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Latex allergy: low prevalence of immunoglobulin E to highly purified proteins *Hevea brasiliensis* 2 and *Hevea brasiliensis* 13

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Clinical and Experimental Allergy

Summary

Background *Hevea brasiliensis* (Hev b) 2 and Hev b 13 have recently been identified as major latex allergens by detecting specific IgE antibodies in >50% of sera from Hev b latex-allergic individuals.

Objective We assessed the prevalence rates for sensitization to extensively purified latex allergens in patients from three diverse geographical areas.

Methods Native Hev b 2, Hev b 5, Hev b 6.01 and Hev b 13 were purified by non-denaturing chromatography and were used in ELISAs to assess sera from 215 latex-allergic patients and 172 atopic non-sensitized controls from Finland, Spain and the United States to detect allergen-specific IgE antibodies.

Results Unexpectedly, even highly purified Hev b 13 contained epitope(s) to which Hev b 6-specific human IgE antibodies bound effectively. Further purification, however, reduced the prevalence of IgE antibody reactivity to low levels: 15%, 5% and 11% for Hev b 2, and 18%, 30% and 27% for Hev b 13 among latex-allergic Finnish, Spanish and American patients, respectively. Interestingly, Finnish patients had a lower prevalence of Hev b 5-specific IgE antibody (28%) as compared with Spanish (49%) and American (71%) patients. The prevalence of Hev b 6.01-specific IgE reactivity was uniformly >50% in all three populations.

Conclusion Neither Hev b 2 nor Hev b 13 appear to be major latex allergens when evaluated in serological assays using highly purified allergens. The reason(s) for the observed differences in published sensitization rates in various geographic regions requires further study. The purity of the allergen preparations has a marked impact on the accuracy of latex-specific IgE antibody detection in epidemiological studies and in the serological diagnosis of latex allergy.

Keywords allergens, Hev b 2, Hev b 13, IgE antibodies, latex hypersensitivity, prevalence

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Introduction

Immediate-type hypersensitivity to natural rubber latex (NRL) continues to be an important occupational disease among health-care workers (HCW) and other personnel using NRL gloves [1–4]. Of the more than 250 different polypeptides detected from NRL, only about one-fourth appear to be capable of binding IgE antibodies as assessed by IgE immunoblotting after two-dimensional gel electrophoresis [5–7]. Knowledge of the principal allergens in *Hevea* latex can facilitate the development of more reliable

in vivo and *in vitro* diagnostic reagents for use in assessing individuals with a suspected NRL allergy based on their clinical history. Moreover, knowledge of which *Hevea brasiliensis* (Hev b) allergenic proteins drive the sensitization to NRL aids in the design of assays for monitoring the allergenicity of NRL-containing products. The WHO/IUIS Allergen Nomenclature Committee (www.allergen.org) in February 2006 listed 13 NRL Hev b allergens as having been characterized at the molecular level.

Several NRL proteins reportedly represent clinically important allergens [8–10]. However, the current

knowledge about which allergenic proteins or their fragments are present in extracts of manufactured NRL products is still limited. This has made it difficult to define the relevance of each allergen in the induction of clinical disease. Of the well-characterized NRL allergens, Hev b 6 [Hev b 6.01 (prohevein) and Hev b 6.02 (hevein)] and Hev b 5 have been unequivocally identified as major allergens for adults as evidenced by the presence of specific IgE antibodies in the sera of more than half of the sensitized individuals in any defined population of patients [5, 11–13]. Moreover, Hev b 1 and Hev b 3 are important allergens for children with spina bifida or other congenital malformations, who are sensitized as a result of exposure during their multiple surgeries at an early age [14–17]. Recently, Hev b 2 and Hev b 13 have been reclassified as major allergens in NRL allergy [8, 10, 18], based on a positive skin prick test (SPT) in 63% of NRL-allergic patients. However, preliminary studies in a Finnish population have indicated to the contrary that IgE to purified native Hev b 2 and Hev b 13 are relatively rare (e.g., ~20% of Finnish patients). In this study, we examine the prevalence of IgE antibodies using highly purified Hev b 2 and Hev b 13, and contrast it to the presence of IgE antibody specific for Hev b 5 and Hev b 6.01. Sera from three latex-sensitized populations in diverse geographic areas were evaluated to control for the possibility of differential exposure to latex products containing variable distributions of individual NRL allergens.

Methods

Patients and controls

A total of 215 NRL-allergic adult patients and 172 atopic controls without NRL allergy were included in the study. The demographics of the study subjects, including the proportions of each group who were HCW, are presented in Table 1. For the Finnish patients, the diagnosis of NRL allergy was based on a compatible clinical history, upon exposure to NRL gloves and/or other NRL products in the near past, a positive SPT (Stallergènes, Antony Cedex, France, and/or high-allergen glove extracts) and a positive

latex-specific IgE serology [ImmunoCAP, Pharmacia Diagnostics (currently Phadia), Uppsala, Sweden]. Controls were atopic subjects with positive SPTs to one or more regional aeroallergens but with a negative SPT to NRL.

Descriptions of patients and control subjects from Spain and the USA have been reported in detail elsewhere [8, 19]. Briefly, the American patients had a positive clinical history consistent with an allergic reaction upon exposure to NRL gloves and/or other NRL products, a positive SPT with the Greer latex reagent [non-ammoniated latex (NAL) tested at protein concentrations up to 1000 µg/mL] and a positive latex-specific IgE serology (ImmunoCAP, Phadia, Kalamazoo, MI, USA) and AlaSTAT [Diagnostic Products Corporation (currently Siemens), Los Angeles, CA, USA]. The control subjects had clinical symptoms, a positive SPT for at least one of the 12 regional aeroallergens and a history of exposure to NRL gloves with a negative SPT to the Greer NAL.

The Spanish patients had a positive clinical history of allergic reactions to NRL gloves and/or other NRL products, a positive SPT to a commercial NRL extract (ALK-Abelló, Madrid, Spain) and a positive latex-specific IgE serology [ImmunoCAP, Pharmacia Diagnostics (currently Phadia)]. Atopic controls were defined with a positive SPT to one or more regional aeroallergens or to cockroach, but they had a negative SPT to the ALK-Abelló NRL extract. In all study centres, blood samples were taken from the cases and controls at the time when the latex SPT became an integral part of the diagnostic algorithm. Serum was stored as aliquots at –20 °C before they were tested. This study was approved by ethics committees in Finland (Tampere University Hospital and National Public Health Institute), Spain (Hospital Universitario de Gran Canaria Dr Negrín) and the United States (the Research and Ethics Committee of the Johns Hopkins Medical Institutions, Baltimore, MD, USA).

Purification of natural rubber latex allergens

The source latex for isolation of the NRL allergens (B-serum and C-serum of non-ammoniated NRL, clone RRI 600) was purchased from the Rubber Research Institute

Table 1. Demographic properties of NRL-allergic patients and control subjects

	Finland		Spain		United States	
	Patients	Controls	Patients	Controls	Patients	Controls
No. of subjects	79	73	74	50	62	49
Mean age (years)	39.2	34.2	33.3	31.4	36.1	37.1
Age range	16–63	15–75	17–69	15–54	21–67	28–56
Sex (F/M)	68/11	54/19	66/8	33/17	60/2	35/14
Atopic disease (%)	100	100	61	100	89	100
HCW (%)	23	14	31	0	100	100

NRL, natural rubber latex; HCW, health-care worker; F, female; M, male.

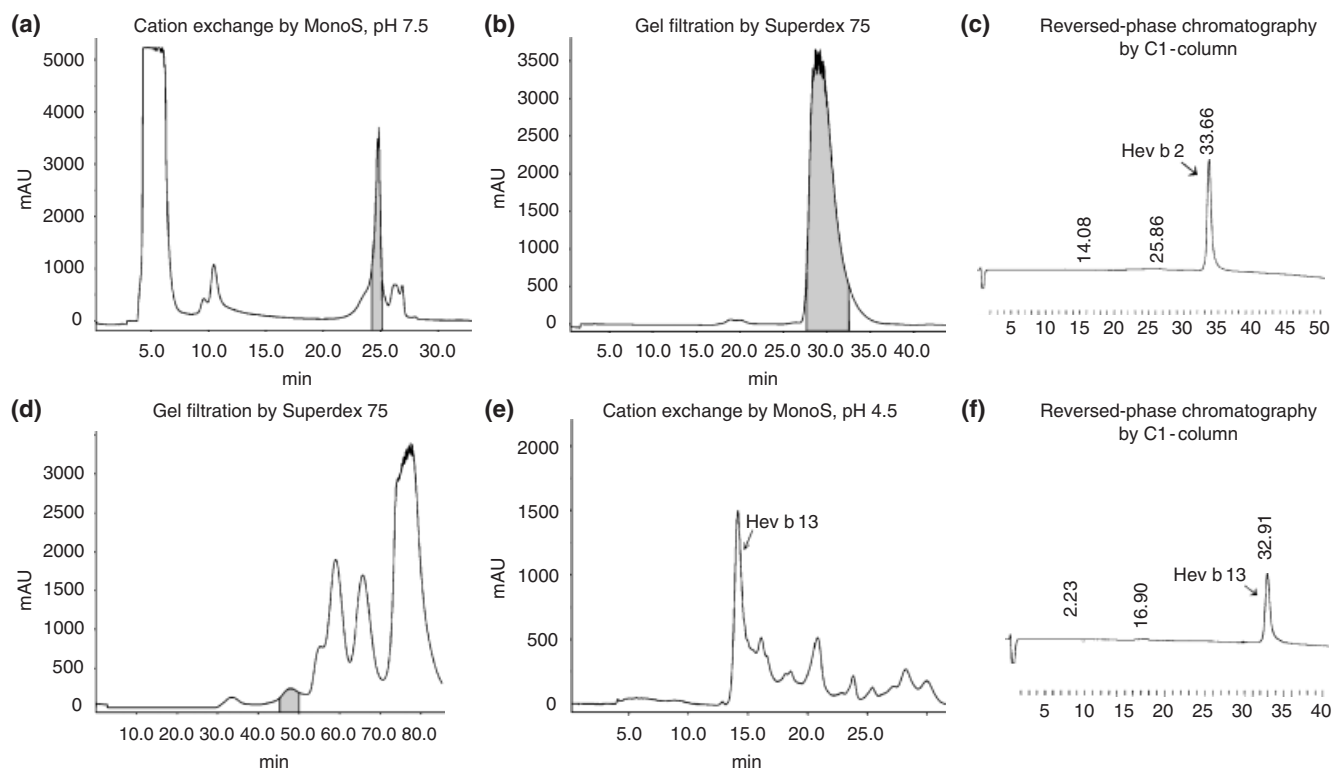


Fig. 1. Purification steps and confirmation of purity by reversed-phase chromatography of *Hevea brasiliensis* (Hev b 2 (a–c) and Hev b 13 (d–f) proteins.

(Kuala Lumpur, Malaysia). Hev b 2, Hev b 6.01 and Hev b 13 were purified from B-serum of NRL (diluted 1 : 5 with 50 mM sodium phosphate, pH 7.5), whereas Hev b 5 was isolated from C-serum [9].

The purification of Hev b 2 began with cation exchange chromatography using a MonoS (Tricorn 4.6/100PE, Amersham Biosciences, Uppsala, Sweden) column that had been equilibrated with 50 mM sodium phosphate, pH 7.5 (Fig. 1a). Elution was performed using a linear gradient of NaCl (0–1 M in 30 min). The flow rate was 1.0 mL/min and the chromatography was monitored at 214 nm. Hev b 2-containing fractions (marked grey in Fig. 1a) were pooled and a concentrated Hev b 2-containing preparation was subjected to gel filtration chromatography on a Superdex 75 HR 10/30 column (Amersham Biosciences) in phosphate-buffered saline (PBS) (Fig. 1b). The flow rate was 0.5 mL/min, and the chromatography was monitored at 214 nm. The Hev b 2-containing fractions (marked grey in Fig. 1b) were again pooled and concentrated.

Hev b 5 was purified by cation exchange chromatography. C-serum was first subjected to a buffer exchange of 20 mM Na-citrate, pH 3.8, using a BioGelP6 desalting gel column (Bio-Rad Laboratories, Hercules, CA, USA). Cation exchange chromatography was then performed on a MonoS 4.6/100 PE column (Tricorn, Amersham Biosciences) that had been equilibrated with 20 mM Na-citrate, pH 3.8. Elution was performed with a linear gradient of NaCl (0–1 M in 30 min) in the equilibration buffer.

The purification of Hev b 6.01 involved gel filtration and anion exchange chromatography [20]. Briefly, gel filtration was performed on a Superdex 75 HR 16/60 column (Amersham Biosciences) in PBS. Prohevein-containing fractions were concentrated and the buffer was changed to 20 mM Tris-HCl, pH 8.5, using a BioGelP6 column. Anion-exchange chromatography was performed on a MonoQ HR5/5 column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 8.5. Elution was performed with a linear gradient of NaCl (0–1 M in 30 min) in the equilibration buffer.

For purification of Hev b 13, 1.5 mL of NRL B-serum was chromatographed on a Superdex 75 (HR 16/60) column at a flow rate of 1.5 mL/min and monitored at 280 nm (Fig. 1d). Hev b 13-containing fractions (marked grey) were pooled and concentrated. The buffer was changed to 50 mM sodium acetate, pH 4.5, on a Bio-GelP6DG column. Hev b 13-containing fractions were applied to a cation exchange column, MonoS (HR 5/5, Amersham Biosciences) (Fig. 1e). Elution was performed using a linear gradient of NaCl (0–1 M in 40 min). The flow rate was 1.0 mL/min and the chromatography was monitored at 214 nm. Hev b 13 eluted as a peak marked in the figure (Fig. 1e).

The purity of all four allergen preparations was ascertained by reversed-phase chromatography on a C1 column (2 × 20 mm, Tosoh Corp., Tokyo, Japan) as described previously [20]. Elution was performed with a linear

gradient of acetonitrile (0–100% in 60 min) in 0.1% trifluoroacetic acid. The flow rate was 0.2 mL/min and detection was performed at 214 nm. The eluted peaks for Hev b2 and Hev b13 are shown in Figs 1c and f, respectively.

Identification and characterization of the purified allergens

Monoclonal antibodies to the four allergens and commercial ELISAs for Hev b 5 and Hev b 6.02 (Fit Biotech Ltd, Tampere, Finland) were used to identify and quantify the purified allergens. For molecular characterization of the purified allergens, the molecular mass and peptide mass fingerprint analyses were performed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and electrospray quadrupole time-of-flight mass spectrometric analyses [21]. N-terminal sequencing was performed as described previously [22].

Immunoglobulin E enzyme linked immunosorbent assay

IgE antibodies to Hev b 5 and Hev b 6.01 were measured by ELISA as described previously [23]. Briefly, Hev b 6.01 and Hev b 5 were separately coated on microtitre plates at 2 µg/mL (100 µL/well). The wells were then blocked with 1% human serum albumin (Red Cross Finland Blood Service, Helsinki, Finland) in 50 mM sodium carbonate buffer, pH 9.6, and 100 µL of patient or control serum (in duplicate, diluted 1 : 10) was added. Biotinylated goat anti-human IgE (Vector Laboratories Inc., Burlingame, CA, USA; diluted 1 : 1000) was added, followed sequentially by streptavidin-conjugated alkaline phosphatase (Bio-Rad Laboratories; diluted 1 : 3000) and a substrate (*p*-nitrophenyl phosphate, ICN Biomedicals Inc., Aurora, OH, USA). When internal pooled control sera had reached predetermined optical density (OD) values, the OD was read at 405 nm with an automated ELISA reader (Titertek Multiscan, Eflab, Turku, Finland). IgE antibodies to Hev b 2 and Hev b 13 were measured using the same assay format, except that the coating concentration of these antigens was 1 µg/mL in PBS, pH 7.4. Positive reactions in each assay were determined as OD values exceeding the 98th percentile of local atopic control subjects, which approximately corresponded to the mean + 3 SD. Inhibition ELISA analyses were performed for specificity assessment as described earlier [24], using different NRL allergens to inhibit the binding of patients' IgE to the solid-phase antigens.

Statistical analyses

Non-parametric statistics (Mann–Whitney *U*-test) were used because the majority of OD values were not normally distributed. A *P*-value of 0.05 or less was considered to be

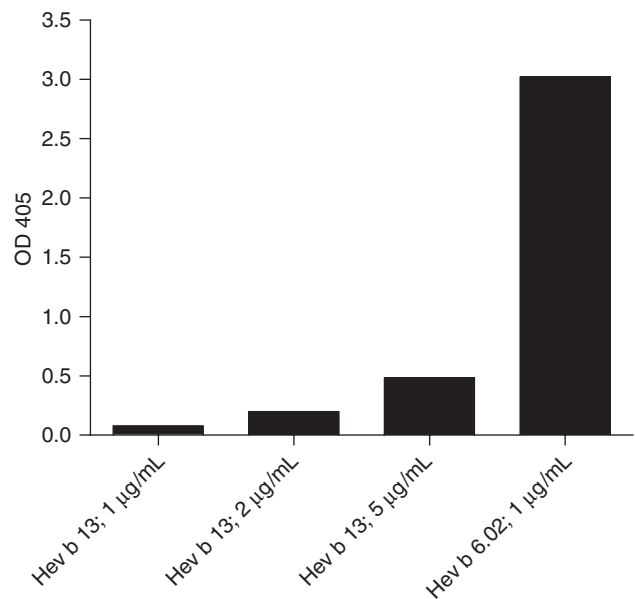


Fig. 2. Dose-dependent binding of a murine monoclonal anti-*Hevea brasiliensis* (Hev b) 6.02 antibody (diluted 1 : 1000) onto Hev b 13-coated (1 µg/mL) microtitre wells. The binding of the antibody to wells coated with purified Hev b 6.02 (1 µg/mL) is shown for comparison. OD, optical density.

a statistically significant difference between groups. The correlation between individual serum IgE antibody levels to the various allergens was assessed by a non-parametric Spearman's *r*-test.

Results

Identification and assessment of the purity of *Hevea brasiliensis* 2 and *Hevea brasiliensis* 13 preparations

The Hev b 2 preparation used in this study for serological evaluation was shown to be homogeneous by reversed-phase chromatography (Fig. 1c). Its identity was further confirmed using monoclonal anti-Hev b 2 antibody reactivity, N-terminal sequence assessment and spectrometric mass fingerprint analysis. Monoclonal antibodies specific for Hev b 6.02, Hev b 5 or Hev b 13 had no detectable immunoreactivity to the finalized native purified Hev b 2 (data not shown).

The Hev b 13 used in the study required extensive re-purification. Monoclonal anti-Hev b 6.02 antibodies and human sera containing IgE antibodies specific for Hev b 6.01 or Hev b 6.02 bound to the Hev b 13 preparations at various stages in the purification process. Figure 2 shows that even after the final purification step, Hev b 6.02 reactivity was still present in the Hev b 13 preparation. However, there was no indication of the presence of mature hevein (Hev b 6.02), prohevein (Hev b 6.01) or other proteins as analysed by reversed-phase chromatography or peptide mass fingerprint analyses in the final

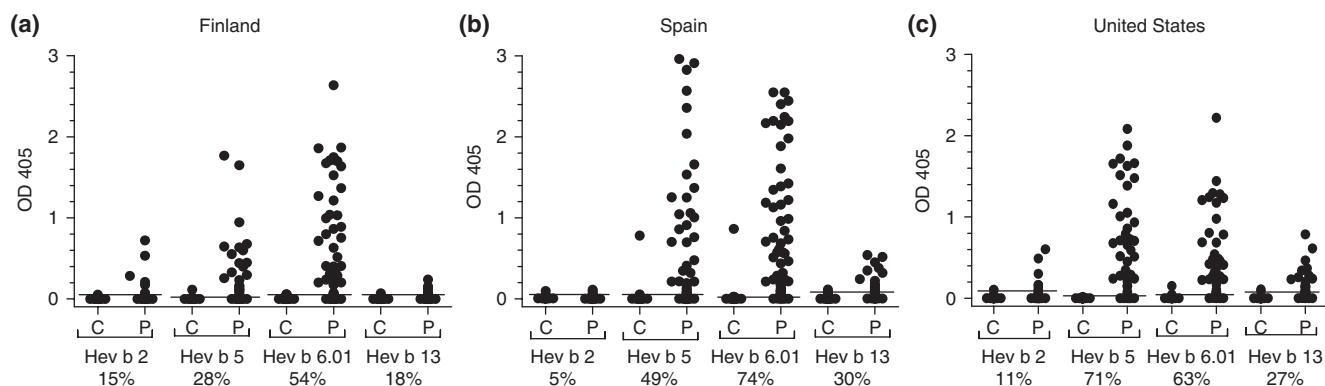


Fig. 3. Distribution of IgE antibodies to *Hevea brasiliensis* (Hev b) 2, Hev b 5, Hev b 6.01 and Hev b 13 in three natural rubber latex (NRL)-allergic patients and non-NRL-allergic control populations from Finland (a), Spain (b) and the United States (c). The frequency of positive reactions (percentage of values exceeding the 98th percentile of respective local control subjects) is given for each allergen on the x-axis. Horizontal bars on the y-axis denote cut-off points for positive reactions, corresponding approximately to the mean + 3 SD of the local atopic controls. OD, optical density.

Hev b 13 preparation. Thus, the nature of the binding of anti-Hev b 6.01 to epitopes in the Hev b 13 remained elusive. Monoclonal antibodies specific for Hev b 13 bound effectively to the purified protein in ELISA while monoclonal antibodies specific for Hev b 2 and Hev b 5 displayed no reactivity to the Hev b 13 (data not shown). Mass fingerprint analyses and N-terminal sequencing confirmed the identity of the purified protein as Hev b 13. Sequence analysis of the purified protein gave an N-terminal sequence of ETCDPFA, which resulted from the cleavage of the Hev b 13's 27 amino acid signal sequence. MALDI-TOF mass spectrometric analysis of the purified Hev b 13 produced an average molecular mass of 43 375 Da. The mass of the protein polypeptide chain, as calculated from the known deduced sequence [18] and the N-terminus determined in the present study, was 40 313.2 Da. This indicated that the protein is post-translationally modified.

The Hev b 13 polypeptide chain contains 3 putative N-glycosylation sites. The last one (N 286) contained at least three different forms of glycans, composed of N-acetyl glucosamine and varying amounts of mannose, fucose and xylose (data not shown). The mass of these glycans was not enough to cover the suggested mass for the post-translational modifications. The tryptic peptide containing the two remaining glycosylation sites was not further analysed due to its low recovery and large size.

Prevalence of immunoglobulin E antibodies to four purified natural rubber latex allergens in three geographic areas

There were well-defined differences in the prevalence of IgE specific for the four NRL allergens in serum from NRL-allergic patients from Finland, Spain and the United States (Fig. 3). IgE antibodies specific for Hev b 2 were rare in Spain (5%), and seen only in a minority of patients from Finland (15%) and the United States (11%). The prevalence

of IgE to Hev b 13 ranged between 18% and 30% in all three geographic regions, suggesting a relatively low overall frequency of sensitization to this allergen. The prevalence of IgE antibodies to Hev b 5 was unexpectedly low in Finland (28%), which contrasted with the sera collected from Spanish and American patients (49% and 71%, respectively). Meanwhile, the frequency of IgE antibodies to Hev b 6.01 was >50% in the serum of NRL-allergic patients in all three countries (Fig. 3).

Significant differences were noted among the three geographic areas in the mean OD levels that reflected the relative quantity of IgE antibodies in the serum that was reactive to the individual NRL allergens (Fig. 4). The highest mean response values for anti-Hev b 2, anti-Hev b 5 and anti-Hev b 13 were seen with sera from the American patients, and the highest mean anti-Hev b 6.01 level was present in the sera from the Spanish patients. The lowest mean anti-Hev b 2, anti-Hev b 5 and anti-Hev b 13 levels were observed with sera from the Finnish patients, and the lowest mean anti-Hev b 6.01 level was present in American sera.

Immunoglobulin E binding to solid-phase *Hevea brasiliensis* 13 can be inhibited by adding soluble *Hevea brasiliensis* 6.01

Purified soluble Hev b 6.01 inhibited the binding of patient-specific IgE to immobilized Hev b 13 in a dose-dependent manner with most of the sera that were studied. Figure 5 depicts representative examples of the three modes of inhibition. Figure 5a shows a 'pure' anti-Hev b 13 IgE response that was not inhibitable by Hev b 6.01. In Fig. 5b, IgE anti-Hev b 13 binding was partially inhibited by the addition of soluble Hev b 6.01. In contrast, Fig. 5c displays the binding of human IgE anti-Hev b 13 that was totally inhibited by Hev b 6.01. Inhibition experiments were performed using eight sera chosen for their moderate

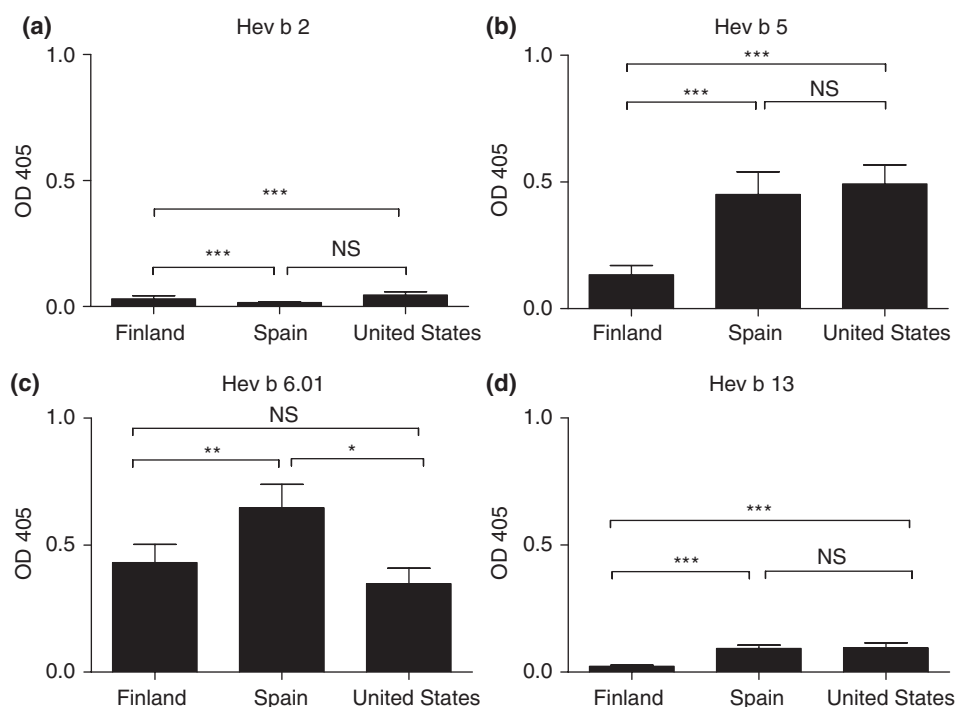


Fig. 4. Comparison of mean IgE antibody levels to *Hevea brasiliensis* (Hev b 2 (a), Hev b 5 (b), Hev b 6.01 (c) and Hev b 13 (d) in three patient populations. The number of patients in Finland, Spain and the United States were 79, 74 and 62, respectively. The columns and the error bars represent means $\pm$ SEMs; * P < 0.05; ** P < 0.01; *** P < 0.001; NS, not significant; OD, optical density.

to strong IgE antibody responses to Hev b 13. Of these, two sera belonged to the Fig. 5a-like category in which soluble Hev b 6.01 at 0.1 μ g/mL produced a 2–4% inhibition of the binding of IgE to solid-phase Hev b 13. Two other sera mimicked the Fig. 5b-like category in which 26–54% inhibition was observed with the addition of soluble Hev b 6.01. The remaining four sera fell in the Fig. 5c-like category with 69–95% inhibition produced by soluble Hev b 6.01 (data not shown).

Discussion

Hev b 6.01 and Hev b 5 have been known to be the clinically most important 'major' NRL allergens [7]. Recent studies have suggested that Hev b 2 [10, 25, 26] and Hev b 13 [18, 27] may also be clinically important NRL allergens. Accordingly, Bernstein *et al.* [8] recently reported that 63% of NRL-allergic patients had a positive SPT to both purified Hev b 2 and Hev b 13. This was similar to the frequencies of positive skin reactions seen with Hev b 6.01. Kurup *et al.* [10] used similar allergen preparations in serological assays and reported that ~70–80% of NRL-allergic adults had IgE antibodies reactive with Hev b 2 and Hev b 13. Importantly, the Hev b 13 that had been produced in *Escherichia coli* by recombinant DNA technology did not bind human IgE [18]. Because the removal of the glycans from native Hev b 13 protein abolished its IgE-binding reactivity, it was proposed that the epitopes mediating IgE reactivity

appeared to reside in the glycan residues of the proteins [18, 28]. However, these epitopes have not been characterized or clearly defined, especially in relation to the relative purity of the proteins that were used to assess their allergenicity. Parenthetically, assessment of the prevalence of IgE antibodies to the 9 Hev b allergens other than Hev b 2, 5, 6.01 and 13 was not undertaken in this study because it is generally agreed, based on the literature, that these allergens represent minor players in terms of inducing IgE sensitization in adult HCW [8, 27, 29].

During our studies involving the purification of multiple NRL allergens, we faced the problem that Hev b 6.02 (mature hevein) readily co-purified with Hev b 13. This unfortunately resulted in false-positive serologic and SPTs results in otherwise IgE anti-Hev b 13-negative individuals. In the present study, we paid exceptional attention to the relative purity of the Hev b 13 and strove to remove all Hev b 6.02 before its use in the serology studies. Our previous experience with partially impure Hev b 13 and Hev b 2 preparations revealed the presence of other NRL allergens, or their fragments such as Hev b 6.02, Hev b 5 and, in some instances, Hev b 1 when analysed using a commercial capture enzyme immunoassay (FIT kit, FIT Biotech Ltd). It can be noted that the isolation and purification procedures for Hev b 13 in the earlier two studies [8, 10] differed from the present study. For instance, the initial ion exchange chromatographic steps were basic (pH 8.6) in the previous studies, while we used ion exchange chromatography under acidic

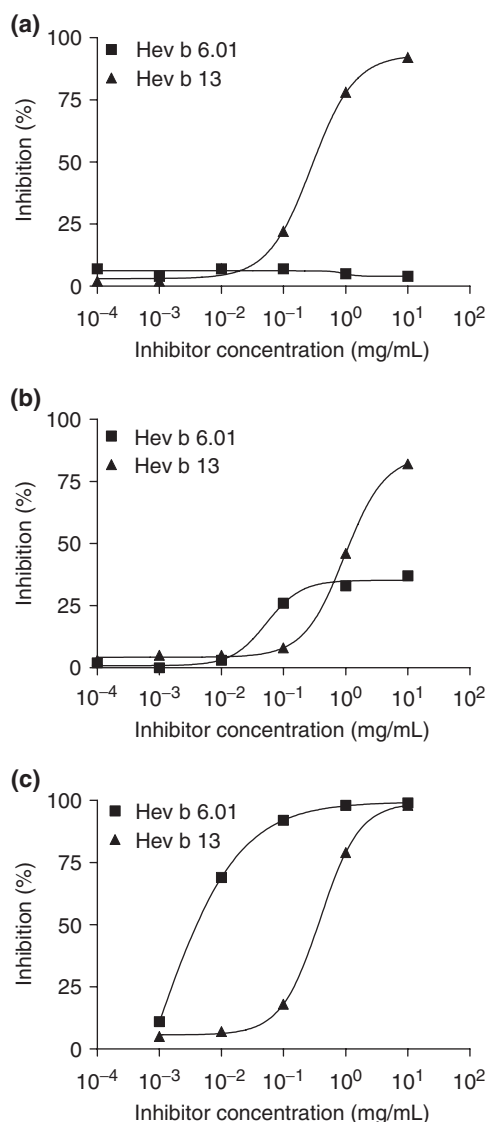


Fig. 5. Illustration of patterns of competitive inhibition of IgE binding to solid-phase *Hevea brasiliensis* (Hev b) 13 (1 µg/mL) with soluble Hev b 6.01 and Hev b 13. Sera from three anti-Hev b 13-positive natural rubber latex-allergic patients were analysed. (a) No inhibition, (b) partial inhibition and (c) total inhibition by the presence of soluble Hev b 6.01.

conditions. Different purification procedures may result in a varying level of purity of the proteins and it is possible that in the previous studies [8, 10] the purification conditions have been less effective to remove the well-defined excess Hev b 6.02 and Hev b 6.01 in NRL serum preparations.

One may argue that the extensively purified allergen preparations were devoid of *in vivo* reactivity and were therefore unsuitable for IgE prevalence assessment. This, however, was shown not to be the case because SPTs performed on eight Finnish NRL-allergic individuals (at an allergen concentration of 50 µg/mL, data not shown) revealed two patients positive to Hev b 2 and four patients

positive to Hev b 13 (diameter of the weal at least 3 mm). Three of the eight patients were positive for Hev b 5 and six for Hev b 6.01. There was no correlation between the SPT weal size produced by Hev b 6.01 and Hev b 13 (Spearman's $r = -0.189$, not significant), suggesting that the low Hev b 6.02-like impurity in the extensively purified Hev b 13 preparation had little, if any, effect on the SPT reactions. Among the four Hev b 13-positive patients, one was negative to Hev b 6.01 and to other allergens tested, suggesting the presence of a pure Hev b 13-specific reaction. The small number of patients precludes a generalized conclusion regarding the *in vivo* reactivity of these extensively purified allergen preparations in the broader latex-allergic patient population.

To assess the effect of alleged impurities in Hev b 13 preparations on the outcome of SPT reactivity, we conducted SPT on eight additional Finnish NRL-allergic patients (all positive in SPT to Hev b 6.01) with an insufficiently pure Hev b 13 preparation (data not shown). This Hev b 13 preparation was taken from the purification protocol before it had been subjected to the final purification procedures. We observed that all eight patients had positive SPT reactions to the insufficiently pure Hev b 13 and there was an almost perfect correlation between the two sets of results (Spearman's $r = 0.974$, $P = 0.0004$). This indicates that Hev b 6.01/6.02 was involved in most, if not all, the reactions produced by the insufficiently pure Hev b 13 preparation.

During the Hev b 13 purification process, binding of anti-Hev b 5 and anti-Hev b 1 progressively decreased to undetectable levels. In contrast, monoclonal anti Hev b 6.02 reactivity to even the final ultrapure Hev b 13 preparation could not be totally abolished. When the purity of the final Hev b 13 preparation was assessed with different protein chemical methods, such as reversed phase chromatography, N-terminal sequencing, electrospray- or MALDI-TOF mass spectrometry or mass spectrometric peptide mass fingerprinting, no structures other than Hev b 13 could be detected. However, the relative Hev b 6.02 levels below approximately 1–2% could not have been detected using these techniques.

There is no apparent homology between the primary sequences of Hev b 6.02 (or Hev b 6.01) and Hev b 13. The exact nature of the binding of the Hev b 6.02 reactive antibodies to the Hev b 13 preparation has not yet been determined. Fortunately, with extensive purification, we were able to reduce this phenomenon from significant to very low levels, enabling the use of this protein to assess human IgE antibodies specific for native Hev b 13 in serological assays. We reasoned that should the binding of anti-Hev b 6.02 to the ultrapure Hev b 13 preparation be due to cross-reactive epitopes on Hev b 13, one would expect to continue seeing the binding of anti-Hev b 6.02 over the course of its increasing purification. Because anti-Hev b 6.02 binding diminished with increasing

purity, it appears unlikely that binding of Hev b 6.02-specific IgE to cross-reactive epitopes plays a significant role in explaining the phenomenon.

We used the 98th percentile of local atopic controls to define a positive threshold for the presence of the Hev b allergen-reactive IgE antibody. This approach can be expected to enable one to better control for possible differences associated with variability resulting from the local geographic environment, such as exposure to different NRL-containing products. Moreover, the actual potency of the immunological response (level of antibody) is unlikely to influence the determination of prevalence. In addition, this study contended with the skewed distribution of the low absorbance values obtained with the sera from the control subjects.

The sensitization rates to purified Hev b 13 were generally low (18–30%, Fig. 3) when the ultra-pure Hev b 13 was used in the analysis. Furthermore, the binding of patients' IgE to Hev b 13 could be partially or totally inhibited by the addition of soluble Hev b 6.01. When the binding of IgE to solid-phase Hev b 13 was not inhibited by Hev b 6.01 (Fig. 5a), the response was considered specific to Hev b 13. However, sera with a clearly defined IgE anti-Hev b 13 response in the absence of measurable IgE anti-Hev b 6.01 antibodies were rare. Inhibition experiments were performed only to a small subset of the sera evaluated in the study, and thus these results should be interpreted with care. Interestingly, the binding of IgE was partially inhibited (up to 54%) by soluble Hev b 6.01 in some sera, suggesting that the measured IgE binding was a result of a mixture of both Hev b 13 and Hev b 6.01-specific antibodies (Fig. 5b). In half of the sera evaluated by inhibition, up to 95% of the binding activity to Hev b 13 was inhibited by the addition of soluble Hev b 6.01 (Fig. 5c). In these cases of essentially complete inhibition, it is likely that the serum does not contain IgE antibodies that are specific for Hev b 13. It is therefore possible that NRL-allergic patients with a restricted Hev b 13-specific IgE antibody response are even rarer than indicated by the present results (18–30%). Consistent with our observations is a recent report that shows that only 10% of Taiwanese HCW had IgE binding to Hev b 13 that had been quality controlled with peptide mass fingerprinting and analysed by immunoblotting after two-dimensional gel electrophoresis [30].

Because the binding of IgE to solid-phase Hev b 13 can be influenced by a minor impurity of Hev b 6.01 or Hev b 6.02, one might expect to see a correlation between the levels of IgE anti-Hev b 13 and anti-Hev b 6.01 in the patients' sera. Even though the numbers of anti-Hev b 13-positive sera were low, a correlation was evident between Hev b 13 and Hev b 6.01 reactive antibody ($n = 215$, $r = 0.517$, $P < 0.0001$). Alternatively, no significant correlation was observed between the presence of anti-Hev b 6.01 and anti-Hev b 2 antibodies (data not shown).

The purification procedure for Hev b 2 generated a clean, native protein that displayed no reactivity with monoclonal antibodies specific for Hev b allergens other than Hev b 2. Using this protein in ELISA and previously in immunoblotting [31], we have verified markedly lower prevalence rates of IgE anti-Hev b 2 in NRL-allergic patients than previously reported elsewhere [8, 10, 28, 29]. The prevalence of IgE to Hev b 2 and Hev b 13 in NRL-allergic patients in the present study also differed considerably across three different geographic areas. Among the Spanish patients, the prevalence of IgE to Hev b 2 and Hev b 13 was exceptionally low (5% and 30%, respectively).

While the present study has focused on the significance of Hev b 2 and Hev b 13 as NRL allergens, we also performed a comparable analysis for the prevalence of IgE antibodies specific for Hev b 6.01 and Hev b 5, which are known to be major NRL allergens. The prevalence of IgE to Hev b 5 was unexpectedly low (28%) in Finland, while it was higher in Spain (49%) and the United States (71%). One possible explanation is the marked decrease in the proportion of medical gloves containing Hev b 5 in Finnish markets between the years 1995 and 2001 from 73% to 23% [32].

In contrast, the prevalence of Hev b 6.01-specific IgE was high (54–74%) in all three geographic areas. The reasons for the different sensitization rates may be due to the different properties of the NRL gloves used in these countries, and differences in glove usage habits. We noted significant differences in not only the prevalence but also in the relative levels of IgE antibodies specific for the four Hev b allergens (Fig. 4). These may be related to differential exposure to these and other possibly cross-reactive allergens. Differences among the study groups may also relate to patient selection and demographic issues. We acknowledge a marked decrease in the average concentration of NRL allergens in medical gloves currently marketed in the United States and most European countries from those available 5–10 years ago. Moreover, the use of powdered gloves has simultaneously decreased. These changes have unquestionably reduced the incidence of NRL allergy in these regions. Precise information on extent of glove usage by both cases and controls providing sera in this study are not possible to accurately determine retrospectively.

In conclusion, the low frequency of IgE antibodies to ultra-purified Hev b 2 and Hev b 13 in the serum of NRL-allergic patients from three diverse geographic areas suggests that the rates of sensitization to these allergens are relatively low in comparison with previous reports. Thus, based on serologic evaluation, these allergens should not be classified as major NRL allergens. Although our purification protocols led to reasonable but not complete purity of Hev b 13 and to chromatographically and immunologically pure Hev b 2, the production of

these allergens in an immunologically active form by recombinant DNA technology is needed to definitively assess their clinical significance in NRL allergy.

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