

Occupational IgE-Mediated Softwood Allergy: Characterization of the Causative Allergen

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Established Facts

- The lack of standardized and commercially available wood dust allergen diagnostic tools complicates the diagnosis of occupational allergy.
- Differentiation between occupational and nonoccupational sensitization is often difficult due to either cosensitization or cross-reactivity.

Novel Insights

- Specifically tailored in vitro diagnosis and allergen identification correlate with wood dust challenge tests and are therefore a valuable tool for a reliable wood allergy diagnosis.
- Specification of IgE binding concerning proteinogenic and glycosylated structures as well as allergen identification allows the elucidation and classification of the source of allergy.

Key Words

Occupational allergy • Softwood • Spruce (*Picea abies*) •
In vitro diagnostics • Allergen identification

Abstract

Allergic reactions to wood dust allergens are rare, and only few in vitro diagnostic tools and information about relevant allergens are available. To differentiate between protein-based allergy and probably clinically silent glycosylated sen-

sitization, it is helpful to characterize the relevant protein allergens and specify IgE binding. The current case report deals with the occupational softwood allergy of a carpenter exposed to different wood dusts. Skin tests and IgE tests against wood were performed with specifically tailored ImmunoCAPs and cross-reactive carbohydrate determinants. Potential allergens were identified by IgE blots and tandem mass spectrometry. The clinical relevance was verified by challenge tests. Specific IgE to softwood (spruce, pine and larch wood), beech wood, natural rubber latex (NRL) and

horseradish peroxidase (HRP) were detected. Allergens in spruce wood, the dominant allergen source, were identified as peroxidases. Softwood were the strongest inhibitors. HRP reduced IgE binding to softwood to <50%, indicating predominantly proteinogenic epitopes, whereas IgE binding to NRL and beech wood was reduced to >50% by HRP, indicating predominantly glycolytic IgE epitopes. Skin and challenge tests underlined that softwoods were the source of sensitization. For the polysensitized patient, a clinically relevant softwood allergy was diagnosed, not only by challenge tests but also with specifically tailored *in vitro* tools.

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Introduction

Occupational exposure to wood dust is known to be a risk factor for respiratory diseases based on different immunological or nonimmunological mechanisms. In a recent analysis [1], the relative risk of asthma among workers exposed to wood dust was calculated to be 1.53 (95% CI 1.25–1.87). IgE-mediated wood dust allergy (type I allergy) is mostly induced by exposure to tropical wood dust [2–5] whereas the prevalence of IgE-mediated softwood sensitization among exposed workers is limited [6]. Even though the prevalence of softwood sensitization seemed to be quite low in the present case, it is important to know the causative allergens to avoid more severe respiratory symptoms. Therefore, it is necessary to clarify the possible health risks based on the relevant wood dust source and cross-reactivity with other substances, and to evaluate prevention and/or rehabilitation strategies to prevent workers from having to leave their jobs in the wood industry or, in the worst case, losing their regular income.

It is often difficult to diagnose an IgE-mediated wood dust sensitization because allergen extracts are rare and in most cases not commercially available, and complex and laborious to prepare [7]. In addition, false-positive IgE results may be caused by cross-reactive carbohydrate determinants (CCDs) in wood dust and other occupational allergen extracts [7, 8]. For an appropriate diagnosis, specifically tailored *in vitro* diagnostic tools are necessary to quantify wood dust-specific IgE. The potential clinical relevance of wood dust sensitization can be corroborated by specification of IgE binding to proteinogenic allergen epitopes.

Case Report

Patient

A 25-year-old carpenter with reported occupational allergic respiratory symptoms during work was investigated. The allergic symptoms affected his nose, eyes and lungs. Mostly processed wood species at the workplace were pine (*Pinus* spp.), spruce (*Picea* spp.), beech (*Fagus* spp.) and oak (*Quercus* spp.). In fact, the patient was not able to specify one single wood species responsible for his allergic symptoms during work because mostly pine, spruce, beech and oak wood were processed in parallel or at least a mixture of all four wood dusts were present at the workplace. Occupational exposure to wood dust had existed for 16 years; exposure to chemicals, like varnish, paint or solvents, was excluded.

Additionally, the patient reported allergic symptoms after eating kiwi and banana, which manifested as swelling of the pharynx and lips as well as eczema. A skin prick test was negative for kiwi and banana extracts but sIgE to kiwi (2.09 kU/l) and banana (1.52 kU/l) were measured. Later on, he also developed sensitization to grass pollen, house dust mites and dog epithelia with clinical symptoms.

Skin Tests and Challenge Test

Wood dust sensitization was tested intradermally with all commercially available extracts of wood allergens (spruce, pine, obeche, beech, oak, mahogany, walnut and fir wood; Allergopharma Joachim Ganzer KG, Reinbeck, Germany). Any skin reaction to wood allergens >1 mm in the intradermal test was regarded as positive.

Environmental allergens were tested by skin prick test solutions (Allergopharma), skin reactions >3 mm being regarded as positive.

The patient underwent a specific inhalation challenge with pine wood from the workplace over 20 min according to Gonsior et al. [9]. Before the challenge, baseline values for nasal resistance, nasal flow using rhinomanometry (Master Scope Rhino, Care Fusion Hoechberg), FEV₁, resistance (R_t) and specific resistance (SR_t) in a body plethysmograph (Master Screen Body Care Fusion, Hoechberg) were determined. The clinical symptoms were documented during the challenge.

Nasal resistance, nasal flow, FEV₁, R_t and SR_t were measured immediately, and 10, 20 and 60 min after the challenge test. Moreover, FEV₁, R_t and SR_t values were defined 5 h later.

Preparation of Wood Dust-Specific ImmunoCAPs

Wood dust samples were extracted as described earlier [7]. Briefly, wood material with defined botanical origins was obtained from a timber specialist. The wooden blocks were grinded to dust and extracted for 6 h under aqueous conditions, followed by an ethanolic extraction overnight. Both wood dust extracts were dialyzed against distilled water, pooled and lyophilized. The protein concentration was determined using a Biorad Protein Assay®. Wood dust extracts were biotinylated and loaded on Streptavidin-ImmunoCAPs (o221, Phadia, Uppsala, Sweden) as described previously [7].

Table 1. Results of serological and intradermal skin testing to relevant allergens

Allergens	Specific IgE (ImmunoCAP) kU _A /l	Intradermal skin test wheal/ erythema, mm
Spruce	9.10 (3)	1/4
Pine	7.41 (3)	3/6
Larch	2.41 (2)	n.d.
Beech	1.00 (2)	0/0
HRP	0.51 (1)	n.d.
Oak	0.13 (0)	0/0
Obeche	0.09 (0)	0/0
Bromelain	0.11 (0)	n.d.
NRL	3.82 (3)	n.d.
rHev b 1	0.05 (0)	n.d.
nHev b 2	1.18 (2)	n.d.
rHev b 3	0.06 (0)	n.d.
rHev b 5	0.01 (0)	n.d.
rHev b 6.01	0.07 (0)	n.d.
rHev b 7	0.03 (0)	n.d.
rHev b 8	0.06 (0)	n.d.
rHev b 10	0.08 (0)	n.d.
rHev b 11	0.08 (0)	n.d.
rHev b 12	0.10 (0)	n.d.

Numerary shown in parentheses are CAP class. Positive values are indicated in bold. n.d. = Not done; r = recombinant; n = native.

Determination of Specific IgE and Reciprocal IgE Inhibition Test

Serum was tested for sIgE to various wood extracts (table 1) with ImmunoCAP 250 (Phadia) using specifically tailored ImmunoCAPs. Additionally, two commercially available CCDs, horseradish peroxidase (HRP; Ro400) and bromelain (k202), were tested as well as latex (k82) and recombinant major allergens of latex (rHev b 1, rHev b 3, rHev b 5, rHev b 6.01, rHev b 7, rHev b 8, rHev b 10, rHev b 11, rHev b 12 and the native (n)Hev b 2 [10]). Allergen-specific IgE values ≥ 0.35 kU_A/l were considered as positive.

To differentiate between primary sensitization and cross-reactivity or cosensitization, a competitive ImmunoCAP inhibition test was performed. All relevant wood dust extracts as well as the glycosgenic substance HRP and natural rubber latex (NRL) proteins from latex C serum [11] served as inhibitors. The serum sample (50 μ l) was mixed with 10 μ l inhibitor solution [spruce (*Picea abies*): 0.5 μ g/ μ l, pine (*Pinus sylvestris*): 0.5 μ g/ μ l, larch (*Larix decidua*): 0.5 μ g/ μ l, beech (*Fagus sylvatica*): 0.5 μ g/ μ l, NRL: 0.5 μ g/ μ l and HRP: 2.5 μ g/ μ l] and incubated for 1 h at room temperature. Subsequently, sIgE binding was measured on a wood dust or latex solid phase. The same volume of PBS instead of the inhibitor solution was taken as a positive control (100% binding, 0% inhibition). The reduction in IgE binding to wood and latex allergens induced by HRP and other inhibitors was calculated as the difference between PBS control and HRP inhibition. Inhibition values $>80\%$ were considered as high cross-reactivity where-

as values $<20\%$ were considered as no inhibition, indicating no cross-reactivity.

Electrophoresis and IgE Blotting

Wood extract proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent silver staining as described earlier [5]. To identify IgE-binding proteins, softwood Western blot was incubated with patient's serum (diluted 1:3) as described in Kespohl et al. [5].

Identification of Proteins by Tandem Mass Spectrometry

IgE-binding spruce proteins were analyzed by tandem mass spectrometry (MS-MS). Therefore, protein bands of interest were cut from SDS-PAGE gels and digested overnight using trypsin (Sigma-Aldrich, Geisenhofen, Germany) as described [12]. The resulting peptides were eluted, concentrated by vacuum centrifugation and analyzed using an LTQ-Orbitrap hybrid mass spectrometer (LTQ Orbitrap XL ETD, ThermoElectron, Bremen, Germany) equipped with a nanoelectrospray ion source (ThermoElectron) coupled online to a nano-HPLC system (NanoLC-Ultra 2D, Eksigent Technologies, Dublin, Calif., USA).

Using the ProteinQuant Suite [13], MS raw data were converted to mascot generic files (mgf). Afterwards a database search was performed with the mgf files, using the MS/MS ion search (MASCOT, <http://www.matrixscience.com>) against all entries of NCBI nr (GenBank) with subsequent parameters: trypsin digestion, up to three missed cleavages, fixed modifications: carbamidomethyl (C) and with the following variable modifications: oxidation (M), peptide tol.: ± 1.2 Da, MS/MS tol.: ± 0.6 Da and peptide charge: +1, +2 and +3.

Results

Clinical Diagnosis of Wood Dust Allergy

Intradermal skin tests to several wood extracts revealed reactions only to two softwood: spruce (1 mm wheal/4 mm erythema) and pine (3 mm wheal/6 mm erythema). Obeche, beech, oak, mahogany, walnut and fir wood extracts did not induce any skin reaction. The specific inhalation challenge with pine wood dust from the workplace showed the following allergic symptoms after application: burning of the eyes, lacrimation, rhinorrhea, sneezing and shortness of breath. Rhinomanometric measurements showed that nasal resistance was markedly increased and nasal flow was reduced. No airway responsiveness was measured.

Determination of Specific IgE to Wood Dust Allergens

Serological tests using specifically tailored ImmunoCAPs demonstrated very strong reactions to spruce, pine, larch and beech wood (table 1). Other wood species, like obeche and oak, were not recognized. IgE binding to CCDs was only measured against HRP (CAP class 1) but not against bromelain.

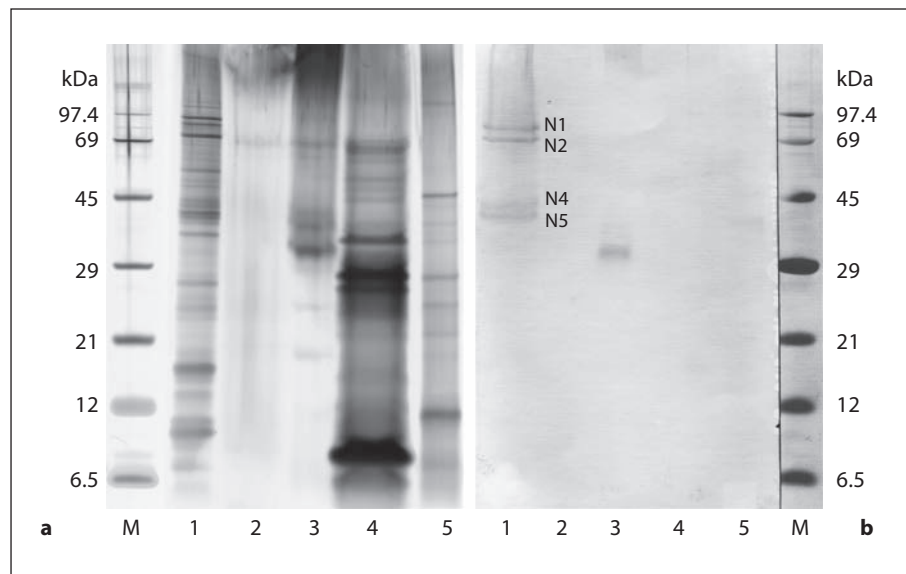


Fig. 1. Silver-stained SDS-PAGE (**a**) and corresponding IgE immunoblot (**b**) of wood and NRL allergens. M = Molecular weight standard; 1 = spruce; 2 = pine; 3 = larch; 4 = beech; 5 = NRL.

The patient exhibited further specific IgE to food (banana and kiwi, CAP class 2), grass pollen (CAP class 4), house dust mites (CAP class 3) and dog dander (CAP class 1). Specific IgE to NRL was measured with CAP class 3. Additional ImmunoCAP testing with the single latex allergens rHev b 1, rHev b 3, rHev b 5, rHev b 6.01, rHev b 7, rHev b 8, rHev b 10, rHev b 11 and rHev b 12 did not show specific IgE to any of the recombinant major latex allergens. A positive IgE value of CAP class 2 was measured for native (n)Hev b 2, the glycosylated latex glucanase. The total IgE concentration was 360 kU/l.

Specification of IgE-Mediated Sensitization by Reciprocal ImmunoCAP Inhibition Tests

When the glycocomponent HRP was used as inhibitor, sIgE binding to beech wood was abolished and sIgE binding to softwood was reduced to 18–41% (table 2). Specific IgE binding to NRL was reduced to 50% by HRP. Beech wood extract as inhibitor did not reduce sIgE binding to spruce and larch wood or NRL, but IgE binding to pine wood was reduced by about 36%. In contrast, NRL almost completely abolished sIgE binding to beech wood but not to softwood. sIgE binding to softwood was only affected when softwood themselves were used as inhibitors. Softwood extracts of spruce and pine reduced sIgE binding to beech and NRL by 67–93%; larch as inhibitor reduced the response to beech wood by about 89% whereas NRL-induced inhibition was only weak (22%). Cross-inhibition between softwood extracts was always >50%.

Table 2. Reciprocal inhibition test

Inhibitor	Solid phase				
	spruce	pine	larch	beech	NRL
Spruce	89	59	64	93	90
Pine	77	85	68	91	67
Larch	52	56	57	89	22
Beech	0	36	12	63	21
NRL	0	27	28	89	77
HRP	30	41	18	94	50

sIgE-binding epitopes were specified by inhibition of the solid phase allergen coupled to ImmunoCAPs with the soluble inhibitor. Values are given in percentages. Inhibitions ≥50% are indicated in bold.

Autoinhibition of spruce and pine wood was >80%, which is defined as complete inhibition. Autoinhibition of larch and NRL was not complete because of a difference between solid-phase and liquid allergens. Surprisingly, autoinhibition to beech wood was only 63% even though the same wood extract was taken as solid and liquid phase.

Detection and Identification of sIgE-Binding Proteins from Softwood (P. abies)

Allergen extracts were analyzed by SDS-PAGE (fig. 1a) exhibiting numerous protein bands in a molecular range

Table 3. Identified peptide sequences in spruce wood

ID	Identification/blast similarity	Species	NCBI accession No.	Mascot score	MW Da	Matching peptides
N1	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase enolase	<i>Picea sitchensis</i>	gi 148909224	95	84,584	19
		<i>Picea sitchensis</i>	gi 148909807	93	48,350	7
N2	peroxidase	<i>Picea sitchensis</i>	gi 55057256	112	84,299	2
N4	peroxidase	<i>Picea abies</i>	gi 55057260	345	38,523	6
	aspartate aminotransferase	<i>Picea sitchensis</i>	gi 148905886	155	46,583	9
	peroxidase	<i>Picea abies</i>	gi 55057256	112	39,161	7
N5	alcohol dehydrogenase	<i>Picea sitchensis</i>	gi 116783917	284	34,039	5
	peroxidase	<i>Picea abies</i>	gi 55057256	151	38,523	10

between 5 and 100 kDa. Spruce wood extract (lane 1) yielded several distinct protein bands at 10 and 100 kDa whereas pine wood extract (lane 2) produced no clear protein bands. From larch wood (lane 3) predominantly proteins of 29–42 kDa were extracted. Beech wood showed major protein bands at 10 and 26/27 kDa and several others. The NRL extract produced three major protein bands at about 10, 27 and 48 kDa.

Allergens were detected by incubation with patient's serum (fig. 1b). Only in softwood extracts of spruce and larch (lanes 1 and 3) were IgE-binding proteins detectable. Spruce wood extract yielded two IgE-binding protein doublets at 35–40 and 70–80 kDa. In larch wood extract, a 29-kDa protein was labeled by sIgE.

Both IgE-binding spruce wood protein doublets were identified by LTQ-Orbitrap. Table 3 summarizes the results of the protein peptide identification. The mascot score was always above 51 for all bands. All identified peptide sequences exhibited enzymatic activity. Proteins of N1 (80 kDa) were identified as methyltransferase (85 kDa) and/or enolase (48 kDa). Peroxidases (34–84 kDa) were the most prominent proteins identified in N2 (70 kDa), N4 (40 kDa) and N5 (35 kDa). The discrepancy between the molecular weights of the IgE-binding spruce wood protein bands from IgE immunoblot and the proteins found by comparison of peptide sequences after trypsin digestion by MS-MS of these bands showed that the similarity within peptide sequences did not include the same molecular weight of the corresponding proteins. Thus, even though peptide sequences – which are mostly conserved regions – were similar or identical, the whole protein molecules can be different.

Discussion

Softwood were identified as the cause of occupational allergic symptoms in this patient, with a diagnostic armamentarium including skin tests, challenge tests and specifically tailored ImmunoCAPs. The relevant IgE-binding protein was identified as peroxidase. Spruce and pine wood were the most potent inhibitors and probably the primary source of sensitization. In contrast, IgE binding to beech wood was based on glycosidic structures, suggesting that the beech wood sensitization is likely a cross-sensitization that did not induce occupational allergic symptoms. This assumption was further supported by incomplete autoinhibition to beech wood of 63% even though the same wood extract was taken as solid and liquid phase. Thus all other wood, NRL and HRP extracts caused higher reductions in IgE binding to beech wood than the allergen itself. A possible explanation may be predominantly glycosidic IgE binding to beech wood allergens. If all other allergen extracts contained higher glycosidic components than beech wood extract, the decrease in IgE binding could be larger than that observed for autoinhibition. For autoinhibition, the same amount of inhibitor was used as in the cross-inhibition study. This may be a limitation in the outcome of the experiments.

The softwood larch was an effective inhibitor for other wood but not for NRL. Regarding the inhibition results with HRP, the sensitization to larch wood was predominantly based on proteinogenic epitopes, maybe on homologue and conserved softwood proteins rather than glycosidic structures.

NRL-specific IgE binding can be ascribed to proteinogenic as well as to glycosidic structures. Regarding

proteinogenic wood dust allergens, it is known that obeche wood allergen Trip s 1 exhibits strong cross-reactivity with NRL allergens, Hev b 6.02 and latex chitinase (Hev b 11) based on homologue protein domains [14].

In fact, the patient did not exhibit the typical NRL sensitization profile seen in NRL-allergic health care workers with most frequently specific IgE to Hev b 5, Hev b 2 and Hev b 6.02 or in NRL-allergic spina bifida patients (NRL allergy induced by multiple operations) with the dominant allergens (Hev b 1, Hev b 2, Hev b 3, Hev b 5) as described [10]. In contrast, sIgE binding was only seen to native, glycosylated Hev b 2, indicating a possible cross-reaction based on either the known food allergy to kiwi and banana or on glycogenic structures from the patient's grass pollen sensitization. Consequently, NRL as inhibitor had no significant effect on sIgE binding to softwood. Therefore, softwood and NRL sensitization seemed to be independent in this case.

IgE-binding proteins in spruce wood extract were identified as peroxidases. Especially class III peroxidases are essential in lignin polymer synthesis and occurred predominantly in the xylem part of spruce [15]. The raw wood material consists almost completely of lignified xylem, containing large amounts of peroxidases. During wood processing, these peroxidases became airborne in the form of associated proteins on wood dust particles. This mostly occupational respiratory exposure might have induced IgE-mediated sensitization with subsequently clinical relevant allergic symptoms in our patient. So far, peroxidases are known as food allergens in tomato (Lyc e peroxidase) [16] and wheat (Tri a and Tri m peroxidase) [17]. When comparing the known

peroxidase sequences from horseradish (*Armoracia rusticana*), tomato (*Lycopersicon esculentum*) and wheat (*Triticum aestivum*) with the newly identified protein sequences of spruce wood (*P. abies*), the identity was between 43 and 52% and the similarity between 59 and 67% (data not shown). Serologically, the patient showed sIgE against tomato allergens (2.07 kU_A/l) and wheat (0.79 kU_A/l) without any clinical symptoms when eating this food, indicating cross-reactivity is predominantly due to plant glycostructures present in Hev b 2. Peroxidase as potential cause of cross-reactivity between wood dust and food should be considered carefully because an amino acid similarity of 60% is low with regard to immunological cross-reactivity. Additionally, recent studies by Giroux et al. [18] demonstrated that peroxidase from horseradish did not cross-react with other common plant sources. The clinically silent sensitization to tomato and wheat may be triggered by CCDs, similar to latex sensitization that is predominantly induced by glycosylated native Hev b 2. Therefore it is important to know the causative allergens involved in softwood dust allergy, first to diagnose clinically relevant softwood dust allergy and second to evaluate potential sources of cross-reactions.

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