therapeutic monoclonal antibodies, the site-specific vaccine-induced antibodies blocked the binding of IgE to the high affinity receptor Fc $\epsilon$ RI, prevented anaphylactic reactions, and did not by themselves cross-link IgE and signal degranulation. Like the passive immunization trials performed in mice [37], the active immunization of dogs resulted in reductions in serum IgE. These reductions were correlated to the anti-IgE response, except in those normal dogs that had low serum IgE at the start of the vaccine trial (data not shown). However, the reduction in total serum IgE may be a species effect since trials in humans have yet to find a reduction in total IgE levels even after 1 year of treatment [21–23] (and unpublished reports).

Homologous target peptide sequences for human, chimp, rat, mouse, dog, cat, horse, pig, and goat IgE are compared in Table 2. In this case, homologous sequences did result

in homologous function since the target sequences from human, murine, and canine epsilon chains all exhibited the same functional antigenicity of blocking the binding of IgE to the high affinity receptor. This broad effect is predictive of efficacy for immunotherapeutic allergy vaccines in multiple species, including humans and dogs.

The vaccine-elicited antibodies blocked the sensitisation of mast cells and basophils by free IgE. However, this action mode alone cannot by itself provide the pharmacological mechanism for therapeutic efficacy by the antibodies or a vaccine. Even if free IgE were neutralized to 99% by the anti-IgE, the therapy still would fail because the few remaining IgE molecules would be sufficient to sensitise the mast cells and basophils [29]. This would be a more serious problem in dogs since levels of IgE are relatively high compared to humans [28]. However, an anti-IgE approach has the potential to provide therapeutic efficacy through other actions. Human basophil studies have shown that FcεRI expression is regulated by the level of free IgE, so that reduced levels of free IgE should lead to lower densities of FcεRI on basophils and mast cells and lowered sensitivities [19,38,39]. And, anti-IgE may lead to the down-regulation of IgE production by eliminating or down-regulating IgE-expressing B cells, perhaps by cross-linking membrane-bound IgE and causing apoptosis or anergy [37,40] or perhaps by complement-mediated and cell-mediated cytolysis [41]. It has been difficult to demonstrate reduction of IgE-producing B cells in the clinical trials with monoclonal antibodies, but other *in vivo* and *in vitro* experimental results are supportive of an anti-IgE-expressing B cell mechanism [18]. Whatever the underlying mechanism, the reduction of IgE levels in the dog trials suggests that tolerance for IgE can be broken and that the appearance of anti-IgE has a dramatic effect on at least one aspect of the immune response. Future studies will be needed to determine whether this characteristic will be found in primates.

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