

Comparative Studies on Tree Pollen Allergens

XI. Trials on the Regulation of IgE Response in Mice Using Modified Birch Pollen Allergens

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Abstract. The major allergen of birch pollen, BV45, was isolated and conjugated to 2-*o*-methoxy polyethylene glycol-4,6-dichloro-5-triazine (mPEG). The molecular-weight variant of 6,000 daltons of the activated mPEG was used in an antigen-specific regulation of IgE response. In these studies 200 genetically high IgE responders (CBA/Ca) female mice were intradermally immunized by the purified BV45, with the use of various adjuvants.

Four different sets of experiments were made. In the first experiment 50 mice were immunized on days 1 and 15 with BV45. This was followed by another booster dose of the native BV45 or the modified mPEG-BV45 in Freund's incomplete adjuvants (FIA) on day 72. In the 2nd experiment, a similar procedure was performed except that booster doses were injected on day 135. Subsequently on day 136, BV45/FIA was intradermally injected. In the 3rd and 4th sets of experiments, 2 groups of 50 mice were daily immunized by intraperitoneal injections of 10 successive doses of BV45. On day 33 a dose of BV45 or mPEG-BV45 respectively, was intraperitoneally injected in each mouse. This injection was followed by other 10 successive doses of BV45. In experiment 3 no adjuvants were used, and in experiment 4 aluminium hydroxide gel was used as adjuvant.

The mice immune response was assessed by analyses of the serum IgG and IgE levels. In all experiments higher IgG concentrations were shown for the immunized mice as compared to the non-treated control animals. Administration of booster doses of BV45 or mPEG-BV45 induced increases in the IgG levels. As shown in experiments 3 and 4, the mPEG-BV45-treated animals gave higher IgG responses than those treated with native allergens. Although the results of the experiments did not show a prominent decrease of IgE, we do not exclude the possibility that modified birch pollen allergens could be superior in hyposensitization therapy. The results obtained suggested within certain limitations that modified birch pollen allergens gave better results in modulating the IgG and IgE synthesis in mice as compared to the non-modified allergens. These observations are partially in agreement with papers earlier published on mPEG-modified allergens from honey bee venom, grass pollen and various tree pollens by other authors.

Introduction

Immediate type hypersensitivity reactions to allergens from environmental sources, such as pollen, are associated with the synthesis of specific IgE and IgG antibodies. Humoral IgE has a unique affinity to bind tightly but reversibly to Fc receptors on the outer membrane of mast cells and basophil granulocytes. The interaction of a polyvalent allergen molecule with 2 adjacent IgE molecules causes the release of pharmacologically vasoactive mediators responsible for the pathophysiological manifestations of allergy

[29]. Immunotherapy of allergic diseases by inducing gradually increasing doses of allergens results in an elevated level of IgG and usually a decreased IgE concentration in serum, with the subsequent relief of symptoms [18, 23]. This experience could not be reproduced for all experiments. Recently, no correlation was found between the severity of the symptoms and Ig titers [22]. The theoretical basis of the mechanisms of the immuno-modulation reactions yet remains uncertain in spite of the large number of papers published on hyposensitization therapy [25].

Seasonal allergic manifestations caused by birch

pollen allergens are common among North European populations, and immunotherapy is a part of the daily practice in allergy clinics. Chemically modified allergens have been shown useful in hyposensitization therapy [2, 16, 26–28, 31]. Unlike the native molecules, they are used for hyposensitization treatment with less risks of unwarranted effects [20, 21, 25]. Chemical alterations of the allergens by covalent binding to polyethylene glycol (PEG) was demonstrated in ragweed [16, 31]. The modified ragweed molecule has also been studied in animal models [30, 39], and recently methoxy-PEG (mPEG)-modified tree and grass pollen allergens have been tested and suggested for clinical use [38].

The isolation and purification of the major allergen from birch pollen have been reported [32, 33]. The copolymerization of birch pollen major allergen (BV45) with mPEG of 6,000 daltons, and the investigation of the immunochemical properties of the reaction products, were examined in a previous publication in this series [34]. In another recent publication, mPEG coupled to honey bee venom (HBV) was used to examine the Ig response in CBA/Ca female mice as compared to uncoupled HBV. By the use of particular immunization schedules and adjuvants, a suppression of IgE response was achieved while the IgG levels were either slightly enhanced or unchanged. The results were encouraging for further trials of the modified HBV in human systems [2]. It was therefore incentive to examine the honey bee model using modified birch pollen allergens.

Material and Methods

Antigen Preparation

The purified birch pollen major allergen (BV45) was isolated and purified from the aqueous crude pollen extract [33]. The mPEG-6,000-daltons modified allergen was prepared according to the procedure reported in an earlier communication [34]. The end product mPEG-6,000-daltons BV45, was composed of 19% dry weight BV45 and 81% mPEG-6,000 daltons as determined from the amino acid compositions and protein analysis [5, 8].

Animals

Two hundred CBA/Ca female mice, apparently healthy and more than 15 g each, were supplied by Anticimex, Stockholm. The animals were placed in groups of 7 mice in separate cages at the Animal Department, University of Bergen, and were fed on laboratory animal pellets and water.

Immunization Schedule

IgG and IgE titers in mice sera were measured during 4 separate

experiments, each including 50 animals. The experiments were numbered 1, 2, 3 and 4.

Experiment 1

Freund's incomplete adjuvant (FIA) was used. Each of 50 mice were subcutaneously immunized on day 1 with 10 μ g BV45 in 50 μ l emulsion of equal volumes of saline and FIA. Subcutaneous injections were repeated using 1 μ g BV45 in the same medium on day 15. Finally, booster doses of 10 μ g BV45 or 10 μ g mPEG-BV45 were intraperitoneally injected on day 72. Blood samples were collected by heart puncture under anesthesia at days 1, 72, 79 and 86.

Experiment 2

All mice were injected with 10 μ g and 1 μ g BV45 on days 1 and 15, respectively, using FIA emulsion. On day 135, 10 μ g BV45, 1 μ g mPEG-BV45 or 10 μ g mPEG-BV45 were given intraperitoneally to the 3 different groups (7 mice in each group). On day 136 all the animals were injected with 1 μ g BV45. Blood samples were collected on days 1, 135, 143 and 150.

Experiment 3

In this experiment no adjuvant was used. The mice were subcutaneously injected 10 times on successive days 1–5 and 8–12 by 100 ng BV45 in 50 μ l of physiological saline solution. On day 33, 10 μ g and 100 μ g of mPEG-BV45, respectively, or 100 ng BV45 were subcutaneously injected to 3 different groups of animals. The procedure was followed by another 10 successive BV45 injections for all the animal groups included, each of 100 ng (days 36–40 and 43–47). Blood samples were collected on days 1, 15, 33 and 50.

Experiment 4

The conditions of experiment 3 were repeated using aluminium hydroxide [Al(OH)₃] gel as adjuvant.

Isolation and Radiolabeling of Immunoglobulins from Rabbit Antiserum

Lyophilized goat anti-mouse IgE serum (GAM) was supplied by Nordic Immunological Laboratories, The Netherlands. The antibodies were reconstituted to the initial volume of 2 ml. The isolation of the IgG/IgA goat anti-IgE was performed according to previously described procedures [12] using ammonium sulphate precipitation. A column of DEAE-Sephadex A-50, dimensions 1 $\times$ 30 cm, was equilibrated with 25 mM sodium acetate before use. The void volume peak, representing the IgG and IgA fractions, were collected, dialyzed against 0.1 M NaCl, lyophilized and stored at -20°C until used.

Bolton and Hunter reagent was used for the radiolabeling and iodination of proteins. The reagent was supplied by Amersham International Ltd., London, UK, and the coupling procedure described by Bolton and Hunter [4] was followed. An amount of reconstituted GAM serum corresponding to 100 μ l, was added to 37 MBq of the iodinated reagent. Desalting of the radiolabeled proteins was performed on a Sephadex G-25 Fine column, LKB, Sweden, dimensions 1 $\times$ 30 cm, equilibrated and eluted with borate buffer, 0.05 M at pH 8.5. The radiolabeled immunoglobulin fraction was eluted in the void volume fractions which were collected and stored at 4°C until used.

Sheep Anti-Mouse IgG

The iodinated ^{125}I sheep anti-mouse IgG was supplied by Amer-

sham, and an initial activity corresponding to 50,000 cpm was utilized.

Quantitation of IgG

The method described by Djurup et al. [7] was followed with minor modifications. Nylon balls, diameter 6.4 mm, were obtained from Precision Plastic Ball Co. Chicago, Illinois, and used for the antigen coupling [7, 15]. For the antigen coating, 1,000 activated nylon balls were incubated in 25 mg BV45 dissolved in 300 ml phosphate-buffered saline (PBS) pH 7.2 for 4 h at room temperature. The collected serum samples were diluted 1:5 with freshly prepared incubation buffer. The antigen-coated nylon balls were individually placed in plastic tubes (dimensions 10 $\times$ 50 mm). Aliquots of 0.2 ml diluted serum were added, and the tubes were incubated for 3 h at 37°C without shaking. To each tube 50 ng ^{125}I -labeled anti-mouse IgG (250 kBq/ μ g) was added in 1 ml incubation buffer (calculated to give 50,000 cpm). All the sera were tested in triplicates and the mean values corrected for the background and unspecific-binding counts. The nylon balls were thoroughly washed, transferred to fresh tubes and finally counted for 1 min in a gamma scintillation counter (NE 1600, Nuclear Enterprises Ltd., UK).

Quantitation of IgE

A modified RAST system using nitrocellulose paper discs as the solid phase, 6 mm in diameter with pores of 0.2 μ m, were used as a modification of procedures described earlier [3, 13, 35, 36]. The discs were incubated with approximately 1 μ mol BV45 solution in Tween/PBS, pH 7.4, for 3 h at room temperature while shaking. They were dried for 1 h at room temperature and incubated at 37°C in 3% bovine serum albumine (BSA) in PBS. Single discs were separately placed in each of the plastic tubes. They were thoroughly washed with 0.5% Tween 20 and 0.05% BSA in PBS, then transferred to fresh tubes and further incubated with 25 μ l of 2-fold diluted mice serum in PBS for 5 h at room temperature while shaking. Washing was repeated, and 50 μ l ^{125}I anti-IgE corresponding to 5×10^4 cpm added to each tube for an incubation period of 16 h. Uncoupled excess anti-IgE proteins were then removed by washing and the total counts per minute estimated and corrected for unspecific binding to the NC discs and background [3, 6].

Results

Estimations of IgG Values

In experiment 1 (fig. 1), the sera of the mice immunized at days 1 and 15 were collected and assayed at day 72. The estimated IgG results showed relatively increased levels. The analysis performed at day 79 showed a further increase in IgG. The animals injected with BV45 at day 72 showed the highest rise in IgG as compared to those immunized with mPEG-BV45. The IgG values measured on day 86 showed gradual declines until reaching the initial preimmunization levels. At this point the animals with the lowest estimated values were those injected with native BV45 on day 72. In other words, the animals injected with

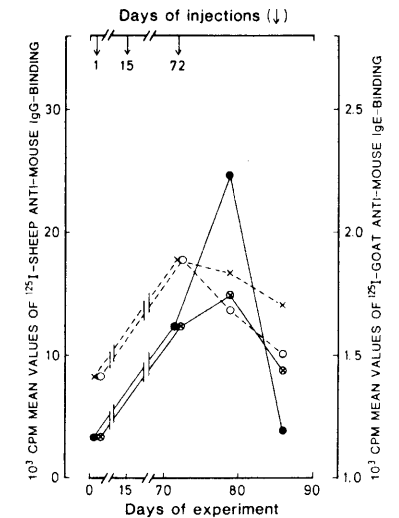


Fig. 1. A combined illustration of the IgG and IgE values obtained from the analyses of sera in experiment 1. In all figures, unbroken lines represent IgG and broken lines IgE. ● = IgG level using allergen BV45; ⊗ = IgG, 1 μ g mPEG-BV45; ○ = IgE level using BV45; × = IgE, 1 μ g mPEG-BV45.

BV45 produced the highest IgG response as compared to mPEG-BV45. The same animals have also shown the most rapid decline of antibody concentration until reaching the basic preimmunization levels.

The results from experiment 2 showed an increase in the IgG levels until day 135 (fig. 2). The elevation was valid for all the 3 tested groups of animals. At the same day, a booster injection of BV45 or mPEG-BV45 was given. On day 136, injections of 1 μ g BV45 were given to all the animals. Consequently a general increase in the IgG levels was found on days 143 and 150. The native BV45 gave the highest IgG values estimated on day 150.

In experiment 3 an increase in the IgG levels following the first 10 successive BV45 injections was measured. Further increases in the IgG levels were estimated on day 33, on which the mice were treated with native or mPEG-BV45. All the mice were further injected with 10 daily successive injections of BV45. All 3 animal groups showed an increase in the estimated IgG levels. The mice treated with mPEG-BV45

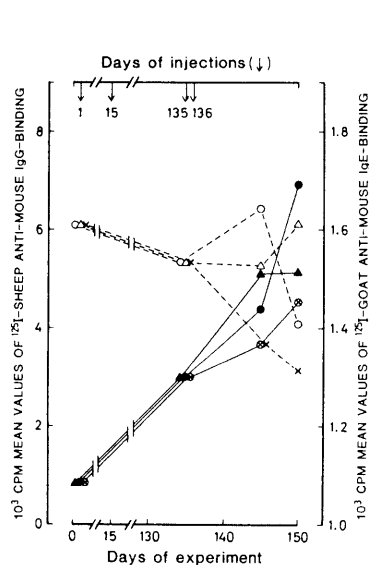


Fig. 2. Similar figure to the above one showing a combined illustration of the IgG and IgE values obtained in experiment 2. Symbols as in figures 1 and 3.

on day 33 responded with the highest IgG increases measured (fig. 3).

Experiment 4 has further confirmed the elevation of the IgG level for the BV45-injected animal groups. On day 33 the IgG values were lower than those of the control animals. After a 2nd period of 10 daily injections, an increase in IgG levels could clearly be observed, most dominant for the 2 animal groups preinjected with 10 or 100 μ g mPEG-BV45 (fig. 4).

Estimation of Serum IgE Values

The results obtained in experiment 1 on day 72 showed elevated IgE levels for all the treated animals. The values were thereafter decreased for both the native-pollen- or the mPEG-treated animals. The lowest values were found following the administration of 1 μ g native BV45 on day 72 (fig. 1).

In experiment 2 the IgE levels decreased after 2 injections of 10 and 1 μ g native BV45 on days 1 and 15, respectively. The 3 different allergens were injected

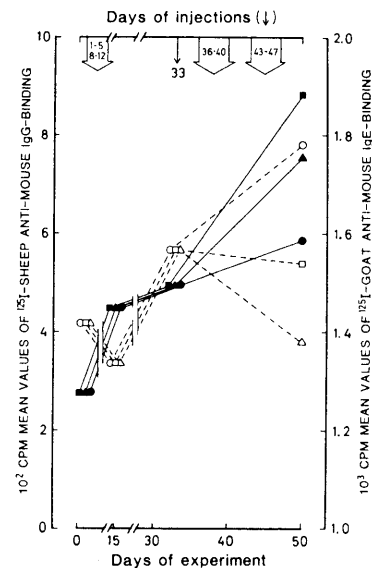


Fig. 3. The cpm levels of both IgG and IgE illustrated from experiment 3. ● = IgG, BV45; ■ = IgG, 100 μ g mPEG-BV45; ▲ = IgG, 10 μ g mPEG-BV45; ○ = IgE, BV45; □ = IgE, 100 μ g mPEG-BV45; △ = IgE, 10 μ g mPEG-BV45.

on day 135. Thereafter, 1 μ g native BV45 was injected in all the animals on day 136. A transient increase in the IgE level was measured on day 143 in the animals treated with BV45. This was followed by a decline in 2 animal groups on day 150. Injections of 10 μ g mPEG-modified allergens on day 135 gave no changes in the IgE levels, while 1 μ g mPEG-BV45 induced a reduced IgE level as estimated 1 and 2 weeks after these injections. Injection of 1 μ g mPEG-BV45 induced the lowest estimated IgE values as analysed on day 150 (fig. 2).

The animals in experiment 3 were given 10 daily successive injections of 100 ng BV45. This has reduced the IgE values on day 15. On day 33 the IgE values were increased 2-fold. Furthermore, the animals were treated with 10 μ g mPEG-BV45 followed by another 10 daily injections of 100 ng native BV45 given on days 36-40 and 43-47 (fig. 3). The IgE levels estimated on day 50 were decreased for the mPEG-pre-treated animals.

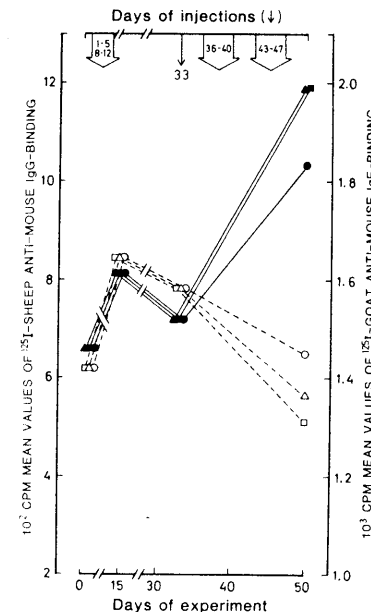


Fig. 4. The result of the IgG and IgE analyses for experiment 4. Symbols identical to figure 3.

In experiment 4 the IgE levels were raised during the first 15 days, after 10 injections of 100 ng BV45; the increases were measured on days 15 and 33. From day 33 on, a decreased IgE level was observed for all the tested animal groups. The lowest IgE values were seen following the injection of mPEG-BV45-modified allergens (fig. 4).

Discussion

Specific immunoglobulin responses during hyposensitization therapy have been studied using several grass pollen and tree pollen extracts. Relationships between the observed relief of allergy symptoms and changes in the IgG and IgE levels during immunotherapy have been reported [1, 10, 18]. The correlation between clinical effects, Ig titer and the injected amount of allergens remains a subject of debate [22, 24].

The increase in specific IgG antibodies under hyposensitization treatment has been suggested to be the contributing factor for the relief of symptoms [10, 19]. However, in other reports the increase in IgG failed to be shown in patients with only pollen allergy which were pretreated with purified allergen extracts. Furthermore, the occurrence of anti-IgG antibodies did correlate neither to relief of symptoms nor to side effects during treatment [22, 37].

The estimated IgE levels during different schedules of hyposensitization therapy have given more divergent observations. In one presentation alum-adsorbed yellow-jacket venom (YJV) caused an increased IgE antibody level in the sera of 9 out of 10 patients, while non-bound aqueous YJV gave unchanged levels when tested 6 months after starting the therapy [6]. By the use of mPEG-modified ragweed allergens in patients with rhino-conjunctivitis, correlations between an increase in ragweed-specific IgG antibodies and relief of symptoms have been estimated [11, 14]. Nevertheless, no correlation between the IgG increase and decrease in IgE levels could be found in ragweed patients treated by mPEG-modified allergen extracts [15]. This is different from the experience in patients hyposensitized with non-modified allergen extracts [9, 17].

Several trials have been made for the production of modified allergens with the same immunogenicity and lower allergenic reactivity than the native products. An mPEG-modified allergen preparation of a mixed tree pollen extract has recently been produced and commercially introduced for clinical immunotherapy on tree pollen-allergic patients [38]. Polymerization of the major allergen of birch pollen, BV45, with methoxy-polyethylene glycol has been reported in a previous paper [34]. In this paper, an experimental animal model for testing the antibody-response using birch pollen extracts under different schedules of administration and adjuvants was examined. The results obtained showed clear increases in IgG synthesis in the CBA/Ca mice as induced by minute doses of native birch pollen allergens (table I). The administration of the native or mPEG-modified birch pollen allergens to the 3 groups of mice during a period of 135 days induced an increase in specific IgG synthesis for all the animal groups tested. The high IgG titers were retained for at least 14 days after the injection of an antigen booster dose. Furthermore, the mPEG-modified allergens induced higher IgG responses than BV45. The administration of mPEG-BV45

Table 1. A concluding table summarizing the data obtained from the four experiments performed; the amounts and types of antigens used and the corresponding IgE-response are shown

Experiment, No.	Antigen	Doses μ g	Adjuvant	IgG	IgE
1	A	10, 1, 10	FIA	+	-
1	B	10, 1, 10	FIA	+	-
2	A	10, 1, 10, 1	FIA	+	+
2	B	10, 1, 1, 1	FIA	+	-
2	B	10, 1, 10, 1	FIA	+	-
3	A	0.1 $\times$ 10, 0.1, 0.1 $\times$ 10	0.9% NaCl	+	+
3	B	0.1 $\times$ 10, 10, 0.1 $\times$ 10	0.9% NaCl	+	-
3	B	0.1 $\times$ 10, 100, 0.1 $\times$ 10	0.9% NaCl	+	0
4	A	0.1 $\times$ 10, 0.1, 0.1 $\times$ 10	Al(OH) ₃	+	-
4	B	0.1 $\times$ 10, 10, 0.1 $\times$ 10	Al(OH) ₃	+	-
4	B	0.1 $\times$ 10, 100, 0.1 $\times$ 10	Al(OH) ₃	+	-

All the injected animal groups responded with an increased IgG-synthesis while the IgE levels were reduced after these treatments except for those injected by native BV45 in FIA in experiment 2 and in 0.9% NaCl in experiment 3. A = BV45; B = mPEG-BV45.

caused decreases in serum IgE level in these experiments.

The use of different adjuvants other than incomplete Freund adjuvant seemed to have a small influence on the estimated IgE levels. In experiments 1 and 2, FIA induced a slightly higher increase in the serum IgG levels than the control or Al(OH)₃ gel. However, the IgE levels were similarly decreased after the various immunization regimes.

Although the results of the above experiments did not show a prominent decrease of IgE, we do not exclude the possibility that modified birch pollen allergens could be superior in hyposensitization therapy. This conclusion is justified by the analyses data of IgG and IgE levels in the described model. The results obtained from this study suggested within certain limitations that modified birch pollen allergens gave better results in modulating the IgG/IgE synthesis in mice than the non-modified allergens. Other studies will be necessary to indicate whether the similarity between the immune response in rodents and man allow to derive conclusions regarding the practical benefits of the experimental model used.

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