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Profound reduction in allergen sensitivity following treatment with a novel allergy vaccine*

A novel approach is described for the treatment of IgE-mediated allergic reactions which is based on the induction of a strong anti-IgE response in the host. Vaccination of ovalbumin-sensitized rats with constant domains two and three of rat IgE coupled to a heterologous carrier protein resulted in a profound decrease in serum levels of IgE, and later in a nearly complete block of histamine release from mast cells and basophils upon challenge with either a cross-linking polyclonal anti-IgE antiserum or a specific allergen.

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1 Introduction

IgE is, despite its normally very low concentrations in human plasma (10–400 ng/ml), a major cause of allergies found within the human population. Common atopic allergies includes hay fever, fur allergies and many types of asthma, food allergies and dermatitis. This property of IgE is due to its strong interaction with specific high-affinity receptors for IgE on mast cells and basophilic granulocytes.

A region of 76 amino acids at the border between the constant domains CH2 and CH3 of human IgE has been shown to interact with the IgE receptor. This 76-amino acid peptide has the capacity to inhibit the interaction between native IgE and the receptor with approximately the same efficiency as a protein containing the entire CH2-CH3-CH4 region of human IgE [1, 2].

When IgE binds to its receptor, interactions with IgG antibodies directed against the CH2-CH3 region of the whole IgE molecule are prevented. In contrast, anti-IgE antibodies directed against regions of the IgE molecule outside of the receptor binding region, can bind to an IgE molecule while it is bound to its receptor. This results in cross-linking of receptor-bound IgE, producing an anaphylactic shock in animals in which such antibodies are injected systemically. Anti-IgE antibodies directed against the region participating in receptor binding do not have the capacity to cross-link receptor-bound IgE, but might instead have the ability to cross-link free circulating IgE, thereby stimulating removal of IgE from the plasma. Such clearance would lead to a nearly complete depletion of circulating IgE and is likely to result in a profound reduction in the levels of receptor-bound IgE, thus reducing the sensitivity of the mast cells to allergen challenge.

Based on these assumptions we initiated a study to investigate the possibility of inducing a strong anti-IgE response against an organism's own circulating IgE and to analyze the effect of this type of vaccination on allergen-specific IgE-mediated inflammatory reactions. The likelihood of achieving a successful vaccination is highly dependent on the concentration of the molecule targeted for clearance from the patients circulation. High concentrations of the target molecule may be difficult to eliminate. For example, it would be much more difficult to remove all circulating IgG molecules because the serum concentration of IgG is normally five to six orders of magnitude higher than the concentration of IgE in serum. IgE is an ideal target because of its low plasma concentration, which in normal individuals is in the range of 10–400 ng/ml. This corresponds to approximately 0.01 % of the total immunoglobulin content of human blood. The levels are sometimes elevated in allergic patients, but the concentration seldom exceeds 5 µg/ml. From a study in mice, it has been shown that a nearly complete clearance of circulating antibodies containing the kappa light chain can be obtained by treatment with a monoclonal antibody directed against this light chain protein [3]. This example shows that it is possible to obtain a nearly complete removal of a plasma protein which is present at a concentration approximately one million times higher than that of IgE.

2 Materials and methods

2.1 Production and purification of fusion protein for immunization studies

A 636-bp HincII-Eco47 III cDNA fragment encoding the CH2-CH3 domains of rat IgE [4] was ligated into the fusion protein vector pGEX-5T [5]. The CH2-CH3 region is directly linked in this vector to the gene encoding a 26-kDa glutathione-S-transferase (Sj26) protein from *Schistosoma japonicum*. The glutathione-S-transferase (GST) gene in this vector contains six histidines in its N-terminal region, facilitating purification of the fusion protein on Ni²⁺-chelating columns. The rat CH2-CH3-fusion protein (GST-rat C2C3) has a very low solubility and readily precipitates within the bacterial cells. However, the aggregates can be dissolved by incubation for 30 min at 80 °C in a solution containing PBS (pH 7.5), 8 M urea, 0.5 % SDS and 1 mM 2-mercaptoethanol. To evaporate excess of 2-mercaptoethanol, the tube containing the dissolved fusion protein was left open at room temperature for 1 h. The extract was then separated on a nickel-chelating column using a solution

[I 12373]

* This work was supported by grants from the Swedish Natural Science Research Council.

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Abbreviations: CH2: Constant domain 2 of an immunoglobulin GST: Glutathione-S-transferase

Key words: Vaccine / Allergy / IgE / Autoimmunity

containing PBS (pH 5.0), 8 M urea and 0.5 % SDS and yielded virtually 100 % pure fusion protein. After dialysis against PBS (pH 7.5) the fusion protein was soluble at room temperature at a concentration of more than 2 mg/ml.

2.2 Immunization procedure and ELISA measurement of the anti-IgE response in different strains of rat

A total of 16 rats of four different strains (4 rats from each strain: Lewis, Sprague Dawley, Wistar and Brown Norway) were injected s.c. with one of three different test solutions, each mixed with CFA, according to the following scheme: 8 rats were vaccinated with the GST-rat C2-C3 fusion protein, 4 rats were injected with human IgG and 4 rats with only PBS. In this study each rat which received i.p. injections of protein was immunized with approximately 100 µg of antigen dispersed in 0.2 ml 1:1 CFA/PBS. Three weeks later all rats were given a booster injection with the same amount of antigen in IFA/PBS. One week after this second immunization blood samples were taken and measured as described below.

The levels of IgG anti-IgE antibodies directed against native rat IgE, or antibodies directed against human IgG in a control experiment, were measured by an ELISA. Native rat IgE was used at a concentration of 5 µg/ml for coating ELISA plates. Successive 1/5 dilutions of serum from each rat were tested.

2.3 Sensitization and vaccination procedures

All rats shown in Figs. 3–6 had been sensitized to ovalbumin by weekly injections of ricin and ovalbumin according to the protocol described in [6] (ricin, L-1090, was purchased from Vector Laboratories, Burlingame, CA). In brief: during the first injection, 50 ng of ricin and 1 µg of ovalbumin were administered i.p. at separate injection sites. This was followed by weekly injections of 10 ng ricin and 1 µg of ovalbumin, starting 3 weeks after the initiation of the sensitization program. Eight rats were each sensitized for 8 weeks with ricin and ovalbumin to obtain a high, persistent and antigen-specific (ovalbumin) IgE response before initiating the vaccination program. Four out of these rats were vaccinated with 500 µg of pure GST-rat C2C3 fusion protein in 200 µl 1:1 CFA/PBS and four rats were blank immunized with 200 µl 1:1 CFA/PBS alone. Four weeks after the primary immunization the rats were boosted with 100 µg of GST-rat C2C3 fusion protein in 200 µl 1:1 IFA/PBS. Control rats received an identical booster injection but without a vaccine component. A second booster, identical to the first booster dose, was given four weeks later. The ricin/ovalbumin treatment was continued during the entire 10 weeks of vaccine treatment, with weekly injections of 10 ng of ricin and 1 µg of ovalbumin.

2.4 Radioimmunoassay (RIA) of total IgE levels in rat serum

The total IgE levels were measured by an RIA, using purified rat IgE diluted in horse serum as standard (IgE from the rat immunocytoma IR-162 was a kind gift from Dr.

H. Bennich, Uppsala). The RIA was based on commercially available antibodies and used Dynal magnetic beads as a solid support (tosyl-activated M-280, Dynal A.S., Oslo, Norway). The antibody bound to the magnetic beads was a polyclonal goat anti-rat IgE Fc (7S) (Nordic, Tilburg, Netherlands) and the tracer was a mouse monoclonal antibody directed against rat IgE Fc (Sera Lab, Crawley Down, GB; MAS 314p). The tracer antibody (50 µg) was labeled with 500 µCi of ¹²⁵I by the chloramine T method. Blood samples were taken every 3 weeks starting 1 day prior to the initiation of the vaccination program. The detection limit of the RIA was approximately 10–20 ng/ml when analyzing 100 µl of rat serum.

2.5 Assay of skin reactivity by Evans blue administration

As an assay system, a polyclonal cross-linking anti-IgE antiserum [polyclonal goat anti-rat IgE Fc (7S; (Nordic); diluted 1/5 in PBS] or a 1 or 5 mg/ml solution of ovalbumin was used to provoke a strong IgE-mediated inflammatory reaction under the skin of test animals. Sterile PBS was used as a negative control. Approximately 50 µl of the appropriate solutions was injected at each point under the skin, followed by an injection of 500 µl 2 % Evans blue solution into the blood stream of the animals via the jugular vein. The rats were kept under Inactin anesthesia for an additional 2 h, after which the rats were killed and the skin removed to measure the size of the blue zones.

3 Results

3.1 Induction of an autoimmune anti-IgE response in an experimental animal

To study the possibility of inducing a strong IgG response to circulating IgE in an experimental animal model, the CH2-CH3 domains of IgE were coupled to a foreign carrier protein and used as an antigen. This procedure may circumvent the tolerance against the animal's own IgE molecules by the recruitment of a non-tolerized T cell population which normally would have given help only to B cells directed against the carrier, but which, due to coupling of the CH2-CH3 domains to the carrier also supports B cells with specificities directed against the IgE molecules of the vaccinated subject. The antigen used in these experiments was a bacterially expressed fusion protein consisting of the CH2-CH3 domains of rat IgE fused to the GST polypeptide. As shown in Fig. 1, the GST-rat C2C3 antigen was first purified practically to homogeneity by affinity chromatography. Different strains of rat were used as animal models to study the immune response against self IgE. A preparation of native human IgG was used as a control antigen to study the normal immune response against a foreign antigen. An experiment was performed with these antigens in four different strains of rat. Four weeks after immunization with the fusion protein, a strong immune response against native rat IgE was detected by ELISA measurements in three out of four strains of rat (Fig. 2). The fourth strain did not appear to mount a response to this particular antigen (a non responder), a phenomenon not infrequently seen when using inbred strains of animals. As measured by ELISA, the immune response in the three remaining strains of rat, was

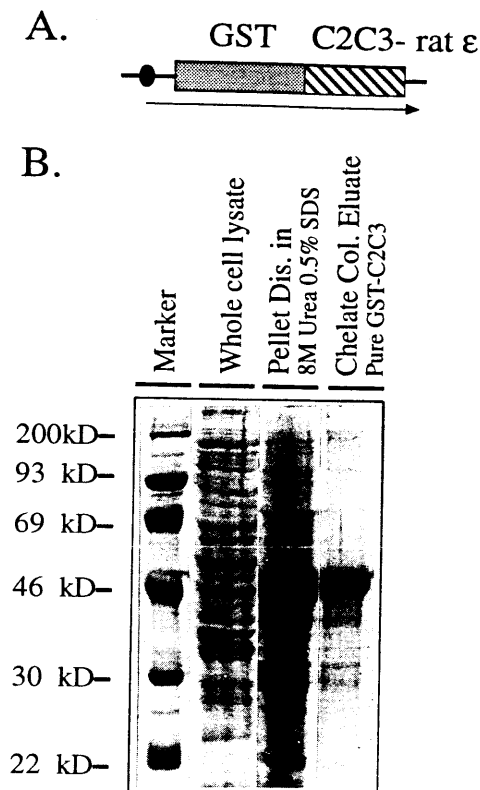


Figure 1. SDS-PAGE analysis of purified or partly purified, bacterially expressed fusion protein, containing the CH2-CH3 domains of rat IgE [4] linked to the 26-kDa glutathione-S-transferase (Sj26). **Panel A** shows a schematic representation of the fusion protein gene in the vector pGEX-5T. The arrow shows the orientation of transcription. **Panel B** shows an SDS-PAGE analysis of total soluble material from the different steps of the purification procedure.

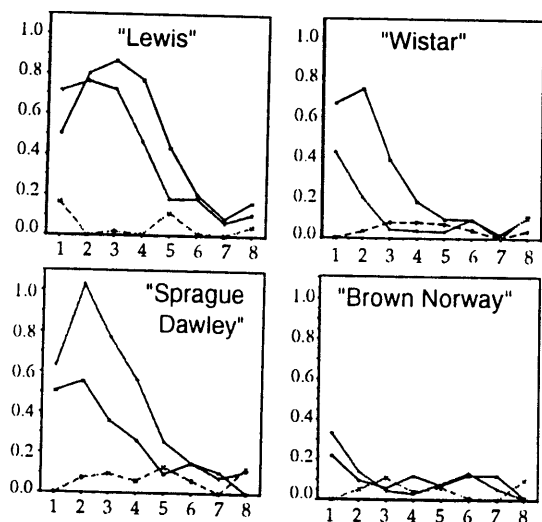


Figure 2. Analysis of the immune responses against the GST-rat C2-C3 fusion protein in a panel of four different strains of rats. The level of IgG anti-IgE was measured by an ELISA. Native rat IgE was used at a concentration of 5 µg/ml for coating of ELISA plates. Successive 1/5 dilutions of serum from each of the individual rats were tested by color reaction in the ELISA (1 in the Fig. represents undiluted serum, 2 a 1/5 dilution in PBS, 3 a 1/25 dilution and so on). Two GST-C2C3 vaccinated rats were analyzed together with one control rat from each strain. The results from the four control rats, which were injected with adjuvant and PBS alone, are shown by a dotted line.

of a magnitude close to, or only slightly lower than, that of an immune response directed against a foreign protein, in this case human IgG (data not shown).

3.2 An animal model to study the effect of the allergy vaccination on an allergen-specific inflammatory reaction

Although rodents have been the most frequently used animal models in immunological studies, it has previously been shown that it is quite difficult to mount a strong and relatively long-lasting antigen-specific IgE response in most strains of rat and mouse. However, a potent sensitization protocol has recently been developed for rats [6]. By repeated injections of the toxin ricin together with low amounts of a specific antigen, high titers of both total and antigen-specific IgE can be obtained in rats after only 3-4 weeks of treatment. To obtain long-lasting high IgE titers, we sensitized rats for 8-9 weeks before initiating the vaccination program. Wistar rats were chosen for further studies because this strain has been shown to respond well to the sensitization protocol [6].

To obtain a model system which mimics as closely as possible the situation in an allergic human patient with a deregulated control of IgE expression, rats were injected every week with low levels of ovalbumin and ricin during the entire vaccination program.

The IgE response is highly IL-4 dependent, as has been shown from studies of mice, transgenic for IL-4, in which IgE levels are drastically up-regulated. All animals expressing the IL-4 gene in their T cell population displayed characteristic symptoms of IgE-mediated inflammatory reactions [7]. The IL-4-dependent IgE response is influenced by IFN-γ [8, 9]. Freund's adjuvant has been shown to increase IFN-γ production, which thus could inhibit the sensitization of the test animals [10]. An equal number of control rats were, therefore, immunized with Freund's adjuvant and PBS alone.

Four pairs of sensitized rats were used to evaluate the effect of the vaccine on total serum IgE levels and later to examine hypersensitivity in the skin upon stimulation with both a polyclonal mast cell activator (in this case a cross-linking anti-IgE serum) and an antigen-specific activator (ovalbumin) (see Sect. 2.3).

3.3 RIA of IgE levels in vaccinated and control animals

To study the influence of the vaccination on total IgE levels, blood samples were taken every 3 weeks during the vaccination program from each of the eight rats. The first blood sample was taken 1 day prior to the initial vaccination. Total IgE levels were measured by an RIA. The levels of IgE in plasma drastically decreased in the vaccinated rats (Fig. 3). As expected, Freund's adjuvant alone also reduced the IgE levels in the sensitized animals (possibly due to IFN-γ production caused by the adjuvant). However, in all the blank-vaccinated animals the IgE levels were nevertheless significantly higher than in the vaccinated animals, in which IgE levels were below the limit of detection within 6 weeks of vaccination.

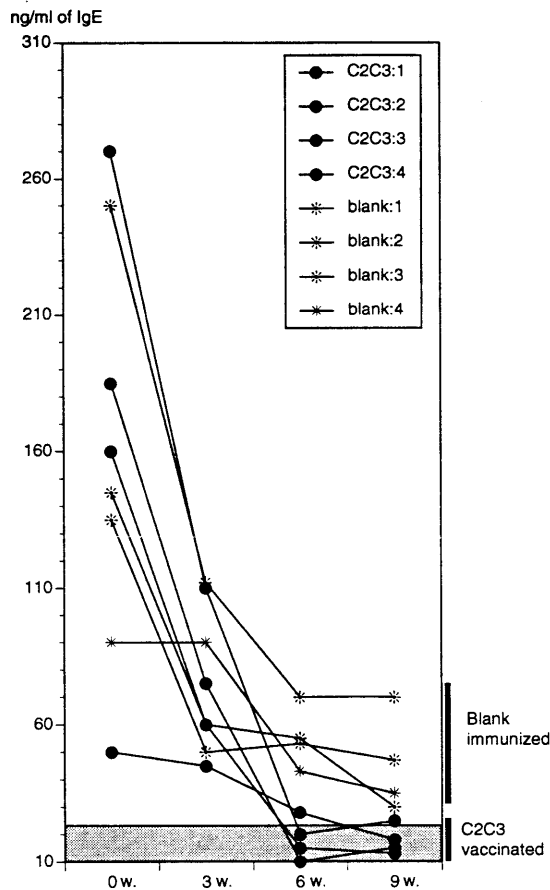


Figure 3. Effect of the allergy vaccination on total IgE levels in a panel of eight different rats. The total IgE levels were measured by an RIA with purified rat IgE diluted in horse serum as standard. The gray zone at the bottom of the Fig. shows the detection limit of the RIA. This was approximately 10–20 ng/ml when analyzing 100 μ l of rat serum.

3.4 Assay of skin reactivity in vaccinated and control animals

To study the effect of the vaccination on the inhibition of an IgE-mediated inflammatory reaction the animals were analyzed 2 weeks after the third injection by i.d. injections either with anti-IgE antisera (four separate injections in each animal) (Figs. 4 and 5) or with ovalbumin in PBS (Figs. 4 and 6), followed by an i.v. injection of Evans blue (see Sect. 2.5). The size of the Evans blue zones are proportional to the extent of induced inflammation. Injection of pure PBS, which was used as a control, gave no measurable blue zone. The polyclonal anti-IgE antiserum and the ovalbumin gave rise to large blue zones, due to the fact that the antibodies or the allergen cross-linked receptors on the surface of mast cells in the skin. This cross-linking causes an intensive granule release, and the material released from the mast cells increases the permeability of blood components including the injected Evans blue, thus facilitating their entry into the skin.

A nearly complete suppression of the IgE-mediated inflammatory response was seen after vaccination with the rat allergy vaccine, indicating that practically no IgE was present on the surface of mast cells in the skin of the vaccinated rats (Figs. 4–6).

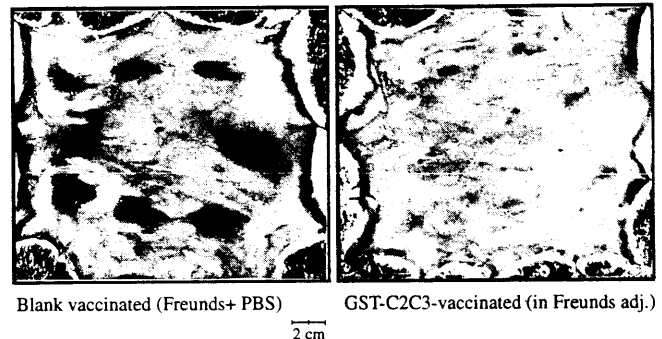
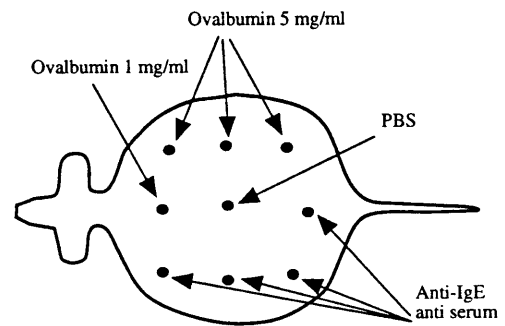


Figure 4. Analysis of reactivity against a polyclonal anti-IgE antiserum and a specific allergen (ovalbumin) in the skin of Wistar rats. The upper part of the Fig. shows a schematic presentation of the injection pattern of the skin of the rats used in the study to analyze the potential of the vaccine to suppress an IgE-mediated inflammatory reaction. The lower panels show the result from two rats, one vaccinated with the GST-C2C3 fusion protein in CFA and one rat injected with Freund's adjuvant and PBS alone (control). The experiment was performed as described in the text.

3.5 Mast cell numbers in vaccinated and blank-immunized animals

To study the number of mast cells in the tissue of vaccinated and control animals, skin samples were taken and analyzed by Alcian blue staining. The concentration of mast cells was found to be almost identical for both groups of animals (data not shown). In addition, four additional sensitized rats were used as positive controls for the presence of mast cells in both vaccinated and control rats by a simultaneous injection of rat IgE and the anti-IgE antiserum. Both vaccinated and blank-immunized rats showed a similar reaction after Evans blue administration (data not shown). This shows that the lack of reactivity in the skin was not due to the absence of mast cells in the skin of the vaccinated animals.

4 Discussion

I describe a novel approach for the reduction of total and receptor-bound IgE in an experimental animal, a procedure which may have the potential for use in the treatment of atopic allergies in man.

The vaccination programme was maintained for 10 weeks before assaying the reactivity since even very small residual amounts of IgE bound to mast cells are sufficient to induce mediator release. Just a few cross-linked receptors are

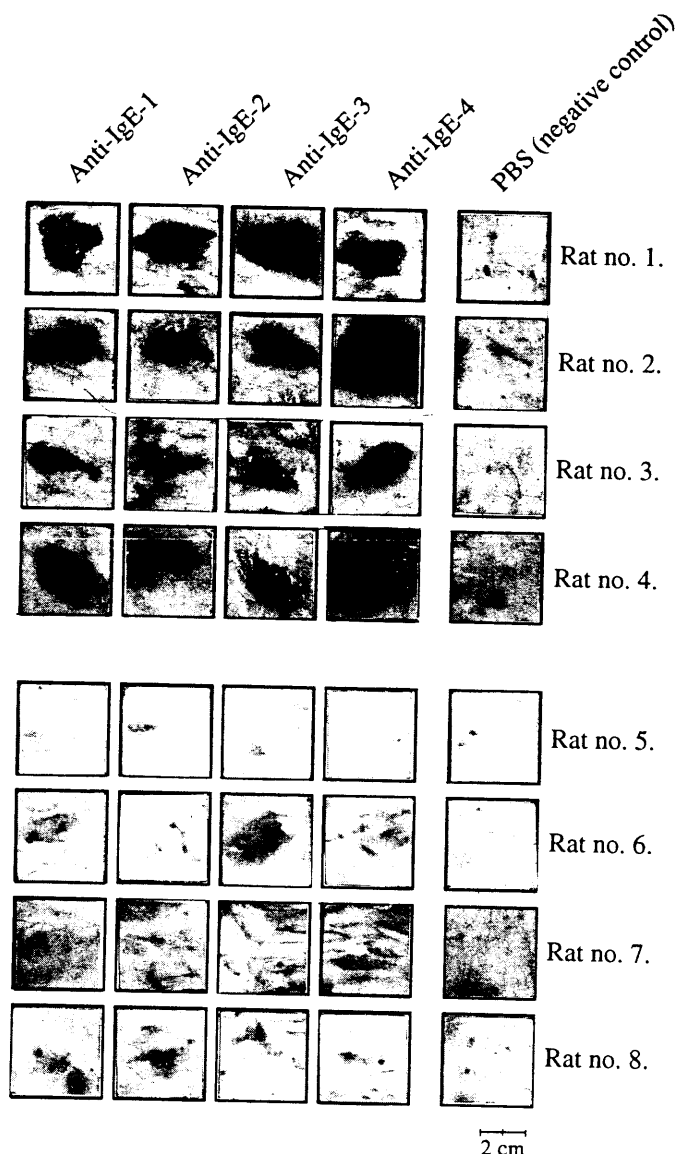


Figure 5. A study of the suppression of an anti-IgE-mediated inflammatory reaction by the anti-allergy vaccine in eight different rats. The experiment was performed as described in the text. Rats nos. 1–4 were blank-immunized (Freund's adjuvant + PBS); rats nos. 5–8 were immunized with rat GST-C2C3 fusion protein in Freund's adjuvant. To appreciate the size of the blue zones a scale bar is shown in the bottom of the figure.

sufficient for activation of nearly complete release of the entire pool of granules stored within a mast cell. In addition, the half-life of receptor-bound IgE has been estimated to approximately 14 days. Even if we could block the IgE production completely from the first day of treatment, the remaining receptor-bound IgE might still be sufficient to activate mast cells for several months. This is due to the fact that mast cells have a relatively high number of receptors for IgE, estimated as 3×10^4 – 3×10^5 /cell, and that a large percentage of these receptors are occupied by IgE. The nearly complete inhibition of the sensitivity to both a polyclonal mast cell activator and a specific allergen found here strongly indicates that the free and the receptor-bound pools of IgE in these animals are virtually non-existent. All the control rats injected with Freund's adju-

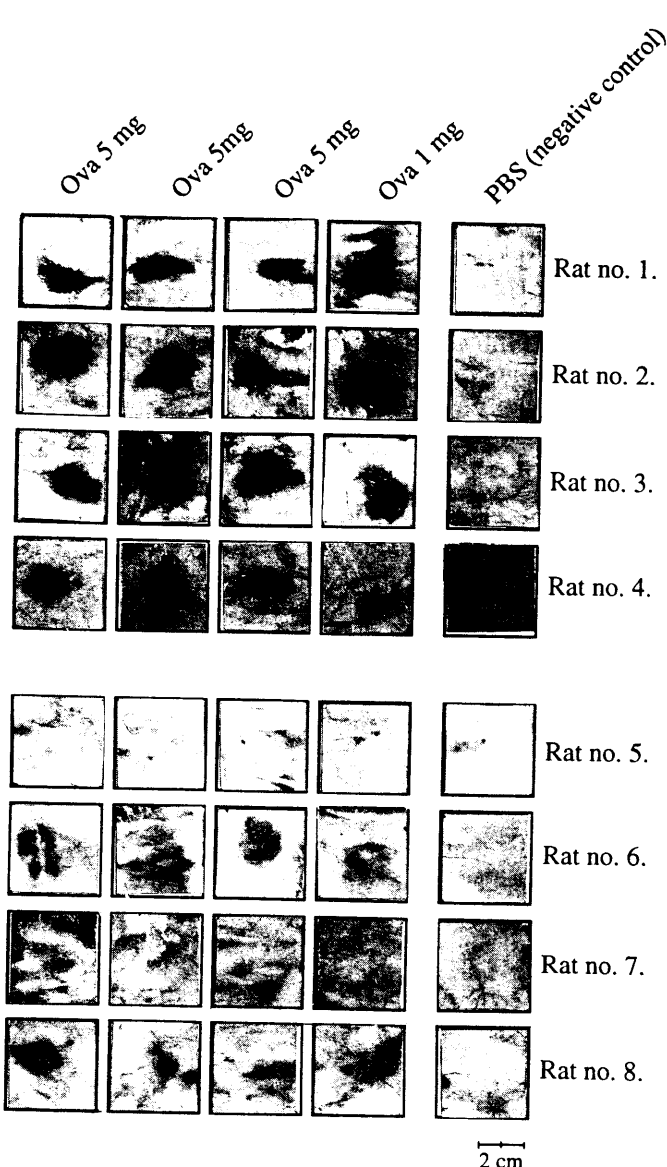


Figure 6. A study of the suppression of an allergen (ovalbumin)-mediated inflammatory reaction by the anti-allergy vaccine in eight different rats. The experiment was performed as described in the text. Rats nos. 1–4 were blank-immunized (Freund's adjuvant + PBS); rats nos. 5–8 were immunized with rat GST-C2C3 fusion protein in Freund's adjuvant.

vant and PBS responded strongly to both the anti-IgE sera and the specific allergen (Figs. 4–6). This shows that, even if the Freund's adjuvant down-regulates the total IgE levels, the adjuvant has not by itself the capacity to block the response to an anti-IgE antiserum or to a specific allergen.

The inflammatory reaction induced by a specific allergen may to some extent be caused by an IgG-, IgM- and complement-dependent mechanism. This is due to the generation of small amounts of the anaphylotoxins C3a and C5a. The allergen-induced inflammation actually showed some weak residual activity in two out of four rats (Fig. 4–6). The sensitization protocol based on weekly injections of a specific allergen and ricin has been shown to

increase the levels of both IgE and IgG [11]. The IgG levels are not, however, reduced by the allergy vaccination, so the residual activity seen in Fig. 6 is probably caused by the IgG anti-ovalbumin antibodies still present in the circulation.

Several other alternative methods for treatment of atopic allergies in man are presently under investigation in various laboratories. An alternative approach to reduce allergen sensitivity is the injection of anti-IgE mAb into the allergic patient [12]. However, this strategy has several disadvantages compared to the present vaccination protocol, since monoclonal antibodies from mouse or even "humanized" antibodies contain multiple new epitopes to which the human immune system has never been exposed. Antibodies directed against these new epitopes or an anti-idiotypic response may result in very severe immune complex formation. To avoid these complications a large panel of different monoclonals may have to be used and a careful program for successive administration of these monoclonals during the treatment would have to be maintained.

Dr. Stanworth and his collaborators have for a number of years been working on a peptide vaccine based on the use of a ten amino acid peptide from an internal region of the CH4 domain of human IgE. This peptide has been coupled to a heterologous carrier protein and used as an antigen. However, no detectable anti-IgE or anti-peptide titers have, in their experiment been detected by ELISA in rats, even when using a non-species-specific peptide [13]. For a successful vaccination it is probably essential to obtain a strong immune response which reacts predominantly with the native IgE molecule (surface epitopes). This is required to achieve efficient competition with the IgE receptor for free IgE, as the interaction between the IgE molecule and its specific receptor is very strong (2.6×10^{-10} [14]). As seen from the present study, the anti-IgE antibodies produced after vaccination are very efficient in competing with the IgE receptors for free IgE and have, after 10 weeks of treatment, nearly completely blocked the responsiveness of mast cells to both a cross-linking anti-IgE antiserum and to the specific allergen, indicating that the anti-IgE response induced in the animal is capable of efficiently competing with the IgE receptors for free IgE and may even remove IgE which is already bound to its receptor.

Using only the CH2-CH3 domains, the resulting anti-IgE antibodies react predominantly with regions involved directly in receptor binding. This reduces the risk of generating cross-linking anti-IgE antibodies. In addition, the anti-IgE antibodies are continuously produced during the immune response to the vaccine and the concentration of these antibodies builds up slowly in the blood. The newly formed antibodies slowly reduce the free plasma pool of IgE so that no sudden change in concentration of anti-IgE

antibodies occurs, thus preventing the occurrence of life-threatening immune complex formations. Compared to the strategy of using monoclonal antibodies in allergy treatment, this last feature is a great advantage.

In the present study no deleterious side effects have been observed, even in rats which had highly elevated IgE titers prior to vaccination. Also, this type of vaccination procedure is directed against the entire free pool of IgE and is, thereby, not limited to a specific allergen. The vaccine, therefore, has the potential of being used in man for the treatment of a large variety of different atopic allergies.

I would like to thank Maria Aveskog for skilful technical assistance and for fruitful and stimulating discussions, Alkvin Wanders for preparing tissue sections and for performing Alcian blue staining to evaluate mast cell numbers, and Stanley Burnett for linguistic revision of the manuscript.

Received October 4, 1993; in revised form October 30, 1993; accepted November 4, 1993.

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