

Comparative Studies on Tree Pollen Allergens

VII. Monomethoxy Polyethylene Glycol Conjugation of the Major Allergen of Birch Pollen (*Betula verrucosa*)

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Abstract. The major allergen of birch pollen (BV45) was conjugated to 2-0-methoxy polyethylene glycol-4,6-dichloro-5-triazine (mPEG). Three molecular weight variants of 4,000, 6,000 and 20,000 daltons, respectively, of the activated PEG were used. The modified preparations were labelled by ^{125}I and both the native and the radiolabelled protein conjugates were purified by gel filtration chromatography. The relative molecular weights of the purified two peaks were preliminarily estimated by high performance liquid chromatography (two populations of $\approx 100,000$ or 20,000 daltons). The amino acid composition of the acid hydrolysates of the three conjugated proteins indicated that 5 residues of lysine were modified by mPEG. Other charged amino acid side chains could also be bound to the activated PEG. The immunochemical properties of the copolymers were studied. The immunogenicity and antigenicity were examined by immunizing rabbits with ^{125}I -mPEG 4,000 daltons BV45 and ^{125}I -mPEG 20,000 daltons BV45 and subsequently crossed immunoelectrophoresis. The clearance of the radiolabelled protein showed normal pattern. A sharp decline in the radioactivity could be measured. At days 10-12, the remaining radioactivity was below 2%.

Preliminary studies showed that the modified proteins were immunogenic in rabbits. The findings were demonstrated by crossed immunoelectrophoresis of the ^{125}I -mPEG 4,000, 6,000 or 20,000 daltons BV45 and the corresponding autoradiographs. Apparently, the immunogenicity and antigenicity of the preparations were qualitatively unaltered. The allergenicity of the modified preparation was measured in vitro by RAST and RAST inhibition. The results indicated that the allergenic reactivity of the preparation was reduced by at least 25%. Similar results were shown in vivo by skin prick test (SPT) and PK technique. The reduced allergenic reactivity is likely to be attributed to masking of the immunoreactive determinants of the allergen by attachment to an inert net of polyethylene glycol.

These studies showed that conjugation of the major allergen of birch pollen (BV45) resulted in a reduction of the allergenicity. Although the immunogenic and antigenic reactivities seemed qualitatively unaltered, a similar reduction could not be excluded. The data provide an experimental model for the modification of tree pollen allergens, suitable for further application on the manipulation of antibody synthesis in animals.

Introduction

Chemically modified allergens were shown to be useful in immunotherapy of allergy. The ideal benefit of these allergens is their selective suppression of IgE synthesis and enhancement of that of other Ig classes [Marsh et al., 1970, 1981; Norman et al., 1982; Patterson et al., 1974, 1978; Ahlstedt et al., 1983]. Chemical alterations of allergens by covalent binding to polyethylene glycol (PEG) was demonstrated in ragweed

antigen E. The modified molecule has since been a subject of intensive studies in clinical allergy and in animal models [King et al., 1979; Sehon et al., 1981; Lee and Sehon, 1978a, b]. During hyposensitization therapy of allergic diseases, the relief of symptoms is usually accompanied by relative decrease in reaginic IgE and increase in precipitating antibodies. However, the immunological basis of the mechanisms behind such relief remains uncertain.

Seasonal allergic manifestations caused by birch

pollen allergens are common among North European populations, and immunotherapeutic treatment is a common practice in allergy clinic. So far no chemical modification studies on tree pollen allergens have been reported. The copolymerization of birch pollen major allergen (BV45) with mPEG and the investigation of the immunochemical properties of the reaction products, could provide an adequately modified birch pollen antigen superior in experimental immunotherapy. The aim of the present paper is to establish the experimental conditions for the modification of birch pollen allergen and to investigate the effect of the modification on some of the immunochemical properties.

Material and Methods

Preparation of Birch Pollen mPEG-BV45

Birch pollen BV45 was isolated and purified from the crude birch pollen aqueous extract as described by Vik et al. [1982a, b]. Attachment of 2-O-methoxy polyethylene glycol-4,6-dichloro-5-triazine (mPEG), molecular weight 4,000, 6,000 and 20,000 daltons, respectively, was performed as follows: The purified allergen preparation BV45 (1 µmol) dissolved in 2 ml 100 mmol sodium borate buffer at pH 9.30 was slowly added to mPEG (100 µmol) while stirring. The pH was retained constant for 60 min in a pH stat. The copolymer was dialyzed overnight against three changes of phosphate-saline buffer (75 mmol, pH 7.20) and finally against distilled water. The dialyzate was then lyophilized and stored at -20°C until further use.

Gel Filtration Chromatography

The copolymer mPEG BV45 was fractionated on an Aca-22 column, LKB, Bromma, Sweden, 15 × 1,000 mm, using phosphate-saline buffer, at the same molarity and pH mentioned before. The molecular weights of the copolymerized proteins were preliminarily estimated using a molecular weight marker of globular proteins, as described below.

Iodination of mPEG BV45

Bolton and Hunter reagent for iodination of proteins, N-succinimidyl 3-(4-hydroxy-5-¹²⁵I-iodophenyl) propionate, was supplied by Amersham International Ltd., London, UK and used for the radioactive labelling of 0.5 µmol of BV45 dissolved in 100 µl distilled water as described by Bolton and Hunter [1973]. Preliminary purification of the radiolabelled protein was performed on Aca-54 column, LKB, Bromma, Sweden (15 × 180 mm), using phosphate-saline buffer, 75 mmol at pH 7.5. The iodinated protein resolved at the first eluted peak was dialyzed against distilled H₂O, lyophilized and stored at -20°C until used. The grade of binding of ¹²⁵I to mPEG-BV45 was checked by counting the fractions eluted from an Aca-22 column (molecular weight ranges 10,000–120,000 daltons; 15 × 100 mm) on a gamma scintillation counter (NE 1600, Nuclear Enterprises Ltd., UK). The radioactive protein fractions were treated as previously outlined.

Molecular Weight Estimation

In addition to the molecular weight estimation by conventional gel filtration chromatography, the results were supported by high performance liquid chromatography. This was performed on a silica-based column 'Protein Column, I-125' supplied by Waters Associate, USA, by the method described earlier. A series of marker proteins: bovine serum albumin (67,000 daltons), pepsin (34,000 daltons), trypsin (24,000 daltons), β-lactoglobulin (18,400 daltons), Allergen M (12,328 daltons) [Elsayed and Bennich, 1975], ribonuclease (12,000 daltons) and insulin (6,000 daltons) were used for the molecular weight determination as described previously by Vik et al. [1982a, b].

UV Spectra

Ultra violet spectra of the native and the modified proteins were scanned at 225–340 nm.

Amino Acid Analysis

The acid hydrolysates of BV45 and the conjugated derivatives were analysed by the method described earlier [Elsayed and Bennich, 1975].

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was performed using 7.5% acrylamide and sodium dodecyl sulfate (SDS) at neutral pH according to Weber and Osborn [1969, 1975] and according to O'Farrell [1975]. For the three ¹²⁵I-labelled preparations some of the discs were stained by Coomassie stain while the rest were sliced at 2.5 mm thickness and the radioactivities of the discs were counted. The corresponding peaks which bound the ¹²⁵I-reagent were located and used for further studies of the immunochemical reactivities.

Immunization Schedule

Antibodies against the mPEG copolymers were raised in albino rabbits weighing approximately 3 kg each. Immunization was initiated by the intravenous injection in the lateral ear vein of 5 mg ¹²⁵I-mPEG (4,000, 6,000 and 20,000 daltons) BV45 dissolved in 1 ml physiological saline solution. A supporting dose of 2 mg of the protein conjugate in 500 µl saline buffer 75 mmol, pH 7.2 in 500 µl complete Freund's adjuvant was given intramuscularly in three sites. By the end of experiment each rabbit had received 11 mg protein. Three booster doses were given, the first at 10 days after the primary immunization and then repeated twice at 2-weeks intervals. The animals were bled and sacrificed by cardiac puncture under anesthesia 10 days after the last injection.

Crossed Immunelectrophoresis (CIE), Fused Rocket Immunelectrophoresis (FRIE) and Autoradiographic Preparations (CRIE)

CIE and CRIE were performed on mPEG 4,000 daltons BV45, mPEG 6,000 daltons BV45 and mPEG 20,000 daltons BV45 according to Vik et al. [1982a, b] and Löwenstein [1978]. For autoradiography, the plates were placed on a DuPont Chronex 4 film at room temperature and the set was fitted into an intensifying screen cassette (Siemens Special, calcium tungstate Ca W O₂) for 30 min, 1, 3, 6, 18 and 24 h. Exposed films were developed on a Kodak RP X-Omat automatic processor for 90 s at 35°C by the method described by the suppliers.

Skin Prick Test (SPT) and Passive Cutaneous Transfer Test (PK)

Direct SPT was performed in 2 birch pollen allergic patients with unequivocal clinical history of tree pollen allergy. Both patients showed positive skin reactions of more than one histamine equivalent prick (HEP) [Elsayed et al., 1976], to the two purified birch pollen allergen BV45 at a concentration of 200 µg · ml⁻¹. The selected reaginic sera gave positive RAST (class 2 and 4) with birch allergosorbent. A volunteer tolerating birch pollen was used for control and a sterile physiological saline solution was used as a negative control. The reactions were read and recorded after 20 min and the wheal areas were calculated relative to histamine control. The BV45 and the conjugated proteins were used at similar concentrations of 10 nmol · ml⁻¹.

PK test was performed using a serum from a birch pollen allergic patient and one of the modified preparations. Diluted serum (1 + 5) was injected intradermally (100 µl) to a nonallergic person. A negative control serum was also used. The sites were pricked 24 h after injection with BV45 and mPEG 6,000 daltons BV45.

Radioallergosorbent Test (RAST) and RAST Inhibition

RAST was performed according to the procedures outlined by Yman et al. [1975]. ¹²⁵I-anti-IgE and the birch pollen allergosorbents used for control were purchased from Pharmacia AB., Sweden. Twofold serial dilutions of the allergen BV45 or conjugate mPEG (4,000, 6,000 and 20,000 daltons) BV45 were used, respectively, for the coupling of 5 discs for each dilution. Initial concentrations of 10, 5 or 1 mg · ml⁻¹ were, respectively, utilized to obtain approximately equal molar concentrations of the various preparations (molecular weights 100,000, 20,000 and 10,000 daltons, respectively).

RAST inhibition was performed as described by Apold and Elsayed [1979] and Florvaag et al. [1982]. A serum pool of birch pollen allergic patients was selected after the same criteria described in SPT and PK test. Aliquots of 50 µl of 1 + 1 of the diluted pool was incubated with 100 µl of allergen or conjugate for 4 h at room temperature prior to coupling of the allergosorbents, Pharmacia®, Sweden. The rest of the experimental conditions were run as described by the manufacturer and the relative inhibition was calculated as previously outlined [Apold and Elsayed, 1979].

Results

Gel filtration on Aca-22 column showed two peaks for all the modified proteins, a relatively small one with higher molecular weights in case of 4,000 and 6,000 daltons and a larger peak with a relatively lower molecular weight (fig. 1). The peaks with the higher UV absorption were used for the study of the immunochemical reactivities outlined above. Radioactive labelling of the BV45 protein and the copolymers with Bolton and Hunter reagent was possible at the experimental conditions utilized; desalting of the labelled protein was carried out by gel filtration. One major protein peak and a smaller one of polymorphic isomorphous compounds could be seen. The peak

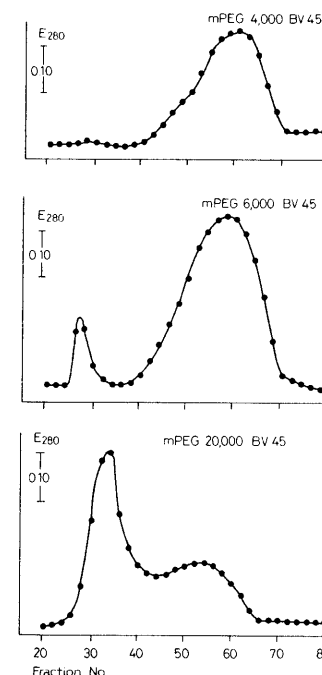


Fig. 1. Elution diagram of gel fractionation chromatography of mPEG 4,000 daltons BV45, mPEG 6,000 daltons BV45 and mPEG 20,000 daltons BV45 on an Aca-22 column. The three peaks with the high UV absorption were pooled and used for the immunochemical reactivities. The peaks with the highest UV absorption were the ones which bound the ¹²⁵I reagent during the labelling procedure. This was shown by counting the radioactivity of the eluted fractions. The free ¹²⁵I was retarded and eluted together with the salt and contaminants peak.

containing the radioactivity was the one with the high UV absorption, salts and rest of free ¹²⁵I were retarded.

Immunogenicity and Antigenicity

FRIE was run on the fractions eluted from Aca-22 gel filtration column on all the modified preparations against birch pollen antibodies in gel. Figure 2a illustrates a representative run performed on ¹²⁵I-mPEG 20,000 daltons BV45, where the antigenic proteins were in the area of fractions 31–57. This area corresponds to peak I and a part of peak II in figure 1c. An

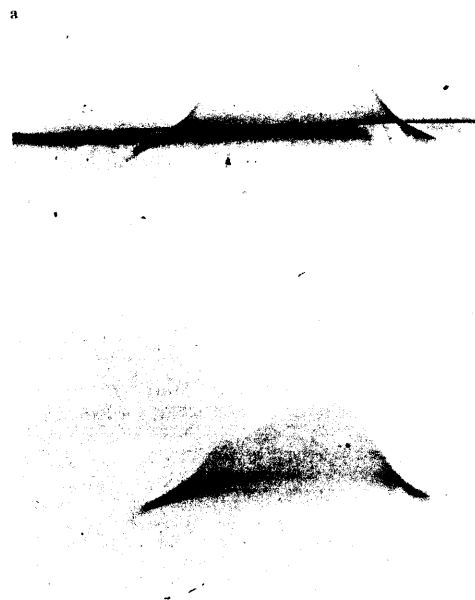


Fig. 2. a Fused rocket immunoelectrophoresis (FRIE) of the fractions of mPEG 20,000 daltons BV45 eluted from Aca-22 column. Fractions were applied (12 μ l) in the wells and the rabbit anti-BV crude pollen extract (800 μ l) was incorporated in the antibody gel. One line of antigenic precipitation could be seen in the area of fractions 31–57. This area is corresponding to the peak with the high UV absorption on the gel filtration column. b An autoradiographic representation of the FRIE in a. ^{125} I-mPEG 20,000 daltons BV45. The plate was placed on the film as described in the methods. The radiostaining in this experiment was visible after 30 min; the figure shows a 3-hour exposure preparation.

autoradiographic registration of this preparation is presented in figure 2b. The antigenic precipitate of the CIE preparation of mPEG 4,000 daltons BV45 was weak as no coomassie staining could be obtained. This precipitate could similarly be visualized by its corresponding autoradiography.

Metabolic Clearance of ^{125} I-mPEG 4,000 Daltons BV45 and ^{125} I-mPEG 20,000 Daltons BV45

The clearance was determined by counting the radioactivity of the blood collected at days 2, 5, 9 and 12. A sharp decline in radioactivity starting from day 5 was observed in the case of the rabbit immunized

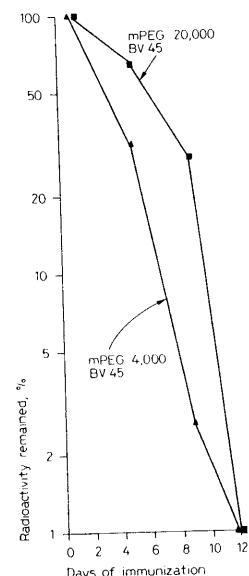


Fig. 3. The metabolic clearance as demonstrated by ^{125} I-mPEG 4,000 daltons BV45 and ^{125} I-mPEG 20,000 daltons BV45 performed on 2 rabbits. The activity declined faster in case of the rabbit immunized by the 4,000 dalton-preparation. A parallel decline was observed for the two rabbits; however, the clearance in case of the animal immunized by the higher molecular weight polymer (20,000 daltons) was at a moderately lower rate.

with mPEG 4,000 daltons BV45 as demonstrated in figure 3. At day 12, the remaining radioactivity was under 1%. The clearance of the mPEG 20,000 daltons BV45 was moderately lower than that of the mPEG 4,000 daltons BV45 preparation. At day 9, approximately 28% of the radioactivity of the 20,000-dalton preparation remained and could be detected in the circulation as compared to approximately 2.5% of the 4,000-dalton preparation.

Radioallergosorbent Test (RAST)

A decrease in RAST uptake was found for the mPEG 4,000 daltons BV45, mPEG 6,000 daltons

Table 1. The values of RAST uptake for the crude extract, BV-spek. (a fraction including the allergenic proteins of birch pollen extract exclusively) [Vik et al., 1982], and two of the modified preparations mPEG 6,000 daltons BV45 and mPEG 20,000 daltons BV45

Protein	Mean highest uptake cpm	Mean percentage of	
		BV crude	BV45
BV crude	15,183	100	—
BV-spek. [Vik et al., 1982]	1,127	74.2	—
BV45	12,334	81.2	100
mPEG 6,000 daltons BV45	7,500	49.3	60.8
mPEG 20,000 daltons BV45	5,000	32.9	40.5

The modified preparations could bind ^{125} I-anti-IgE at a lower level (60.8 and 40.5%) than the value obtained for BV45.

BV45 and mPEG 20,000 daltons BV45 as compared to the unconjugated allergen BV45. The RAST uptake of the crude mPEG 6,000 daltons BV45 and the Aca-22 purified copolymer were examined and were shown to be very similar at lower concentrations (fig. 4). The results in table I showed that at equal concentrations of 50 $\text{nmol} \cdot \text{ml}^{-1}$ of the purified native and modified preparations, a remarkable decrease of binding ^{125} I-anti-IgE was obtained in RAST. Figure 4 showed that at high concentrations a difference in the activities between the crude and the purified preparations was remarkable. At 2-fold dilutions the activities of either preparation were very similar, showing that peak II of the Aca-22 column contained the conjugated BV45 under investigation. The RAST uptake of peak I in the case of mPEG 6,000 daltons BV45 and peak II in the case of mPEG 20,000 daltons BV45 (fig. 1), showed no binding of ^{125} I-anti-IgE and the cpm obtained were equal to the background counts.

RAST Inhibition Test

Preliminary experiments shown in figure 5, demonstrated that mPEG 6,000 daltons BV45 has about 80% inhibition when used at a molar concentration 2-fold higher than BV45. At 1-fold dilution, the inhibition of the copolymer was approximately half that of BV45. At the lowest concentrations used (1.25 and 2.5 nmol , respectively) no inhibition could be measured for either the native or the modified. The results in figure 6 show that high inhibition was obtained by the copolymers at concentrations of approximately

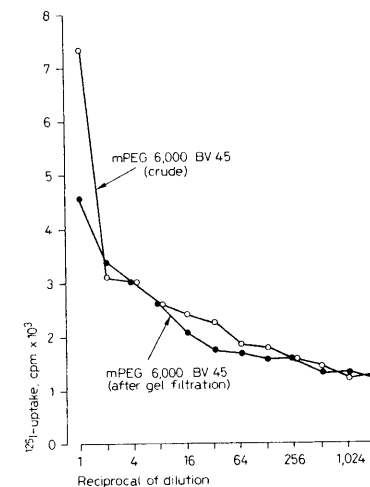


Fig. 4. Results of direct RAST using mPEG 6,000 daltons BV45 before and after gel filtration chromatography. The initial concentration used for the allersorbents was 60 $\text{nmol} \cdot \text{ml}^{-1}$.

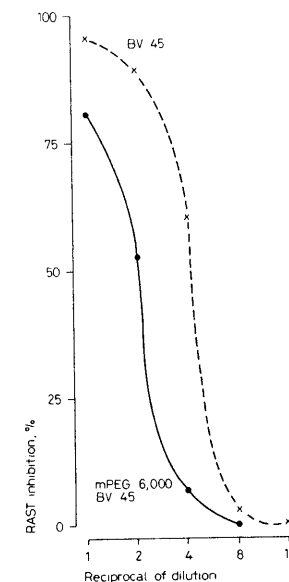


Fig. 5. RAST inhibition of BV45 and mPEG 6,000 daltons BV45 at initial molar concentrations of 10 and 20 nmol , respectively.

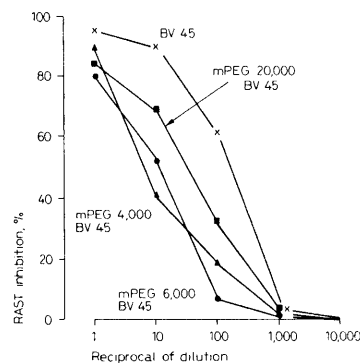


Fig. 6. RAST inhibition of the three conjugates of BV45 as compared to the native allergen. The starting concentration of BV45 was 100 nmol while these of 4,000-, 6,000- and 20,000-dalton preparations were, respectively, 120, 140 and 70 nmol. At the starting concentrations, the inhibition percentage was over 80% for all the four proteins. At lower concentrations, the RAST inhibition values of the copolymers were less than the native BV45. At 1/4 of the initial concentration the RAST inhibition capacity of the modified preparation was much more reduced than that at the initial concentration.

Table II. The results of SPT were demonstrated as wheal areas and HEP values

Protein	Patient E.N.		Patient N.T.	
	wheal areas, mm ²	HEP	wheal areas, mm ²	HEP
BV45	125	5	60	4
mPEG 4,000 daltons BV45	20	0.8	15	1
mPEG 6,000 daltons BV45	20	0.8	-	-
mPEG 20,000 daltons BV45	10	0.4	0	0

The allergenicity of the modified preparations was shown to be diminished. A clear representation was obtained by patient E.N. for all the modified derivatives.

100 μ mol. At lower concentrations, approximately 1 μ mol, mPEG 6,000 daltons BV45 was the weakest inhibitor, the mPEG 4,000 daltons BV45 inhibiting capacity was reduced by approximately 70%, while mPEG 20,000 daltons BV45 inhibition was reduced by approximately 50% and mPEG 6,000 daltons by 30%.

SPT and PK Test

The results of the SPT (table II) indicated that the allergenicity of the mPEG BV45 products was reduced as compared to the native BV45 in either of the patients tested (E.N., N.T.). In PK test fraction, mPEG 6,000 daltons BV45 was shown to elicit a wheal reaction of 1 HEP after challenging of the sensitized sites; the corresponding values for BV45 was 4–5 HEP.

Amino Acid Analysis

A reduction of approximately 5 lysine residues per mole due to mPEG modification was obtained. The rest of the amino acid residues had values very similar to these depicted in the composition of the nonmodified BV45 [Vik et al., 1981].

Discussion

The pilot experiments performed in this work were designed to provide a model preparation of methoxy polyethylene glycol modified birch pollen allergen and to investigate the immunochemical properties of the products. The application of the modified allergen for immunotherapy as well as the studies on the regulation of IgE synthesis are beyond the scope of this communication. Fraction BV45 was shown to have a molecular mass of approximately 10,000 daltons and contains approximately 88 amino acid residues together with 14 other residues of carbohydrates. The covalent binding of mPEG to BV45 increased the molecular size of the polymerized molecule substantially. A molecular weight of approximately 100,000 daltons was demonstrated using a reversed phase HPLC protein column for the three modified preparations. In addition, a protein peak of approximately 20,000 daltons was shown. Polymers of higher molecular weights than 100,000 daltons could not be resolved by the column used and were eluted together. The modified plant proteins with the hydrophilic mPEG covalently attached, have different hydrodynamic properties from those of the globular animal-derived native proteins used as markers. The molecular weight estimation in this study is therefore tentative. Amino acid analysis of the acid hydrolysates of the conjugates showed a reduction of 5 residues of lysine, i.e. approximately 60% of the lysine of BV45 was modified under the experimental conditions. These residues

were modified by binding mPEG at their free epsilon NH₂ groups. In view of the papers published on the allergenic determinants of Allergen M [Elsayed, 1983], all the allergenic epitopes shown encompassed at least 1 Lys in addition to other charged residues such as Glu, Asp and Ser. All these residues could similarly bind activated polyethylene glycol explaining the diminishing of allergenicity and the higher molecular weight obtained. Nevertheless, the products were easily soluble at the required physiological conditions despite their high molecular weights. Moreover, the coupling of the activated mPEG to BV45 was also shown by masking of the peak of the aromatic amino acids in UV spectral analysis as well as by the previously mentioned amino acid analysis. The iodination by the method described gave ¹²⁵I derivatives with reasonable specific activities as shown by the radioactivity of the fractions eluted from gel filtration after desalting. Rabbits were injected with the radiolabelled modified BV45 to demonstrate the rate of the metabolic clearance of the antigen versus the animals' immune response. A normal pattern of reticuloendothelial function of the animals with sharp decline in the remaining radioactivity in both preparations is shown in figure 4. At the time the antibody response was detectable, the concentration of the radiolabelled antigen was < 2%. The results obtained by CIE and FRIE showed relatively weak binding of rabbit anti-crude pollen extract as compared to the antiserum against the native BV45. The reduction of the affinity of the derivatives rather than that of BV45 could be induced by a steric hindrance due to the large polymer groups, reducing the accessibility of the active determinants to the immune competent cells.

Acknowledgement

The present study was supported by the Research Fund of the Norwegian Asthma and Allergy Federation.

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Received: September 11, 1983

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