

Interest of Two-Dimensional Electrophoretic Analysis for the Characterization of the Individual Sensitization to Latex Allergens

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Key Words

Latex allergens · Immunoblotting · Two-dimensional gel electrophoresis · Individual therapy monitoring

Abstract

Background/Objective: Latex allergy is a type 1 hypersensitivity reaction that mainly affects high-risk populations such as health care workers, spina bifida-affected or multiply-operated children. Ten molecules have so far been identified and registered as latex allergens (Hev b 1 to Hev b 10). The aim of the present investigation was to identify the major latex allergens by an individual analysis of the IgE response of latex-allergic patients to latex proteins separated by two-dimensional (2-D) gel electrophoresis. **Materials and Methods:** Latex proteins from a sap or a glove extract were separated by 2-D electrophoresis and transferred to a nitrocellulose membrane. Each membrane was incubated with the serum of one latex-allergic patient. The most frequently recognized latex allergens were characterized in sap and glove extracts using monoclonal antibodies or amino acid microsequencing. **Results:** The one-dimensional screening of 54 patient sera revealed 4 major bands recognized by IgE. The 2-D analysis of the sensitization to latex allergens allows the identification of allergen isoforms and the characterization of an individual response diversity. Hev

b 6.01 was recognized by 88.9% of the patients. Protein spots around 14 kD were recognized by 48.1% of the patients and corresponded to Hev b 6.03 as well as other proteins. A not yet characterized doublet of acidic proteins with molecular masses of 43 and 94 kD was recognized by 20.4% of the sera. Only 5.5% of the sera did not recognize any of these 4 major allergens. Hev b 1 is the main protein from the glove extract but was not constantly found in sap extracts. **Conclusions:** One-dimensional electrophoretic analysis of the allergen is usually not sufficient to characterize the individual specificity of the IgE response to latex allergens. Latex-glove proteins which are allergens can be absent from the sap extracts and the sensitization to these allergens could be underestimated. Individual 2-D analysis of the sensitization to latex allergens is useful to define the best allergen mixture required for diagnosis and needed for individual therapy monitoring.

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Introduction

Latex allergy is a well-characterized entity which is related to type I hypersensitivity. Several reports have described 3 major allergens: rubber elongation factor (Hev b 1), prohevein (Hev b 6.01) or its N-terminal end

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hevein (Hev b 6.02) and Hev b 5 [1–4]. These major allergens are usually recognized by high-risk populations such as health care workers and children with spina bifida. Two of these major allergens were detected by monoclonal antibodies on a one-dimensional (1-D) SDS-PAGE immunoblot of a standardized latex sap extract and displayed apparent molecular masses around 14 kD (Hev b 1) and 25 kD (Hev b 6.01) [5]. However, the two-dimensional (2-D) gel electrophoretic analysis of the latex extract shows several protein spots corresponding to these molecular masses. In the 14-kD range, 4 allergens obtained a nomenclature designation: Hev b 1 (14.6 kD), Hev b 6.03 (14 kD), Hev b 8 (14 kD) and Hev b 5 (16 kD). The allergens Hev b 3 and Hev b 10 were described in a molecular mass range of 23–27 kD [6–8]. Although Hev b 5 has a molecular mass of 16 kD, it was detected at 24 kD in a nonammoniated latex extract separated by SDS-PAGE [4]. Moreover, the existence of various isoforms of Hev b 8 and Hev b 10 has been suggested and submitted to the Allergen Nomenclature Committee (www.allergen.org).

In 1996, Kurup et al. [9] demonstrated that, in blots of latex sap proteins separated by 2-D gel electrophoresis (2-D blots), 56 protein spots were recognized by IgE from pooled latex-allergic patient sera. However, also in 2-D blots, only a few allergens were detected in manufactured latex products [5].

Several reports suggested that various symptomatic profiles in latex-allergic individuals, such as those with latex-fruit syndrome, spina bifida, or health care workers, are associated with the sensitization to different latex allergens. For example, the latex hevein and class I chitinases, which contain an hevein domain, have been demonstrated as major cross-reacting allergens in latex fruit syndrome [3, 10, 11]. On the other hand, Hev b 3 is an allergen strongly associated with spina bifida patients [12–14], while Hev b 1 is widely found in health care workers and spina bifida patients [2]. Such results suggest that some allergens could be more or less important, according to the origin of the sensitization. If we add the fact that crude or even standardized extracts can vary from one batch to another, the identification of the most relevant allergens is an important goal for future diagnosis or vaccination.

Thus the present study was undertaken to characterize individual responses to latex allergens isolated from crude or standardized latex sap extracts, and to compare these allergens with those extracted from manufactured latex products. For this purpose, the IgE specificity of selected human sera was individually analyzed by 2-D blots and compared to the analysis obtained with the CAP system using recombinant latex allergens.

Materials and Methods

Human Sera

Fifty-four human sera were obtained from latex-sensitive patients selected on the basis of positive values for serum-specific IgE to latex (Pharmacia CAP system ≥ 5 kU/l). These sera were used as immunochemical reagents to identify allergens by their ability to bind to IgE.

Latex Extract

The latex sap extracts used were from Stallergènes (Antony, France). Two different batches were used: a crude extract and a standardized extract used for skin testing.

Latex gloves were cut into small pieces, frozen in liquid nitrogen and crushed using a pestle and mortar. The proteins were then extracted from the powder with 8 M urea containing 2% CHAPS (2 ml of extracting solution/1 mg powder) for 3 h at 22°C. The extracted solution was filtered first through glass wool and then through a 0.8- μ m pore size filter (Sartorius, Göttingen, Germany).

Screening of Human Anti-Latex IgE

The human sera were screened against a latex crude extract. Briefly, latex proteins were separated by SDS-PAGE (8–18% gradient gel, ExcelGel, Amersham-Pharmacia Biotech, Uppsala, Sweden) and blotted onto a nitrocellulose (NC) sheet, reference BAS 83 (Schleicher & Schuell, Dassel, Germany). Blot strips (4 mm wide) were successively blocked with 1% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20 (PBS-BSA-Tw) for 1 h at 22°C, and incubated with 0.5 ml of individual human sera diluted at 1:10 in PBS-BSA-Tw (overnight, 22°C). After extensive washing (PBS-Tw), the NC strips were incubated for 2 h at 22°C with alkaline phosphatase-conjugated goat anti-human IgE (1:700 in PBS-BSA-Tw, Sigma Biochemicals, St. Louis, Mo., USA). The alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma Biochemicals) in 0.1 M Tris buffer pH 9.5.

2-D Gel Electrophoresis

Latex proteins were separated by isoelectrofocusing (IEF) in a 4% polyacrylamide gel containing 5% v/v Servalyt® pH 2–11 (Serva, Heidelberg, Germany) on a flat bed apparatus. Two 4-mm-wide strips of the focused extract were cut and submitted to SDS-PAGE separation on an 8–18% gradient gel. One of the 2-D gels was silver stained and the other one submitted to a Western blot.

Western Blot

After a 2-D gel electrophoresis, the proteins were electroblotted onto an NC sheet (Schleicher & Schuell) with a semi-dry Novablot apparatus (Amersham-Pharmacia Biotech), according to the manufacturer's instructions (1 h, 1 mA/cm²). Just after blotting, the NC sheets were blocked with PBS-BSA-Tw for 1 h at 22°C, and then incubated with 3 ml of either mouse monoclonal antibodies II4F9 directed against Hev b 1 [2, 15] (1:50 in PBS-BSA-Tw, overnight, 22°C), monoclonal antibodies 8013G3 directed against Hev b 6.02 (1:100 in PBS-BSA-Tw, 2h, 22°C), latex-sensitive human sera (1:10 in PBS-BSA-Tw, overnight, 22°C), or birch profilin-sensitive human sera (1:10 in PBS-BSA-Tw, overnight, 22°C). After extensive washing, the NC sheets were incubated for 2 h at 22°C with alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:15,000 in PBS-BSA-Tw, Sigma Biochemicals) or with alkaline phosphatase-conjugated goat anti-human IgE (1:700 in PBS-BSA-Tw, Sigma Biochemicals).

Table 1. Summary of the screening of the human anti-latex IgE

	25 kD	14.4 kD	43 kD + 94 kD	25 kD + 14.4 kD	All four allergens
General expression	48 (88.9)	26 (48.1)	11 (20.4)		
Alone	23 (42.6)	1 (1.8)	2 (3.7)	–	–
Combined expression	–	–	–	17 (31.5%)	8 (14.8%)

Four major bands were identified as the most frequently recognized allergens. The 43- and 94-kD bands were always recognized simultaneously. Out of the 54 sera, only 3 did not recognize any of these allergens. Numbers in brackets are percentages.

The alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma Biochemicals) in 0.1 M Tris buffer pH 9.5.

Recombinant CAP-System Characterization

Four sera analyzed in 2-D blots were also characterized with the Pharmacia CAP system (Pharmacia Diagnostics, Uppsala, Sweden) on the recombinant allergens rHev b 1, rHev b 2, rHev b 3, rHev b 5, rHev b 6.01, rHev b 6.02, rHev b 6.03, rHev b 8 and rHev b 10. All recombinant allergens were produced as fusion proteins containing the maltose-binding protein (MBP) and coupled to CAP by MIAB (Uppsala, Sweden; Dr M. Lundberg). An MBP-CAP was used as negative control.

Amino Acid Sequence Analysis

After IEF separation, the bands which corresponded to the pIs of the proteins of interest were cut out and submitted to SDS-PAGE in an 8–18% gradient gel (double 1-D electrophoresis according to Altland [16]). For N-terminal amino acid sequencing, the proteins were electrotransferred overnight onto a ProBlott membrane (Applied Biosystems, Weiterstadt, Germany) in 50 mM borate, 50 mM Tris (pH 8.3). After Coomassie staining, the bands of interest were cut out and submitted to microsequencing. Amino acid sequence similarity with already-known proteins was sought by using the Basic Local Alignment Search Tool (BLAST) algorithm [17] (PIR, Swiss-Prot and Genpept databases).

Results

1-D Screening of Human Sera

The result of 1-D screening of human sera is summarized in table 1 and its photomontage is shown in figure 1. The screening of the latex-sensitive sera with latex proteins from a crude extract separated by SDS-PAGE showed that 4 bands were the most intensely and frequently recognized: a 25-kD band was recognized by 88.9% of the sera, a band around 14.4 kD was recognized by 48.1% of the sera, and a doublet corresponding to 43- and 94-kD bands was recognized by 20.4% of the sera. Only 3 sera (5.5%) did not recognize any of these allergens. The recognition of these bands by individual sera

was as follows: the 25-kD band was the only band intensively recognized in 42.6% of the cases, the 25- and 14.4-kD bands together were recognized by 31.5% of the sera, whereas the 14.4-kD band alone was recognized by only 1 serum (1.8%). The 43- plus 94-kD doublet together with the 25- and 14.4-kD bands were recognized in 8 cases out of the total 11 sera positive for the 43- plus 94-kD doublet.

This 1-D screening allowed the identification of 4 main profiles of sensitization: 14 kD alone, 25 kD alone, 25 plus 14 kD, 43 plus 94kD alone, and the 4 bands together. These 4 profiles were further analyzed for a 2-D characterization of the allergens.

2-D Analysis of the Individual Sensitization to Latex Proteins

Representative results of the 2-D screening are shown in figure 2. The allergens were identified using monoclonal antibodies or amino acid microsequencing, and the result of this identification is summarized in figure 3a.

In all sera studied, the 25-kD band represented a series of spots in a pH range of 4.8–6.5 and 7.5–9.5. This series of spots was observed whatever the 25-kD band, it was recognized alone (fig. 2a) or together with the other major bands (fig. 2b–d). The characterization of this band by monoclonal antibodies to Hev b 6.02 showed that all these spots were recognized and that they belong to the hevein-containing proteins (fig. 3d).

The 43- plus 94-kD doublet was always identified as a doublet with an acidic pI around 5 (fig. 2c–e). This doublet was not further characterized. In one case (fig. 2c), the 43-kD band in 1-D was not only related to the lower spot of the doublet, but also to a large spot with a basic pI (>8.3). The N-terminal sequence of this spot was GGIAIYW. This sequence is 100% identical to the N-terminal sequence of latex heveamine A (fig. 3a).

The band around 14 kD displayed various 2-D profiles. The main profile corresponded to a series of spots in

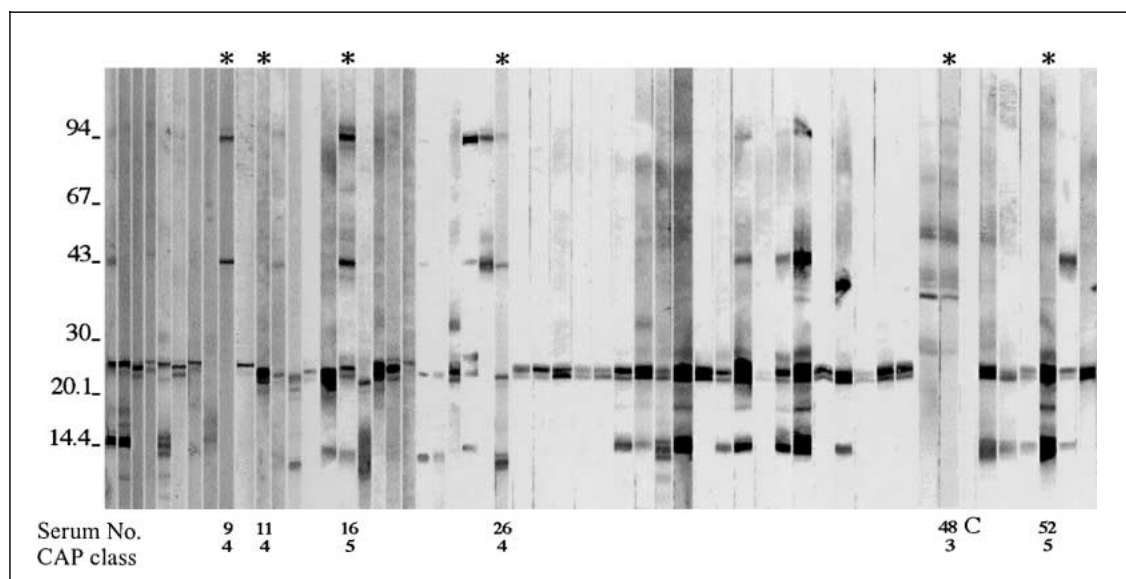


Fig. 1. Western blot analysis of SDS-PAGE-separated latex allergens. Latex proteins were separated by SDS-PAGE and transferred by Western blot onto an NC sheet. Each NC strip was incubated with the serum of a latex-allergic patient and anti-human IgE. All 54 sera screened are shown on this photomontage; the sera analyzed by 2-D gel electrophoresis are indicated by an asterisk (*), their number and CAP class value are given below each strip. Molecular masses are shown on the left. A control strip (C) was obtained by incubating the strip in PBS-BSA-Tw instead of human sera.

the 6–8.5 pH range (fig. 2b, d). The sequence of the six N-terminal amino acids of 2 spots of the series were GGSASN. These amino acids are 100% identical to the ones in position 67–72 of the prohevein (Hev b 6.01). Moreover, the sera which recognized this series of spots also recognized the recombinant C-domain of the prohevein (data not shown). A completely different profile was observed in one case (fig. 2c), with five spots grouped in the 4.75–5.65 pH range. These spots were not revealed by monoclonal antibodies to Hev b 1 (fig. 3b), and the anti-birch profilin serum cross-reacted with a latex molecule situated lower than 14 kD and in a pH range lower than 4.75 (fig. 3c).

One of the 4 sera which did not recognize any of the major allergens was further analyzed for 2-D characterization (fig. 2f, serum No. 48). Several spots with various pIs and molecular masses were stained. None of the allergens identified with the other sera in 2-D was recognized by this serum.

CAP System on Recombinant Allergens

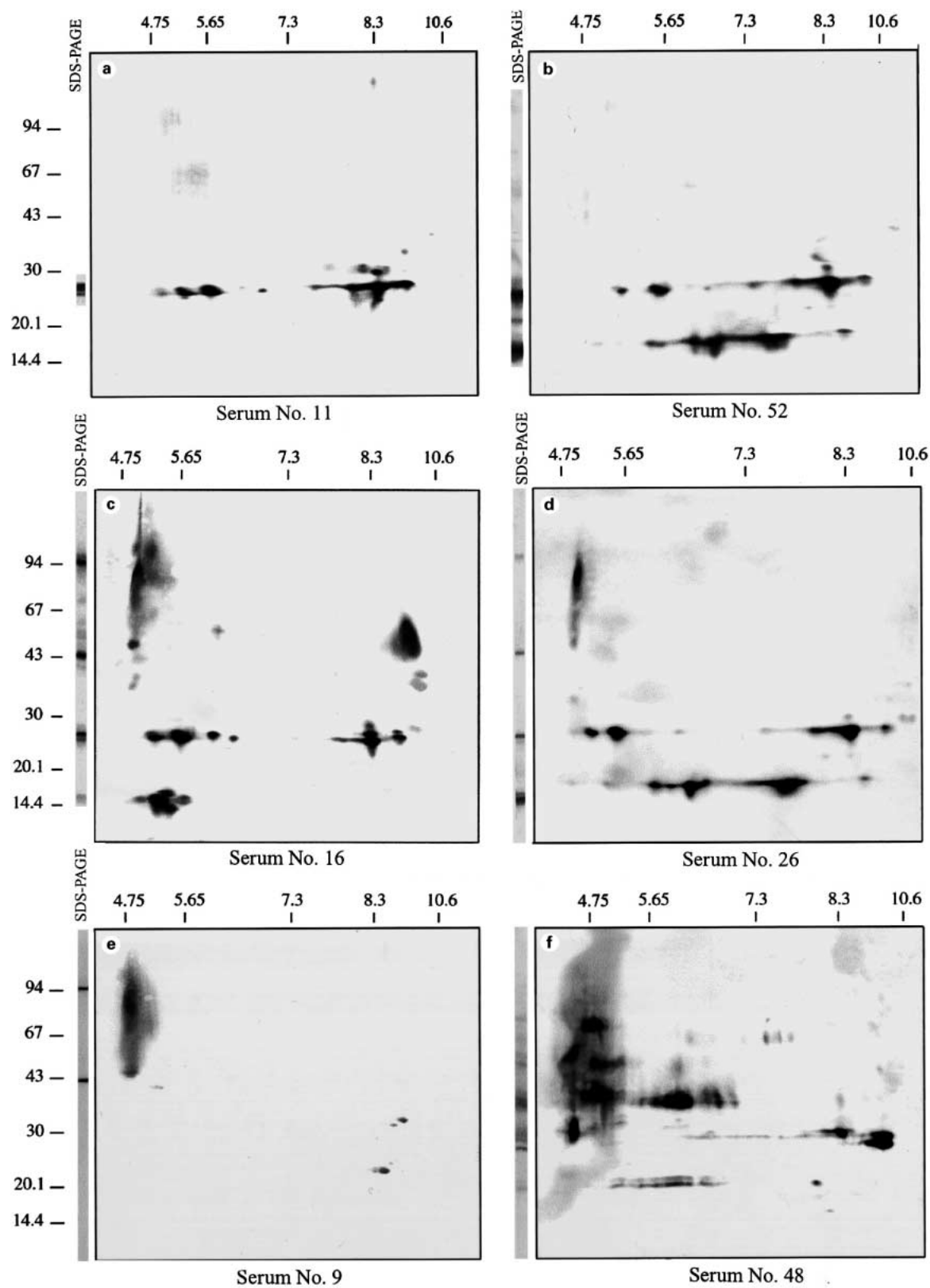
Sera No. 11, 16, 26 and 48 analyzed in 2-D blots were also characterized by CAP system on recombinant allergens. The result of this characterization is shown in

table 2. Serum No. 11 was monosensitized to the hevein and recognized only rHev b 6.01 and 6.02. Serum No. 16 recognized the hevein and Hev b 5. Serum No. 26 recognized the prohevein by its hevein domain and its C-terminal domain, and it was also positive to Hev b 5. Serum No. 48 appeared to be only faintly positive to Hev b 5.

2-D Comparison of Latex Sap and Glove Extracts

The 2-D comparison of various batches of latex extract showed some differences in the total protein (data not shown) and allergen content. In particular, monoclonal antibodies to Hev b 1 recognized acidic spots in the extract shown in figure 3a, a 'standardized' extract used

Fig. 2. Western blot analysis of latex allergens separated by 2-D gel electrophoresis. Latex proteins were separated by an IEF followed by SDS-PAGE and transferred by Western blot onto an NC sheet. Each sheet was incubated with the serum of a latex-allergic patient and anti-human IgE. The 1-D SDS-PAGE spectrum of each serum is shown on the left of each 2-D sheet. Molecular masses are given on the left and isoelectric points on the top. a Serum No. 11. b Serum No. 52. c Serum No. 16. d Serum No. 26. e Serum No. 9. f Serum No. 48 (see fig. 1).



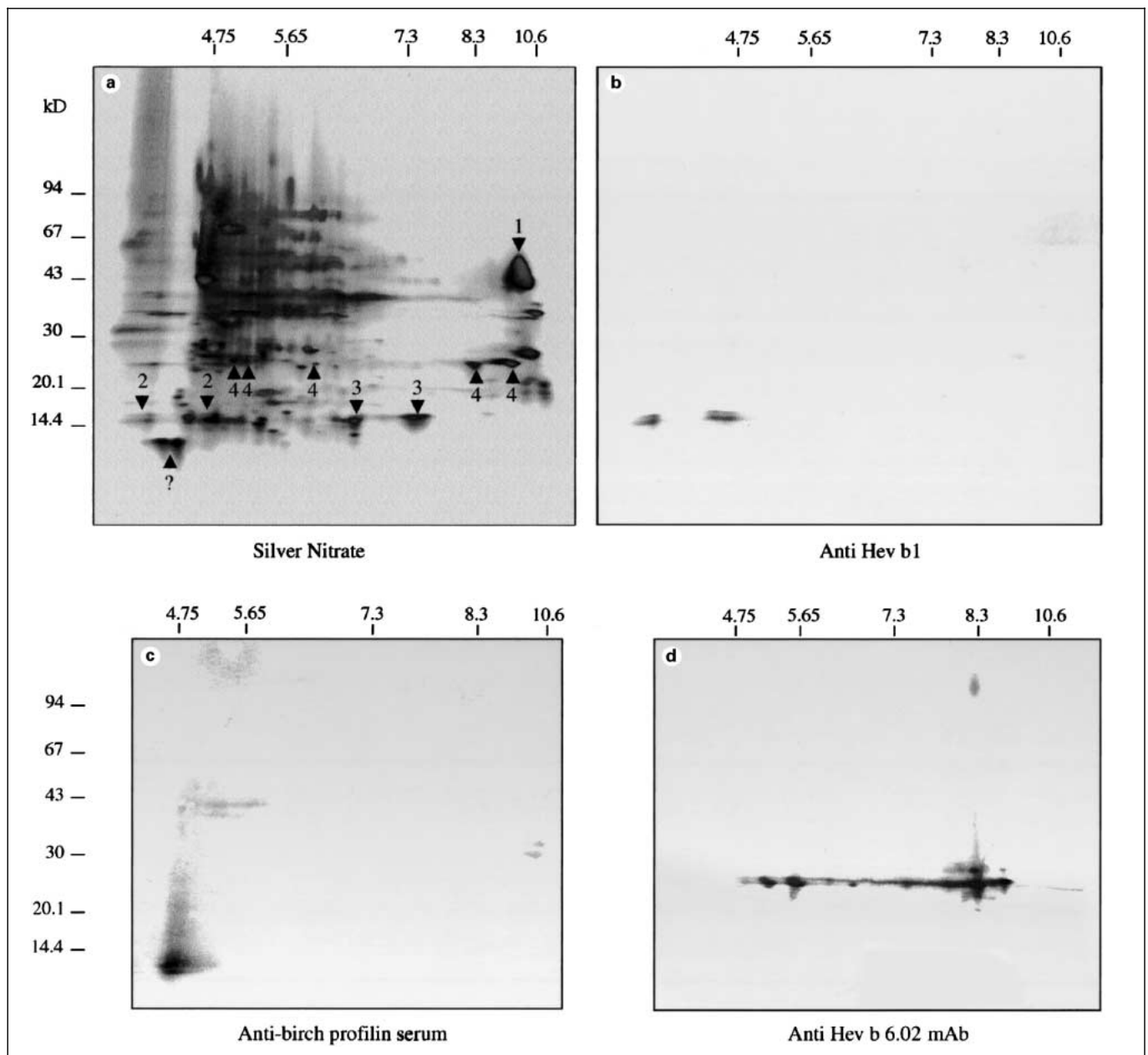


Fig. 3. Identification of the latex allergens by 2-D gel electrophoresis. Latex proteins were separated by an IEF followed by a SDS-PAGE. The gels were either stained with silver nitrate (a) or submitted to a Western blot. The NC sheets were incubated with monoclonal antibodies against Hev b 1 (b) or Hev b 6.02 (d) or incubated with a human serum anti-birch profilin (c). a Identification of the allergens on the silver stained 2-D gel. 1 = Hevamine A; 2 = Hev b 1; 3 = hevein C-domain (Hev b 6.03); 4 = prohevein (Hev b 6.01). The question mark (?) indicates the possible location of the latex profilin. Molecular masses are given on the left and isoelectric points on the top.

for skin testing (fig. 3b), but failed to reveal any molecule in a 2-D blot of a crude extract from the same origin (data not shown).

The identification of the major allergen in latex gloves is shown in figure. 4. The silver-stained 2-D gel demon-

strated that the major proteins extracted from gloves were located around 14.4 kD and varied in pIs from less than 4 to more than 9. All these spots were recognized by an anti-Hev b 1 monoclonal antibody.

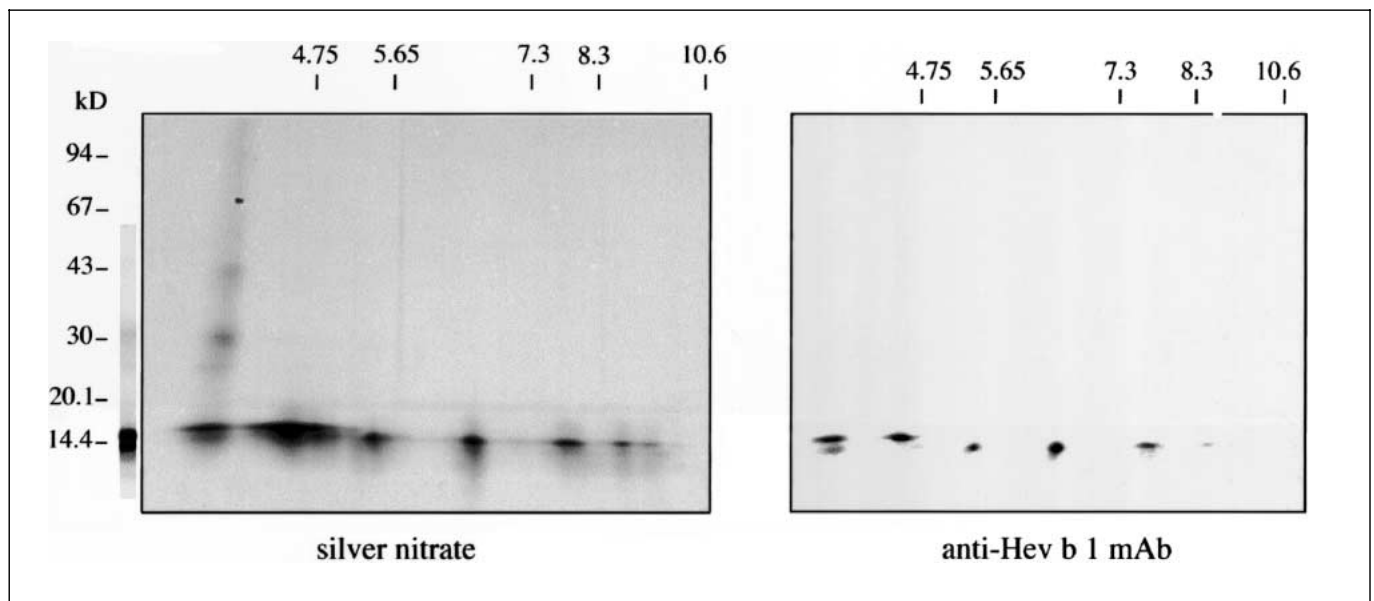


Fig. 4. 2-D characterization of latex glove allergens. Latex proteins were separated by an IEF followed by SDS-PAGE. The gels were either stained with silver nitrate or submitted to a Western blot (not shown). The NC sheet was incubated with monoclonal antibodies against Hev b 1. Molecular masses are given on the left and isoelectric points on the top.

Table 2. Results of the screening with the Pharmacia-CAP system on recombinant allergens

Serum	Latex	rHev b 1	rHev b 2	rHev b 3	rHev b 5	rHev b 6.01	rHev b 6.02	rHev b 6.03	rHev b 8	rHev b 10	MBP
11	10.90/3	<0.35/0	<0.35/0	<0.35/0	<0.35/0	12.8/3	6.72/3	<0.35/0	<0.35/0	<0.35/0	<0.35/0
16	23.40/4	<0.35/0	<0.35/0	<0.35/0	25.7/4	7.23/3	8.44/3	<0.35/0	<0.35/0	<0.35/0	<0.35/0
26	4.52/3	<0.35/0	<0.35/0	<0.35/0	2.74/2	1.18/2	0.49/1	0.37/1	<0.35/0	<0.35/0	<0.35/0
48	1.56/2	<0.35/0	<0.35/0	<0.35/0	0.41/1	<0.35/0	<0.35/0	<0.35/0	<0.35/0	<0.35/0	<0.35/0

Four sera analyzed in 2-D immunoblots were screened with recombinant CAP system. All recombinant latex allergens are fusion proteins with MBP. The values were established with sera diluted 1:2. The MBP-CAP values served as negative controls. The values are indicated in kU/I/class.

Spots with acidic pIs and molecular masses around 30 and 43 kD were also seen after silver staining, and a faint spot with an acidic pI was detectable around 25 kD. These 3 acidic proteins were not recognized by an anti-Hev b 1 monoclonal antibody.

Discussion

The present study analyzes the individual 2-D specificity of various latex-allergic patient sera. One acidic doublet of proteins with apparent molecular masses of respectively 43 and 94 kD was recognized by 20% of the sera. The recognition of this acidic doublet is consistent with

the description of Beezhold et al. [18], who found a strong IgE reactivity with 2 protein bands at 46 and 110 kD and further characterized the 46 kD protein as Hev b 7 [19].

Our results also show that approximately 90% of the sera have IgE to the prohevein which is recognized by its N-terminal end hevein in, at least, half the cases. The prohevein displays an apparent molecular mass of 25 kD and pIs which vary from 4.75 to more than 9. In previous reports [7, 20, 21], prohevein was described as having a molecular mass of 20 kD and a pI of 4.7 or 5.6. The present results clearly establish that, whether in crude or in standardized sap extract, the prohevein presents various isoforms recognized by monoclonal antibodies to hevein and by IgE from latex-allergic individuals. Two

isoforms were in the previously described acidic zone, but the other ones were in a basic pH zone. Several reports indicated that the main IgE-binding epitopes of the prohevein were located in the N-terminal fragment, hevein [22–25]. The present results demonstrate that at least half of the sera which recognize the prohevein do not recognize its C-terminal domain. We also analyzed the reactivity of the sera positive to a 25- and a 14-kD band in 1-D by testing them with 2-D gels. We observed that the great majority of these sera recognize the C-domain of the prohevein, indicating a positivity of approximately 35% to this molecule.

Hev b 5, a proline-rich protein, has also been suggested to be an important allergen, with a calculated molecular mass of 16 kD and a pI of 3.5 [26]. The Pharmacia Diagnostics Company proposed to add recombinant Hev b 5 to enhance the performance of their latex immunoCAP [27]. In our study, 1 patient (serum No. 16) was determined to be strongly sensitized to Hev b 5 by recombinant CAP, and he had an unusual but very strong recognition pattern in an acidic 14.4-kD zone on 2-D blot. This pattern was difficult to characterize because it did not correspond to silver-stained proteins on 2-D gels. Beezhold et al. [28] suggested that Hev b 5 is a strong antigen present in small amounts in natural latex extracts, and Slater et al. [4] suggested that Hev b 5 migrates at 24 or 36 kD rather than at its calculated molecular mass. Discrepancies between the apparent molecular masses in SDS-PAGE and the calculated molecular masses for prohevein (25 instead of 20 kD) and heveamine A (43 instead of 30 kD) were also observed in this study [20].

Hev b 1 is another latex allergen with a molecular mass of 14 kD that has been described as a major allergen [1, 2, 29]. In the present study, we show that the Hev b 1 content varies from one extract to another. We tested two sap extracts from the same company and observed striking differences in the Hev b 1 content: the crude extract contained no detectable Hev b 1, whereas the 'standardized' version displayed a weak, but clear, reactivity. Hev b 1 is quantitatively an important protein in latex gloves, and it has also been demonstrated in other latex products with various isoforms corresponding to an amino-terminal degradation of the native protein [5]. The fact that Hev b 1 is not constantly present in latex sap extracts but is probably altered during the manufacturing procedure can result in an underestimation of the sensitization to this allergen or its altered forms. Several reports have emphasized the importance of obtaining safe and accurate extracts for latex allergy diagnosis [30–32], and recent studies propose the use of purified or recombinant latex

allergens [33, 34]. Yip et al. [34] showed that an association between recombinant Hev b 5, Hev b 6 and Hev b 7 can be used to diagnose latex allergy by skin prick tests with 93% sensitivity and 100% specificity. However, if we consider the present results in the light of a previous study showing that 80% of the spina bifida and 50% of the health care workers allergic to latex have IgE against Hev b 1 [2], we can conclude that this allergen is certainly important in diagnosis and should be taken into account especially in therapy.

Finally, we found that 5.5% of the sera have a faint and unusual profile in 1-D. We observed that the sera with this unusual specificity do recognize the major allergens, with the exception of a faint reaction to Hev b 5 detected by the CAP system. So it can be assumed that in these cases, 2-D analysis is recommended when a therapy is indicated. Moreover, a further characterization of these unusual allergens is needed.

Recently, Grüber et al. [35] focused on the use of immunoblotting for the detection of latex allergy in spina bifida patients. They concluded that immunoblotting was not superior to other tests in terms of diagnosis, but could have a valuable role in monitoring therapy. In the present work we demonstrated that a 1-D analysis is not always sufficient for individual monitoring. It seems that a 1-D analysis is sufficient in certain cases, e.g., when a mono-specificity to prohevein is determined. However, when the 1-D profile is unusual, or when the recognized allergens are located in a zone containing various molecules (e.g., in the 14 kD range), 2-D analysis is needed to identify the allergens to which the patient is sensitized.

In conclusion, and from a diagnostic point of view, it can be assumed that 2-D analysis of the individual specificity is useful at research level in order to determine which allergens must be purified or produced as recombinants to obtain the most relevant diagnosis mixture. Moreover, in terms of therapy, if we assume that all the recognized allergens have to be used together in order to have an efficient vaccination, individual 2-D analysis should be done to determine to which allergen(s) the patient has to be desensitized. The major allergens are recognized by the majority of patients, but we have to take into account that there are also 'unusual' allergens that are determining the reaction of patients sensitized to them.

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