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Significance of Rubber Elongation Factor as a Latex Allergen

Abstract

IgE-mediated hypersensitivity to proteins eluting from surgical gloves and other natural rubber products is a well-recognized allergic condition with special impact to health care workers and sensitized patients. Since rubber elongation factor (REF) has been suggested to be the major latex allergen (Hev b 1) we purified REF from rubber particles using electrophoresis and electroelution and reevaluated the occurrence of IgE antibodies to purified REF. The purified protein was subjected to tryptic digestion, peptide separation and amino acid sequencing. Amino acid sequences of 9 tryptic peptides from the purified 14-kD protein gave 100% homology to REF. In immunoblotting, sera from 4 of 6 latex-allergic children with spina bifida or other congenital anomalies exhibited IgE antibodies against REF whereas only 1 serum from the 30 other latex-allergic patients had similar antibodies. In IgE ELISA 8/45 (18%) latex-allergic patients had IgE antibodies to purified REF, whereas 30 (67%) of the same patients had IgE antibodies to purified prohevein, another rubber protein, recently shown to be a major natural rubber latex allergen. The observed overall low frequency of IgE antibodies to purified REF, as measured by two specific IgE assays, indicates that REF is one but not the most significant allergen among the natural rubber latex proteins.

Key words

Latex allergy
 Rubber elongation factor
 Latex proteins
 Amino acid sequence
 IgE antibodies

Introduction

Rubber elongation factor (REF) is an important protein in the milky latex of rubber tree, *Hevea brasiliensis*. The function of REF has been suggested to facilitate prenyl-transferase, another important rubber protein, to add multiple *cis*-isoprene units to rubber molecules [1]. The primary structure of REF has been determined [2] and it has also been cloned [3]. The REF is a highly hydrophobic protein. It is tightly bound to rubber particles but can be solubilized e.g. by detergents [1].

REF was recently suggested to be the major allergen in latex (Hev b 1) and the only allergen present in one latex surgical glove brand analyzed [4]. In the study of Czuppon *et al.* [4] a protein of 58-kD with an isoelectric point of 8.45

was purified from ammoniated latex and from latex gloves. A 14.6-kD monomer of this protein was identified as the REF by its amino acid sequence and it was reported to induce symptoms in latex-allergic patients and to block completely latex-specific IgE antibodies in all 13 sera from adult latex-allergic patients tested. Given the large number of allergens found by other investigators in natural rubber latex (NRL) [5–8] it is, however, difficult to conceive that only one glove allergen, such as REF, could be responsible for hypersensitivity symptoms in most if not all latex-allergic patients.

The purpose of the present study was to isolate and purify REF from rubber particle fraction of freshly collected NRL and to reevaluate the occurrence of IgE antibodies to REF in sera from a large group of latex-allergic patients.

Materials and Methods

Patients and Sera

Sera were obtained from 45 latex-allergic patients and from 24 adult control subjects (including 9 atopic individuals) with no history of latex allergy. Of the patients 31 were adults and 14 were children. Four of the children had spina bifida and 2 had other congenital anomalies and histories of multiple surgeries. Clinical histories of the patients are outlined in table 1. All patients had positive and the controls negative reactions in skin prick testing using an eluate of latex gloves (Exona®, Semperit, Vienna, Austria). The glove extract was prepared, and prick test performed as previously described [9]. Latex-RAST (CAP method, Pharmacia, Uppsala, Sweden) was positive (cutoff level 0.4 kU/l) in 33 patients (73%) and negative in all controls. Total IgE level was available for 41 patients; it was elevated in 21 adult patients and in 7 children.

Purification of REF

Fresh NRL of rubber tree (*Hevea brasiliensis*) was harvested and a sample was centrifuged during the day of collection at 43,000 g for 1 h. The rubber particle fraction (top layer) was then washed 2 times by centrifugation with ammonia (final concentration of NH₃ 0.7%) to remove loosely bound rubber proteins. To solubilize REF, the rubber particle fraction was centrifuged in the presence of sodium dodecyl sulfate (SDS; final concentration 2%) and the supernatant was collected and stored at -20°C.

Proteins in the REF-enriched supernatant were further separated by SDS-polyacrylamide gel electrophoresis (PAGE) according to a method described previously in detail [7]. When the sample was used in immunoblot studies the supernatant was diluted 1:4 in water and further 1:2 in electrophoresis buffer containing 2-mercaptoethanol and then boiled for 3 min. When the sample was processed to be used in enzyme-linked immunosorbent assay (ELISA) studies the supernatant was treated similarly except that no 2-mercaptoethanol was used in electrophoresis buffer and it was not boiled. The samples were then applied to a 16% polyacrylamide gel (24- μ l sample/6-mm-wide well in 0.75-mm-thick gel) and electrophoresis was carried out for 45 min at a constant voltage of 200 V (Mini Protean II Cell; Bio-Rad, Richmond, Calif., USA). Molecular weight protein markers (2.35–46 kD, Amersham) were run along with samples. After electrophoresis, gels were stained with Coomassie brilliant blue. The clear-cut band with an apparent molecular weight of 14-kD was excised from 32 lanes and subjected to electroelution (28 lanes) and tryptic protein digestion in gel (2 lanes).

Electroelution was performed according to manufacturer's instructions using a commercial electroeluter (Model 422 Electro-Eluter, Bio-Rad). Gel slices from 28 lanes were electroeluted at a constant current of 9 mA for 3 h against 50 mM ammonium bicarbonate containing 0.1% SDS using 12.5 kD cutoff membrane caps. Removal of SDS was performed after electroelution by replacing the elution buffer with fresh 50 mM ammonium bicarbonate buffer without SDS and running gel slices containing tubes for 2 more hours. Gel slices were removed from the tubes and the remaining aqueous fractions were combined and concentrated by vacuum centrifugation and used for immunoblotting or ELISA and ELISA inhibition assays.

For comparison, also prohevein [purified as described in ref. 10], another NRL allergen, was electroeluted after gel electrophoresis as described above. Protein concentration of purified prohevein was adjusted to approximately 1 mg/ml before electrophoresis in 16% polyacrylamide gels. Further steps were identical to those used in electroelution of REF.

Tryptic Cleavage, Peptide Separation and Amino Acid Sequencing

In-gel tryptic digestion of the 14-kD protein was performed as described by Hellman et al. [11]. However, the gel piece was dried in a vacuum centrifuge instead of drying under a stream of nitrogen. The extracted peptides were separated by reversed phase chromatography on a Rexchrom C8 (5 μ m, 300 Å, Regis Chemical Company, USA) column on a Smart™ system (Pharmacia, Uppsala, Sweden) using a linear gradient of acetonitrile (0–40% in 160 min) in 0.1% trifluoroacetic acid. Peptides were automatically collected and subjected, after concentration, to amino acid sequencing on ABI 494 A Procise™ Sequencer (Applied Biosystems, Foster City, Calif., USA).

Immunoblotting

SDS-PAGE was performed as described above. The electroeluted proteins were diluted 1:3 into sample buffer, boiled for 3 min and then applied to a 16% polyacrylamide gel (350- μ l sample/ 0.75-mm-thick gel). Electrophoresis was carried out for 45 min at a constant voltage of 200 V (Mini Protean II Cell; Bio-Rad). Immunoblotting was performed as described earlier [7]. Briefly, the proteins were transferred from the gel to a 0.2- μ m pore size nitrocellulose filter (Trans-Blot; Bio-Rad) by electroblotting at 100 V for 1 h (Mini Trans-blot cell, Bio-Rad). Successful transfer of proteins to the membrane was confirmed by Ponceau-S red staining. The membrane was then cut into 3-mm strips, washed with Tris-buffered saline (TBS) containing 0.1% Tween-20 and incubated for 1 h in a blocking buffer containing 5% nonfat milk powder in TBS. The strips were incubated overnight at 4°C with latex-allergic patient sera (diluted 1:5). The strips were then washed and incubated with biotinylated goat antihuman IgE antibody (Vector, Burlingame, Calif., USA) diluted 1:1,000. An extra blocking was carried out for 2 min after washing and the membranes were then washed again. Streptavidin-conjugated alkaline phosphatase (Zymed, Camarillo, Calif., USA), diluted 1:24,000 in TBS was added and the strips were incubated for 30 min at room temperature. The strips were placed in a color development solution (Bio-Rad) after washing, and the reaction was stopped after 8 min by rinsing in tap water.

Latex-ELISA

Electroeluted REF or prohevein (protein concentrations adjusted to 1.2 μ g/ml in 50 mM carbonate buffer, pH 9.6) was applied onto 96-well polystyrene microtiter plates (100 μ l/well; Nunc, Roskilde, Denmark) and incubated for 3 h at room temperature and overnight at +4°C. Wells were then emptied and postcoated for 1 h with 100 μ l of 50 mM carbonate buffer, pH 9.6, containing 1% human serum albumin (HSA). After rinsing 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T), 100 μ l of serum (diluted 1/20 with PBS-T containing 0.2% HSA; PBS-T-HSA) was added to the wells and incubated for 2 h at room temperature. Wells were then washed 3 times with PBS-T and biotinylated antihuman IgE (Vector; diluted 1/1,000 with PBS-T-HSA) was added and incubated for 1 h. Wells were again washed 3 times and streptavidin-conjugated alkaline phosphatase (Bio-Rad, diluted 1/3,000 with PBS-T-HSA) was incubated for 1 h. After 3 washes, the substrate development solution (Sigma, St. Louis, Mo., USA) was added and the developed color was read at 405 nm using an automated ELISA-reader (Titertek Multiskan, Eflab, Espoo, Finland). Mean OD value plus three standard deviations of the control subjects was chosen as cutoff limit for positivity.

Table 1. Clinical data and laboratory results of 45 latex-allergic patients

Patient No	Sex	Age years	Main symptoms	Associated condition	Latex prick ¹	Serum total IgE kU/l	Latex RAST kU/l	IgE-immunoblot reaction to REF ²	IgE-ELISA to REF ²
1	F	41	CU,E	—	2+	n.d.	3.5	—	—
2	F	38	CU,E,FE,GU,A	—	4+	396	20	+	+
3	F	48	CU,E	—	4+	797	5	—	—
4	M	32	CU	—	2+	17,200	2.2	—	—
5	F	40	AR	—	2+	641	3.3	—	—
6	F	33	CU,E	—	4+	1,842	3	—	—
7	F	44	CU,E,FE	—	4+	102	4.7	—	—
8	M	38	CU	—	2+	1,890	<0.4	—	—
9	F	32	CU,E	—	4+	7,075	18.	—	+
10	F	31	CU,FE,GU,AR	—	3+	2,867	18	—	—
11	F	36	CU,E	—	2+	28	<0.4	—	—
12	F	34	CU,FE	—	4+	259	8.3	—	—
13	F	34	CU,ES,FE	—	3+	47	<0.4	—	—
14	F	34	R	—	3+	287	<0.4	—	—
15	F	43	CU,ES,FE,R	—	3+	1,546	3.9	—	—
16	F	32	CU	—	3+	136	2.7	—	—
17	F	34	CU	—	2+	19	<0.4	—	—
18	F	45	CU,E	—	3+	11	<0.4	—	—
19	F	35	CU,E	—	3+	165	<0.4	—	—
20	F	45	CU,E,ES	—	3+	6	<0.4	—	—
21	M	62	CU,E	—	3+	5,320	68.3	—	—
22	M	14	R	—	3+	934	1	—	—
23	M	8	A,E	—	2+	282	0.9	—	—
24	M	15	ES,FE,A	—	2+	891	2.3	—	—
25	F	12	R,E	—	3+	144	<0.4	—	—
26	F	34	ES	—	3+	n.d.	<0.4	—	—
27	F	23	CU	—	2+	1,100	10	—	—
28	F	29	CU,E,AR	—	2+	43	<0.4	—	—
29	F	34	CU	—	3+	n.d.	6.9	—	—
30	F	56	E	—	2+	n.d.	0.5	—	—
31	M	15	GU,FE,R,A	SB	3+	110	2.2	+	+
32	F	9	ES,R,FE	SB	3+	39	1.6	—	—
33	F	12	CU,E	SB	2+	98	2.2	—	—
34	F	8	CU,E	SB	3+	286	70	+	+
35	F	12	AR,ES	CoA	3+	193	<0.4	+	+
36	F	10	CU,FE	CoA	4+	139	11.2	+	+
37	F	46	CU,E	—	3+	3,400	24	n.d.	—
38	F	32	CU,E	—	3+	1,390	2.7	n.d.	—
39	F	23	CU	—	3+	3,090	30.7	n.d.	—
40	F	27	E	—	4+	1,540	30.2	n.d.	—
41	M	7	CU	—	2+	4,930	0.9	n.d.	+
42	M	2	CU	—	3+	166	6.7	n.d.	—
43	F	3	CU	—	4+	2,510	27.5	n.d.	+
44	M	7	CU	—	3+	300	7.2	n.d.	—
45	F	25	CU,GU	—	4+	682	15.7	n.d.	—

CU = Contact urticaria; E = eczema; ES = eye symptoms; FE = facial edema; GU = generalized urticaria; R = rhinitis; A = asthma; AR = anaphylactic reaction; SB = spina bifida; CoA = congenital anomalies; n. d. = not done.

¹ Skin prick test scores 0–4+.

² Patient showing IgE binding to purified REF is indicated as + and patient not showing IgE binding to REF is indicated as –.

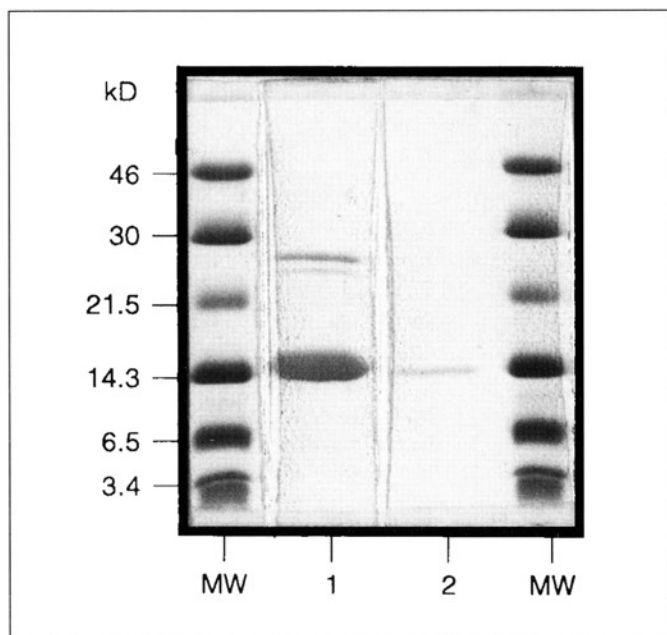


Fig. 1. SDS-PAGE analysis of proteins (1) extracted from rubber particle fraction of NRL and (2) electroeluted 14-kD protein. The proteins were stained with Coomassie brilliant blue. MW = Molecular weight markers (kD).

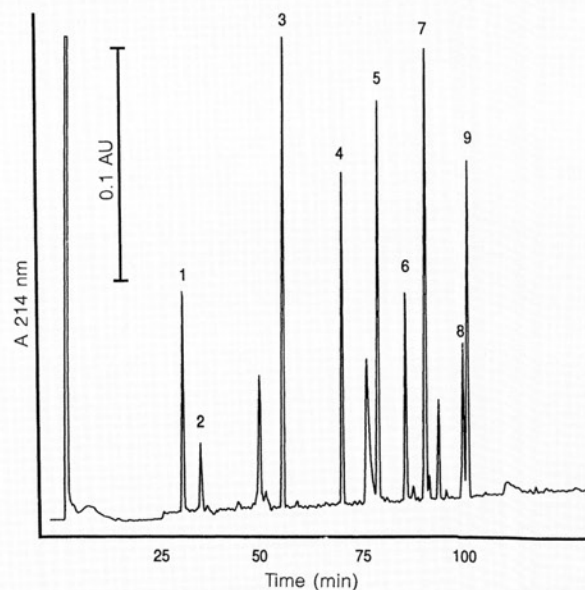


Fig. 2. Reversed phase chromatography of tryptic peptides from purified 14-kD protein on a Rexchrom C8 column. Peptides were collected and subjected to amino acid sequencing. Peak numbers refer to the sequencing results shown in table 2.

ELISA-inhibition

Coating with REF and postcoating were performed as described for ELISA assays. After postcoating, the wells were rinsed 3 times with PBS-T and then 50 μ l of reference patient serum (diluted 1:5 with PBS-T-HSA), previously shown to contain IgE antibodies to purified REF, was incubated for 1 h at room temperature with an equal volume of inhibitor solution (at concentrations of 1 and 0.1 μ g/ml of electroeluted REF and prohevein in PBS-T-HSA, respectively). To control possible nonspecific inhibition the reference patient serum was inhibited also with an unrelated polypeptide (ovalbumin, Sigma; at concentrations of 1 and 0.1 μ g/ml). The inhibitor solution was replaced with an equal volume of PBS-T-HSA in control experiments. The mixture containing the serum and the inhibitor (or serum without inhibitor) was then added to the wells and incubated for 2 h at room temperature. Wells were then washed 3 times with PBS-T and biotinylated anti-human IgE was added and the assay continued as described above for latex-ELISA.

Results

Results from SDS-PAGE analysis of SDS-extracted rubber particle proteins and the electroeluted 14-kD band are shown in figure 1. Amino acid sequences of 9 tryptic peptides (figure 2) obtained from the purified 14-kD protein revealed 100% sequence homology with REF (table 2).

Five (14%) of the 36 latex-allergic patient sera tested showed IgE antibodies to purified REF in immunoblotting. Four of these 5 sera (fig. 3) were from a subgroup of 6 latex-allergic children with spina bifida and/or other congenital anomalies and histories of multiple surgical procedures. Thirty of the 36 patients had suffered mainly from local cutaneous symptoms; only 1 of these 30 sera showed IgE to purified REF (fig. 3). All 7 control sera used in immunoblotting gave negative results.

In ELISA against purified REF, 8/45 (18%) latex-allergic patients had IgE antibodies to REF whereas 30 of the same 45 patients (67%) had IgE antibodies binding to purified prohevein (fig. 4). Four of the 39 (10%) patients without congenital malformations showed IgE to REF in ELISA. Of these 4 patients 2 showed borderline reactions, 1 patient (showing a faint reaction in immunoblotting) had low levels of IgE to REF and 1 patient had moderate levels of IgE to REF in ELISA. All control subjects studied (12 sera were examined against REF and 24 against prohevein) gave negative results in ELISA. Examination of the results for the 36 patients analyzed for anti-REF antibodies by both immunoblotting and ELISA revealed concordant results with one exception (patient No. 9 was negative in immunoblotting

Table 2. Results from sequence analysis and comparison of 9 tryptic peptides from the purified 14-kD rubber particle protein to REF

14 kD	Peptide 8 D K S G P L Q P G V D I I E G P V K															
REF	I I I I I I I I I I I I I I I I I															
position	D K S G P L Q P G V D I I E G P V K 40 57															
14 kD	Peptide 9 S G P L Q P G V D I I E G P V K												Peptide 5 N V A V P L Y N R			
REF	I I I I I I I I I I I I I												I I I I I I I I			
position	S G P L Q P G V D I I E G P V K 42 57												N V A V P L Y N R 58 66			
14 kD	Peptide 7 F S Y I P N						Peptide 6 D A S I Q V V S A I R						Peptide 1 A A P E A A R			
REF	I I I I I I						I I I I I I I I I I						I I I I I I I I			
position	F S Y I P N 67 72						D A S I Q V V S A I R 99 109						A A P E A A R 110 116			
14 kD	Peptide 4 S L A S S L P G Q T K										Peptide 2 I L A K			Peptide 3 V F Y G E N		
REF	I I I I I I I I I I										I I I I			I I I I I		
position	S L A S S L P G Q T K 117 127										I L A K 128 131			V F Y G E N 132 137		

Peptide numbers refer to the corresponding numbers in figure 3.

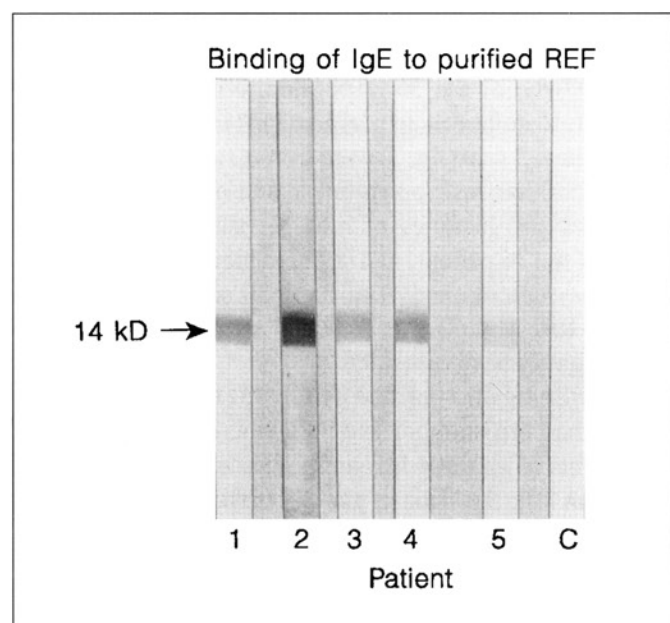


Fig. 3. Immunoblot analysis of binding of IgE antibodies to purified REF. Patients 1–4 are children and patient 5 (weak reaction) is an adult. C = Nonallergic control subject.

but showed borderline reaction in ELISA, table 1). Specificity of the ELISA was confirmed by inhibition experiments: Purified REF at a final concentration of 500 ng/ml inhibited 94% of the binding of IgE to solid-phase REF and at a concentration of 50 ng/ml the inhibition was still 67%. The inhibition produced by ovalbumin or prohevein at a concentration of 500 ng/ml was not significant (<5%).

Discussion

In the present study we purified a 14-kD latex protein from the rubber particle fraction and identified it by amino acid sequencing as REF. IgE reactivity to purified REF, as measured by IgE immunoblotting and ELISA, appeared to accumulate into a subgroup of latex-allergic patients with spina bifida or other pediatric patients with histories of multiple surgeries. A faint reaction in immunoblotting was seen in only 1 serum among 30 mainly adult latex-allergic patients who did not have any disorders requiring repeated surgical procedures. In line with these observations, only 4/39 (10%) patients without congenital malformations showed IgE to REF in ELISA. These results are in contrast to those published by Czuppon et al. [4], who reported that all 13 adult patients tested had IgE antibodies to purified

Scatter plot showing A405 values (Y-axis, 0.00 to 2.25) for various patient groups (X-axis) under REF and Prohevein conditions. The groups are SB or CoA, Other patients, and Controls. A dashed horizontal line indicates a baseline A405 value of approximately 0.05. The 'Other patients' group under 'Prohevein' shows a significant increase in A405 values, with many points above 1.00 and one near 2.00.

Another reason for the discordant results may be in differences in patient materials. It is likely that our patient group ($n = 45$) is more representative of the general population of latex-allergic patients than that of Czuppon et al. [4], which consisted of 13 patients with occupationally induced latex allergy. Our series comprised both adults and children

The antigen used by Czuppon *et al.* [4] for EAST inhibition and skin prick testing was a fraction from an anion exchange chromatography of glove extract, that, according to the figure of the article, resembles an exclusion peak containing all material not binding to the column matrix. Although this fraction obviously contained REF, evidenced by sequence analysis of an excised band at 14-kD obtained after SDS-PAGE, data was not given to assure that the fraction comprised only one protein. Thus, the possibility cannot be excluded that the fraction contained small molecular weight latex peptides other than REF which are still capable of inhibiting the binding of IgE and eliciting skin prick test reactions but escape demonstration in SDS-PAGE. In the present study, we used rubber particle fraction, containing REF as the predominant protein [1], as the source material for the purification. The rubber particle-bound proteins were solubilized by SDS, electrophoresed and the distinct band of 14-kD was excised and electroeluted. The eluted protein, used as antigen in immunoblotting and ELISA, was considered to represent highly purified REF since it showed after tryp-

tic digestion and peptide separation only sequences with a 100% match to REF. The divergent results on the occurrence of IgE antibodies to REF of the present study compared to those of Czuppon *et al.* [4] may thus be partly explained also by the different source material and purification procedures.

In view of recent studies by us and others, it appears that several latex proteins could be important allergens [14]. We recently assigned the highest overall significance to prohevein, a 20-kD protein in NRL [10]. The present study confirms this since the majority of patients exhibited IgE antibodies to purified prohevein also in ELISA. We also reported recently partial primary structure of a unique 27-kD latex allergen recognized only by IgE antibodies from a subgroup of latex-allergic patients with spina bifida or other congenital malformations [15]. Interestingly, IgE reactivity against

REF within this group of patients was very similar to their reactivity against the purified 27-kD allergen, but at the individual level there were different patterns. Furthermore, partial homology between one peptide of the 27-kD protein and REF at amino acid positions from 40 to 54 was observed [15] and it would be of interest to investigate whether this homology will explain the co-occurrence of IgE antibodies to REF and to the 27-kD NRL allergen.

Several latex allergens have recently been characterized at molecular level and the new information has both added considerably to the understanding of the overall picture of latex allergy and given basis for comparison of results between various groups of researchers. Accumulated evidence from the present and previous studies support the view that REF is one but not the most significant allergen among the NRL proteins.

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