



MAILI LEHTO

Natural Rubber Latex Allergy

Cutaneous and Airway Responses in Mouse Models
and Immune Responses in Latex-Allergic Patients



ACADEMIC DISSERTATION

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1 LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, referred to in the text by Roman numerals (I-IV).

- I Lehto M, Koivuluhta M, Wang G, Amghaiab I, Majuri ML, Savolainen K, Turjanmaa K, Wolff H, Reunala T, Lauerma A, Palosuo T, Alenius H (2003): Epicutaneous natural rubber latex sensitization induces T helper 2-type dermatitis and strong prohevein-specific IgE response. *J Invest Dermatol* 120:633-640.
- II Lehto M, Haapakoski R, Wolff H, Majuri ML, Mäkelä MJ, Leino M, Reunala T, Turjanmaa K, Palosuo T, Alenius H (2005): Cutaneous, but not airway, latex exposure induces allergic lung inflammation and airway hyperreactivity in mice. *J Invest Dermatol* 125:962-968.
- III Lehto M, Kotovuori A, Palosuo K, Varjonen E, Lehtimäki S, Kalkkinen N, Palosuo T, Reunala T, Alenius H (2007): Hev b 6.01 and Hev b 5 induce pro-inflammatory cytokines and chemokines from peripheral blood mononuclear cells in latex allergy. *Clin Exp Allergy* 37:133-140. The definitive version is available at www.blackwell-synergy.com
- IV Palosuo T, Lehto M, Kotovuori A, Kalkkinen N, Blanco C, Poza P, Carrillo T, Hamilton RG, Alenius H, Reunala T, Turjanmaa K: Latex allergy: low prevalence of IgE to highly purified Hev b 2 and Hev b 13 (*submitted for publication*).

2 ABBREVIATIONS

ACD	allergic contact dermatitis
ACU	allergic contact urticaria
AE	atopic eczema
AHR	airway hyperreactivity
APC	antigen-presenting cells
BAL	bronchoalveolar lavage
CC	chemokine β -family (cysteine-cysteine)
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CLA	cutaneous lymphocyte antigen
CXC	chemokine α -family (cysteine-amino acid-cysteine)
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	dendritic cells
EC	epicutaneous
ELISA	enzyme-linked immunosorbent assay
Fc ϵ RI	high affinity IgE receptor
Foxp3	forkhead box 3 transcription factor
HCW	health care workers
H&E	haematoxylin and eosin
HPF	high power field
HPLC	high performance liquid chromatography
IC	intracutaneous
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IN	intranasal
IP	intraperitoneal
MCh	methacholine
mRNA	messenger ribonucleic acid
NRL	natural rubber latex
PAS	periodic acid-Schiff
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCD	protein contact dermatitis
PCR	polymerase chain reaction
Penh	enhanced pause
PHA	phytohaemagglutinin
RU	relative units (relative differences compared with the calibrator)
SEB	<i>Staphylococcus aureus</i> enterotoxin B
SPT	skin prick test
TCR	T-cell receptor
TGF	transforming growth factor
Th1	T helper lymphocyte type 1
Th2	T helper lymphocyte type 2
TNF	tumor necrosis factor
Tregs	regulatory T cells

3 ABSTRACT

Natural rubber latex (NRL) allergy has been an important health issue for two decades. Gloves and other NRL products cause contact urticaria, rhinitis and asthma in NRL-allergic health care workers (HCW) and may sensitize patients during surgical operations. In addition to type I immediate symptoms, NRL-allergic subjects often suffer from hand dermatitis. A major goal of the present study was to investigate cutaneous and airway routes in sensitization and the subsequent inflammatory responses to NRL in the skin and lungs. The specific goals included (i) characterization of cutaneous inflammatory response to NRL allergens (ii) examination of the impact of cutaneous exposure route in the development of airway hypersensitivity to NRL in mouse models of NRL allergy and, (iii) investigation in NRL-allergic patients of cytokine and chemokine responses of circulating mononuclear cells to two of the most important NRL allergens, Hev b 6.01 and Hev b 5. Finally, the study aimed to determine IgE antibody prevalence rates in NRL-allergic patients to Hev b 2 and Hev b 13, two proteins, which have recently suggested to be major NRL allergens.

When BALB/c mice were exposed to NRL patches for three one-week periods, the NRL exposed skin exhibited a Th2-type dermatitis, characterized by infiltration of CD3+CD4+ T cells, eosinophils and degranulated mast cells (I). This inflammatory response was associated with enhanced expression of IL-1 β and IL-4 mRNA, and with increased mRNA expression of CC chemokines CCL2, CCL3, CCL4 and CCL11. Cutaneous NRL exposure induced significant total and Hev b 6.01- specific IgE responses, whereas intraperitoneal exposure produced Hev b 1-specific and NRL-specific IgG2a responses.

Airway hyperresponsiveness in mice was measured by whole-body plethysmography (II). Airway NRL-challenge (0.5% in PBS) after intracutaneous (IC) and intraperitoneal (IP) sensitizations, but not after intranasal (IN) sensitization, induced a significant airway hyperreactivity, lung mucus production and influx of mononuclear cells and eosinophils into the lungs. Th2 cytokines (IL-4 and IL-13) and several CC chemokines (CCL1, CCL3, CCL8, CCL11, CCL17 and CCL24) and chemokine receptors (CCR1, CCR3, CCR4 and CCR8) were similarly induced in the lungs after IC and IP sensitization. IN sensitization of mice increased expression of regulatory cytokine TGF- β 1 and regulatory T-cell marker Foxp3 mRNA in the lungs and produced a negligible amount of Hev b 6.01-specific IgE in the blood in contrast to IC and IP sensitization.

In human studies, PBMC from NRL-allergic patients showed significant proliferation responses to Hev b 6.01, but not to Hev b 5 compared with control subjects (III). Both allergens induced a significant mRNA expression of proinflammatory (TNF, IL-12p40) and Th2 (IL-13) cytokines. Regulatory cytokine expression in PBMC of NRL-allergic patients exhibited diametrical

changes; IL-10 was increased but TGF- β 1 mRNA was decreased compared with control subjects. NRL allergens induced significant expression of inflammatory chemokines (CCL3, CCL4, and CCL20). In addition, CCR4 expression was increased on CD3+CD8⁻ T cells whereas CXCR3 expression decreased on CD3+CD8⁺ T cells.

IgE antibody prevalence to Hev b 2 and Hev b 13, and also to Hev b 5 and Hev b 6.01 was examined in the sera of 215 NRL-allergic patients and 172 control subjects from Finland, Spain and the USA (IV). The allergens were purified by chromatography under non-denaturing conditions. A special effort was made to extensively purify native Hev b 2 and Hev b 13. By using these purified allergens in ELISA, IgE prevalence was found to be relatively low both to Hev b 2 (from 5% to 15%) and Hev b 13 (from 18% to 30%). In contrast, IgE prevalence was high both to Hev b 6.01 (from 54% to 74%) and Hev b 5 (from 28% to 71%) confirming that these NRL allergens are the major allergens in Europe and in the USA.

In conclusion, the present experiments in mouse NRL allergy models reveal that cutaneous NRL exposure induces Th2-type skin and lung inflammation and strong humoral allergen-specific IgE responses. Cutaneous exposure to NRL allergens could therefore be involved in the development of hand eczema in NRL-allergic patients and predispose sensitized subjects to asthma. The observed PBMC responses to major NRL allergens suggest that allergen-specific induction of inflammatory cytokines and chemokines is important in the pathophysiology of NRL allergy. These findings also emphasize the important role of the regulatory cytokines. The IgE antibody prevalence study emphasizes the importance of using highly purified NRL allergens and furthermore provides clear evidence that neither Hev b 2 nor Hev b 13 can be considered as major NRL allergens.

TIIVISTELMÄ

Luonnonkumiallergia on ollut merkittävä terveysongelma jo kahden vuosikymmenen ajan. Kumikäsineet sekä muut luonnonkumivalmisteet herkistävät ihokosketuksessa ja aiheuttavat nokkosihottumaa, allergista nuhaa ja astmaa erityisesti terveydenhuollon työntekijöille. Toistuvasti leikatut potilaat voivat myös herkistyä luonnonkumille. Luonnonkumiallergikoilla on usein välittömien (tyypin I) allergisten oireiden lisäksi käsi-ihottumaa, jonka syynä saattaa olla viivästynyt allergia. Nykyään tiedetään melko vähän siitä, mitkä ovat luonnonkumiallergian iho- ja hengitystieherkistymiseen johtavat tulehdusvasteet. Tämän tutkimuksen yhtenä tavoitteena oli selvittää hiirimallin avulla, millaisia ovat luonnonkumin aiheuttamat tulehdusvasteet iholla sekä vasta-ainevasteet seerumissa. Toinen hiirimallin avulla toteutettu tutkimuskohde oli selvittää luonnonkumin ihoaltistuksen mahdollinen vaikutus hengitysteiden herkistymiseen. Kolmantena päämääränä oli tutkia sytokiini- ja kemokiinivasteita luonnonkumin kahdella pääallergeenilla Hev b 6.01:lla ja Hev b 5:lla. Vastikään on saatu tuloksia, joiden mukaan myös Hev b 2 ja Hev b 13 voisivat olla pääallergeeneja luonnonkumiallergiassa. Yksi tutkimuksen tavoitteista olikin määrittää näille spesifisten IgE vasta-aineiden esiintyvyys luonnonkumiallergikoilla.

BALB/c hiiriä herkistettiin luonnonkumille ihoon kiinnitettyjen testilappujen avulla (I). Herkistämisaika oli kolme viikon pituista jaksoa. Altistettuun ihokohtaan kehittyi ihottuma, jota luonnehtivat CD4 positiiviset T solut, eosinofiilit ja degranuloituneet syöttösolut. IL-1 β :n ja IL-4:n sekä CC kemokiinien (CCL2, CCL3, CCL4, CCL11) lähetti RNA pitoisuudet olivat merkittävästi koholla tulehtuneella ihoalueella. Kokonais-IgE sekä Hev b 6.01:lle spesifisten IgE vasta-aineiden pitoisuudet nousivat merkitsevästi. Vatsaonteloon tehty altistus käynnisti Hev b 1-spesifisen sekä luonnonkumille spesifisen IgG2a vasteen.

Hengitysteiden reaktioherkkyys (hyperreactivity) hiirillä mitattiin koko kehon pletysmografialaitteella (II). Ihon ja vatsaontelon kautta herkistetyt hiiret reagoivat voimakkaasti luonnonkumilla tehdyn hengitystiealtistuksen jälkeen. Se aiheutti merkittävän hengitysteiden metakoliinivasteen, liman erittymisen keuhkoihin sekä mononukleaaristen solujen ja eosinofiilien kerääntymisen keuhkokudokseen. Th2 sytokiinien (IL-4, IL-13), useiden CC-kemokiinien (CCL1, CCL3, CCL8, CCL11, CCL17, CCL24) sekä kemokiinireseptoreiden (CCR1, CCR3, CCR4, CCR8) lähetti RNA määrät nousivat merkittävästi ihon ja vatsaontelon kautta herkistetyillä hiirillä. Nenän kautta herkistettyjen hiirten keuhkoissa todettiin immuunivastetta hillitsevien tekijöiden (TGF- β 1 ja Foxp3) lähetti RNA pitoisuuksien nousevan ja seerumissa esiintyvän vain hyvin matalia, kontrollien tasolla olevia Hev b 6.01-spesifisiä IgE vasta-ainepitoisuuksia.

Ihmistutkimuksissa luonnonkumiallergikkojen mononukleaariset solut (PBMC) lisääntyivät merkittävästi enemmän Hev b 6.01:llä aktivoinnin, mutta ei Hev b 5:llä aktivoinnin jälkeen (III). Kumpikin allergeeni käynnisti proinflammatoristen (TNF, IL-12p40) ja Th2 sytokiinien (IL-13) lähetti RNA:n tuotannon. Säätelysytokiineista IL-10:n määrä suureni, kun taas TGF- β 1:n lähetti RNA:n määrä oli vähäinen. Luonnonkumiallergeenit aiheuttivat potilaiden PBMC soluissa merkittävän CC tulehduskemokiinien (CCL3, CCL4, CCL20) tuotannon. Lisäksi havaittiin, että kemokiinireseptori CCR4:n määrä oli suurempi allergikkojen CD3+CD8- soluissa ja CXCR3:n määrä pienempi CD3+CD8+ soluissa verrattuna kontrolleihin.

Hev b 2- ja Hev b 13-spesifisten IgE vasta-aineiden esiintyvyyttä tutkittiin 215 luonnonkumille allergisen potilaan ja 172 verrokin seeruminäytteistä, jotka olivat peräisin Suomesta, Espanjasta ja Yhdysvalloista (IV). Nämä luonnonkumin allergeenit puhdistettiin kromatografisin menetelmin ei-denaturoivissa oloissa. Tämän jälkeen IgE vasta-aineet mitattiin ELISA:lla seerumeista käyttämällä näitä puhdistettuja allergeeneja, joille spesifisiä IgE vasta-aineita esiintyi vähän; Hev b 2 vastetta oli 5 %- 15 %:lla ja Hev b 13 vastetta 18 % -30 %:lla potilaista. Hev b 6.01 (54 % -74 %) ja Hev b 5 (28 % -71 %) IgE vasta-aineiden esiintyvyys oli sen sijaan selvästi korkeampi kaikissa kolmessa potilasaineistossa.

Luonnonkumiallergian hiirimalleja tutkimalla saadut tulokset osoittavat, että ihon kautta tapahtuva herkistyminen aiheuttaa voimakkaan serologisen allergeeni-spesifisen IgE vasteen ja altistaa hiiret allergiselle, Th2 tyyppin iho- ja keuhkotulehdukselle. Ihon altistuminen luonnonkumille saattaa samalla mekanismilla aiheuttaa luonnonkumiallergoille käsi-ihottumaa ja toisaalta lisätä myös alttiutta luonnonkumin aiheuttamalle astmalle. Luonnonkumiallergikkojen PBMC solujen vasteet luonnonkumin pääallergeeneille osoittavat, että tulehdussytokiinit ja kemokiinit sekä säätelysytokiinit ovat todennäköisesti merkittäviä osallisia luonnonkumiallergian aiheuttamassa tulehdustapahtumassa ja sen säätelyssä. Tutkimus IgE vasta-aineiden esiintyvyydestä osoittaa, että on tärkeää käyttää mahdollisimman huolellisesti puhdistettuja luonnonkumiallergeeneja. Tulokset myös merkitsevät, että Hev b 2:ta ja Hev b 13:a ei voida pitää luonnonkumiallergian pääallergeeneina.

4 INTRODUCTION

A world-wide epidemic of allergic diseases is on-going in the developed countries (Asher et al. 2006). The most prevalent phenotypes are allergic rhinitis, atopic eczema, and asthma. An international prevalence study showed that in Finland as many as one in every five adolescents suffers from an allergic disease (ISAAC 1998). Environmental allergens such as pollens, house dust mites, pet animals and food are the major sensitizing agents. Genetically predisposed individuals are mainly affected and they exhibit pronounced IgE antibody production and inflammatory responses with the appearance of eosinophils and T lymphocytes in the target tissues (Kay 2001a).

The epidemic of allergy to natural rubber latex (NRL) has been depicted as a special “man-made” disease. This allergy has been an important health issue during the past 20 years among health care workers (HCW) and other individuals in NRL-glove using occupations (Sussman et al. 2002). A recent meta-analysis found 4.3% prevalence of NRL allergy in HCW and 1.4% prevalence in the general population (Bousquet et al. 2006). The onset of NRL allergy epidemic seems to be linked to the HIV epidemic which demanded that health care personnel had to take more careful personal protection and this led to a vast increase in the use of NRL gloves (Ownby 2002). In the mid 1990’s, the first sensitizing NRL allergens were identified and their presence in the gloves documented. Soon after this, the policy of glove usage changed in many countries towards powder-free gloves whose manufacture had simultaneously started to increase. The second more difficult goal for quality control purposes was to develop reliable methods to measure the NRL allergen content in the gloves (Palosuo et al. 2002).

Symptoms of NRL allergy range from contact urticaria, rhinitis and asthma to anaphylaxis (Turjanmaa et al. 2002b, Vandenplas et al. 2002). In addition to type I immediate symptoms, many NRL-allergic HCW suffer from hand eczema which may disappear if they can avoid any exposure to NRL gloves (Taylor and Praditsuwan 1996, Turjanmaa et al. 2002b). This suggests that one phenotype of NRL allergy could be so called protein contact dermatitis (PCD). This condition occurs particularly in food handlers, bakers and other occupations where animal or plant proteins have repeated contact with the skin (Janssens et al. 1995). One fourth of the NRL-allergic HCW may suffer from rhinitis and a smaller proportion from asthma (Turjanmaa et al. 2002b, Bernstein et al. 2003a). Though skin is an obvious target for exposure to NRL allergens in HCW and other individuals using gloves, the sensitization routes and mechanisms involved in NRL-induced rhinitis and asthma are still poorly understood. In this thesis, mouse models for NRL allergy were used to study whether repeated cutaneous application of NRL evokes specific sensitization and the development of eczema, i.e. PCD, on the skin. The second approach using

mouse models for NRL allergy was to examine the significance of different NRL allergen exposure routes in the development of lung inflammation and airway hyperreactivity.

The major NRL allergens in HCW are hevein (Hev b 6.02) and Hev b 5 (Wagner and Breiteneder 2005). IgE antibody responses to these allergens are well defined but knowledge about NRL-induced cell-mediated immunity and especially to the chemokine responses are, however, scanty. Therefore, one goal of the present thesis was to characterize PBMC responses in NRL-allergic patients to the major NRL allergens Hev b 5 and Hev b 6.02. Recent studies have indicated that Hev b 2 and Hev b 13 could also be major NRL allergens (Bernstein et al. 2003a, Kurup et al. 2005). Confirmation of this claim would be important both for diagnostic and NRL glove quality control purposes. Therefore, the final goal of the present thesis was to examine the prevalence of IgE antibodies to Hev b 2 and Hev b 13, in comparison to Hev b 6.01 and Hev b 5, by using highly purified native allergens to screen a large collection of sera from NRL-allergic patients from three different countries.

5 REVIEW OF THE LITERATURE

5.1 Allergy and allergens

Allergy (Greek *allos*, other and *ergon*, work) means symptoms or signs initiated by exposure to common environmental antigens, which are tolerated by normal persons (Kay 2001a). Any substance capable of eliciting an adaptive immune response is referred to as an antigen (*antibody generator*). Allergy can be considered as a type of immune response commonly known as hypersensitivity reactions (Janeway Jr et al. 2005). These reactions are classified by mechanism: type I allergic reactions involve IgE antibodies; type II reactions involve IgG and/or IgM antibodies; type III reactions involve antigen-antibody complexes; and type IV reactions are T cell-mediated (Coombs and Gell 1964). Type I reactions are often referred to as immediate allergy and type IV reactions as delayed allergy. Allergic reactions can only occur in individuals who have mounted specific immune responses. The term atopy means an increased personal and / or familial tendency to become sensitized and to produce IgE antibodies to common environmental allergens (Kay 2001a, Johansson et al. 2004). In addition to specific genes, environmental factors contribute to the development of atopy and atopic diseases through their ability to influence gene expression (Kay 2001a).

In immediate allergy an allergen is any substance stimulating the production of IgE in a genetically susceptible individual (Aalberse 2000, Pomes 2002, Stewart and Robinson 2003). Most allergens are proteins from complex sources (such as animals and plants) and their molecular weight varies from 5 to 100 kDa. Recent data suggest that also carbohydrate components of glycoproteins may be allergic, although IgE is usually produced against the protein part of glycoproteins. Allergens that are recognized by more than 50% of allergic individuals are termed as major allergens, and those recognized by less than 50% of these patients are considered as minor allergens. The World Health Organization/International Union of Immunological Societies (WHO/IUIS) maintains an official database of all identified allergens and their isoallergens at <http://www.allergen.org>. In addition to proteins, other molecules can also function as allergens. Small molecules (called haptens) such as nickel can bind to a carrier protein (for instance albumin) and this complex can also sensitize subjects and elicit antibody (type I) and T-cell (type IV) mediated hypersensitivity reactions (Divkovic et al. 2005).

5.2 Mechanisms of allergic reactions

The first phase of an allergic reaction is called the sensitization phase, in which contact with allergens leads to the activation of antigen presenting cells and subsequent generation of allergen

specific effector and memory T-cells (Kay 2001a, Janeway Jr et al. 2005, Larche et al. 2006) (Fig.1A). A subpopulation of specific T-cells facilitates B-cell activation and this in turn leads to the production of allergen-specific IgE and IgG antibodies (Fig. 1A). IgE antibodies bind to IgE receptors on mast cells in the tissue and on basophils in the circulation. In the elicitation phase, cross linking of cell-bound IgE by allergens induces the release of bioactive mediators which elicits the appearance of the immediate allergic symptoms (Fig. 1B). Secretion of inflammatory mediators from IgE-activated mast cells also results in recruitment of allergen specific T-cells into the inflammation site. Allergen activated T-cells proliferate and secrete a variety of inflammatory mediators which attract other leukocytes, e.g. eosinophils, to the inflammatory site. The coordinated action of different leukocytes results in the clinical features that are characteristic of late phase reaction (Fig. 1C).

Although innate immunity also plays an important role in the sensitization and elicitation phases, as well as in the regulation of allergic responses, this thesis will concentrate on adaptive immune responses.

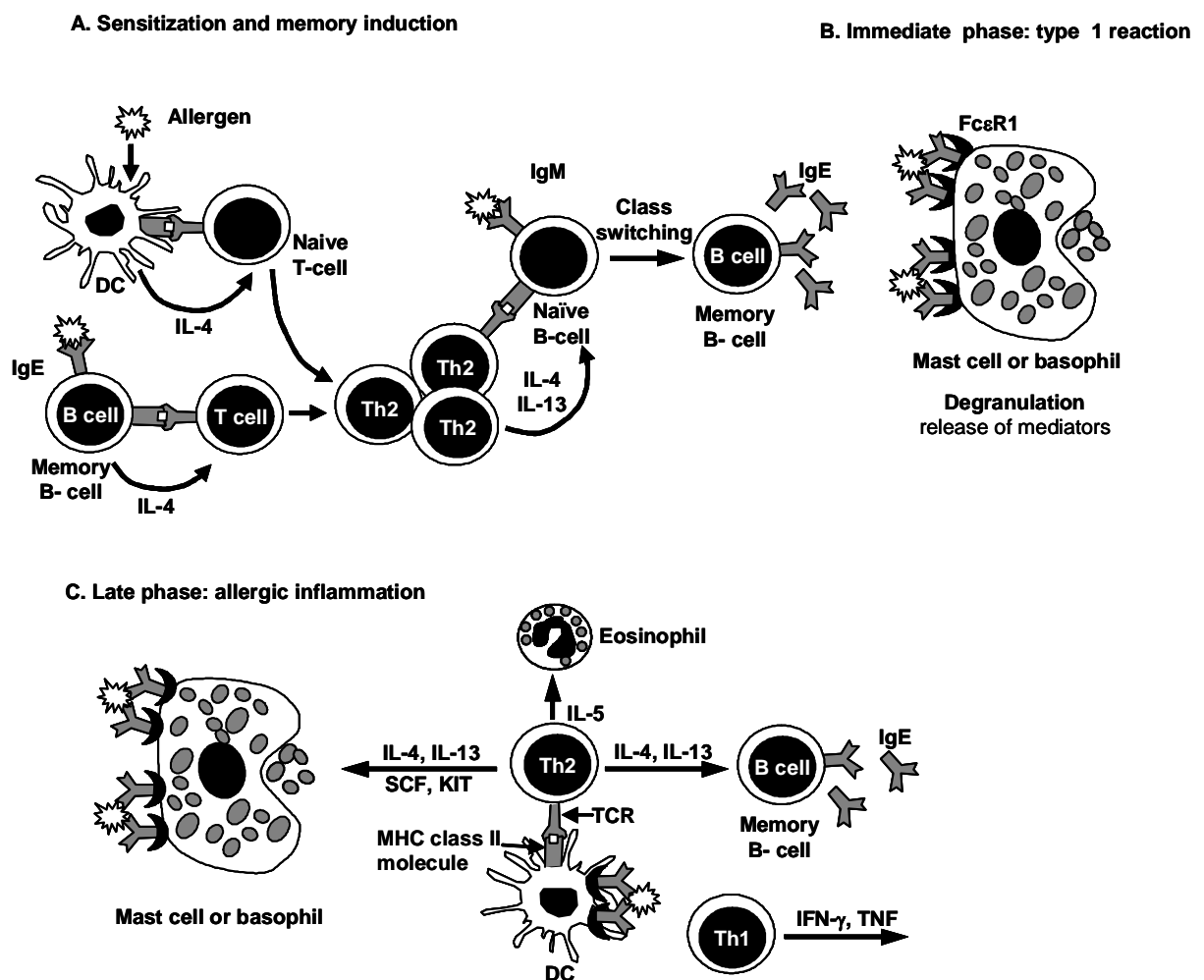


Figure 1. Mechanisms of allergic reactions. Modified from Larche et al. (2006).

5.2.1 Antigen presenting cells and T cells

Skin and epithelial surfaces of airways and the gastrointestinal tract represent the first line of defence since they are constantly exposed to environmental antigens such as allergens. Dendritic cells (DC) which reside in the epithelial surfaces are professional antigen presenting cells (APC) that are key players in the initiation of immune responses (Banchereau et al. 2000, Guemmonprez et al. 2002) (Fig. 1). These cells engulf and process foreign substances and they have the unique capacity to stimulate naive T cells to differentiate into effector or regulatory cells. Immature DC internalize antigens in peripheral tissues, process them into peptides, and finally load these peptides onto major histocompatibility complex (MHC) class I and II molecules present on the cell surface. Antigen processing activates DC maturation during which DC begin to express co-stimulatory molecules on their surface. Maturing DC migrate to secondary lymphoid organs, where fully matured DC present antigens to naive T cells that recognize the processed membrane-bound allergens through their antigen-specific T cell receptors (TCR) and thereby initiate antigen-specific immune responses. In the elicitation phase of allergy, antigen presentation by DC also occurs in various inflammatory sites such as in the dermis, in addition to regional draining lymph nodes.

Naive T-cells that are activated by antigen-loaded DC start to proliferate and differentiate into the effector cells capable of destroying the antigen, e.g., a micro-organism (Lanzavecchia and Sallusto 2001, Kaech et al. 2002, Janeway Jr et al. 2005). The effector T cells can be divided into CD8⁺ cytotoxic T cells (CTL) and CD4⁺ T helper (Th) cells. CD4⁺ T-helper cells can further differentiate into Th1 or Th2 effector cells (Fig. 2). Th1 cells characteristically secrete interferon- γ (IFN- γ), whereas Th2 cells primarily secrete interleukins 4, 5 and 13 (IL-4, IL-5 and IL-13). Th1 cells are responsible for cell-mediated immune responses against intracellular pathogens, whereas Th2 cells account for humoral immunity and direct immune responses against intestinal helminths. The mechanisms of CD4 T-cell differentiation are not yet fully defined; however, it has been demonstrated that local cytokines selectively induce expression of specific transcription factors leading to the development of different Th cell types: IL-4, IL-13, GATA-3, and STAT-6 are involved in Th2 development and IFN- γ , IL-12, T-bet, STAT-1 and STAT-4 are involved in Th1 development (Murphy and Reiner 2002, Wahl et al. 2004, Vercelli 2005).

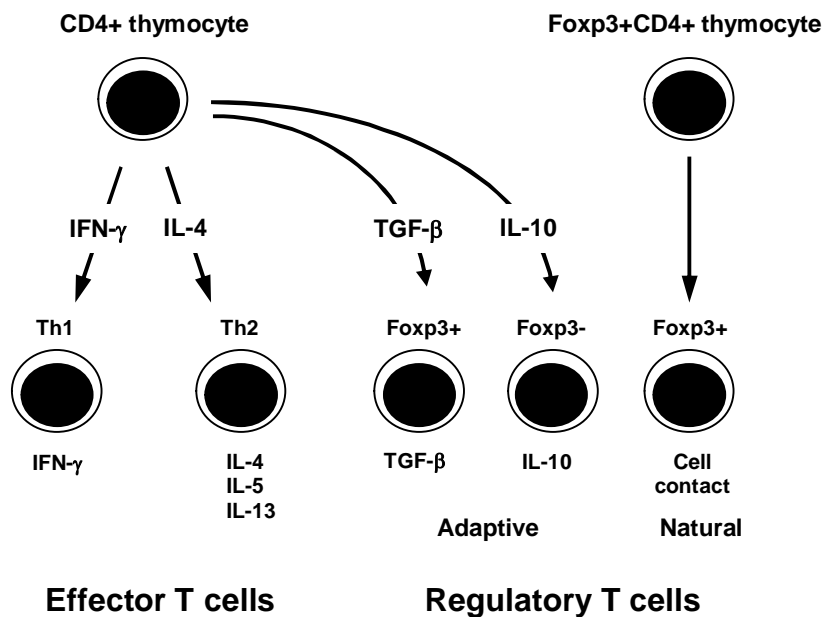


Figure 2. Different CD4+ T cell lineages. Modified from Weaver et al. (2006).

T cells can differentiate also into regulatory T cells (Tregs) which play a major role in the maintenance of tolerance against self- or harmless foreign proteins such as allergens by down-regulating effector T cell responses (Weaver et al. 2006) (Fig. 2). Several subtypes of Tregs have been described, including thymus-derived CD4+CD25+ (called natural Tregs) that express transcription factor forkhead box 3 (Foxp3), and adaptive Tregs that develop in the periphery on exposure to exogenous antigens or allergens. The regulatory function of Tregs is believed to be at least partially mediated by two regulatory cytokines, IL-10 and TGF-β, though a contact-dependent mechanism of suppression has also been reported to play an important role. Tregs can directly or indirectly suppress different cells such as APC, Th2 cells, Th1 cells, mast cells, basophils, and eosinophils. In addition, they have a role in the suppression of allergen-specific IgE and induction of IgG4, and/or IgA (Akdis et al. 2005). It has been shown that Foxp3 and TGF-β1 can promote the differentiation of Tregs (Wahl et al. 2004, Li et al. 2006a, Li et al. 2006b, Weaver et al. 2006).

In addition to effector T cells, memory T cells are also crucial in adaptive immune responses. Memory T cells are derived from the clonal expansion and differentiation of antigen-specific T lymphocytes and they ultimately persist for a lifetime. Memory T cells can be divided into two subsets: effector and central memory cells (Sallusto et al. 2004). Effector memory T cells migrate to inflamed tissues and show effector function, whereas central memory T cells travel to the secondary lymphoid organs, and have minor effector function. However, central memory T cells differentiate to effector cells after antigenic stimulation.

5.2.2 Immunoglobulin E in the allergic reaction

B cells produce antibodies against foreign agents and maintain a pool of memory cells (Manz et al. 2005, Kalia et al. 2006, Radbruch et al. 2006). When a B lymphocyte has recognized its antigen, with the help of T helper cells, it becomes activated and is transformed into a lymphoblast that starts to divide. The dividing lymphoblast produces a clone of cells with identical specificity that in the end differentiates into antibody secreting plasma cells. Antibodies are secreted into blood and other extracellular fluids where they affect by binding to their corresponding antigens. This marks the antigen for elimination by other cells. After the antigen has been eliminated, a part of the differentiated plasma cells remains, building up the so called immunological memory. These memory lymphocytes react more rapidly and effectively to the presence of the antigen in secondary infections. In mammals, there are five classes of antibodies, IgG, IgM, IgD, IgA and IgE, each with its own class of heavy chain - γ , μ , δ , α , and ϵ , respectively. The basic structural unit of an antibody molecule consists of four polypeptide chains, two identical light chains and two identical heavy chains.

Immunoglobulin E (IgE) is the key molecule mediating type I allergic reactions, such as allergic asthma, allergic rhinitis and insect venom allergy (Platts-Mills 2001, Geha et al. 2003, Gould et al. 2003). IgE is a Y-shaped molecule with two distinct regions. One is the constant region (Fc), which determines the type of immunoglobulin (e.g. Fc ϵ for IgE) and the other is the variable region that forms two identical antigen-binding sites (Fab). The Fc region switches on the effector mechanisms that are activated by antigens. The concentration of IgE in serum is the lowest of the five immunoglobulins and its half-life in human serum is only 2 days. On the other hand, IgE is mostly bound to tissue mast cells as well as to circulating basophils and activated eosinophils and therefore its half-life is considerably longer. The synthesis of IgE by B cells occurs at a low rate compared with that of the other antibodies, even in allergic subjects.

Most IgE is bound to cells via its high-affinity receptor, Fc ϵ RI, expressed by tissue mast cells and circulating basophils (Galli et al. 2005a, Galli et al. 2005b, Gibbs 2005). Cross-linking of IgE bound to Fc ϵ RI on these cells by specific antigen results in a local release of inflammatory mediators (for example, histamine, prostaglandins and leukotrienes), enzymes and cytokines, such as IL-4, -5, -6, -10, and -13 and TNF, which are important in the pathogenesis of various allergic reactions. In particular, IL-5 production is reported to be critical for the pathogenesis of eosinophilic inflammation in the lung (Foster et al. 1996, Hamelmann and Gelfand 2001, Rothenberg and Hogan 2006). In addition, the low-affinity IgE receptor, Fc ϵ RII (CD23), which is expressed by a wide variety of immune cell types, including B cells, macrophages and dendritic cells can also bind IgE (Geha et al. 2003). IgE bound to the high-affinity IgE receptor

FcεRI or FcεRII can also facilitate allergen uptake by antigen-presenting cells (APC) and augment secondary immune responses.

5.2.3 Cytokines and chemokines

Cytokines

Various cells secrete soluble proteins or glycoproteins called cytokines (Greek *cyto*, cell, and *kinesis*, movement) that act as intercellular (between cells) mediators or signaling molecules (Prescott et al. 2002, Borish and Steinke 2003, Steinke and Borish 2006). Cytokines affect growth, differentiation and activation properties of cells, regulating the nature of immune responses. They participate in nearly every aspect of immunity and inflammation, and their production is induced by a variety of non-specific stimuli such as, different infections, cancers and inflammations, or by specific interactions between T cells and antigens. The biological functions of cytokines are expressed when they bind to their high-affinity receptors on target cells. In general, cytokines function locally, but some cytokines can affect distant cells because of their ability to enter the circulation. Cytokines can operate in an autocrine way (on the cell producing them), in a paracrine way (on adjacent cells) or in an endocrine way (on the cells attainable via the circulation) (Prescott et al. 2002, Borish and Rosenwasser 2003).

Cytokines can be divided roughly according to their major functions into different subtypes e.g. chemokines, proinflammatory cytokines, growth factors, regulatory cytokines, Th1 type and Th2 type cytokines. However, it is not always clear to what category each cytokine belongs and sometimes it is possible to allocate the same cytokine into more than one category (Borish and Steinke 2003). Main cytokines and chemokines contributing to the allergic reaction and which are relevant to the present study are presented in Table 1.

Table 1. Simplified presentation of main cytokines and chemokines in allergy.

Cell type	Production and function	Cytokine/chemokine involved
Th2 cells	Main cytokine products	IL-4, IL-13, IL-5
	Development and maintenance	IL-4
	Recruitment	CCL1, CCL17, CCL22
Th1 cells	Main cytokine products	IFN-gamma, IL-2
	Development and maintenance	IL-12, IL-18, IL-23, IFN-gamma
	Recruitment	CXCL9, CXCL10, CCL3, CCL4, CCL5
Regulatory T cells (Tregs)	Main cytokine products	IL-10, TGF-beta
	Development and maintenance	IL-2, IL-10, TGF-beta, FOXP3
	Recruitment	Not well defined
Eosinophils	Main cytokine products	IL-3, IL-5, IL-13, GM-CSF, TGF-beta
	Development and maintenance	IL-5, IL-3, GM-CSF
	Recruitment	IL-5, CCL11, CCL24, CCL26, CCL5, CCL13
Mast cells	Main cytokine products	TNF, IL-3, IL-4, IL-5, IL-6, IL-13, GM-CSF
	Development and maintenance	Stem cell factor (SCF), IL-3, IL-9, NGF
	Recruitment	CCL2, CCL3, CCL5, CCL7

Proinflammatory cytokines, such as IL-1 α , IL-1 β , IL-6, TNF, IL-12 and IL-18, of which IL-12 and IL-18 also act as Th1 cytokines, are important in the early stages of the inflammatory reaction. IL-1, IL-6 and TNF activate their target cells specifically through their receptors. Activated cells then produce more chemokines, proinflammatory cytokines and other biologically active mediators. The amount of surface antigens and adhesion molecules increases on stimulated cells and this strengthens the interactions between different cell types.

Th2 type cytokines are important in antibody mediated immune responses. IL-4 is a central Th2 cytokine which influences the differentiation and growth of B cells and Th2 cells. In addition, Th2 cells produce IL-5, IL-10 and IL-13 cytokines. All of these Th2 cytokines further strengthen Th2-polarisation. IL-13 is important in the differentiation and growth of B cells and it enhances the production of IgG4 and IgE. Th2 cytokines IL-4 and IL-13 are especially important in class switching of IgE antibodies. IL-5 specifically acts on eosinophils, enhancing their differentiation and survival.

Th1 type cytokines (IL-2, IL-12, IL-18 and IFN- γ) promote differentiation of Th1 cells and strengthen cell-mediated immunity. IL-12 is especially important in Th1 polarization, since it regulates the differentiation and growth of Th1 cells. IL-18 participates in the differentiation

of Th1 cells and, in addition, it stimulates the production of chemokines and proinflammatory cytokines. Two cytokines, IL-12 and IL-18, act synergistically to increase IFN- γ production. IFN- γ is the most important Th1 cytokine produced, especially by Th1 cells. IFN- γ activates macrophages and inhibits the growth of Th2 cells.

Regulatory cytokines IL-10 and TGF- β weaken the intensity of the inflammatory response. They are especially produced by regulatory T-cells. These cytokines diminish the production of proinflammatory cytokines and reduce cell stimulation by IL-12 and IFN- γ .

Chemokines

Chemokines are members of a family of chemoattractant cytokines released by tissues in the early phases of inflammation (Zlotnik and Yoshie 2000, Rot and von Andrian 2004, Esche et al. 2005, Pease and Williams 2006). This family contains over 40 proteins which are divided into four subclasses, on the basis of the arrangement of the first two amino terminal cysteine residues: CXC (α -family), CC (β -family), C (γ -family), and CX3C (δ -family) (where "X" is an amino acid). Chemokines interact with G protein-coupled receptors that initiate signal transduction pathways leading to a plethora of cellular responses, in particular leukocyte chemotaxis and adhesion. Chemokine receptor expression patterns differ between different leukocyte types and their maturation levels. In general, the expression of CCR7 (receptor of CCL19/MIP-3 β and CCL21/6Ckine) guides immature leukocytes (naive T cells, B cells, immature DC) toward the lymph nodes. Cells of inflammatory tissues (keratinocytes, endothelial cells, T cells) produce various sets of chemokines, which attract different effector cells, such as T cells, mast cells, eosinophils and APC cells.

After allergen contact in the lymph nodes, naive T cells differentiate and start to express the appropriate chemokine receptors that help them to travel to the sites of infection. Th1 cells express preferentially CXCR3 (receptor of CXCL9/Mig, CXCL10/IP-10, CXCL11/I-TAC) and CCR5 (receptor of CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, CCL11/eotaxin, CCL14/HCCI, CCL16/HCC4), whereas Th2 cells express CCR4 (receptor of CCL17/TARC, CCL22/MDC) and CCR8 (receptor of CCL1/I-309) (Bonecchi et al. 1998). Eosinophils and mast cells express a wide range of chemokine receptors of which CCR3 has been most extensively studied in the context of allergy. CCR3 is a receptor for CCL11/eotaxin, CCL24/eotaxin-2, CCL26/eotaxin-3, CCL7/MCP-3, CCL5/RANTES, CCL8/MCP-2, CCL13/MCP-4) and plays critical role in the recruitment of eosinophils to the site of allergic inflammation. In addition to the cell-specific expression of chemokine receptors, also tissue-specific expression of chemokines exists. For example, epidermal keratinocytes express selectively CCL27 which attracts the skin-

homing CCR10 positive T cells to the inflamed skin (Sallusto and Mackay 2004, Homey et al. 2006).

5.3 Allergic disorders

Allergens can enter the body through skin, airways and gastrointestinal tract and in sensitized individuals they evoke a variety of allergic manifestations (Kay 2001a, Kay 2001b). Allergic contact urticaria (ACU) and contact dermatitis (ACD) are typical cutaneous responses to topical allergen exposure. Aeroallergens cause airway responses such as allergic rhinitis and asthma. Ingested food allergens can cause gastrointestinal, cutaneous or more generalized symptoms. The most severe systemic reaction is anaphylaxis which may occur for example in peanut, bee sting and NRL allergies (Chiu and Kelly 2005, Sicherer and Leung 2006). In addition to IgE-mediated allergies, there are allergic disorders with delayed symptoms. ACD is a typical example which is a cell-mediated, type IV hypersensitivity reaction (Saint-Mezard et al. 2004b). Delayed symptoms are seen also in protein contact dermatitis (PCD) but the pathophysiological events leading to this disorder are not fully understood (Doutre 2005). Atopic eczema (AE) is often associated with food allergy, especially in children, and it often precedes allergic rhinitis and asthma. AE is, however, best regarded as a delayed, T cell dependent cutaneous inflammation (Leung and Bieber 2003).

5.3.1 Cutaneous contact allergies

Intact skin barrier is important in the protection of the human body from the outer environment. Small chemical molecules can easily penetrate into the skin, but penetration of larger protein molecules usually requires that the skin barrier is damaged (Smith Pease et al. 2002). The entry of allergens into the skin in a sensitized subject can cause an immediate type I reaction manifesting as ACU or a delayed response such as ACD. The pathophysiology of AE is complex and seems to depend on genetic, immunological and environmental factors (Leung and Bieber 2003, Homey et al. 2006, Simpson and Hanifin 2006).

Contact urticaria and protein contact dermatitis

Contact urticaria can be caused either by allergic or nonallergic mechanisms (Doutre 2005). The wheal and flare reaction occurs in nonallergic contact urticaria after nonimmunologic release of vasoactive substances from mast cells, whereas in ACU, previous sensitization to an allergen is mandatory. After sensitization, allergen binding to IgE molecules on the mast cell surfaces leads to release of histamine and other mediators (Geha et al. 2003, Gould et al. 2003, Galli et al. 2005a,

Galli et al. 2005b). When allergy is severe, the wheal and flare reaction can be associated with systemic symptoms such as generalized urticaria, asthma and even anaphylaxis. Contact urticaria syndrome is the term which covers the whole range of symptoms in ACU (Maibach and Johnson 1975). A contact urticaria reaction can sometimes be followed by delayed symptoms, such as local erythematic swelling, appearing a few hours after the wheal and flare reaction. This delayed reaction may be a late-phase type I hypersensitivity reaction attributable to the influx of eosinophils, basophils, mast cells and other lymphocytes (Macfarlane et al. 2000, Larche et al. 2006).

Protein contact dermatitis (PCD) is a rather new term and its pathophysiology is far from clear (Janssens et al. 1995). By definition, PCD is caused by protein contact and the phenotype is dermatitis, i.e. eczema, which is a delayed inflammatory response in the skin. Since PCD occurs mostly in atopic subjects with IgE antibodies, it could be a delayed IgE-associated reaction caused by penetration of protein allergens through damaged skin (Hjorth and Roed-Petersen 1976, Johansson et al. 2004). Epidermal Langerhans cells (and other cutaneous DC) express high affinity IgE receptors (FcεRI) on their surface and possibly participate in this allergic disorder. These cells can internalize allergens via FcεRI-associated IgE and further process them for antigen presentation to T cells. IgE binding to cutaneous FcεRI expressing Langerhans cells has been detected in patients with active AE, asthma or rhinitis, but not in individuals with disease remission or healthy controls (Semper et al. 2003). FcεRI expression of these cells are specifically found in the lesional skin of AE and they seem to affect the outcome of T cell responses in AE (Novak et al. 2004). Patch testing with mite, pollen and food allergens on the intact skin has often revealed delayed eczematous reactions in sensitized atopic subjects (Darsow et al. 2004, Turjanmaa 2005). In addition to subjects with IgE antibodies, positive atopy patch test reactions can also be seen in AE patients who have no IgE antibodies to the allergen tested, a typical example being a positive reaction to cow's milk (Isolauri and Turjanmaa 1996). Positive atopy patch test reactions to protein allergens have been shown to be associated with allergen-specific T cell responses, suggesting that these eczematous reactions could be caused by T-cell mediated immune reactions (Wistokat-Wulfing et al. 1999, Johansson et al. 2002).

ACU and PCD are particularly prevalent in occupations where animal or plant proteins are handled (Doutre 2005). Examples of this can be found in the food industry (fish handlers, butchers), in bakeries (exposure to wheat, egg) and in kitchen work where the skin is exposed to all kinds of foods including also fruits and vegetables (Hjorth and Roed-Petersen 1976, Hannuksela and Lahti 1977, Janssens et al. 1995, Wuthrich 1996). Farmers, veterinarians and laboratory workers may acquire ACU and PCD from animal allergens, such as cow's dander and rat urine (Janssens et al. 1995). ACU is a prominent symptom in NRL-allergic health care workers and these subjects often present also with hand eczema. Recently, Turjanmaa and co-workers

(Turjanmaa et al. 2002b) reported that after avoiding the use of NRL gloves, the hand eczema seemed to heal, suggesting that eczema could also be a PCD caused by NRL allergens.

Allergic contact dermatitis

Allergic contact dermatitis ACD (also called contact hypersensitivity in animal models) is caused mostly by small molecules (haptens), such as nickel, which penetrate into the skin, combine with protein and then cause a two-phase immunological response leading to ACD (Divkovic et al. 2005). Most contact allergens are themselves irritant, providing both antigen and danger signals when they contact the skin. In the sensitization phase, epidermal Langerhans cells (and possibly other cutaneous DC) take up and process the allergen and then migrate to regional lymph nodes where they can activate naive T cells after their maturation (Girolomoni et al. 2004, Saint-Mezard et al. 2004a, Saint-Mezard et al. 2004b, Cavani 2005). The subsequent event is the production of allergen-specific memory and effector T cells. A further allergen exposure leads to the elicitation phase in which hapten-specific T cells migrate to the site of hapten challenge and are activated. Activated CD4⁺ and CD8⁺ T cells then release cytokines, such as IFN- γ (Grabbe and Schwarz 1998, Girolomoni et al. 2004). Experiments in animals have indicated that allergen-specific type 1 CD4⁺ and CD8⁺ T cells act as effector cells and type 2 CD4⁺ T cells act as regulatory cells in ACD (Cavani et al. 2001, Kimber and Dearman 2002, Vocanson et al. 2006). Keratinocytes are also activated in the elicitation phase of ACD; after activation they release cytokines such as IL-1, IL-6, TNF and GM-CSF, and also CXC chemokines, such as CXCL8 (IL-8), CXCL9 (Mig) and CXCL10 (IP). A further event is the influx of monocytes and their maturation to macrophages which then secrete inflammatory mediators, such as IL-1 and TNF (Janeway Jr et al. 2005). Results obtained from human skin biopsy samples suggest that certain DC subgroups also participate in the pathogenesis of ACD (Bangert et al. 2003). The end point of this complex cascade of inflammatory events is ACD. This appears at the site of allergen contact but in severe allergy, simultaneous flare-ups can also occur in other areas of the skin. ACD can easily be diagnosed by patch testing in which the suspected allergen in petrolatum is applied on the skin for 48 hours (Mowad 2006). When positive, an eczema reaction appears at the test site and is still present at day 5 when the last reading is performed.

5.3.2 Atopic eczema

Atopic eczema (AE, also called as atopic dermatitis) is a chronic, very itchy and relapsing inflammatory skin disease characterized by typically distributed eczematous skin lesions and dry skin in the non-involved areas (Leung and Bieber 2003, Leung et al. 2004, Simpson and Hanifin

2006). AE is a very common disease that affects people in all age groups worldwide (Williams 2000, Schultz Larsen 2002, Williams and Flohr 2006). Its prevalence varies between 7% and 17% in children and 1-3% in adults and its incidence has been on the rise in parallel with asthma (Schultz Larsen 2002, Kupper and Fuhlbrigge 2004, Boguniewicz 2005, Simpson and Hanifin 2006). AE is often the initial step in the so-called atopic march, i.e. about a half of young children with AD subsequently develop allergic rhinitis or asthma (Spergel and Paller 2003, Hahn and Bacharier 2005). AE is classified into two main types: an extrinsic type associated with the presence of IgE antibodies to various environmental allergens; and an intrinsic type in which there is no evidence of IgE-mediated sensitization (Akdis and Akdis 2003). According to the new nomenclature, the term AE can only be used for those patients who have associated IgE antibodies and the term non-AE is reserved for those patients who have no evidence of IgE antibodies to environmental allergens (Akdis and Akdis 2003, Johansson et al. 2004).

Clinically, AE is characterized by the development of erythematous, exudative lesions in the skin folds that are associated with intense itching. Histopathological specimens show perivascular infiltration of lymphocytes and macrophages in the dermis, and lymphocytes and spongiotic vesicles in the epidermis. In acute AE, there is a prominent infiltration of T cells whereas in more chronic lesions, various amounts of eosinophils are also seen (Akdis et al. 2006). In the acute phase of AE, Th2-type cytokines, IL-4, IL-5 and IL-13 characterize the inflammatory response in the inflamed skin. In the chronic phase of the disease, however, Th1-type cytokines, IL-12 and IFN- γ , are highly expressed and predominate over Th2 cytokines.

Since approximately 70-80 % of patients with AE have elevated levels of serum IgE and specific IgE antibodies to environmental allergens, this points to an important role for allergens in AE. It has been hypothesised that aeroallergens, such as house-dust mite and pollens allergens, may penetrate into the skin in sensitized atopic subjects, bind to IgE receptors on the Langerhans cells and then cause eosinophil and T cell mediated inflammatory responses leading to the eczema reaction (Bruijnzeel-Koomen et al. 1989). In support of this theory, house-dust mite specific T cells have been found in the inflamed and non-inflamed skin of AE patients sensitized to this mite (Bohle et al. 1998). There is a substantial body of evidence that specific elimination diets can be beneficial in those AE children who are sensitized to food allergens (Sicherer and Sampson 1999). Moreover, double-blind placebo-controlled oral food challenges have confirmed that flare-ups of eczema are specific reactions to food allergens, such as cow's milk, egg and wheat (Isolauri and Turjanmaa 1996, Niggemann et al. 2001), but the mechanism by which they are transferred from the gut to the skin is at present incompletely understood.

The skin of more than 90 % of AE patients is colonized with superantigen-producing strains of *Staphylococcus aureus*, whereas only 5-10 % of healthy individuals carry these micro-

organisms (Leyden et al. 1974, Aly et al. 1977, Michie and Davis 1996, Breuer et al. 2000). It is possible that an innate immunity reaction initiated by bacterial superantigens leads to the release of inflammatory cytokines and then to new lesions in the skin of subjects with AE. Topical exposure to staphylococcal superantigens may also critically contribute to the development of Th1 type skin inflammation in AE patients during the chronic phase of the disease (Breuer et al. 2005). A deficiency in the expression of antimicrobial peptides (e.g. β -defensin and cathelicidin) in inflamed skin may contribute to the increased susceptibility to *S. aureus* colonization in patients with AE (Michie and Davis 1996, Ong et al. 2002).

Although significant progress has been made in the understanding of AE, its cause is still unknown, and much remains to be learned about the complex interrelationship of genetic, environmental and immunological factors in this disease (Leung et al. 2004).

5.3.3 Airway allergies

Airways are continuously exposed to inhaled particles, microbes and harmless antigens to which either immunity or tolerance is induced. Allergic rhinitis and allergic asthma are two important allergic disorders, which affect the upper and lower parts of the respiratory tract, respectively (Howarth 2003, Cohn et al. 2004, Greiner 2006). Similar inflammatory features are found in these diseases, such as vasodilation and local infiltration of mast cells, macrophages, eosinophils, dendritic cells and T cells. However, the importance of affected structures differs: smooth muscles are essential components in lower airways and blood vessels in upper airways. In addition, the surface of the epithelium plays an important role in the development of asthma (Cookson 2004).

Allergic rhinitis

Allergic rhinitis is a common, but often underestimated, inflammatory condition of the nasal mucosa characterized by itching, sneezing, increased nasal secretion and nasal stuffiness. If the symptoms occur at a particular time of the year, e.g. pollen-induced allergic rhinitis, it is called seasonal allergic rhinitis. In perennial allergic rhinitis, the individual is sensitized all year round to allergens, such as dust mites, pets, cockroaches and molds. Epidemiological studies support the concept that allergic rhinitis is a part of the systemic inflammatory process and is associated with other mucosal inflammatory diseases, such as asthma, rhinosinusitis and allergic conjunctivitis (Bousquet et al. 2003, Greiner 2006, Watelet et al. 2006).

The basis for the development of allergic rhinitis is the overproduction of IgE and the interaction between IgE and allergens. Most atopic subjects exhibit a systemic sensitization, where the B cell synthesizes IgE in the draining lymph nodes, when the antigen has been presented to T

cells by APC within the nasal mucosa. However, the B cell can synthesize IgE also locally within the nasal mucosa (Durham et al. 1997). The local production of IgE provides an explanation for the rarely encountered individuals who have a history of seasonal rhinitis but exhibit negative skin prick tests. In addition to these local events within the nose, there is also a systemic component to the allergic response, with stimulation of the synthesis and maturation of bone marrow precursors for eosinophils, basophils, and mast cells. Consistent with this concept, it has been demonstrated that nasal allergen challenge can increase inflammatory cell recruitment within the lower airways, and that a lower airway allergen challenge enhances upper airway cell recruitment in allergic rhinitis (Braunstahl et al. 2001a, Braunstahl et al. 2001b).

Asthma

Asthma is commonly divided into IgE-mediated allergic asthma and non-IgE mediated asthma (nonallergic asthma) (Humbert et al. 1999, Johansson et al. 2004). Eighty percent of childhood asthma and over 50% of adult asthma has been reported to be allergic in this manner. The mechanisms initiating nonallergic asthma are not well-defined, although similar inflammatory changes occur in both forms of asthma.

Asthma is a phenotypically heterogeneous disorder that results from complex interactions between environmental and genetic factors (Maddox and Schwartz 2002, Umetsu et al. 2002, Bel 2004, Wills-Karp and Ewart 2004). Allergic asthma is triggered by allergen-induced activation of submucosal mast cells in the lower airways (Bousquet et al. 2000, Busse and Lemanske 2001, Cohn et al. 2004). This leads to immediate bronchial constriction and amplified secretion of fluid and mucus, causing respiratory distress. A central aspect of asthma is chronic airway inflammation which is characterized by the continuous presence of Th2 lymphocytes, eosinophils, neutrophils, and other leukocytes. These cells work together to cause improper remodelling of the airways, accompanied by augmented mucus production. Th2 cytokines such as IL-13 may directly affect airway epithelial cells and cause the induction of goblet-cell metaplasia and the secretion of mucus (Kuperman et al. 2002). Bronchial epithelial cells express the chemokine receptor CCR3 and also produce at least two of the ligands for this receptor - CCL5 (RANTES) and CCL11 (eotaxin 1). These chemokines attract more Th2 cells and eosinophils to the damaged lungs which can increase the Th2 response. Additionally, recent studies indicate that CCL11 has a profibrogenic effect on human airway epithelial cells and fibroblasts through the chemokine receptor CCR3 (Beck et al. 2006, Puxeddu et al. 2006). Th2 cytokines and chemokines also have a direct effect on airway smooth muscle cells and lung fibroblasts leading to airway remodelling. Remodelling comprises thickening of the airway walls by hyperplasia and hypertrophy of the smooth muscle layer and mucous glands, with the final development of fibrosis.

Initially, allergic asthma is driven by a response to a specific allergen, but chronic inflammation seems to continue unabated even in the absence of allergen exposure. The airways become hyperreactive, and factors other than re-exposure to antigen can trigger asthma symptoms. For instance, environmental irritants, such as cigarette smoke, typically induce airway hyperreactivity. In addition, viral or bacterial respiratory infections can worsen asthma by inducing a Th2 dominated local response (Friedlander and Busse 2005, Lemanske and Busse 2006).

5.4 Natural rubber latex allergy

NRL allergy has been an important health issue over the past 20 years (Sussman et al. 2002). The first NRL-allergic patients were described at the beginning of the 80's (Nutter 1979, Turjanmaa et al. 1984). Subsequently, it was noted that sensitization to NRL was common especially in HCW and in patients with spina bifida (Turjanmaa 1988, Slater 1989). The diagnosis of NRL allergy is based on skin prick testing and measuring of IgE antibodies in the blood (Turjanmaa 2001). Awareness of NRL allergens and their quantification in NRL products has significantly improved during the last few years (Palosuo et al. 2002, Tomazic-Jezic and Lucas 2002). However, there is still some dispute about what are the major NRL allergens and furthermore, little is known about the cellular and molecular mechanisms involved in NRL allergy.

5.4.1 Prevalence and risk groups

Estimates of NRL allergy in the general population vary extensively, from less than 1 % to 12 % (Liss and Sussman 1999, Turjanmaa et al. 2002a). In the health care sector, the risk groups for NRL allergy are HCW and multi-operated patients. Up to 22 % of HCW and 60 % children with spina bifida have been reported to have become sensitized to NRL (Kelly et al. 1994, Ylitalo et al. 1997, Poley and Slater 2000, Mazon et al. 2005). A recent meta-analysis comparing the prevalence of NRL allergy in HCW and in the general population (Bousquet et al. 2006) found NRL allergy in 4.3% of HCW and in 1.4% of the general population. Sensitization to NRL was even more common, since positive SPT varied from 6.9% to 7.8% in HCW and from 2.1% to 3.7% in the general population. The NRL sensitized or allergic HCW showed an increased risk of hand dermatitis (OR 2.5), asthma or wheezing (OR 1.6) and rhinoconjunctivitis (OR 2.7) (Bousquet et al. 2006). Recent German and Italian studies have indicated that education about NRL allergy combined with the use of powder-free NRL gloves with reduced protein levels have led to a steady decline in the numbers of sensitized HCW (Allmers et al. 2002, Allmers et al. 2004, Filon and Radman 2006).

NRL products in the general environment are considered nowadays as an important source of sensitization. NRL cleaning gloves, balloons, condoms, pacifiers and hot-water bottles can evoke allergic reactions (Axelsson et al. 1988, Wrangsjö et al. 1988, Levy et al. 1992, Ylitalo et al. 2000, Wakelin 2002). It seems evident that more attention should be paid to the protection of NRL-allergic children in the domestic environment (Ylitalo et al. 2000).

5.4.2 Symptoms, diagnosis and outcome

The symptoms of NRL allergy vary from local signs to severe systemic reactions. The most common reaction is contact urticaria (Table 2). This becomes visible within a few minutes after contact with a glove or other NRL product and disappears in about 30 minutes. Swelling and itching are mucosal symptoms from NRL contact in the mouth, vagina or rectum (Turjanmaa and Reunala 1989). Systemic reactions like generalized urticaria and anaphylaxis can appear after mucosal or cutaneous exposure (Leynadier et al. 1989, Laurent et al. 1992). Airborne glove powder contaminated with NRL allergens has been reported to evoke allergic rhinitis, conjunctivitis and asthma (Carrillo et al. 1986, Baur and Jäger 1990, Tarlo et al. 1990, Vandemplas et al. 2002).

Table 2. Symptoms in 160 Finnish NRL-allergic patients (Turjanmaa et al. 2002b).

	%
Contact urticaria	64
Hand eczema	37
Eye symptoms	23
Facial edema	21
Generalized urticaria	10
Rhinitis	14
Asthma	8
Anaphylaxis	8

The diagnosis of NRL allergy is based on the clinical history of the patient and specific tests. The most reliable method to confirm the diagnosis is SPT (Turjanmaa 2001). Commercial NRL reagents are available; in one study the sensitivity of SPT was from 90% to 98% and the specificity was 100% (Blanco et al. 1998). *In vitro* tests such as RAST, AlaSTAT or ELISA measuring circulating IgE to NRL provide additional diagnostic information. The performance of these IgE assays can vary, and specific IgE was found in between 50% to 90% of the patients with positive SPT

(Hamilton et al. 1999). NRL allergens produced by recombinant technology are becoming commercially available and their use seems to increase the sensitivity of the IgE testing (Lundberg et al. 2001). When SPT or IgE assays produce results suspected to be false-negative or -positive, the final diagnosis of NRL allergy should be based on a provocation or usage test (Turjanmaa 2001). This test is started by placing a piece of high-allergenic NRL glove on the moistened skin. If the test is negative in this phase, it is continued by putting the whole glove on the hand. The appearance of a wheal and flare reaction on the test site confirms that the patient is allergic, i.e. reacts clinically to NRL. When needed, NRL challenges can also be performed in the airways (Vandenplas et al. 2002).

The outcome of NRL-allergic HCW is generally favourable after the institution of intervention measures such as use of non- or low-allergen NRL gloves. A follow-up study in a Finnish University Hospital showed that utilization of NRL gloves of a low allergen category made it possible that all NRL-allergic employees, whose most common symptom was contact urticaria, could continue working in their previous positions (Turjanmaa et al. 2002b). In a European study, the outcome of NRL-induced asthma in HCW was favourable after reduction of exposure to NRL (Vandenplas et al. 2002). In a study from the USA, a minority of HCW, especially those with asthma, had to change to NRL-safe workplaces, resulting in a mean 24% reduction in their annual income (Bernstein et al. 2003b).

Treatment options for NRL allergy are at present limited. Trials have been performed with anti-IgE (Omalizumab) treatment and with immunotherapy, but the clinical efficacy has been rather low (Sutherland et al. 2002b, Leynadier et al. 2004). Crude NRL preparations used in immunotherapy have produced frequently adverse events (Rolland et al. 2005, Sastre et al. 2006). Tailored hevein molecules with a low potential for adverse events have been generated but not yet evaluated in immunotherapy trials (Karisola et al. 2004).

5.4.3 Natural rubber latex allergens

Natural rubber is a highly processed plant product derived from the cytosol, or latex, of the commercial rubber tree, *Hevea brasiliensis*. Noncoagulated, ammoniated latex is mostly used in the manufacture of rubber gloves and other soft rubber products such as condoms and balloons. The natural function of latex is to seal damaged sites on the surface of the rubber tree (Ko et al. 2003). Sealing is a coagulation process involving aggregation of rubber particles by hevein (Hev b 6.02) and rubber elongation factor (Hev b 1) (Dennis and Light 1989). Prenyltransferase and Hev b 3 link short isoprene units into cis-1,4-polyisoprene chains, which are responsible for the structural integrity of latex (Oh et al. 1999). In addition to these proteins involved in rubber latex

biosynthesis, NRL contains between 200 - 250 different polypeptides (Alenius et al. 1994, Posch et al. 1997, Petsonk 2000), many of which have unknown functions. Analysis of public domain databases of known *H. brasiliensis* proteins revealed 102 different polypeptide amino acid sequences (Guarneri et al. 2006).

At present (February 20, 2006), the WHO/IUS Allergen Nomenclature Committee (www.allergen.org) has listed 13 NRL Hev b allergens which have been characterized at the molecular level (Table 3).

Table 3. Natural rubber latex allergens from *Hevea brasiliensis* (www.allergen.org).

Allergen	Significance	Biochemical		MW** (kDa)	Homologues / cross-reactivity
		name	Function		
Hev b 1	Major/Minor*	elongation factor	biosynthesis	58	papain
Hev b 2	Major ?	1,3-glucanase	plant defence	34	other glucanases
Hev b 3	Major/Minor	small rubber particle protein	biosynthesis, coagulation	24	
Hev b 4	Minor	component of microhelix complex	plant defence	100	lecithinase
Hev b 5	Major	acidic protein		16	kiwi acidic protein
Hev b 6.01	Major	prohevein	plant defence	20	chitin-binding proteins
Hev b 6.02	Major	hevein	plant defence	5	
Hev b 6.03	Minor	prohevein C-domain	plant defence	14	win 1, win 2
Hev b 7	Minor	patatin	biosynthesis inhibitor	42	plant patatins
Hev b 8	Minor	profilin		14	pollen-food allergen
Hev b 9	Minor	enolase		51	mold enolases
Hev b 10	Minor	superoxide-dismutase		26	mold SO-dismutases
Hev b 11	Minor	chitinase		33	class I chitinases
Hev b 12	Minor	lipid transfer protein		9	food lipid transfer protein
Hev b 13	Major ?	esterase		42	patatin

* Major = recognized by over 50% of the NRL-allergic patients

** MW = molecular weight; kDa = kilodalton

Hev b 1, a non-soluble rubber elongation factor, was the first identified latex allergen (Czuppon et al. 1993). IgE antibodies to Hev b 1 were found in 81% of NRL-allergic spina bifida patients and 52% of HCW (Chen et al. 1997a). Hev b 1 exhibits cross-reactivity with papain (Baur et al. 1995). Linear IgE epitopes of Hev b 1 have been mapped and the T cell epitopes identified (Chen et al.

1996, Raulf-Heimsoth et al. 1998). IgE antibodies to Hev b 3, a small rubber particle protein, have been found in 76-83 % of NRL-allergic spina bifida patients (Alenius et al. 1993, Lu et al. 1995, Wagner et al. 1999). The hydrophobic NRL allergens Hev b 1 and Hev b 3 can sensitize spina bifida and other multi-operated patients, apparently because NRL catheters and other devices containing these allergens are often in direct contact for long times with the mucosal membranes (Yeang et al. 1998).

Hev b 5, a major NRL allergen, was reported to be recognized by sera from 56 % of spina bifida patients and 92 % of HCW suffering from latex allergy (Slater et al. 1996). During glove manufacture, highly acidic and proline rich Hev b 5 may degrade and form aggregates and interact with other NRL proteins, leading up to the high levels of Hev b 5 present in NRL gloves (Sutherland et al. 2002a). The B cell and T cells epitopes of Hev b 5 have been mapped and this knowledge has been used to generate a pilot immunotherapy molecule for NRL allergy (Beezhold et al. 1999, Slater et al. 1999, de Silva et al. 2000, Beezhold et al. 2001).

Hevein (Hev b 6.02) at the N-terminus of prohevein (Hev b 6.01) is a major NRL allergen, recognized by over 50% of all kinds of NRL allergic patients. Initial studies indicated that 83 % of NRL allergic patients, 75 % of HCW and 27 % of spina bifida patients possessed IgE to prohevein (Alenius et al. 1995, Chen et al. 1997b). The water soluble hevein is abundant and easily eluted from NRL gloves (Alenius et al. 1996a). The immunological role of hevein has been well characterized e.g. the B-cell and T-cell epitopes have been identified (Banerjee et al. 1997, Beezhold et al. 1997, de Silva et al. 2004), and the B cell and T cell responses clarified (Raulf-Heimsoth et al. 2004).

Hev b 4, a component of the microhelix complex, is a glycoprotein and its IgE reactivity is dependent upon glycosylation (Sunderasan et al. 2005). SPT with native Hev b 4 has been positive in 39 % of NRL-allergic HCW (Bernstein et al. 2003a). Hev b 7 isoallergens exhibit structural homology with potato patatins (Kostyal et al. 1998, Sowka et al. 1998), but cross-reactivity between these proteins is still somewhat unclear (Sowka et al. 1999, Seppala et al. 2000). Hev b 7 is classified as a minor NRL allergen, although it may sensitize patients with spina bifida similarly to Hev b 1 and Hev b 3 (Sowka et al. 1998, Kurup et al. 2000, Wagner et al. 2001a, Bernstein et al. 2003a). Hev b 8 (NRL profilin) and rubber tree enolase, Hev b 9, are minor allergens in NRL. Hev b 8 is recognized by 20 % of HCW and by 12% of spina bifida patients (Rihs et al. 2000), whereas 14.5 % of NRL allergic patients recognize Hev b 9 (Wagner et al. 2000). Sensitization to birch and other plant profilins can result in false-positive IgE tests to NRL according to a recent study (Ebo et al. 2004). Hev b 10, a manganese superoxide-dismutase, is a minor NRL allergen which exhibits cross-reactivity to molds (Posch et al. 1997, Wagner et al. 2001b). Recombinant Hev b 10 was recognized only by two out of 20 spina bifida patients and by

none of the 20 HCW (Rihs et al. 2001). Hev b 11 is also a minor NRL allergen that has recently been cloned and shown to be a hevein-like protein belonging to class I chitinases (O'Riordain et al. 2002). Recombinant Hev b 11 was recognized by 19 % and 29% of NRL-allergic patients (O'Riordain et al. 2002, Rihs et al. 2003). Hev b 12, a lipid transfer protein, is a minor NRL allergen, which in immunoblot analysis was recognized by 24 % of NRL-allergic patients (Beezhold et al. 2003). Potential new NRL allergens have recently been sought by proteomic analysis. This identified five new allergen candidates i.e. UDP-glucose pyrophosphorylase, isoflavone reductase, rotamase, thioredoxin and citrate-binding protein (Yagami et al. 2004).

Up to 50% of NRL-allergic patients have been reported to react with lip swelling or other immediate allergic and even systematic symptoms to several fruits and vegetables (Makinen-Kiljunen 1994, Beezhold et al. 1996, Blanco et al. 1999, Blanco 2003). This reactivity is now termed as latex-fruit syndrome and is most often evoked by consumption of banana, kiwi fruit, chestnut and avocado (Beezhold et al. 1996, Blanco et al. 1999, Wagner and Breiteneder 2002). The molecular basis for this latex-fruit syndrome is well documented. NRL allergens such as Hev b 2, Hev b 5, Hev b 6.01, Hev b 7, Hev b 8 and Hev b 9 have homologous proteins in other plants (Table 3). Class I chitinases in avocado and banana and other fruits are the most likely allergens involved in the latex-fruit syndrome (Chen et al. 1998, Diaz-Perales et al. 1998, Mikkola et al. 1998). In these fruits, the N-terminal chitin-binding hevein-like domains exhibit 70-80 % identity to hevein (Wagner and Breiteneder 2002). Homologous class-II chitinases, lacking N-terminal chitin-binding hevein-like domains, are also reported to be responsible for allergic cross-reactions (Subroto et al. 1999). A recent study revealed that isolated hevein-like domains but not intact endochitinases are responsible for the IgE mediated *in vitro* and *in vivo* reactions in the latex-fruit syndrome (Karisola et al. 2005). It is also known that Hev b 5 shows homology with an acidic protein present in kiwi fruit (Slater et al. 1996) and there is Hev b 7 homology with patatin (Sol t1) in potato (Seppala et al. 2000).

At present there is a general agreement that the most important NRL allergens for HCW and other adult NRL-allergic subjects are Hev b 6.02 and Hev b 5 (Alenius et al. 1996a, Slater et al. 1996, Chen et al. 1997b). The major allergens for the patients with spina bifida and other congenital anomalies are Hev b 1 and Hev b 3 (Alenius et al. 1993, Lu et al. 1995, Chen et al. 1997a, Rihs et al. 1998, Wagner et al. 1999). There is also a general agreement that in medical NRL gloves, the most abundant allergens are Hev b 6.02 and Hev b 5 but Hev b 3 and Hev b 1 are also commonly found (Kujala et al. 2002, Palosuo et al. 2002, Sutherland et al. 2002a, Koh et al. 2005). It has recently been suggested that the major NRL allergens should be expanded to include Hev b 2 and Hev b 13 (Table 3) (Yeang 2004). The role of Hev b 2 and Hev b 13 as major NRL allergens is, however, controversial and needs to be confirmed in further studies.

5.4.4 Peripheral blood mononuclear cell responses

There are much fewer studies on NRL-induced cell-mediated immunity than there are for IgE antibody responses. Proliferation of peripheral blood mononuclear cells (PBMC) in NRL-allergic patients have been performed using NRL extracts, purified native or recombinant allergens (Murali et al. 1994, Raulf-Heimsoth et al. 1996, Ebo et al. 1997, Johnson et al. 1999, Raulf-Heimsoth et al. 2004). Purified Hev b 1 was shown to induce lymphocyte proliferation in 52 % of patients sensitized to NRL and in 25% of NRL-exposed subjects (Raulf-Heimsoth et al. 1996). T cell responses to Hev b 3 have been studied in NRL-allergic patients with spina bifida using poly-, oligo-, and monoclonal T lymphocyte cultures (Lu et al. 1995, Bohle et al. 2000). Further studies with Hev b 5 have indicated that certain Hev b 5 peptides induce proliferation of T cell lines from NRL-allergic patients (de Silva et al. 2000) as well as from NRL-sensitized mice (Slater et al. 1999).

5.5 Mouse models for allergic diseases

Experimental animal models are crucial in elucidating the pathophysiology of different diseases and in the development of novel therapies. Different animal species such as dog, monkey, rat and guinea pig have been used as models for allergic disorders but mouse models are most commonly used, probably due to the availability of immunological tools, such as transgenic and knock out mouse strains and antibodies. Mice have other advantages e.g. short breeding periods, a well-known genome, a fully characterized immune system and relatively low maintenance costs (Gutermuth et al. 2004, Taube et al. 2004).

AE is a pruritic inflammatory skin disease characterized by dermal infiltration of lymphocytes and eosinophils, expression of Th2 cytokines and chemokines and increased levels of total and specific IgE (Leung et al. 2004). Several experimental models have been developed and used to examine specific features of AE, for instance genetic regulation, immune deficiencies, the significance of various cytokines and the role of bacteria in the course of inflammation (Gutermuth et al. 2004, Shiohara et al. 2004). Mouse models of AE can be roughly categorized into the following groups: a) mouse models (NC/Nga) with spontaneous development of AE (Matsuda et al. 1997); b) genetically modified mice, transgenic or knockout e.g. with regard to IL-4 and IL-18 (Chan et al. 2001, Konishi et al. 2002) c) AE-like skin lesions after epicutaneous ovalbumin sensitization (Wang et al. 1996, Spergel et al. 1998) and d) humanized mouse models of AE such as the SCID mutant mice (Carballido et al. 2003).

For the time being, the limited availability and the lack of standardized mice housing conditions make the use of NC/Nga mice inconvenient. Humanised SCID and genetically modified mice are important tools when examining specific mechanisms of diseases. On the other hand, a mouse model making use of repeated epicutaneous protein exposure (Spergel et al. 1998) imitate reasonably well naturally occurring cutaneous sensitization. In this model, tape stripping is an important feature provoking the development of general skin injury characteristic of AE. This protein exposure model has been modified by using NRL instead of ovalbumin to study the cutaneous route exposure to NRL allergens in this thesis (I).

Allergic asthma is a complex condition characterized by increased amounts of systemic IgE, elevated allergen-specific Th2 cells and their products, airway hyper-reactivity (AHR), and structural changes in the lung (Cohn et al. 2004). Animal models of both acute and chronic allergic airway responses have been described, although none of these models encompasses all aspects of human asthma (Kips et al. 2003, Epstein 2004, Taube et al. 2004, Boyce and Austen 2005, Fulkerson et al. 2005). Moreover, considerable differences in the airway reactivity between different mice strains exist (Takeda et al. 2001, Whitehead et al. 2003). The most widely used mouse model of acute asthma involves intraperitoneal injections with ovalbumin combined with alum, followed by repeated ovalbumin challenges intratracheally, intranasally, or by inhalation. This evokes a marked eosinophilic inflammation and AHR, which is independent of IgE, B cells, or mast cells, but dependent on CD4+ T cells (Corry et al. 1998). The presence of effector T cells is essential and sufficient to provide the necessary Th2 cytokines to evoke both histological changes and induced AHR (Boyce and Austen 2005). Chronic asthma models with remodelling of lung tissue have been reported but these models are difficult to standardize (McMillan and Lloyd 2004, Fulkerson et al. 2005, Wegmann et al. 2005).

A few studies have used mouse models to examine the pathological mechanisms involved in NRL allergy (reviewed in (Meade and Woolhiser 2002, Herz et al. 2004). BALB/c mice have been most commonly investigated mice strain, but in two studies also C57BL/6 and B6C3F1 mice were used (Kurup et al. 1994, Woolhiser et al. 1999). Crude NRL has been the allergen used in almost all studies (Kurup et al. 1994, Thakker et al. 1999, Woolhiser et al. 1999, Xia et al. 1999) but recombinant Hev b 5 has also been administered in two studies (Slater et al. 1998, Slater et al. 1999). One group has been interested in the effects of NRL in the lungs which they have investigated by using intranasal and intraperitoneal sensitization (Kurup et al. 1994, Thakker et al. 1999, Xia et al. 1999). They found increased levels of total IgE, IL-4, IL-5 and eosinophils in the blood (Kurup et al. 1994). Eosinophils were detected in the lungs and AHR occurred after methacholine (MCh) challenge (Thakker et al. 1999, Xia et al. 1999). Experiments in IL-4 knockout BALB/c mice showed no eosinophils nor IgE antibodies, confirming the importance of this

cytokine in the initiation of this response (Xia et al. 1999). It is, however, of interest that IL-4 knock-out mice exhibited a weak AHR to MCh (Thakker et al. 1999, Xia et al. 1999).

Woolhiser et al. (1999) studied the effects of IN, subcutaneous and EC exposure by using crude NRL as allergen. They found increased total IgE levels by all exposure routes and detected the presence of several IgE bands with cutaneous exposure. However, the results of different exposure routes are not comparable because NRL dosage and exposure times were different. Slater and co-workers (Slater et al. 1998, Slater et al. 1999) used recombinant Hev b 5 with maltose binding protein to sensitize BALB/c mice by the IN route. Hev b 5 specific IgG1 or IgE antibodies were found in the blood. A significant lymphoid cell infiltration, but no mucus secretion, was seen in the lungs when the allergen was given together with LPS. These mouse model studies indicate that IgE antibodies appear after cutaneous, intranasal and intraperitoneal exposure to NRL. It seems evident that IN exposure to NRL can evoke eosinophilic lung inflammation and AHR in the sensitized mice. None of these mouse experiments have examined cutaneous inflammation to NRL and studied whether sensitization through the skin can predispose the animal to NRL-allergic asthma.

6 AIMS OF THE STUDY

The aims of the present study were:

1. To examine the role of cutaneous exposure to NRL in the development of skin inflammation and the humoral IgE antibody response in mice.
2. To study the effect of different exposure routes of NRL in the development of lung inflammation and airway hyperreactivity in mice.
3. To characterize cytokine and chemokine profiles to major NRL allergens Hev b 5 and Hev b 6.01 in peripheral blood mononuclear cells of NRL-allergic patients.
4. To investigate the prevalence of IgE antibodies to highly purified Hev b 2 and Hev b 13 in relation to Hev b 5 and Hev b 6.01 in NRL-allergic patients from Finland, Spain and the USA.

7 MATERIALS AND METHODS

7.1 Natural rubber latex allergens

7.1.1 NRL (I - III)

NRL (Yeang et al. 2002) was obtained from fresh Malaysian non-ammoniated latex (Ansell International, Melbourne, Australia) (I, II). The liquid latex from *H. brasiliensis* was collected and stored at -70°C . For the extraction of soluble proteins, latex was diluted 1:2 with phosphate-buffered saline (PBS) and centrifuged at 100,000 *g* for 90 min after which supernatant was stored at -70°C (Alenius et al. 1993). Buffer exchange to PBS was achieved by high-performance liquid chromatography (HPLC) on a Fast Desalting Column HR 10/10 (Amersham Biosciences, Uppsala, Sweden) for mice experiments (I, II). Pooled protein fractions were concentrated in a vacuum centrifuge (SpeedVac, Savant Instruments, Inc., Farmingdale, NY, USA) (I, II). Native proteins were used for human studies and buffer exchange was conducted on a BioGelP6 desalting gel column (Bio-Rad Laboratories, Hercules, CA, USA) in PBS (III). The source material for isolation of native NRL allergens (B-serum and C-serum of non-ammoniated NRL, clone RRI 600) was obtained from the Rubber Research Institute of Malaysia (Kuala Lumpur, Malaysia) (III, IV).

7.1.2 Purified NRL allergens (I - IV)

Hev b 1 (I)

Hev b 1 was purified from NRL with electroelution using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Alenius et al. 1996b).

Hev b 2 (IV)

Native Hev b 2 was isolated and purified from B-serum of NRL (Yeang et al. 2002). The first purification step was cation exchange chromatography on a MonoS (4.6/100 PE, Tricorn) column (Amersham Biosciences) equilibrated with 50 mM sodium phosphate, pH 7.5 and eluted with a linear gradient of NaCl (0 to 1 M in 30 min) at a flow rate of 1.0 ml/min (IV, Fig. 1A). Gel filtration chromatography of pooled and concentrated Hev b 2 fractions was conducted on a Superdex 75 (HR 10/30) column (Amersham Biosciences) in PBS (0.5 ml/min) (IV, Fig. 1B). Monitoring of these chromatographs was made at 214 nm.

Hev b 5 (III, IV)

The starting material for native Hev b 5 was the C-serum of NRL (Yeang et al. 2002) and purification was undertaken via buffer exchange and cation exchange chromatography. A BioGelP6 desalting gel column in 20 mM Na-citrate, pH 3.8 was used for buffer exchange and cation exchange was performed on a MonoS (4.6/100 PE) column equilibrated with the same buffer. Elution was done with a linear gradient of NaCl (0 to 1 M in 30 minutes) in the equilibration buffer.

Hev b 6.01 (I-IV)

For purification of Hev b 6.01, chitin-binding proteins of NRL were attached to chitin (Sigma Chemical Co, St.Louis, MO, USA) and detached from it with 0.25 M and 0.6 M acetic acid (Hanninen et al. 1999) (I, II). Hev b 6.01 fractions were then obtained by HPLC on a ReSource (3 ml) column (Amersham Biosciences) (linear gradient of 0-60% acetonitrile in 30 min, flow rate 1 ml/min). The fractions were pooled and concentrated in a vacuum centrifuge. For human studies (III, IV) native Hev b 6.01 was purified from the B-serum of NRL by gel filtration and anion exchange chromatography (Karisola et al. 2005). Gel filtration was performed on a Superdex 75 (HR 16/60) column (Amersham Biosciences) in PBS. Prohevein fractions were concentrated and the buffer was changed to 20 mM Tris-HCl, pH 8.5, by means of a BioGelP6 column. Anion-exchange chromatography was conducted on a MonoQ (HR5/5) column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 8.5 and eluted with a linear gradient of NaCl (0 to 1 M in 30 minutes) in the same buffer.

Hev b 13 (IV)

B-serum of NRL was at first chromatographed on a Superdex 75 (HR 16/60) column to obtain Hev b 13 containing fractions; flow rate was 1.5 ml/min and with monitoring at 280 nm (IV, Fig. 1D). Buffer exchange to 50 mM sodium acetate, pH 4.5 was then made for pooled and concentrated Hev b 13 containing fractions on a BioGelP6 column. Subsequently, a cation exchange column, MonoS (HR 5/5, Amersham Biosciences) was used for further purification (IV, Fig 1E). Elution was performed with a linear gradient of NaCl (0 to 1 M in 40 min) at a flow rate of 1.0 ml/min and the chromatography was monitored at 214 nm.

Identification and characterization of the purified allergens (III, IV)

Identity of the purified native allergens Hev b 2, Hev 5, Hev b 6.01 and Hev b 13 was confirmed with monoclonal antibodies and commercial ELISA kits for Hev b 5 and Hev b 6.02 (Fit Biotech Ltd., Tampere, Finland). In addition, reversed-phase chromatography on a C1 column (2 x 20 mm, Tosoh Corp, Tokyo, Japan) was used to check the purity and to quantify the purified allergens

(Karisola et al. 2005) (IV, Figs 1C and IF). Elution was performed using a linear gradient of acetonitrile (0-100 % in 60 min) in 0.1% TFA at a flow rate of 0.2 ml/min, monitoring at 214 nm. Molecular characterization was performed using molecular mass determination and peptide mass fingerprinting by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Ylonen et al. 1999, Poutanen et al. 2001). N-terminal sequencing was performed essentially as previously described (Ylonen et al. 1999). Hev b 2 and Hev b 13 were further analyzed by electrospray quadrupole time-of-flight (Q-TOF) mass spectrometric analyses (Poutanen et al. 2001).

7.2 Experiments in mice

Female BALB/cJBom mice) were purchased from Taconic M&B A/S (Ry, Denmark) and used when they were six-to eight-weeks-old (I, II). All mouse experiments were approved by the Animal Care and Use Committee of the National Public Health Institute (I) or by Social and Health Services of Finland; Provincial Office of Southern Finland (II).

7.2.1 Dermatitis model for NRL allergy (I)

Epicutaneous NRL exposures were performed as earlier described (Spergel et al. 1998). Briefly, Avertin (2,2,2 tribromoethanol, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used to anaesthetize mice. After that, the skin on the back of the mice was shaved and tape-stripped with transparent IV dressing (Tegaderm, 3M Health Care, St.Paul, MN, USA) four times to remove the stratum corneum to mimic scratching, which is one symptom of atopic eczema. A sterile gauze patch soaked with NRL (100 µg /100 µl PBS) or PBS (100 µl) was fastened onto the skin with Tegaderm. The patches remained on the backs of mice for three one-week periods separated by a two-week rest time. Mice were sacrificed one day after the end of the epicutaneous exposure protocol and samples (blood and skin) were obtained for analysis (Fig.3).

Intraperitoneal (IP) exposure of mice consisted of two injections of 20 µg NRL in 2.25 mg alum (Imject Alum, Pierce, Rockford, IL, USA), or PBS with alum. The injection volume was 100 µl and injections were given on days 0 and 7. The mice were sacrificed and blood was taken for serum antibody analysis on day 19.

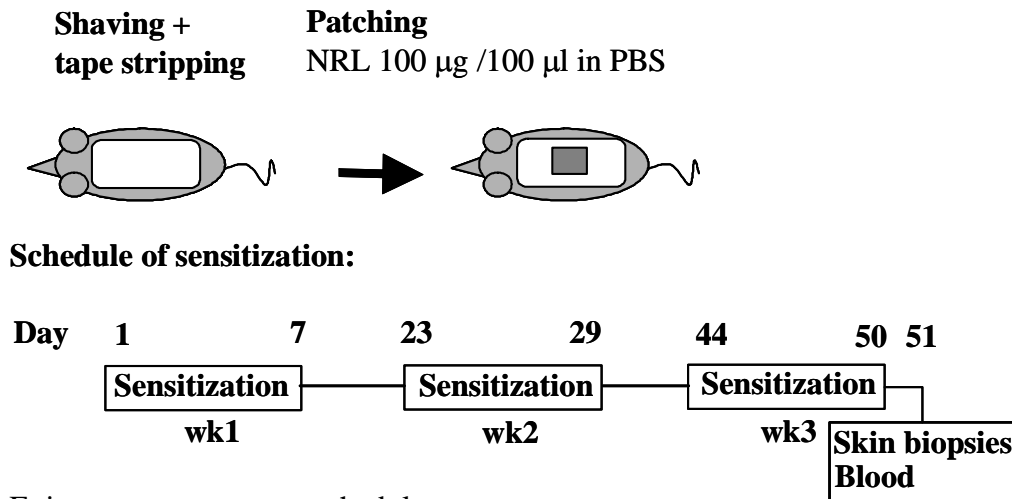


Fig. 3. Epicutaneous exposure schedule.

7.2.2 Asthma model for NRL allergy (II)

Total exposure time to NRL (NRL groups) or PBS (SAL or saline groups) via intracutaneous (IC), intraperitoneal (IP) and intranasal (IN) routes lasted for four weeks (II, Fig 6). Anaesthesia with isoflurane (Abbott Laboratories Ltd, Queenborough, UK) was used, when IC or IN administration of NRL or PBS was made. Before the IC exposures, the backs of the mice were shaved and tape-stripped as earlier described for epicutaneous exposures. Mice were given once a week NRL (40 µg /100 µl PBS) during the IC exposures. Intranasally exposed mice received also 40 µg NRL in 50 µl PBS once per week. IP exposure consisted injections of 80 µg NRL in 2 mg alum (Pierce, Rockford, IL, USA) on days 1 and 14 or 100 µl PBS with alum. IC and IN control mice received 100 µl PBS instead of NRL. Inhalation challenges of NRL (0.5% in PBS for 20 min) were achieved with an ultrasonic nebulizator (Aerogen Ltd., Galway, Ireland) on days 28, 29, and 30.

Airway hyperreactivity

Airway hyperreactivity (AHR) to methacholine (MCh) (Sigma-Aldrich Co, St.Louis, MO, USA) was assessed 24 hours after the last NRL challenge (on day 31) by whole-body plethysmography (Buxco Electronics, Inc., Sharon, CT, USA) using MCh concentrations 0 - 100 mg/ml. The average enhanced pause (Penh) values were measured by a exposure time of 5 min to each concentration. The results are expressed as compared to Penh values following PBS exposure.

The mice were killed with carbon dioxide and samples (blood, BAL fluids and lung biopsies) collected. The blood was used to measure antibodies, BAL fluids to analyze inflammatory cells and tissue biopsies to study mRNA expression and inflammatory cells.

7.2.3 Cytological, histological and immunohistological methods (I, II)

BAL cytology (II)

Bronchoalveolar lavage (BAL) samples were prepared by lavaging lungs once with 0.8 ml of PBS via the trachea. The samples were cytocentrifuged (Shandon Scientific Ltd., Runcorn, UK; 150 g, 5 min), and stained with May-Grünwald-Giemsa. Cells were counted in 40 high-power fields (HPF) at x 1000 using light microscopy.

Lung histology (II)

The lung samples in 10% buffered formalin were embedded into paraffin, cut into sections of 4 µm, and stained with haematoxylin and eosin (H&E) or with periodic acid-Schiff (PAS) solution. Lung inflammation was scored after H&E staining (1: slightly inflamed tissue; 2: inflammation of the area over 20%; 3: inflammation of the area over 50%; 4: inflammation of the area over 75%; 5: entire tissue inflamed). Mucus producing cells per 100 µm were counted after PAS staining. The slides were analyzed in a blind manner with a Nikon Eclipse E800 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Skin histology (I)

Skin biopsies from patch areas were treated like the lung samples and after that the slides were stained with H&E or o-toluidin blue for mast cell counting. Inflammatory cell types were counted at x 1000 and mast cells at x 400 magnification. A scaled ocular lens was used to measure the thickness of the skin. The measurements were adjusted for the magnification optics and expressed in micrometers. The slides were examined in a blind manner using a light microscope (Leitz Dialux 20 EB, Wetzlar, Germany).

Skin immunohistology (I)

Skin samples were embedded in a Tissue-Tek OCT compound (Sakura, AA Zoeterwoude, The Netherlands) on dry ice. Subsequently, skin sections (4 µm) were stained immunohistochemically. Briefly, the sections were incubated with rat anti-mouse monoclonal antibodies (anti-CD3, anti-CD4, anti-CD8) (BD PharMingen) followed by biotinylated rabbit anti-rat Ig (Vector Laboratories, Burlingame, CA, USA). Peroxidase-blocking solution (Dako, Carpinteria, CA, USA) was used to block endogenous peroxidase activity. Sections were then incubated with peroxidase-conjugated streptavidin (Dako) and stained in a substrate solution containing hydrogen peroxide (Dako) and 3-amino-9-ethylcarbazole (Dako), followed by haematoxylin incubation (Dako). Mounting of the skin sections was achieved with Aquamount (BDH, Gurr, Poole, UK). Cell counting was done in 10 HPFs at x 1000 with positively stained cells being expressed as cells per HPF.

7.2.4 Cytokine and chemokine measurements by PCR (I, II)

RNA isolation (I, II)

Skin and lung biopsies were removed and immediately frozen in dry ice. Total RNA was isolated using Trizol (Gibco BRL, Paisley, UK) according to the manufacturer's instructions. Homogenization of the samples was done by Ultra-Turrax T8 (IKA Labortechnik, Staufen, Germany). After RNA isolation, genomic DNA was removed with DNaseI (RNase-free) (Gibco BRL) treatment and final RNA (in nuclease-free water) was obtained on the Trizol according to guidelines.

cDNA synthesis and real-time quantitative PCR (I, II)

Total RNA was reverse-transcribed into cDNA with MultiScribe™ Reverse transcriptase (PE Applied Biosystems, Foster City, CA, USA) and random hexamers as primers according to the manufacturer's instructions. The real-time quantitative polymerase chain reaction (PCR) was performed with an AbiPrism 7700 Sequence Detector System (SDS) (PE Applied Biosystems). PCR primers and probes were pre-developed reagents or designed by PrimerExpress (version 2.0) software and obtained from PE Applied Biosystems. The PCR products were detected by monitoring fluorescence levels of the reporter dyes, FAM or VIC™. The FAM dye of the sample cDNA was standardized to the VIC dye of the endogenous reference (18S rRNA) to control sample loading and to allow normalization between samples according to the manufacturer's instructions (PE Applied Biosystems). The final results were expressed as relative units (RU) i.e. ratio of PCR amplification efficiency in the sample cDNA compared to that of the calibrator. These were calculated by the comparative C_T method according to the manufacturer's instructions. An FAM C_T value of 40, reflecting the situation in which the gene is not expressed at the mRNA level, was used as the calibrator.

7.2.5 IgE and IgG2a antibody measurements (I, II)

Total IgE (I, II)

Total IgE levels in serum were measured by ELISA. Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with rat anti-mouse IgE mAb (BD PharMingen, San Diego, CA, USA). Diluted serum samples were added to the plate and incubated overnight. Bound IgE was detected using biotin-conjugated rat anti-mouse IgE (BD PharMingen), streptavidin-conjugated horseradish peroxidase (BD PharMingen) and peroxidase substrate reagents (KPL, Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Absorbance values at 405 nm were obtained by an ELISA

plate reader (Titertek Multiscan, Eflab, Turku, Finland). Total IgE levels were calculated from IgE standard curve.

Specific IgE and IgG2a (I)

For measurement of specific IgE and IgG2a antibodies, plates were coated (50µl/well) with purified antigen (Hev b 6.01 or Hev b 1, 2µg/ml) or 20µg/ml of NRL in 0.05M NaHCO₃, pH 9.6 at 4⁰C overnight. Briefly, the plates were then blocked (2 hours) and 100 µl of serial dilutions of sera were incubated overnight at 4⁰C. Subsequently, the biotin-labeled anti-mouse isotype-specific antibody (BD PharMingen, 2 µg/ml) was added. Streptavidin-horseradish peroxidase (BD PharMingen) was used for detection and finally peroxidase substrate (KPL) was added, and absorption at 405 nm was read with an automated ELISA reader.

Specific IgE to Hev b 6.01 (II)

A slightly modified specific ELISA for Hev b 6.01 was used in the second study (II) compared to the first study (I). Plates were first coated for 3 hours at +20⁰C and then overnight at +4⁰C. The blocking time was 1 hour and 1:10 diluted samples were incubated for 2 hours at +20⁰C. Biotin-labeled anti-mouse isotype-specific antibody (BD PharMingen) was added. Streptavidin-conjugated alkaline phosphatase (Zymed, San Francisco, CA, USA) and colour substrate (p-nitrophenyl phosphate, Sigma) were used for detection and absorption values were read at 405 nm.

7.3 Studies in patients allergic to natural rubber latex

7.3.1 Patients and controls (III, IV)

Fourteen NRL-allergic patients participated in the study of PBMC responses (III) (Table 2). All of them were occupational NRL-glove users and six of them were HCW. Altogether eleven of these individuals suffered from different atopic diseases (asthma, allergic rhinitis and atopic eczema). All patients had a positive skin prick test to a commercial latex extract (Stallergenes, Antony Cedex, France) and a positive NRL glove challenge test (Turjanmaa et al. 2002b). Induction of cytokines, chemokines and chemokine receptors was investigated in 10 NRL-allergic patients with high levels of antigen-specific IgE, as well as positive SPT and PBMC proliferative responses to NRL allergens. Ten healthy subjects served as controls. The study was approved by the ethics committee of Helsinki University Central Hospital.

Table 4. Natural rubber latex allergic patients and controls in the studies III and IV.

	Study III		Study IV					
	Finland		Finland		Spain		USA	
	Patients	Controls	Patients	Controls	Patients	Controls	Patients	Controls
No. of subjects	14	10	79	73	74	50	62	49
Mean age (years)	45.5	42.3	39.2	34.2	33.3	31.4	36.1	37.1
Age range (years)	32-73	27-54	16-63	15-75	17-69	15-54	21-67	28-56
Females/Males	13/1	9/1	68/11	54/19	66/8	33/17	60/2	35/14
Atopic disease (%)	79	30	100	100	61	100	89	100
Health care workers (%)	43	70	23	14	31	0	100	100

In total, 215 NRL-allergic patients and 172 atopic controls without NRL allergy participated in the prevalence study (IV) (Table 2). The diagnosis of Finnish NRL-allergic patients was based on a clinical history, positive SPT (Stallergenes, Antony Cedex, France, and/ or high-allergen glove extracts), and a positive IgE antibody reactivity (latex- ImmunoCAP, Pharmacia Diagnostics, Uppsala, Sweden). Spanish and American patients and control subjects have been described in detail elsewhere (Bernstein et al. 2003a, Blanco et al. 2004). This study was approved by local ethics committees in Finland, Spain and USA.

7.3.2 Skin prick tests and IgE ELISA (III, IV)

SPT (III)

Purified NRL extract (500, 50, 5 and 0.5 µg/ml in PBS), Hev b 6.01 (50, 5 and 0.5 µg/ml in PBS) and Hev b 5 (50, 5 and 0.5 µg/ml) was used for SPT. Histamine (10 mg/ml; ALK, Copenhagen, Denmark) served as a positive control, PBS as a negative control. The wheal area was measured after 15 minutes and calculated by a mathematical formula: $\pi \times r^2$, where r = radius of wheal. A greater wheal area than 9 mm² was considered as positive.

Allergen-specific IgE (III, IV)

IgE antibodies to NRL allergens were measured by ELISA. Briefly, the coating concentration for Hev b 6.01 and Hev b 5 was 2 µg/ml, for NRL 20 µg/ml in 50 mM sodium carbonate buffer, pH 9.6, and for Hev b 2 and Hev b 13, 1 µg/ml in PBS, pH 7.4. The wells were then blocked with 1% human serum albumin (Red Cross Finland Blood Service, Helsinki, Finland), and diluted (1:10, in duplicate) patient or control sera were added. Biotinylated goat anti-human IgE (Vector Laboratories, Inc, Burlingame, CA, USA), streptavidin-conjugated alkaline phosphatase (Bio-Rad,

Hercules, CA, USA) and color substrate (p-nitrophenyl phosphate, ICN Biomedicals Inc., Aurora, Ohio, USA) were used to detect bound IgE. The absorbance values were read at 405 nm, when pooled control sera had reached predetermined OD-values. Positive reactivity was defined as absorbance values exceeding the 98th percentile of local atopic controls (approximately the mean + 3 SD) (IV).

Inhibition ELISA tests (IV)

The assay was performed using the allergen-specific ELISA protocol described above, except that a competitive inhibition step was added (Mikkola et al. 1998). Inhibition was performed by incubating sera (final dilution 1/10) for 1 hour at room temperature with an equal volume of inhibitor solution. Different NRL allergens were used to inhibit the binding of patients' IgE to solid-phase antigens (IV).

7.3.3 PBMC proliferation assay (III)

PBMC were cultured in complete RPMI 1640 containing 5% heat inactivated human AB serum as earlier described (Lehto et al. 2003). Briefly, Ficoll gradient centrifugation of peripheral blood was used to obtain PBMC. Isolated cells were washed twice with PBS. PBMC were stimulated alone or together with allergens (NRL 100, 50, 10, 5, 1 µg/ml, Hev b 6.01 10, 5, 1 and 0.1 µg/ml, Hev b 5 10, 5, 1 and 0.1 µg/ml). Phytohaemagglutinin was used as a positive control (PHA, 45 µg/ml; Murex Biotech Ltd., Dartford, UK). Proliferation was determined by tritiated methyl-thymidine (Amersham Biosciences, Little Chalfont, UK) incorporation after 6 days of culturing. The proliferation results were estimated as stimulation indexes (uptake of isotope in stimulated culture/uptake of isotope in non-stimulated control culture).

7.3.4 Cytokine and chemokine assays (III)

Cell culture

PBMC were cultured in complete RPMI 1640 on 24-well plates (Costar, Corning Incorporated, Corning, NY, USA) with or without Hev b 6.01 (10 µg/ml) or Hev b 5 (10 µg/ml). PHA (45 µg/ml) and *Staphylococcus aureus* enterotoxin B (SEB; 1 µg/ml; Sigma-Aldrich Co Ltd., Gillingham, UK) were utilized as polyclonal stimulators of PBMC. The cell culture time was 6 hours for flow cytometry analysis and isolation of RNA, whereas cell culture supernatants were collected after 24 hours.

Cytokine and chemokine mRNA measurements by PCR

Total RNA was isolated from PBMC according to Trizol instructions (Gibco, BRL). Subsequently, synthesis of cDNA and real-time quantitative PCR with AbiPrism 7700 Sequence Detector System (Applied Biosystems) were performed as earlier described (I, II). PCR primers and probes were obtained from Applied Biosystems, where they were also designed. Cytokine and chemokine mRNA results have been expressed as relative units (RU), which were calculated by the comparative C_T method as earlier described (I).

Cytokine and chemokine protein measurements by ELISA

Commercial ELISA kits were used to measure protein levels of TNF, IL-10 and CCL3. The sensitivities of TNF and IL-10 kits were 4 pg/ml and 2 pg/ml, respectively (eBioscience, Inc.; San Diego, CA, USA). The lower detection limit of the CCL3 kit (R&D Systems, Minneapolis, MN, USA) was 8 pg/ml.

7.3.5 Flow cytometry (III)

Measurements of T-cell surface markers were made by FACSCalibur (BD Biosciences, San Jose, CA, USA) using FACSComp software version 4.01 (BD Biosciences). Monoclonal antibodies and corresponding isotype controls were obtained from BD Biosciences. In brief, PBMC were incubated with the fluorochrome-conjugated monoclonal antibodies, washed, and fixed in 1% paraformaldehyde (BD Biosciences). A total of 20 000 events within the lymphocyte gate were collected. The numbers of CCR4 and CXCR3 positive cells within the CD3+, CD3+CD8+ and CD3+CD8- populations were analyzed by BD CELLQuestPro software (BD Biosciences).

8 RESULTS

8.1 Experiments in mice

8.1.1 NRL exposure to the skin: cutaneous and systemic responses (I)

Repeated EC exposure to NRL induced strong epidermal and dermal thickening in sensitized skin sites in mice (I, Figs 1 and 2). Infiltration of inflammatory cells, especially eosinophils, increased significantly into the NRL-sensitized skin sites, when compared to PBS-treated sites (I, Fig. 3). A significant increase in the number of degranulated mast cells was also seen in NRL-sensitized skin sites, although the total number of mast increased insignificantly (I, Figs 4 and 5). In addition, a significant 5-fold increase in the numbers of CD4 positive cells and a 7-fold increase in CD3 positive cells were observed after EC NRL exposure (I, Fig. 6).

Epicutaneous exposure to NRL caused enhanced expression of proinflammatory and Th2 type cytokines. After NRL sensitization, mRNA expression of IL-1 β and IL-4 increased significantly in skin, whereas the enhancement of IFN- γ mRNA expression was insignificant as compared to PBS-treated skin (I, Fig. 7). In agreement with the evidence of inflammatory cell recruitment into NRL-treated skin, mRNA expression of several chemokines increased significantly in NRL exposed skin sites. Expression of CCL3, CCL4, CCL2, and CCL11 mRNA was induced significantly after epicutaneous sensitization compared to PBS-treated skin (I, Fig. 8). On the other hand, mRNA expression of CCL17 and CCL24 was moderate and comparable in both mice groups, whereas expression of CCL27 mRNA was strong, but there were no differences between the animal groups.

In contrast to intraperitoneal exposure, epicutaneous exposure to NRL induced high amounts of total and specific IgE to NRL and Hev b 6.01 (I, Figs 9 and 10). Both EC sensitization and IP immunization with NRL induced low but comparable levels of Hev b 1-specific IgE antibodies. On the other hand, intraperitoneal, but not epicutaneous, NRL-treatment caused enhancement of the NRL and Hev b 1-specific IgG2a antibody response.

8.1.2 NRL exposure routes: respiratory and systemic responses (II)

IC and IP exposure caused perivascular and peribronchial inflammation of lung tissues after airway NRL challenge, whereas after IN exposure, only a slight, mostly perivascular inflammation was detected (II, Figs 1A and S1). The number of eosinophils increased dramatically after IC treatment,

but not after IP or IN exposure in lung tissues, whereas significant amounts of eosinophils and lymphocytes were seen in BAL fluid samples of IC and IP NRL-exposed mice (II, Fig. 1B). In line with the histological results, eosinophils were virtually absent and only a few lymphocytes were present in the BAL of intranasally exposed mice.

Airway hyperreactivity (AHR) to inhaled MCh increased vigorously in response to IC and IP NRL exposure followed by airway challenge (II, Fig. 2A). In contrast, IN NRL exposure did not induce AHR and the control mice group exhibited a slightly elevated but statistically non-significant response to inhaled MCh. PAS-staining of lung sections showed that animals treated intracutaneously or intraperitoneally with NRL displayed a significant increase in mucus production, as seen in the high amount of goblet cells around the bronchiolar lumen (II, Figs 2B and S2). IN NRL exposure induced only a slight and insignificant enhancement of mucus production in the airways.

Expression of mRNA of several CC chemokines, which attract eosinophils (CCL3, CCL8, CCL11 and CCL24) or Th2 (CCL1 and CCL17) cells was measured. It was noted that IC, IP and IN exposure and airway challenge elevated significantly mRNA expression of CCL1, CCL8, CCL11, CCL17, and CCL24 relative to the PBS-treated control mice (II, Fig. 3). Expression of CCL3 mRNA significantly increased after IC and IP NRL exposure, but not after IN exposure. In general, expression of most chemokines was significantly higher after IC NRL exposure (CCL1, CCL3, CCL8, CCL17, and CCL24) or IP exposure (CCL1, CCL3, CCL8, CCL17, and CCL24) compared to IN treatment. Expression of chemokine receptors (CCR1, CCR3, CCR4, CCR8), which bind these CC ligands was also analyzed (Fig. 4). All exposure routes increased significantly mRNA expression of these chemokine receptors. Expression of CCR1 and CCR8 mRNAs was significantly higher after IC and IP sensitization compared to intranasally treated mice. A markedly higher level of CCR3 mRNA was seen in intracutaneously sensitized mice compared to the corresponding intranasally sensitized group.

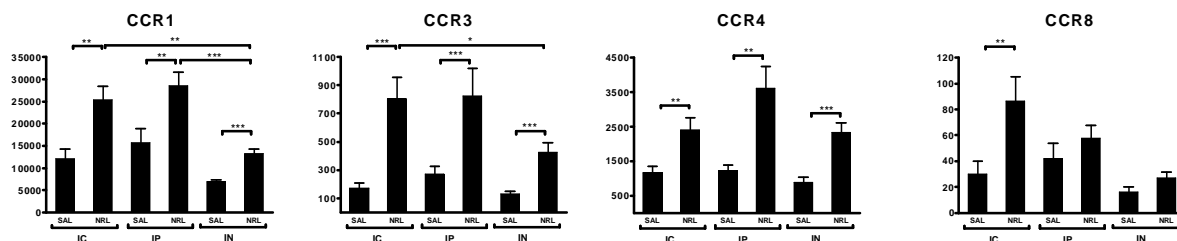


Figure 4. Chemokine receptor mRNA expression in lung samples after exposures to PBS (SAL) or NRL. RU (relative units) is expressed as relative differences to the calibrator. The columns and error bars represent means \pm SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 12-15$ mice per group (II, Fig. S3).

The cytokine expression profile at the mRNA level revealed differences between different exposure routes. IC and IP exposure to NRL clearly increased the expression levels of IL-4 and IL-13 mRNA, but only IC exposure increased significantly the expression of IL-5 mRNA in lung compared to PBS treated controls (II, Fig. 4A). In addition, the IL-13 mRNA level was elevated (8-fold) in the intranasally NRL exposed mice in comparison with the controls. IC exposure elicited significantly higher expression of IL-4, IL-5, and IL-13 mRNA compared to IN group. The mRNA levels of regulatory cytokine IL-10 enhanced significantly in all NRL exposed groups, especially in intranasally treated mice (II, Fig. 4B). On the other hand, a significant elevation in the levels of TGF- β 1 mRNA was observed after IN and IP NRL exposure but not after IC exposure. In addition, the TGF- β 1 mRNA levels were significantly higher in IN and IP exposed mice than in IC exposed mice (II, Fig. 4B). Forkhead box 3 (Foxp3) transcription factor mRNA expression also increased significantly after IP and IN NRL administration but not after IC exposure (II, Fig. 4B) with the elevation in expression being most marked after IN NRL exposure.

Total and Hev b 6.01-specific IgE antibodies were strongly elevated after IC and IP exposure, but not after IN exposure to NRL (II, Fig. 5).

8.2 Studies in patients allergic to natural rubber latex

8.2.1 PBMC responses to Hev b 5 and Hev b 6.01: proliferation, cytokines and chemokines (III)

Two highly purified native NRL allergens Hev b 5 and Hev b 6.01 were used to stimulate PBMC of NRL-allergic patients and controls. IgE reactivity of the study groups was studied by SPT and allergen-specific IgE ELISA. All 14 patients had positive (wheal area ≥ 9 mm²) SPT to NRL, 12 to Hev b 6.01 and 4 to Hev b 5. In addition, 2 patients showed borderline positive SPT responses (wheal area 7-9 mm²) to Hev b 5 (III, Fig. 1A). In ELISA, 13 patients demonstrated increased IgE to NRL, 12 to Hev b 6.01 and 3 to Hev b 5 (III, Fig. 1B). All control sera were negative against all NRL allergens (III, Fig. 1B)

Proliferative responses of PBMC to Hev b 6.01 were significantly higher in NRL-allergic patients than in controls, whereas stimulation indexes against NRL and Hev b 5 revealed no differences between the two groups (III, Fig. 1C). There was a strong proliferative response against PHA but no differences were seen between responses in patients and controls.

Hev b 6.01, and to a lesser extent Hev b 5, stimulated PBMC of the NRL-allergic patients and were found to express more TNF mRNA and protein on PBMC compared with the

non-allergic controls (III, Figs 2A, 2B). They also clearly induced the expression of IL-12p40 mRNA in NRL-allergic patients (III, Fig. 2C). In addition, expression of the Th2 cytokine IL-13 mRNA increased significantly after allergen-specific stimulation (III, Fig. 2E), whereas the expression of the Th1 cytokine, IFN- γ , did not change (III, Fig. 2D). Finally, PBMC from NRL-allergic patients expressed significantly lower amounts of the regulatory cytokine TGF- β 1 mRNA (III, Fig. 2F) after allergen stimulation compared with the controls, while the expressions of IL-10 mRNA and protein (III, Figs 2G and H) were upregulated. Polyclonal stimulators (PHA and SEB) induced the expression of several cytokines (i.e., TNF, IFN-gamma, IL-13, and IL-10) in NRL-allergic patients as well as in control subjects (III, Figs 2 A, B, D, E, G, H), but the only significant difference was that SEB down-regulated the expression of TGF- β 1 mRNA in the patients' PBMC (III, Fig. 2F).

Hev b 6.01 and Hev b 5 stimulation of PBMC caused significantly higher mRNA expression of the proinflammatory chemokines CCL3 (both mRNA and protein), CCL4 and CCL20 in NRL-allergic patients than in controls (III, Fig. 3). On the contrary, expression of the Th1-type chemokine CXCL10 mRNA was significantly induced only after polyclonal stimulation, and there were no differences in the expression levels between patients and the controls (III, Fig. 3E). Expression of the Th2-type chemokine CCL17 mRNA was enhanced to a lesser degree after allergen-specific stimulation compared to the situation after polyclonal stimulation (III, Fig. 3F). Levels of CCL17 mRNA were similar in the patient and control groups (III, Fig. 3F).

Surface expression of the chemokine receptors CCR4 and CXCR3 on CD3+CD8- and CD3+CD8+ T cells did not change after any stimulation (III, Figs 4A and B). However, baseline expression of CCR4 on CD3+CD8- T cells was significantly enhanced and that of CXCR3 on CD3+CD8+ T cells was significantly reduced in the patient group compared to the control group (III, Fig. 4).

8.2.2 Prevalence of IgE antibodies to Hev b 2 and Hev b 13 (IV)

One important aspect in the prevalence studies of allergen-specific IgE antibodies is the identity and purity of allergen preparations. Homogeneity of Hev b 2, Hev b 5, Hev b 6.01, and Hev b 13 was ensured by reversed-phase chromatography (IV, e.g. Fig. 1), and their identity was confirmed using monoclonal antibodies specific for Hev b 2, Hev b 5, Hev b 6.01, and Hev b 13. It is of interest, however, that low binding of monoclonal anti-Hev b 6.02 antibodies to purified Hev b 13 was seen indicating that minute amounts of Hev b 6.02 reactivity were still present even in the highly purified Hev b 13 (IV, Fig. 2).

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 USA (IV, 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 USA (11%). The prevalence of IgE to Hev b 13 ranged between 18 to 30% in all three countries, 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%), in contrast to 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 (IV, Fig. 3).

Inhibition ELISA tests established that 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 studied sera. There were three different modes of inhibition (IV, Fig. 5). Either soluble Hev b 6.01 could not inhibit anti-Hev b 13 IgE binding (IV, Fig. 5A) or inhibited binding partially (IV, Fig. 5B) or totally (IV, Fig. 5C). Inhibition experiments were carried out for 8 sera, which displayed moderate to strong IgE antibody response to Hev b 13. Two of these sera produced a 2-4% inhibition of the binding of IgE to solid-phase Hev b 13, when the concentration of Hev b 6.01 was 0.1 µg/ml (III, such as Fig. 5A). Two other serum samples produced 26-54% inhibition (IV, such as Fig. 5B), and with the remaining four sera the inhibition was between 69 to 95% after exposure to soluble Hev b 6.01(IV, like Fig. 5C).

9 DISCUSSION

The peak of the epidemic of NRL allergy in the health care sector seems to have passed in Western Countries but this allergy is still a problem among HCW and people using gloves or other NRL products (Ahmed et al. 2003, Reunala et al. 2004). Proteins eluting from NRL gloves cause in the skin type I immediate symptoms such as contact urticaria but hand eczema may also be one phenotypic manifestation of NRL allergy (Turjanmaa et al. 2002b). On the other hand, airborne glove powder contaminated with NRL allergens can cause asthma and allergic rhinitis in sensitized subjects (Vandenplas et al. 2002). In this thesis, the significance of different exposure routes in the development of NRL-allergy was examined by using mouse models. The findings highlight the importance of cutaneous exposure in inducing a strong allergen-specific IgE antibody response, development of Th 2-type dermatitis and marked airway sensitization predisposing to NRL-allergic asthma (I, II). Cellular immune mechanisms in NRL-allergic patients were studied by investigating PBMC responses to the major NRL allergens, Hev b 6.01 and Hev b 5. The results demonstrate that allergen-specific stimulation of PBMC induces upregulation of several inflammatory cytokines and chemokines. The prevalence of IgE antibodies was studied in NRL-allergic patients from Finland, Spain and USA using highly purified native NRL allergens (IV). In contrast to Hev b 6.01 and Hev b 5, the prevalence rates of IgE antibodies to Hev b 2 and Hev b 13 were in the range of 5 - to 30% showing that these two allergens cannot be considered as major NRL allergens.

9.1 Experiments in mouse models of natural rubber latex allergy

9.1.1 Cutaneous responses to NRL (I)

Repeated EC exposure to NRL led to dermatitis characterized by skin thickening and dermal infiltration of eosinophils and CD4⁺ T cells. Degranulated mast cells were also increased in NRL-sensitized skin, suggesting that these cells may also participate in the development of dermatitis. In support of this theory, it has been previously reported that mast cells crucially affect IFN-gamma expression in a mouse model (Alenius et al. 2002). EC exposure to NRL also induced significant expression of mRNA of proinflammatory (IL-1beta) and Th2 (IL-4) cytokines. A previous study using a mouse model of allergic dermatitis showed that eosinophils were decreased and T cells increased in the skin of ovalbumin-sensitized IL-4 deficient mice suggesting that this cytokine is important in the modulation of protein induced skin inflammation (Spergel et al. 1999).

In line with enhanced traffic of inflammatory cells to the sensitized skin, epicutaneous NRL exposure induced higher mRNA expression of CCL2, CCL3 and CCL4, which attract T cells,

monocytes, NK cells, and dendritic cells. Expression of CCL11, which is especially important in the recruitment of eosinophils, mast cells and Th2 cells (Luster and Rothenberg 1997, Rot and von Andrian 2004, Charo and Ransohoff 2006), was also enhanced in NRL sensitized skin. In addition, mRNA expression of CCL17 and CCL27 was found, although the expression levels were similar between control and NRL sensitized groups. CCL27 and CCL17 are important chemoattractants for CCR10+CLA+T cells and CCR4+Th2 cells, respectively, during skin inflammation (Reiss et al. 2001, Homey et al. 2002). Cutaneous expression of CCL17 and CCL27 can then attract NRL-specific CD4+Th2 cells from the circulation to the inflamed skin. In summary, our results show that EC exposure to NRL significantly enhances the expression of various chemokines in the skin contributing to the recruitment of different inflammatory cells, especially T cells, eosinophils and mast cells to the NRL-sensitized skin sites.

Disruption of the skin barrier is likely to be an important factor influencing the development of dermatitis in NRL allergy. Stratum corneum, the outer layer of the skin normally prevents foreign substances from penetrating into and through the skin (Cookson 2004, Elias 2005, Cork et al. 2006). In our mouse model, mechanical skin injury is induced by tape stripping mimicking the effects of skin scratching in humans. Scratching is a common consequence of pruritus, typical symptom of eczema patients and is thought to participate in the pathophysiology of AE (Homey et al. 2006). Moreover, a dysfunction of skin barrier function of stratum corneum strongly associates with AE (Cork et al. 2006, Morar et al. 2006). Especially two loss-of-function mutations of the epidermal barrier protein filaggrin predispose an individual to AE (Hudson 2006, Marenholz et al. 2006, Palmer et al. 2006, Weidinger et al. 2006).

Epicutaneous sensitization to NRL proteins described here presents a model for protein contact dermatitis. This model incorporates several features that are likely to be important in the pathogenesis of NRL allergy in humans. Induction of dermatitis manifested by epidermal and dermal thickening and characterized by the dermal accumulation of eosinophils and degranulated mast cells required cutaneous barrier disruption by tape stripping (injury) and repeated application of NRL proteins. NRL-sensitized skin exhibited an increase in the expression of Th2 cytokine mRNA. These results in mouse indicate that epicutaneous exposure to protein antigens induces dermatitis with a striking Th2 bias. To our knowledge, no biopsy studies have been performed from hand eczema (dermatitis) in NRL-allergic patients. However, NRL patch testing has evoked eczematous reactions (Sommer et al. 2002). This suggests that repeated exposure to NRL proteins similarly to the present mouse NRL model in humans could also cause delayed allergic responses in skin, such as hand eczema or protein contact dermatitis. This is indirectly further supported by the clinical findings in NRL-allergic patients in most of whom avoidance of high-allergenic NRL gloves has resulted in improvement of hand dermatitis (Turjanmaa et al. 2002b).

9.1.2 Airway responses to NRL; effects of different exposure routes (II)

Sensitization to NRL proteins may derive from the use of NRL gloves through different exposure routes, such as via skin and airways in HCW, and via internal mucous membranes during surgical procedures in frequently operated patients. However, little is known about the importance of different exposure routes in the elicitation of clinical symptoms. The second study in mouse NRL model was performed to examine the significance of intracutaneous (IC), intraperitoneal (IP) and intranasal (IN) exposure routes in the development of airway hyperreactivity and lung inflammation.

In the present study in mice (II), IC and IN exposures were performed with the same amount (160 µg) of NRL without adjuvants. When the airways were challenged with NRL in IC exposed mice, a more intense lung eosinophilia was found than in IN and IP exposed mice. IC and IP exposures, but not IN exposure, induced significant mucus production and AHR to inhaled MCh. In agreement with these findings, a previous study revealed that topical NRL exposure to tape stripped skin increased also AHR to inhaled MCh, whereas intratracheal exposure of NRL had no influence on the airway response (Howell et al. 2002, Howell et al. 2004). These results in mice clearly indicate that repeated cutaneous NRL exposure can efficiently sensitize the lungs and therefore, predispose this tissue to NRL-allergic asthma.

Th2 cytokines IL-4 and IL-13 are most important regulators of IgE class switching (Geha et al. 2003) and IL-13 also participates in AHR and mucus production (Kuperman et al. 2002). In the present experiments, all three (IC, IP and IN) NRL exposure routes were able to upregulate IL-4 and IL-13 mRNA in the lungs of the mice. The increase was, however, highest after IC exposure and lowest after IN exposure in mice. It is known that IL-5 is especially involved in the recruitment and survival of eosinophils (Hamelmann and Gelfand 2001). In the present study, IL-5 mRNA expression was increased significantly after IC but not after IP or IN exposure. One effect of IL-5 could then be the influx of eosinophils into the lungs; a finding which was especially marked in IC exposed mice. In line with the present findings, a previous study documented that IP sensitization with NRL glove extract or Hev b 5 can induce IL-5 protein into the BAL fluid (Hardy et al. 2003). It is of interest that mRNA expression of eosinophil attracting chemokines (CCL3, CCL8, CCL11, CCL24) and their receptors (CCR1, CCR3) was significantly higher in the lungs of IC exposed mice compared to the lungs of IN exposed mice. CCL1 and CCL17 chemokines are known to attract Th2 cells (Panina-Bordignon et al. 2001). Expression of these chemokine mRNAs was also higher after IC and IP exposure compared to IN exposure in mice, and the same was true for Th2 associated chemokine receptor CCR8. In summary, these findings indicate that repeated

cutaneous NRL allergen exposure sensitizes effectively also the lungs. Thereafter, NRL airway challenge is able to upregulate different inflammatory chemokines and chemokine receptors. The next step seems to be the influx of eosinophils and Th2 lymphocytes into the lung tissue, the cells which are important in allergic airway inflammation.

Airway inflammation, mucus production and AHR were substantially lower in IN exposed mice compared to intracutaneously exposed mice. Therefore we measured the mRNA levels of regulatory cytokines IL-10 and TGF- β 1 (Terui et al. 2001, Akbari et al. 2003, Nagler-Anderson et al. 2004). Interestingly, expression of IL-10 was higher after IN exposure than after IC and IP exposure in mice. In addition, both TGF- β 1 and Foxp3, which is a marker for CD4+CD25+ T regulatory cells (Fontenot et al. 2003, Khattri et al. 2003), were significantly upregulated after IN exposure. These findings in mice suggest that an allergen exposure to the airways, but not to the skin, could activate also regulatory mechanisms for the inflammation and in this process IL-10 and TGF- β 1 as well as Foxp3+ regulatory T-cells could be the key players. This suggestion is in line with the concept that the mucosal immune system is programmed to induce tolerance to ingested or inhaled allergens (Macaubas et al. 2003, Mayer and Shao 2004, Holmgren and Czerkinsky 2005, Lefrancois and Puddington 2006). A recent NRL mouse study provided further evidence for the fact that tolerance can take place when allergen exposure initially occurs in the nasal mucosa (Hufnagl et al. 2003). These investigators observed that IN exposure, when performed before IP exposure, prevented antibody responses to Hev b 1 and Hev b 3. A similar induction of tolerance has also been observed in mice after IN administration of the birch pollen allergen Bet v 1 (Winkler et al. 2002, Winkler et al. 2006). The IN administration suppressed both the allergic immune responses and airway inflammation and the induction of tolerance seemed to be due to Foxp3+ CD4+ T cells (Winkler et al. 2006).

9.1.3 IgE and IgG2a antibody responses (I, II)

In addition to examining cutaneous and lung responses in mouse NRL models, specific IgE and IgG2a antibodies were also measured in the peripheral blood. Epicutaneous NRL exposure was found to induce IgE antibodies to Hev b 6.01, but not to Hev b 1(I). In comparison, IP exposure enhanced IgG2a antibodies to Hev b 1, but not to Hev b 6.01. In line with these observations, a previous study (Woolhiser et al. 2000) showed that topical NRL exposure induced IgE antibodies to Hev b 6 and subcutaneous NRL injection to Hev b 1 and Hev b 3. In the present study, IP exposure caused a marked IgG2a response to Hev b 1, but no IgE to Hev b 1 was found. The amount of Hev b 6 in the NRL extracts is about 75-fold higher than that of Hev b 1 (Yeang et al. 2006). This concentration difference could be one reason for the different IgE responses to Hev b 6.01 and Hev

b 1 in the present study. However, different antibody responses could be explained even better by biochemical properties of the NRL allergens. Hev b 6.01 is hydrophilic and can therefore easily penetrate into the skin. In contrast, Hev b 1 is hydrophobic and seems to be capable of causing marked IgE response only when injected into the skin or intraperitoneally. In agreement with these findings in the mouse model, Hev b 6.01 is known to be a major sensitizing allergen for glove-using HCW, whereas it seems that Hev b 1 needs to be delivered from NRL gloves or catheters to the peritoneal or other mucous membranes before it can cause a significant IgE response. Apparently this is the reason, why Hev b 1 is a significant allergen for spina bifida and other multi-operated patients (Alenius et al. 1996a, Alenius et al. 1996b, Chen et al. 1997a).

In the second study (II), IP exposure induced specific IgE antibodies to Hev b 6.01 in contrast to the first study (I). One possible explanation for this discrepancy could be different IP exposure protocols. In the first study (I), a booster injection was given after one week and serum was obtained on day 19. In the second study (II), a booster injection was provided after two weeks and the blood was collected after airway challenge on day 31. Therefore, in the first study, the IP immunisation time may have been too short to elicit proper IgE responses. Another difference was the amount of NRL antigen used during the IP exposures. The total amount was 40 µg in the first and 160 µg in the second study. In line with the results in the second study (II), NRL specific IgE antibodies have also been found after IP exposure in two other studies (Hardy et al. 2003, Hufnagl et al. 2003).

9.2 Studies in patients allergic to natural rubber latex

Earlier studies on cell-mediated immune responses with NRL-allergic patients have focused on the measurement of PBMC proliferation responses against different NRL allergens using crude NRL extracts or purified native proteins or recombinant NRL allergens. One goal of these studies has been to resolve, which allergens are relevant for clinical and diagnostic purposes. One aim of this thesis was to obtain information about allergen-specific cytokine and chemokine responses in NRL-allergic patients by stimulating their peripheral blood mononuclear cells (PBMC) with highly purified native major allergens Hev b 5 and Hev b 6.01. An additional aim was to assess IgE class antibody prevalence to two recently characterized NRL allergens, Hev b 2 and Hev b 13, in three different geographical areas to resolve whether these proteins truly meet the criteria for major allergens.

9.2.1 PBMC responses to Hev b 5 and Hev b 6.01 (III)

In NRL-allergic patients, diagnosed by positive SPT and glove challenge test, Hev b 6.01, but not Hev b 5, induced significantly higher PBMC proliferation when compared to the control subjects. This finding suggests that the patients had been mainly sensitized to Hev b 6.01. This result is in agreement with the results of the fourth study of the present thesis (IV) which revealed that Finnish NRL-allergic patients had a markedly higher IgE antibody prevalence to Hev b 6.01 than to Hev b 5. A previous study indicated that the NRL proliferation test had a low sensitivity (39%) but high specificity (95%) in NRL allergy (Ebo et al. 1997). It is also well documented that cell-mediated responses do not correlate with SPT reactivity or specific IgE antibody levels (Raulf-Heimsoth et al. 1996, Ebo et al. 1997, Johnson et al. 1999).

Manifestations of allergic symptoms are greatly dependent on the cytokine and chemokine milieu, where allergen-specific cells meet their corresponding allergens (Borish and Rosenwasser 2003, Borish and Steinke 2003, Homey et al. 2006). The present study revealed that the major NRL allergens Hev b 6.01 and Hev b 5 induced expression of proinflammatory cytokines (TNF and IL-12p40) and Th2-type cytokine IL-13. Interestingly, NRL allergen stimulation did not induce IFN- γ mRNA, although IL-12 was upregulated and this cytokine is known to be a major inducer of IFN- γ (Hunter 2005). Most of the present NRL-allergic patients were atopic subjects, and it is of interest that after IL-12 stimulation, IFN- γ production has been shown to be low especially in atopic patients (Matsui et al. 2000). Moreover, Th2 cell differentiation can occur even though in the presence of IL-12 signalling (Nishikomori et al. 2000). Thus, IL-12 activation is clearly required for Th1 immune responses, but it is not sufficient to suppress Th2 responses (Heath et al. 2000, Nishikomori et al. 2000, Berenson et al. 2004). However, IL-12 expression was measured at the mRNA level using primers and probes specific for the IL-12p40 subunit. Human IL-12p40 subunit is a part of heterodimeric proinflammatory cytokines IL-12 and IL-23, both of which induce Th1-type immune responses (Hunter 2005). Further studies on protein expression level are needed to clarify which one of these related cytokines or perhaps both of them are important in the outcome of NRL allergy. Overall, the present results imply that, in addition to Th2 cytokines (IL-13), also proinflammatory cytokines (IL-12/IL-23 and TNF) are involved in the induction of NRL-specific immune responses.

IL-10 and TGF- β 1 are important suppressors of immune responses and TGF- β signaling by T cells is essential for tolerance and T cell homeostasis (Jutel et al. 2003, Hawrylowicz 2005, Hawrylowicz and O'Garra 2005, Horwitz 2006, Li et al. 2006a, Li et al. 2006b, Marie et al. 2006). The present finding of decreased expression of TGF- β 1 mRNA both after allergen and SEB stimulations focuses attention onto the role of this regulatory cytokine in NRL allergy. On the other

hand, IL-10 mRNA expression increased significantly in PBMC after allergen stimulation. This suggests that IL-10 may downregulate Th1 responses and as a result, the Th2 response is more prominent in NRL-allergic patients. In agreement, a recent study with IL-10 deficient mice in a mouse model of allergic dermatitis revealed that IL-10 was crucial for the development of cutaneous Th2 response and eosinophilia (Laouini et al. 2003).

Allergen stimulation markedly increased the expression of the proinflammatory chemokines CCL3, CCL4 and CCL20 mRNA in the NRL-allergic patients. CCL3 seems to be important for optimal mast cell degranulation (Miyazaki et al. 2005) and CCL3 and CCL4 co-stimulate T cell responses (Molon et al. 2005, Trautmann 2005). It is of interest that allergen-specific stimulation did not induce the Th2-type chemokine CCL7 or the Th1-type chemokine CXCL10. These results indicate that proinflammatory chemokines, rather than Th1 or Th2 chemokines, seem to play an important role in the immune responses in NRL allergy.

Flow cytometric analysis of CCR4 (Th2 type) and CXCR3 (Th1 type) chemokine receptors on the T cells did not exhibit changes after stimulation with NRL-allergens. However, there were differences in the expression pattern of these chemokine receptors in the T cell subpopulations. NRL-allergic patients showed increased CCR4 expression on CD3+CD4+ T cells, whereas CXCR3 expression was decreased on CD3+CD8+ T cells. In agreement with these findings in NRL-allergic patients, previous studies in AE have been shown an increase of CCR4 expression on peripheral blood CD4+ T cells (Wakugawa et al. 2001) and a decrease of CXCR3 in PBMC (Hatano et al. 2001).

9.2.2 Prevalence of IgE antibodies to Hev b 2 and Hev b 13 (IV)

In the present study, the prevalence rates of IgE antibodies to Hev b 6.01 were high (54-74%) in Finland, Spain and the USA. This result from these three countries confirms that Hev b 6.01 is the most important NRL allergen, especially in HCW a fact that was also documented in a recent meta-analysis (Bousquet et al. 2006). Hev b 5 is the second major NRL allergen. The prevalence of IgE to Hev b 5 was found to be high in the USA (71%) and Spain (49%) but rather low (28%) in Finland. The reasons for these differences in the patients series may also be due to the variable amounts of Hev b 5 in the NRL gloves used in different countries (Palosuo et al. 2002).

The prevalence rates of IgE antibodies to both Hev b 2 (5-15%) and Hev b 13 (18-30%) were low, showing that these NRL proteins should not be classified as major NRL allergens. These prevalence rates which are obtained by using extensively purified allergens are markedly lower than those previously reported (Kurup et al. 2000, Bernstein et al. 2003a, Yeang 2004, Kurup et al. 2005). It is noteworthy that in all of the studies cited above, the native Hev b 2 and Hev b 13

allergen preparations used were obtained from one single laboratory. In the present study, it was shown that binding of IgE to insufficiently purified Hev b 13 could be partially or totally inhibited in most sera studied by the addition of soluble Hev b 6.01. This suggests that there is some impurity expressing or resembling Hev b 6.01 or Hev b 6.02 epitopes in the conventionally purified Hev b 13 allergen and the high prevalence rates obtained earlier may be explained by contamination by Hev b 6-like components. Since no sequence homology between Hev b 13 and Hev b 6.02 has been found significant cross-reactivity between these proteins is unlikely. Moreover, if cross-reactivity could explain this phenomenon, it should remain at the same or even higher level when the purity of Hev b 13 increases. Consistent with the present results 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 analyzed by immunoblotting after two-dimensional gel electrophoresis (Lee et al. 2006).

10 SUMMARY AND CONCLUSIONS

Allergy to NRL has been an important health issue during the last 20 years. In particular, HCW have been sensitized to NRL proteins diffusing from the protective gloves. NRL causes type I allergy symptoms such as contact urticaria but it is not known whether delayed symptoms such as hand eczema could be one phenotypic manifestation of NRL allergy. Airborne NRL allergens could theoretically cause asthma but little is known about cutaneous and airway route sensitization and inflammatory responses to NRL in the skin and lung. At present, IgE mediated responses are well characterized in NRL allergy but allergen-specific cell-mediated immune responses are poorly understood. Considerable progress has been made in the characterization of NRL allergens and there is agreement that the major allergens present in the gloves are hevein (Hev b 6.02) and Hev b 5. Recently, it has been claimed that Hev b 2 and Hev b 13 could also be major NRL allergens.

Cutaneous inflammatory responses to NRL were explored in a mouse model of NRL allergy (I). Mouse skin exposed to NRL developed a Th2 type dermatitis consisting of CD4+ T cells, eosinophils and degranulated mast cells. This inflammatory response was associated with increased expression of IL-1 β , IL-4 and inflammatory chemokines. Cutaneous NRL exposure induced also significant humoral Hev b 6.01- specific IgE responses. On the basis of these findings, it can be concluded that in addition to producing significant allergen-specific IgE responses, cutaneous NRL exposure can also induce Th2-type dermatitis. Whether the common hand eczema in NRL-allergic patients would have a similar pathophysiology and could be termed as protein contact dermatitis needs to be assessed in human studies. Mouse models have been invaluable in studies on pathophysiology of different diseases. Nonetheless, the many differences existing between mice and human immunological responses necessitate that any results obtained in mouse models need to be confirmed in human studies.

A NRL mouse model was also used to examine the impact of cutaneous NRL exposure in the development of airway hypersensitivity (II). NRL airway challenge caused significant AHR, lung mucus production and influx of mononuclear cells and eosinophils into the lungs in intracutaneously but not in intranasally exposed mice. The expressions of mRNA of Th2 cytokines (IL-4 and IL-13), and several CC chemokines and chemokine receptors were similarly induced in the lungs. Intranasally exposed mice exhibited an increase in the expression of the down-regulatory TGF- β 1 and Foxp3 mRNA in the lungs but negligible levels of IgE to Hev b 6.01 in the blood. These results suggest that repeated cutaneous NRL exposure predisposes mice to NRL-allergic asthma.

In human studies, PBMC from NRL-allergic patients underwent significant proliferation responses to Hev b 6.01 but not to Hev b 5 (III). Both allergens induced significant expression of mRNA of proinflammatory (TNF, IL-12p40) and Th2 (IL-13) cytokines. Regulatory cytokine expression was complex; levels of IL-10 were increased but TGF- β 1 decreased as compared to control subjects. NRL allergens also induced significant expression of mRNA of inflammatory CC-chemokines. It is important that major NRL allergens Hev b 6.01 and Hev b 5 were shown to induce proinflammatory cytokines and chemokines and that regulatory cytokines also seemed to be involved in the NRL-specific immune responses. These findings increase our knowledge of the immune mechanisms involved in NRL allergy and also highlight the possible role of regulatory cytokines in the maintenance of this allergy. Whether tolerance to NRL allergens can be achieved in NRL-allergic subjects by natural mechanisms or by immune therapy remains an open question that needs to be addressed in future studies.

To reassess the importance and role of two NRL allergens, Hev b 2 and Hev b 13, which were recently proposed to be major NRL allergens, a large IgE prevalence study was undertaken in NRL-allergic patients from Europe and North America. In this study, extensively purified native Hev b 2 and Hev b 13 allergen preparations were used. The prevalence was shown to be low to both allergens (from 5% to 30%) in contrast to Hev b 6.01 and Hev b 5 (from 28% to 74%). These results indicate that Hev b 6.01 and Hev b 5, but not Hev b 2 and Hev b 13, are major NRL allergens both in Europe and in the USA. This study highlights the importance of using extensively purified native NRL allergens when characterizing IgE responses in patient populations. Thus caution is necessary in interpretation of results where native NRL allergens have been used for diagnostic purposes. Whether recombinant Hev b 2 and Hev b 13 allergens, if in the future they were produced as immunologically reactive molecules, would exhibit better performance than the native compounds in prevalence assessment and diagnosis of NRL allergy needs to be elucidated in further studies.

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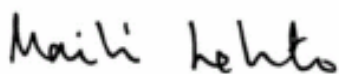
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A handwritten signature in black ink that reads "Maili Lehto". The script is cursive and somewhat informal.

Maili Lehto

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Epicutaneous Natural Rubber Latex Sensitization Induces T Helper 2-Type Dermatitis and Strong Prohevein-Specific IgE Response

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In addition to immediate type I allergy symptoms, natural rubber latex allergy may manifest as protein contact dermatitis on the hands of health-care workers and other natural rubber latex glove users. We examined whether repeated application of natural rubber latex on mouse skin causes sensitization to natural rubber latex and dermatitis. Epicutaneous sensitization with natural rubber latex produced a significant influx of mononuclear cells, CD4⁺ CD3⁺ cells, and eosinophils to the sensitized skin sites. The number of degranulated mast cells in natural rubber latex-sensitized skin sites was significantly higher compared with control sites treated with phosphate-buffered saline. The expression of interleukin-1 β and interleukin-4 mRNA was markedly increased in natural rubber latex-sensitized skin sites. Moreover, significant increases in the mRNA expression of chemokines CCL2 (monocyte chemoattractant protein-1), CCL11 (eotaxin-1), CCL3 (macrophage inflammatory protein-1 α), and CCL4 (macrophage inflammatory protein-1 β) were found. In addition to the cutaneous inflammatory response, epicutaneous sensitization with natural rubber latex induced a striking increase in the total and specific immunoglobulin E levels but not in the immunoglobulin G2a levels.

Intraperitoneal immunization with natural rubber latex induced a strong natural rubber latex-specific immunoglobulin G2a response, but only a weak immunoglobulin E response. We also studied the role of two major natural rubber latex allergens, the highly hydrophilic prohevein and the hydrophobic rubber elongation factor. Cutaneous application of natural rubber latex elicited a strong immunoglobulin E response against prohevein, but not against rubber elongation factor. On the contrary, intraperitoneal immunization with natural rubber latex elicited strong immunoglobulin G2a production to rubber elongation factor but not to prohevein. These results demonstrate that epicutaneous sensitization with natural rubber latex induces T helper 2-dominated dermal inflammation and strong immunoglobulin E response in this murine model of natural rubber latex induced protein contact dermatitis. Epicutaneous sensitization to natural rubber latex proteins eluting from latex gloves may therefore contribute to the development of hand dermatitis and also natural rubber latex-specific immunoglobulin E antibodies. *Key words: chemokines/cytokines/immunohistochemistry/natural rubber latex allergy/protein contact dermatitis. J Invest Dermatol 120:633–640, 2003*

Immediate hypersensitivity to natural rubber latex (NRL) has become increasingly common during the last 20 y and is currently one of the most frequently encountered occupational diseases among health-care workers (HCW) and others using protective gloves (Liss and Sussman, 1999). Several groups at high risk for developing NRL allergy have been defined, including HCW and children with spina bifida or other congenital malformations with histories of multiple

surgeries (Alenius *et al*, 2002b). In addition, hand dermatitis has been proposed to be a risk factor for latex allergy (Nettis *et al*, 2002). The wide spectrum of clinical manifestations of NRL allergy range from contact urticaria and rhinitis to severe systemic reactions, e.g., asthma and anaphylaxis (Alenius *et al*, 2002b).

Previous studies have demonstrated that in addition to type I immediate symptoms such as contact urticaria, at least 50% of NRL-allergic HCW suffer from hand dermatitis (Taylor and Praditsuwan, 1996; Alenius *et al*, 2002b). A recent follow-up study showed that avoidance of NRL gloves containing high levels of allergenic proteins significantly reduced the occurrence of hand dermatitis in HCW (Turjanmaa *et al*, 2002). These results suggest that NRL allergy may also manifest as protein contact dermatitis on the hands of HCW and other NRL glove users. Protein contact dermatitis has been described previously in food handlers, bakers, farmers, and other occupations where animal or plant proteins have repeated contact with the skin (Nethercott and

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Abbreviations: HCW, health-care workers; Hev b1, rubber elongation factor (REF); Hev b6.01, prohevein; HPF, high power field; NRL, natural rubber latex.

Holness, 1989; Susitaival *et al*, 1995); however, the pathomechanism of protein contact dermatitis is still poorly understood (Tosti *et al*, 1990; Janssens *et al*, 1995).

In this study we used repeated applications of NRL extract on to murine skin to assess the role of cutaneous route sensitization to NRL proteins in the development of skin inflammation and antibody responses.

MATERIALS AND METHODS

Mice and sensitization BALB/CJBOM mice were obtained from M&B (Ry, Denmark) and maintained under pathogen-free conditions. All procedures performed on the mice were in accordance with the Animal Care and Use Committee of the National Public Health Institute.

Epicutaneous sensitization of 6–8 wk old female mice was performed as described previously (Spergel *et al*, 1998). Briefly, mice were anesthetized with Avertin (2,2,2 tribromoethanol; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and their backs were shaved with an electric razor. The shaved areas were tape-stripped four times by transparent IV dressing (Tegaderm, 3M Health Care, St Paul, MN) to remove loose hair and to introduce a standardized skin injury. A dose of 100 µg of antigen in 100 µl of phosphate-buffered saline (PBS) or placebo (100 µl of PBS) was placed on a patch of sterile gauze (1 × 1 cm), which was secured to the skin with Tegaderm. The patches were placed for a 1 wk period and then removed. Two weeks later, an identical patch was reapplied to the same skin site. Each mouse had a total of three 1 wk exposures to the patch, separated from each other by 2 wk intervals. Mice were killed and specimens were collected 1 d after the end of the series of three epicutaneous sensitizations. Blood was collected (by cardiac puncture) and used for serum antibody analysis. Skin biopsies from sensitized areas were used for histology, immunohistology, and RNA isolation.

Intraperitoneal (IP) sensitization of mice was carried out by IP injection of 20 µg NRL in 2.25 mg alum (Imject Alum, Pierce, Rockford, IL), or the animals received PBS with alum in a total volume of 100 µl on days 0 and 7. On day 19 the mice were killed and blood was collected for serum antibody analysis.

Antigens NRL used for epicutaneous sensitization and enzyme-linked immunosorbent assay (ELISA) analysis was prepared from fresh Malaysian nonammoniated latex, a gift from Ansell International, as earlier described (Alenius *et al*, 1995). Further purification by high-performance liquid chromatography was performed on a Fast Desalting Column HR 10/10 (Amersham Pharmacia Biotech, AB, Uppsala, Sweden). Protein fractions were pooled and concentrated in a vacuum centrifuge (SpeedVac, Savant Instruments, Inc., Farmingdale, NY).

Purified prohevin (Hev b6.01) and rubber elongation factor (REF or Hev b1) were used as solid phase NRL allergens in ELISA analysis. Hev b6.01 was purified from NRL using the chitin-binding method (Hanninen *et al*, 1999). Briefly, chitin-binding proteins in NRL were attached to chitin (Sigma, St Louis, MO) and detached from chitin with 0.25 M and 0.6 M acetic acid. Supernatants were pooled and filtered (0.22 µm). Hev b6.01-containing fractions were isolated with high-performance liquid chromatography using a ReSource 3 ml column (Amersham Pharmacia Biotech) (linear gradient of 0–60% acetonitrile in 30 min, flow rate 1 ml per min). Hev b6.01-containing fractions were pooled and concentrated in a vacuum centrifuge. Hev b1 was purified from NRL with electroelution as described previously (Alenius *et al*, 1996b).

Antibodies All antibodies were purchased from BD PharMingen (San Diego, CA). Rat and hamster monoclonal antibodies (MoAb) against mouse CD3 (145–2C11), CD4 (H129.19), and CD8 (53–6.7) were used for immunohistochemical analysis. The MoAb used in antigen-specific ELISA were biotin-conjugated rat anti-mouse IgE MoAb clone R35-92 and biotin-conjugated rat anti-mouse IgG2a (clone R19-15). For total IgE measurements, rat anti-mouse IgE MoAb clone R35-72 was used for coating the plates, and biotin-conjugated rat anti-mouse IgE MoAb clone R35-92 was used for detection.

ELISA The standard PharMingen protocol for sandwich ELISA was used to quantify the total amount of IgE in mice sera, and purified mouse IgE (clone C38-2) (BD PharMingen) was used as a standard (200, 100, 50, 25, 12.5, and 6.25 ng per ml). Ninety-six-well microtiter plates (Nunc, Roskilde, Denmark) were coated with rat anti-mouse IgE MoAb (clone R35-72) and bound IgE was detected with biotin-conjugated rat anti-mouse IgE (clone R35-92). Streptavidin-horseradish peroxidase was purchased from BD PharMingen and peroxidase substrate reagents from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

NRL-specific IgE and IgG2a antibodies were measured as described previously (Spergel *et al*, 1998). Plates were coated (50 µl per well) with purified antigen (Hev b6.01 or Hev b1, 2 µg per ml) or 20 µg per ml of NRL in 0.05 M NaHCO₃, pH 9.6 at 4°C overnight. The plates were washed with PBS-Tween 20 (0.05%), blocked with PBS-3% bovine serum albumin for 2 h at 20°C and washed again. One hundred microliters of serial dilutions of sera (1 : 10, 1 : 20, 1 : 40, 1 : 80 for specific IgE and 1 : 60, 1 : 180, 1 : 540, 1 : 1620 for IgG2a) in 1% bovine serum albumin/PBS were incubated overnight at 4°C. After washing with PBS-Tween 20, biotin-labeled anti-mouse isotype-specific antibody (2 µg per ml) was added for 2 h. After washing with PBS-Tween 20, streptavidin-horseradish peroxidase (BD PharMingen), diluted 1 : 4000 in 1% bovine serum albumin/PBS was incubated for 30 min at room temperature. The plates were then washed with PBS-Tween 20, peroxidase substrate was added, and absorption at 405 nm was read with an automated ELISA reader (Titertek Multiscan, Eflab, Turku, Finland).

Histologic analysis For histologic examination, skin biopsies were obtained from patched areas 24 h after the patch from the third sensitization was removed. Specimens were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4 µm sections were stained with hematoxylin and eosin for inflammatory cell counting or with o-toluidine blue for mast cell counting. Individual inflammatory cell types were counted blinded in 15–20 high-power fields (HPF) at × 1000 and expressed as cells per HPF, with mean and SEM calculated. The HPF were placed directly subepidermally in the histologic and immunohistologic analysis. Mast cell counting was made at × 400 magnification. The slides were analyzed with a Leitz Dialux 20 EB microscope (Wetzlar, Germany).

The thickness of the epidermis and dermis was measured by means of an ocular micrometer. One to three sections from each biopsy were evaluated; at least five evaluations were performed per slide. The measured values were adjusted for the magnification optics, and are expressed as the thickness in micrometers, with mean and SEM calculated.

Immunohistologic analysis Skin sections were embedded in Tissue-Tek oxalacetic compound (Sakura, AA Zoeterwoude, the Netherlands) on dry ice. Sections of 4 µm were prepared and stored at –80°C. Sections were stained by a streptavidin-biotin method. Briefly, the sections were fixed in cold acetone for 2 min, air-dried, and incubated with purified rat anti-mouse MoAb (anti-CD3, anti-CD4, anti-CD8) (BD PharMingen) followed by incubation with biotinylated rabbit anti-rat immunoglobulin (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was blocked by incubation with peroxidase-blocking solution (Dako, Carpinteria, CA). Specimens were then incubated with peroxidase-conjugated streptavidin (Dako) and stained with a solution of hydrogen peroxide (Dako) and 3-amino-9-ethylcarbazole (Dako) followed by 1 min incubation in hematoxylin (Dako). The tissue sections were mounted with Aquamount (BDH, Gurr, Poole, U.K.). The positively stained cells were counted in 10 HPF at × 1000 and expressed as cells per HPF, with mean and SEM calculated.

RNA isolation Skin biopsies were obtained 24 h after the third patch was removed and they were immediately frozen in dry ice. To extract the RNA, the samples were homogenized in Trizol (Gibco BRL, Paisley, U.K.) using a Ultra-Turrax T8 (IKA Labor Technik, Staufen, Germany). Further RNA extraction was performed according to the Trizol instructions. Contaminating genomic DNA was removed from isolated RNA with DNaseI (RNase-free) (Gibco BRL) treatment, after which RNA was extracted with phenol/chloroform (1 : 1) (Gibco BRL) and reprecipitated, washed, and redissolved according to the Trizol instructions. The quantity and purity of the RNA was determined by measuring the absorbance at A260 and A280 nm in a spectrophotometer [GeneQuant II, Pharmacia Biotech (Biochrom) Ltd, Cambridge, U.K.]. Isolated total RNA was stored at –70°C in nuclease-free water.

cDNA synthesis and polymerase chain reaction (PCR) amplification of reverse-transcribed cDNA cDNA was synthesized from 1 µg of total RNA in a 50 µl reaction mix using MultiScribe[™] Reverse transcriptase and random hexamers (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The reaction was performed for 60 min at 48°C, and the enzyme was inactivated at 95°C for 5 min in a thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). PCR primers and probes were purchased from PE Applied Biosystems as predeveloped reagents [18S ribosomal RNA, interleukin (IL)-1β, IL-4, interferon (IFN)-γ, CCL2, CCL3] or were designed [CCL4, CCL11, CCL24 (cotaxin-2), CCL17 (TARC), CCL27 (CTACK)]. **Table I** by PrimerExpress version v2.0 software (PE Applied Biosystems, Foster City, CA). Whenever possible, primer pairs were designed to span exon-exon borders. The real-time

Table I. Sequence of mouse primer pairs and fluorogenic probes for real-time quantitative PCR

		Sequence (5'→3')
CCL4	Forward	5'-TGC TCG TGG CTG CCT CT-3'
	Reverse	5'-CAG GAA GTG GGA GGG TCA GA-3'
	Probe	5'-TGC TCC AGG GTT CTC AGC ACC AAT G-3'
CCL11	Forward	5'-ATG CAC CCT GAA AGC CAT AGT C-3'
	Reverse	5'-CAG GTG CTT TGT GGC ATC CT-3'
	Probe	5'-AGC ACA GAT CTC TTT GCC CAA CCT GGT-3'
CCL24	Forward	5'-CGG CCT CCT TCT CCT GGT A-3'
	Reverse	5'-TGG CCA ACT GGT AGC TAA CCA-3'
	Probe	5'-CCC TCA TCT TGC TGC ACG TCC TTT ATT TC-3'
CCL27	Forward	5'-CAG CCT CCC GCT GTT ACT G-3'
	Reverse	5'-TGC TTG GGA GTG GCT GTC TA-3'
	Probe	5'-TCT GCC CTC CAG CAC TAG CTG CTG TAC-3'
CCL17	Forward	5'-CAG GAA GTT GGT GAG CTG GTA TAA G-3'
	Reverse	5'-TGG CCT TCT TCA CAT GTT TGT CT-3'
	Probe	5'-TGT CCA GGG CAA GCT CAT CTG TGC-3'

quantitative PCR was performed with AbiPrism 7700 Sequence Detector System (SDS) (PE Applied Biosystems). For cDNA amplification, a 10 min incubation at 95°C was done to activate AmpliTaqGold; this was followed by 40 cycles with 15 s at 95°C and 1 min at 60°C for each cycle. PCR amplification of the endogenous 18S rRNA was performed for each sample to control sample loading and to allow normalization between samples according to the manufacturer's instructions (PE Applied Biosystems). The results were expressed as relative units (fold differences): i.e., ratio of PCR amplification efficiency of sample cDNA to that of the calibrator (FAM C_T 40, VIC C_T 12.6 → ΔC_T 27.4, where C_T means a threshold cycle).

Relative units (RU) were calculated by the comparative C_T method. First, the C_T for the target amplicon (FAM) and the C_T for the endogenous control (VIC; 18S rRNA) were determined for each sample. Differences in the C_T for the target and the C_T for the internal control, called ΔC_T , were calculated to normalize for the differences in the amounts of total nucleic acid added to each reaction mixture. The ΔC_T of the calibrator was subtracted from the ΔC_T of each experimental sample and termed as $\Delta\Delta C_T$. The amount of target normalized to an endogenous control and relative to the calibrator, was then calculated by the equation $2^{-\Delta\Delta C_T}$.

Statistical analysis Nonparametric Mann–Whitney U test was used to compare the different mice groups. $p < 0.05$ was considered statistically significant.

RESULTS

Epicutaneous NRL sensitization induces dermatitis characterized by skin thickening, mast cell degranulation, and infiltration of inflammatory cells Repeated epicutaneous sensitization of mice with NRL induced strong epidermal and dermal thickening in sensitized skin sites (**Fig 1**). A significant 3.5-fold thickening of the epidermis and a 1.5-fold thickening of the dermis was found in NRL-sensitized skin sites compared with PBS-treated sites (**Fig 2A,B**). The total number of cells infiltrating to the NRL-sensitized skin sites showed a significant, 2-fold increase (**Fig 3A**); and the number of eosinophils a 4-fold increase (**Fig 3B**), when compared with PBS-treated sites. The number of intact mast cells was slightly increased in NRL-sensitized skin sites, but the difference was not statistically significant. A significant increase in the number of degranulated mast cells was, however, seen in NRL-sensitized skin sites (**Fig 4A,B**). The morphologic features of skin mast cells after epicutaneous sensitization with NRL or PBS are shown in **Fig 5**. Only a few CD8-positive cells in the NRL-sensitized skin sites, as well as in PBS-treated skin sites, were observed (data not shown); however, a significant 5-fold increase in CD4-positive cells and a 7-fold increase in CD3-positive cells was observed in NRL-sensitized skin sites

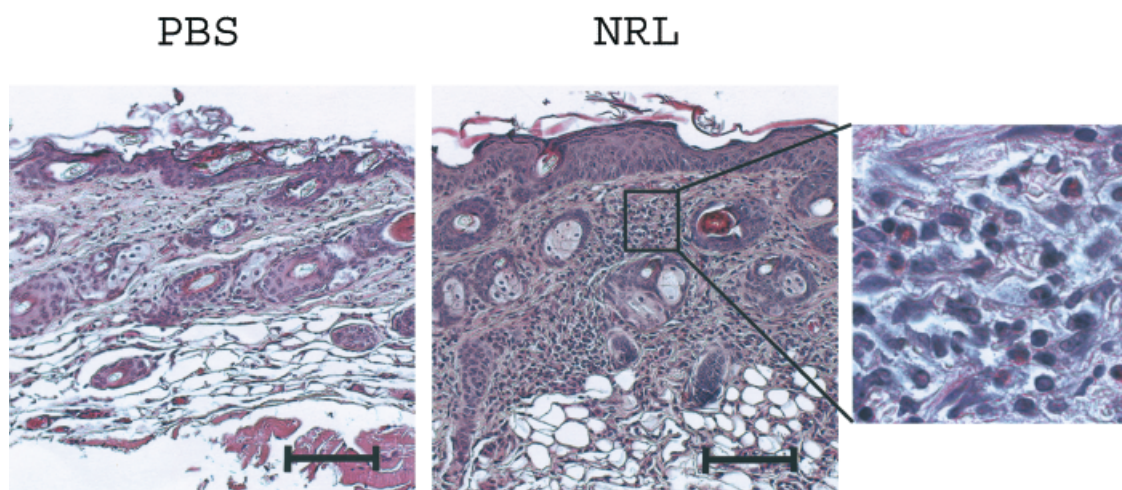


Figure 1. Histologic features of epicutaneous sensitized skin sites in BALB/C mice. Marked infiltration of inflammatory cells can be seen in NRL-sensitized skin sites compared with PBS-treated sites. Scale bar = 100 μ m.

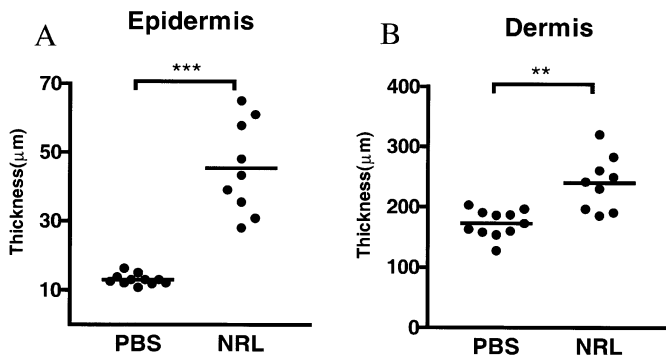


Figure 2. Thickness of epidermal and dermal layers of mice skin after epicutaneous sensitization with NRL and PBS. A significant 3.5-fold thickening of epidermis (A) and a 1.5-fold thickening of dermis (B) were observed in NRL-sensitized sites. ** $p < 0.01$, *** $p < 0.001$.

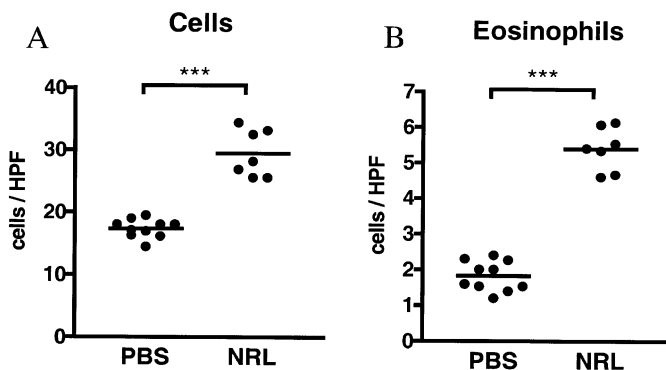


Figure 3. Infiltration of total inflammatory cells (A) and eosinophils (B) in skin after epicutaneous sensitization with NRL and treatment with PBS. The skin infiltrating cells were counted in 15–20 HPF at $\times 1000$ and expressed as cells per HPF. *** $p < 0.001$.

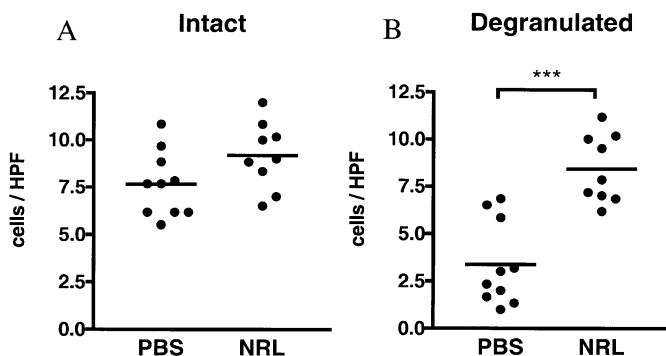


Figure 4. The number of mast cells in epicutaneous sensitized skin sites. (A) The number of intact mast cells and (B) degranulated mast cells in NRL-sensitized and PBS-treated skin sites. The skin infiltrating mast cells were counted in 15–20 HPF at $\times 400$ and expressed as cells per HPF. *** $p < 0.001$.

compared with PBS-treated skin sites (Fig 6A,B). The overall histologic features of the skin after epicutaneous sensitization with NRL or PBS are shown in Fig 1.

Expression of proinflammatory and T helper (Th)2 type cytokines is significantly induced in skin after epicutaneous NRL sensitization Proinflammatory cytokines are involved in

the development of skin inflammation, whereas Th2 type cytokines are important in the acute phase of atopic dermatitis (Leung, 2000). Following NRL sensitization, a drastic 23-fold increase in IL-1 β mRNA expression was found, whereas only low levels of IL-1 β mRNA were detected in the PBS-treated skin sites (Fig 7A). No detectable levels of IL-4 mRNA were found in the PBS-treated skin sites; however, a strong IL-4 mRNA expression was detected after epicutaneous sensitization with NRL (Fig 7B). Weak expression of IFN- γ mRNA was detected in the PBS-treated skin sites. Epicutaneous NRL sensitization induced only a slight and insignificant increase in IFN- γ mRNA levels in sensitized skin sites, as compared with PBS-treated sites (Fig 7C).

Chemokine mRNA expression is significantly increased in NRL-sensitized skin sites Chemokines are small cytokines that critically regulate recruitment of leukocytes to the site of inflammation (Yoshie *et al*, 2001). Epicutaneous NRL sensitization induced strong, 36–64-fold, expression of CCL3 and CCL4 mRNA in sensitized skin sites, respectively, whereas only low levels of CCL3 and CCL4 were detected in PBS-treated skin sites (Fig 8A,B). The expression of CCL2 mRNA was easily detectable in PBS-treated skin sites but the expression was significantly enhanced after epicutaneous sensitization with NRL (Fig 8C). The expression of CCL11 mRNA was significantly increased in NRL-sensitized skin sites, as compared with PBS-treated skin sites (Fig 8D). Moderate, but comparable, expression of CCL24 (mean for NRL group 462 RU, mean for PBS group 514 RU) and CCL17 (mean for NRL group 98 RU, mean for PBS group 100 RU) mRNA was found at NRL- and PBS-treated skin sites. CCL27 expression was considerably high in both study groups (mean for NRL group 55685 RU, mean for PBS group 60007 RU); however, no significant difference was found between the NRL-treated and PBS-treated skin sites.

Sensitization with NRL via an epicutaneous route induces high levels of total and specific IgE antibodies Most of the patients with NRL allergy have elevated levels of total and NRL-specific IgE antibodies (Alenius *et al*, 2002b). Epicutaneous sensitization with NRL induced a striking, 20-fold, increase in total IgE levels ($31,525 \pm 9414$ ng per ml; $n = 9$) compared with PBS-treated controls (1615 ± 322 ng per ml; $n = 6$) and a 10-fold increase when compared with mice that were immunized IP with NRL (3205 ± 1572 ng per ml; $n = 4$) (Fig 9A). NRL-specific IgE levels were significantly increased in mice after epicutaneous sensitization with NRL as compared with mice that were immunized IP with NRL (Fig 9B). On the contrary, NRL-specific IgG2a levels were significantly higher after IP sensitization than after epicutaneous sensitization with NRL (Fig 9C).

We also examined the antibody responses against two major NRL allergens, Hev b6.01 and Hev b1. Epicutaneous sensitization with NRL induced a significant increase in the levels of Hev b6.01-specific IgE antibodies. In contrast, no detectable levels of IgE to Hev b6.01 were found after IP sensitization with NRL (Fig 10A). Neither epicutaneous sensitization nor IP immunization with NRL induced Hev b6.01-specific IgG2a responses (Fig 10B). On the other hand, IP immunization with NRL induced moderate levels of Hev b1-specific IgG2a antibodies, whereas epicutaneous NRL sensitization did not elicit such antibodies (Fig 10D). Both epicutaneous sensitization and IP immunization with NRL induced low but comparable levels of Hev b1-specific IgE antibodies (Fig 10C).

DISCUSSION

Consensus exists that the principal risk groups for NRL allergy are patients with spina bifida and other subjects, who have undergone multiple surgeries, as well as atopic individuals and HCW

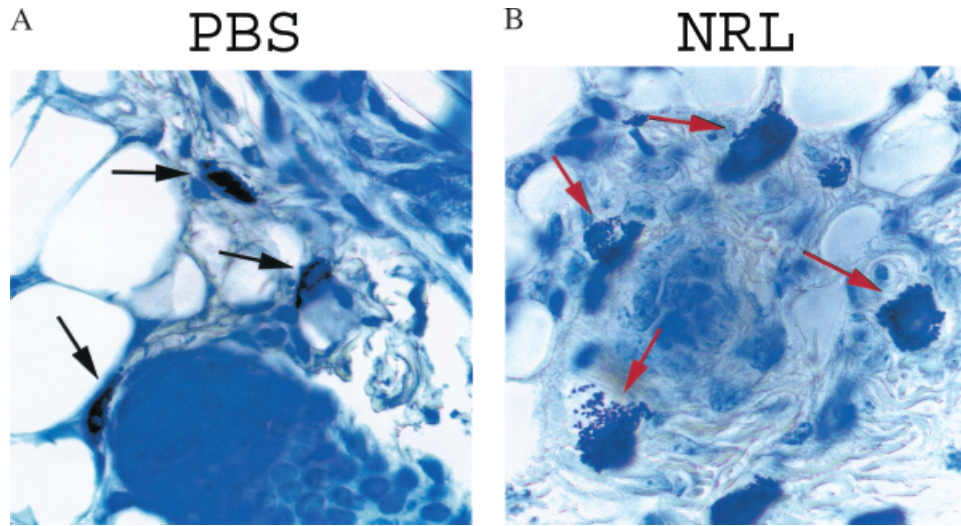


Figure 5. Morphologic features of mast cells after epicutaneous sensitization with NRL and PBS. (A) Intact mast cells (black arrows) in the PBS-treated skin site. Densely stained granules are seen within the cell membrane. (B) Degranulated mast cells (red arrows) in the NRL-treated skin site. Some granules of mast cells are leaking through the cell membrane.

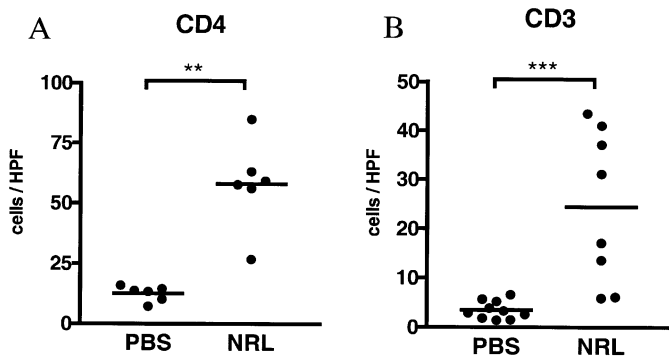


Figure 6. The number of CD4-positive cells (A) and CD3-positive cells (B) in mice skin sensitized with NRL and treated with PBS. The CD3- and CD4-positive cells were counted in 10 HPF at $\times 1000$ and expressed as cells per HPF. ** $p < 0.01$, *** $p < 0.001$.

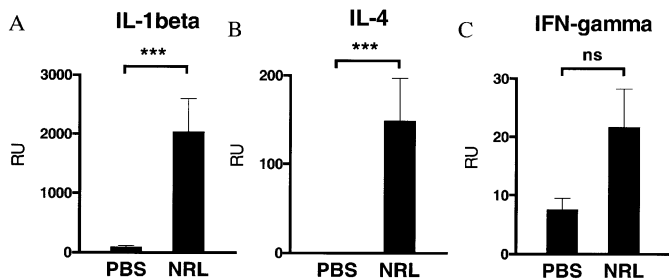


Figure 7. Real-time, quantitative reverse transcription-PCR analysis of IL-1 β (A), IL-4 (B), and IFN- γ (C) mRNA expression levels in skin biopsies of BALB/C mice after epicutaneous sensitization with NRL or PBS. RU are expressed as fold differences relative to the calibrator. The columns and error bars represent mean \pm SEM. $n = 9$ for NRL group and $n = 5$ for PBS group. *** $p < 0.001$, ns = not significant.

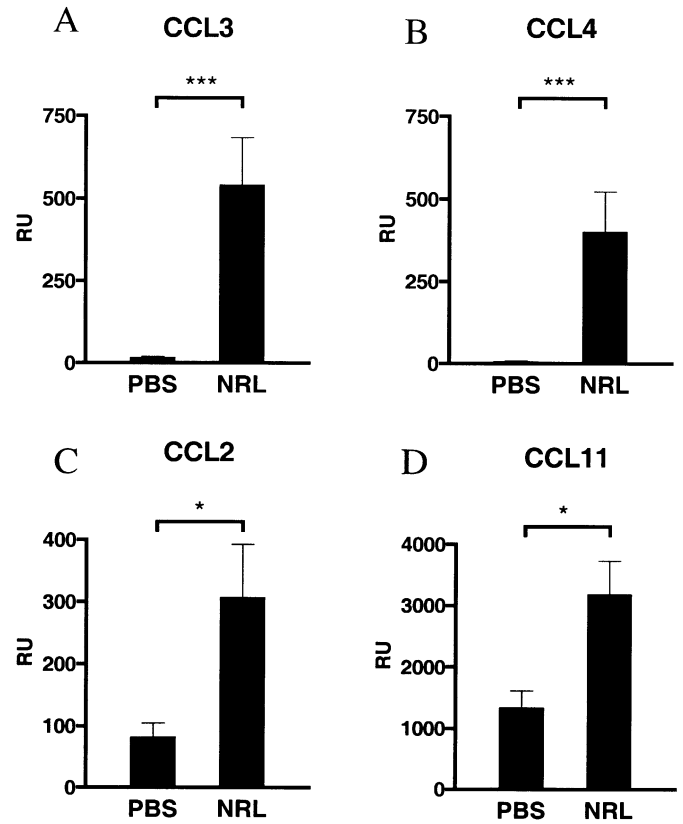


Figure 8. Real-time, quantitative reverse transcription-PCR analysis of CCL3 (A), CCL4 (B), CCL2 (C), and CCL11 (D) mRNA expression levels in skin biopsies from BALB/C mice after epicutaneous sensitization with NRL or PBS. RU are expressed as fold differences relative to the calibrator. The columns and error bars represent mean \pm SEM. $n = 9$ for NRL group and $n = 5$ for PBS group. * $p < 0.05$, *** $p < 0.001$.

in general (Alenius *et al*, 2002b). A recent study shows that HCW form the largest single risk occupation for NRL allergy (Turjanmaa *et al*, 2002). It has been previously reported that, in addition to experiencing immediate hypersensitivity reactions, about 50% of NRL-allergic HCW suffer from hand dermatitis

(Taylor and Praditsuwan, 1996; Alenius *et al*, 2002b). A recent study (Nettis *et al*, 2002) showed that 10% of HCW with glove-related skin symptoms had type IV allergy to rubber chemicals (e.g., thiurams) added to the gloves during manufacture and 9% had type I hypersensitivity to NRL proteins. Pre-existing hand

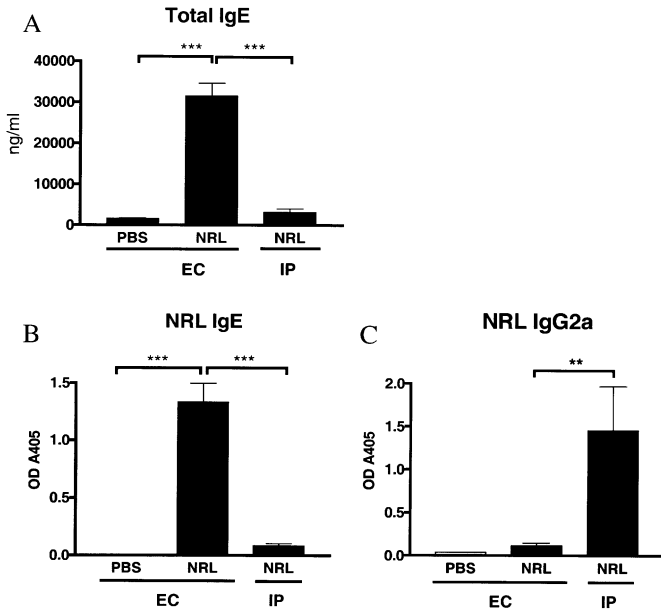


Figure 9. Total IgE and NRL-specific antibody responses in BALB/C mice after epicutaneous or IP sensitization with NRL or treatment with PBS. (A) Total serum IgE levels. (B) NRL-specific IgE serum levels. (C) NRL-specific IgG2a serum levels. Specific IgE and IgG2a antibody levels of the sample are expressed as absorbance units. The columns and error bars represent mean \pm SEM. PBS group n = 5, epicutaneous NRL group n = 9, IP NRL group n = 4. **p < 0.01, ***p < 0.001.

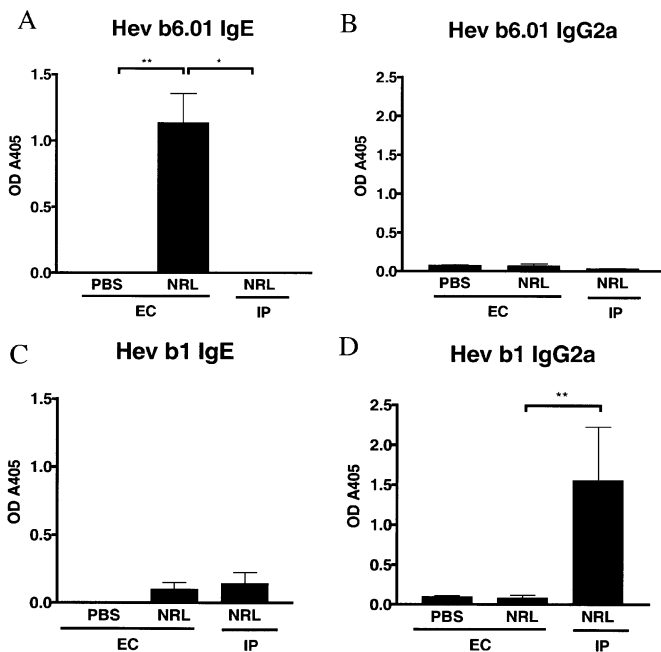


Figure 10. Hev b6.01 and Hev b1 specific antibody responses in BALB/C mice after epicutaneous or IP sensitization with NRL or PBS. (A) Hev b6.01-specific IgE serum levels. (B) Hev b6.01-specific IgG2a serum levels. (C) Hev b1-specific IgE serum levels. (D) Hev b1-specific IgG2a serum levels. Specific IgE and IgG2a antibody levels of the sample are expressed as absorbance units. The columns and error bars represent mean \pm SEM. PBS group n = 5, epicutaneous NRL group n = 9, IP NRL group n = 4. *p < 0.05, **p < 0.01.

dermatitis was a risk factor for the development of IgE-mediated allergy (Nettis *et al*, 2002). Recent indirect evidence supports the view that also proteins eluting from NRL gloves may play an important part in the development of hand dermatitis. We showed that avoidance of NRL gloves containing high levels of allergenic proteins resulted in an improvement of hand dermatitis in 19 of the 30 NRL-allergic HCW (Turjanmaa *et al*, 2002).

In this study, repeated epicutaneous exposure to NRL elicited a local cutaneous inflammatory response in mice. This response was characterized by epidermal and dermal thickening and increased infiltration of the dermal layer with eosinophils and mononuclear cells, including CD3⁺ and CD4⁺ cells. It should be noted, however, that possible irritant properties of NRL may also contribute to the overall development of skin inflammation. The number of degranulated mast cells was also significantly increased in NRL-sensitized skin sites compared with control sites. These findings suggest that, in addition to CD4⁺ CD3⁺ T cells and eosinophils, activated mast cells may be involved in the development and progress of dermatitis. In agreement with this we showed recently that mast cells critically regulate IFN- γ expression in the skin in a murine model of allergic dermatitis (Alenius *et al*, 2002a). To the best of our knowledge, there are no previous reports on the characterization of skin biopsies from NRL-allergic patients or from animal models of NRL allergy; however, histologic changes in lung tissues of NRL-sensitized mice demonstrated interstitial inflammatory infiltrates consisting of lymphocytes, histiocytes, plasma cells, and eosinophils (Kurup *et al*, 1994; Slater *et al*, 1998; Thakker *et al*, 1999; Xia *et al*, 1999).

In order to examine in detail the cytokine milieu in the skin after epicutaneous sensitization with NRL, we studied the expression of major proinflammatory (IL-1 β), Th2 (IL-4), and Th1 (IFN- γ) cytokines. The results revealed that sensitization with NRL induced significant and marked expression of mRNA for IL-1 β and IL-4, but not for IFN- γ . A recent study utilizing a novel murine model of allergic dermatitis suggests that both IL-4 and IFN- γ critically contribute to the development of ovalbumin-induced skin inflammation (Spergel *et al*, 1999). Sensitized skin from IL-4-deficient mice had a reduction in skin eosinophils and a significant increase in infiltrating T cells as compared with wild-type mice. IFN- γ -deficient mice were characterized by reduced dermal thickening (Spergel *et al*, 1999). Among the few cytokine studies dealing with NRL allergy, none have attempted to characterize skin cytokines; however, elevated levels of IL-4 mRNA and protein have been reported in splenocytes from animals sensitized with NRL via intraperitoneal and intranasal routes (Kurup *et al*, 1994).

Recruitment of inflammatory cells to the site of inflammation is mediated by chemokines that regulate the leukocyte traffic (Yoshie *et al*, 2001). In order to investigate further the mechanisms of leukocyte infiltration in our model, we studied several chemokines in the skin after epicutaneous sensitization with NRL. Significant induction of CCL3, CCL4, CCL2, and CCL11 mRNA was found after repeated epicutaneous sensitization with NRL. CCL3 acts as a chemoattractant via CCR1 and CCR5 receptors, CCL4 via the CCR5 receptor and CCL2 through the CCR2 receptor (Proudfoot, 2002). CCR1, CCR2, and CCR5 are expressed on the surface of a variety of cells involved in host defense, such as T cells, monocytes, natural killer cells, and dendritic cells (Proudfoot, 2002). CCL11 is a potent eosinophil chemoattractant via CCR3, which is highly expressed on eosinophils and mast cells (Luster and Rothenberg, 1997). Taken together, our results demonstrate that epicutaneous sensitization with NRL induces the significant induction of chemokines in the skin, which in turn attract inflammatory cells, such as activated T cells and eosinophils, to the sensitized skin sites.

We and others have recently shown that CCL27 and CCL17 play important parts in the recruitment of CCR10⁺ CLA⁺ T cells and CCR4⁺ Th2 cells, respectively, upon skin inflammation (Reiss *et al*, 2001; Homey *et al*, 2002). In this study, expression

of CCL17 was comparable in NRL-sensitized and PBS-treated skin sites. Similarly, strong but comparable expression of CCL27 mRNA was found in the skin of PBS- and NRL-treated groups. Because the majority of skin-homing Th2 cells express CCR10 and CCR4 on their surface (Homey *et al*, 2002; Reiss *et al*, 2001) it is possible that NRL-specific CD4⁺ CD3⁺ Th2 cells are attracted to the inflamed skin partially through local but constitutive expression of CCL27 and CCL17. Thus, infiltration of T cells to the skin may be regulated, not only by the induction of skin chemokines, but also by the expression of chemokine receptors on the surface of antigen-specific skin-homing T cells. On the other hand also other factors, e.g., adhesion molecules and cytokines, contribute to the cell infiltration as well (Harlan and Winn, 2002).

Atopy and the presence of NRL-specific IgE antibodies are strongly associated with NRL allergy (Alenius *et al*, 2002b). In this study, epicutaneous sensitization, but not IP immunization, with NRL elicited a significant elevation of total serum IgE and NRL-specific IgE antibodies. On the contrary, an elevation of NRL-specific IgG2a antibodies was found only after IP immunization. In agreement with the present findings a recent animal study by Woolhiser *et al* (2000) demonstrated that different sensitization routes can induce differences in NRL-specific IgE profiles. Consistent with our results, Kurup *et al* (1994) showed that IP immunization with NRL induced a marked increase in IgG2a antibodies. The present results suggest that epicutaneous sensitization with NRL favors the development of a Th2 type response, whereas IP immunization favors the development of a Th1 type antibody response.

Hev b6.01 is a major allergen for HCW, whereas Hev b1 is a major allergen for children with spina bifida (Alenius *et al*, 1996a,b; Wagner *et al*, 1999). For the time being, no definitive explanation exists for the observed differences in IgE reactivity between the two groups. In this, epicutaneous sensitization with NRL elicited a strong IgE response against Hev b6.01 but not against Hev b1. On the contrary, IP immunization with NRL elicited strong IgG2a production to Hev b1 but not to Hev b6.01. In line with our observations Woolhiser *et al* (2000) showed indirectly by immunoblotting that mice sensitized intratracheally or topically with NRL produced IgE antibodies against Hev b6, Hev b2, and Hev b4 (Microhelix protein complex), whereas those sensitized subcutaneously produced IgE antibodies against Hev b1 and Hev b3 (small rubber particle protein). The different antibody profiles against Hev b6.01 and Hev b1 may reflect differences in the biochemical nature of the allergens. Hev b6.01 is a highly hydrophilic protein, whereas Hev b1 is a highly hydrophobic one. This may cause differences in the penetration of these allergens through the skin. Differences in the concentrations of Hev b6.01 and Hev b1 in NRL extract may also affect the antibody responses. The amount of Hev b6.01 and its N-terminal fragment hevein is about 30-fold higher than that of Hev b1 in fresh NRL (ML, HA, unpublished observation). It has previously been suggested that high protein antigen concentrations favor the development of a Th2 type IgE response, whereas low antigen concentrations preferably induce a Th1 type IgG2a response (Constant and Bottomly, 1997; Jankovic *et al*, 2001).

In conclusion, the present results demonstrate that epicutaneous sensitization with NRL elicits in mice Th2 type systemic immune response and a local Th2 dominating skin inflammation. The inflammatory cell infiltration to the skin correlates with an induction of proinflammatory and Th2 cytokines as well as chemokines. Epicutaneous sensitization to proteins eluting from NRL gloves may therefore play an important part in the development of NRL-specific IgE antibodies. Moreover, NRL proteins, in addition to rubber chemicals, may also have an influence on the development of hand dermatitis. The murine experiments described here present a model for protein contact dermatitis that can be utilized for studying the mechanisms of allergen-induced skin inflammation as well as for determination

of which protein allergens are capable of sensitizing mice via the cutaneous route.

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Cutaneous, But Not Airway, Latex Exposure Induces Allergic Lung Inflammation and Airway Hyperreactivity in Mice

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As respiratory symptoms are common in addition to skin reactions in natural rubber latex allergy, we investigated the significance of different allergen exposure routes in the development of lung inflammation and airway hyperreactivity (AHR). Both intracutaneous (IC) and intraperitoneal (IP) exposure followed by airway challenge with latex proteins induced an influx of mononuclear cells and eosinophils to the lungs. AHR and lung mucus production increased significantly after IC and IP but not after intranasal (IN) exposure. Infiltration of inflammatory cells was associated with the induction of T-helper type 2 (Th2) cytokines and several CC chemokines. Only a marginal induction of these mediators was found after IN exposure. On the contrary, increased levels of transforming growth factor- β 1 and forkhead box 3 mRNA, markers of regulatory activities, were found in the lungs after IN but not after IC exposure. Finally, IC and IP, but not IN, latex exposure induced a striking increase in specific immunoglobulin E (IgE) levels. Cutaneous latex exposure in the absence of adjuvant followed by airway challenge induces a local Th2-dominated lung inflammation and a systemic IgE response. Cutaneous exposure to proteins eluting from latex products may therefore profoundly contribute to the development of asthma in latex allergy.

Key words: asthma/chemokines/cytokines/latex hypersensitivity
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Natural rubber latex (NRL) allergy has been recognized as a major cause of occupational contact urticaria, rhinitis, and asthma in health care workers (Ahmed *et al*, 2003; Reunala *et al*, 2004). People in other occupations requiring the use of latex gloves or who are frequently in contact with other NRL products are also at risk of becoming sensitized to NRL (Turjanmaa and Makinen-Kiljunen, 2002; Ahmed *et al*, 2003; Sparta *et al*, 2004). Although skin is the most frequently reported site of allergic reactions from latex gloves, airway symptoms are also common (Fish, 2002; Nolte *et al*, 2002). In a recent study, one-fourth of the latex-allergic health care workers were reported to suffer from rhinitis and asthma and several of these asthmatic individuals were forced to change their jobs (Bernstein *et al*, 2003). Sensitization routes and pathomechanisms of NRL-induced rhinitis and asthma, however, are still poorly understood.

Sensitization to NRL proteins may occur through various exposure routes (Weissman and Lewis, 2002). Skin is an obvious target for exposure to latex proteins in people using latex gloves (Bernstein *et al*, 2003) but NRL allergens also

become easily airborne in association with glove powder, thereby permitting exposure and sensitization via the airways (Charous *et al*, 2002). On the other hand, children with spina bifida are thought to become sensitized through mucous membranes to NRL proteins eluting from surgeon's gloves during operations (Weissman and Lewis, 2002). Today, little is known on how these different exposure routes interact during the development of NRL allergy or what is their importance in the elicitation of clinical symptoms.

In this study, we used mouse models of latex allergy to investigate the significance of different exposure routes, i.e. intracutaneous (IC), intraperitoneal (IP), and intranasal (IN) routes, to the development of lung inflammation, airway hyperreactivity (AHR), and antibody production to NRL allergens.

Results

IC exposure and challenge with NRL induces a vigorous influx of eosinophils into the lungs H&E staining of lung tissues revealed enhanced peribronchial and perivascular cell infiltrates consisting primarily of lymphocytes and eosinophils after IC and IP exposure (Fig 1A and S1). Only a slight, mostly perivascular, inflammation was observed after IN exposure. The number of infiltrating eosinophils both in perivascular and in peribronchial areas of lung tissues showed a dramatic increase in intracutaneously

Abbreviations: AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; Foxp3, forkhead box 3; Hev b6.01, prohevein; IC, intracutaneous; IgE, immunoglobulin E; IL, interleukin; IN, intranasal; IP, intraperitoneal; MCh, methacholine; NRL, natural rubber latex; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; Penh, enhanced pause; RU, relative units; TGF- β 1, transforming growth factor- β 1; Th2, T-helper type 2

¹These authors contributed equally to this work.

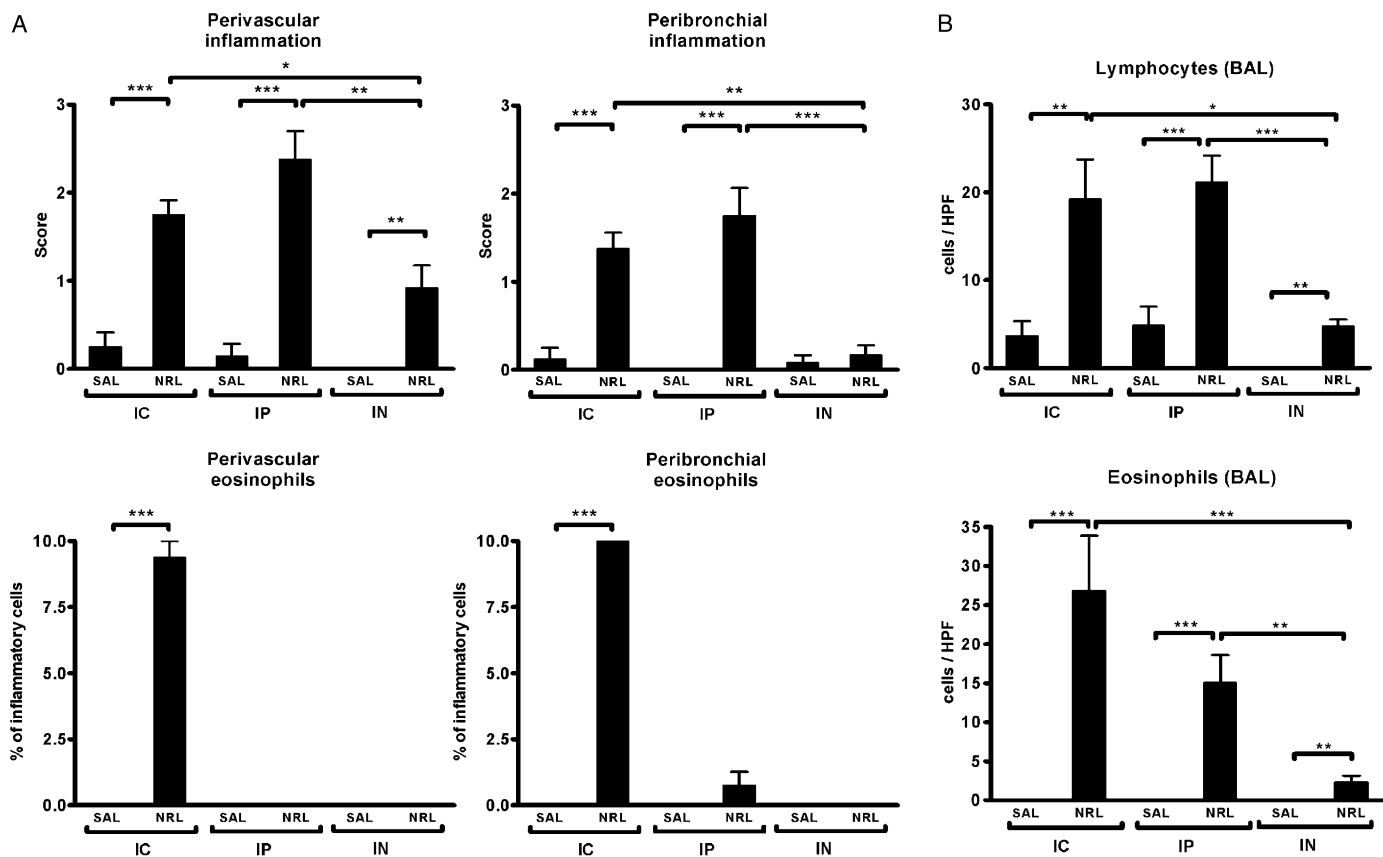


Figure 1
Lung inflammation. (A) Inflammation was expressed as score values and eosinophils as percentages of inflammatory cells (n = 7–12 mice per group). (B) Lymphocytes and eosinophils in bronchoalveolar lavage (BAL) fluids were counted per high-power field (n = 12–16 mice per group). The columns and error bars represent mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

exposed (p < 0.001) but not in intraperitoneally or intranasally exposed mice (Fig 1A and S1).

Differential cell counts of bronchoalveolar lavage (BAL) fluid samples indicated a significant eosinophil and lymphocyte recruitment into the lungs of IC and IP NRL-exposed mice as compared with phosphate-buffered saline (PBS)-treated mice (Fig 1B). In contrast, eosinophils were virtually absent from the BAL of the mice treated intranasally with NRL, and only a few lymphocytes were detectable (Fig 1B).

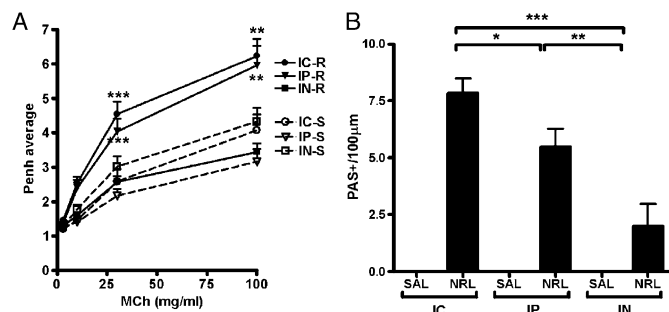


Figure 2
Airway hyperresponsiveness and lung mucus production. (A) Airway hyperresponsiveness was expressed by average enhanced pause (Penh) values in relation to increasing doses of aerosolized methacholine. Data represents mean ± SEM. (B) The number of mucus-producing cells was represented as periodic acid-Schiff (PAS)-positive cells. The columns and error bars represent mean ± SEM; ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001; n = 12–16 mice per group.

We rarely detected any neutrophils or any significant changes in the number of macrophages in the BAL in any group of mice investigated (data not shown).

IC exposure and challenge with NRL induces marked AHR and lung mucus production AHR to inhaled methacholine (MCh) was analyzed in order to evaluate changes in airway resistance in the response to allergen exposure and challenge. IC and IP NRL exposure followed by airway challenge induced a strong and significant increase in airway reactivity to inhaled MCh (Fig 2A). In contrast, IN NRL exposure failed to induce AHR, whereas control mice showed a slightly elevated but statistically non-significant response to inhaled MCh.

Airway mucus overproduction contributes significantly to the pathophysiologic changes in asthma (Maddox and Schwartz, 2002). Lung sections were stained with periodic acid-Schiff (PAS) to quantify the amount of mucus-producing cells around the bronchioles. As shown in Fig 2B and S2, animals treated intracutaneously or intraperitoneally with NRL displayed a significant increase in mucus production, as seen in the high amount of goblet cells around the airway lumen. IN NRL exposure, on the other hand, induced only a minor and insignificant increase in mucus production in the airways.

Expression of several CC chemokine mRNA is strongly upregulated in lung tissue after IC and IP exposure to

NRL We analyzed several CC chemokines known to play an important role in allergic airway inflammation (Lukacs, 2001; Bisset and Schmid-Grendelmeier, 2005). Following IC, IP, and also IN NRL exposure and challenge, significant increases in CCL1, CCL8, CCL11, CCL17, and CCL24 mRNA expressions were detected in the lungs relative to PBS-treated mice (Fig 3). The level of CCL3 mRNA was significantly elevated in the lungs after IC and IP NRL exposures, whereas IN exposure did not elicit induction of this chemokine (Fig 3). Expression levels of most of the chemokines were significantly higher after IC latex exposure (CCL1, CCL3, CCL8, CCL11, and CCL24) and IP exposure (CCL1, CCL3, CCL8, CCL17, and CCL24) compared with intranasally exposed mice. CCL3 mRNA levels were significantly higher in intraperitoneally exposed mice compared with intracutaneously exposed mice. In addition, mRNA expression levels of chemokine receptors corresponding to the investigated chemokine ligands are shown in Fig S3.

IC NRL exposure induces strong expression of T-helper type 2 (Th2)-type cytokine mRNA, whereas IN exposure elicits marked induction of transforming growth factor- β 1 (TGF- β 1) and forkhead box 3 (Foxp3) mRNA in the airways Elevated expression of Th2-type cytokines interleukin (IL)-4, IL-5, and IL-13 in lung tissues is a characteristic feature of the pulmonary allergic response (Oettgen and Geha, 2001). IC and IP exposure to NRL elicited clear increases in the expression levels of IL-4 and IL-13 mRNA, but only IC exposure increased expression of IL-5 mRNA

significantly in lung tissue compared with PBS-treated controls (Fig 4A). Moreover, an 8-fold enhancement in the level of IL-13 mRNA in IN NRL-exposed mice was observed in comparison with controls. IC exposure elicited significantly higher levels of IL-4, IL-5, and IL-13 mRNA compared with the intranasally exposed group. In addition, IL-4 mRNA levels were significantly elevated in intraperitoneally exposed mice compared with intranasally exposed mice.

A significant enhancement in the mRNA levels of regulatory cytokine IL-10 was observed in all NRL-exposed groups, especially in intranasally treated mice (Fig 4B). Interestingly, a significant elevation in the levels of TGF- β 1 mRNA was observed after IN and IP NRL exposure ($p < 0.001$ and $p < 0.01$, respectively) but not after IC exposure. The TGF- β 1 mRNA levels were significantly higher in IN and IP mice when compared with intracutaneously exposed mice (Fig 4B). In addition, Foxp3 transcription factor mRNA expression also enhanced significantly after IP and IN NRL administration but not after IC exposure (Fig 4B). Expression increased most significantly after IN latex exposure.

Total and Hev b 6.01-specific immunoglobulin E (IgE) levels are strongly elevated after IC and IP NRL exposure Allergen-specific IgE antibodies are the hallmark of an immediate allergic reaction (Geha *et al*, 2003). Both IC and IP exposure to NRL elicited the strong induction of total IgE antibodies (Fig 5). On the contrary, total serum IgE remained in the baseline level after IN NRL exposure. IC and IP exposure induced high levels of IgE antibodies against major

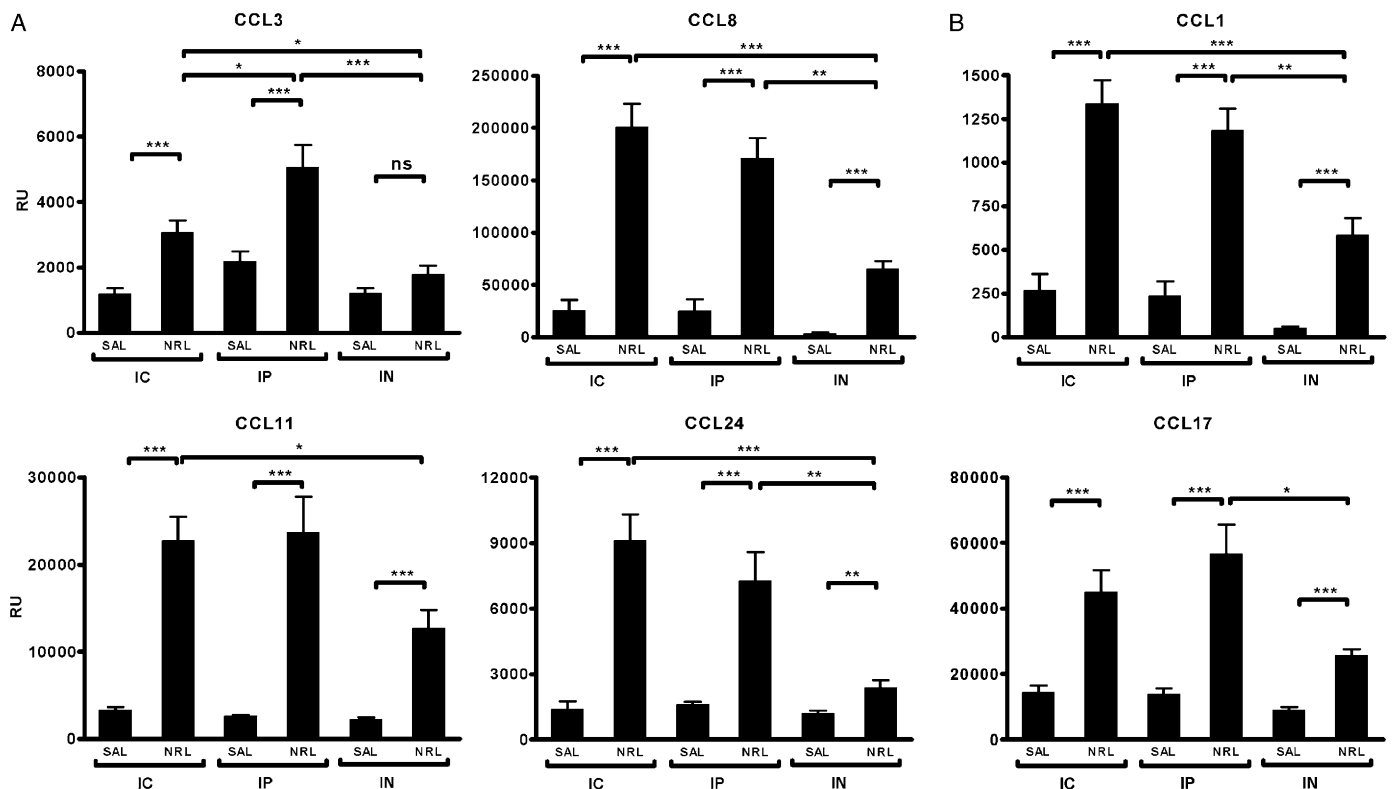


Figure 3
Chemokine mRNA exposure. mRNA expression of chemokines, which attract especially eosinophils (A) or T-helper type 2 cells (B) in lung samples after exposures to vehicle (saline (SAL)) or natural rubber latex (NRL). Relative units (RU) are relative differences compared with the calibrator. The columns and error bars represent mean \pm SEM; ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 12-15$ mice per group.

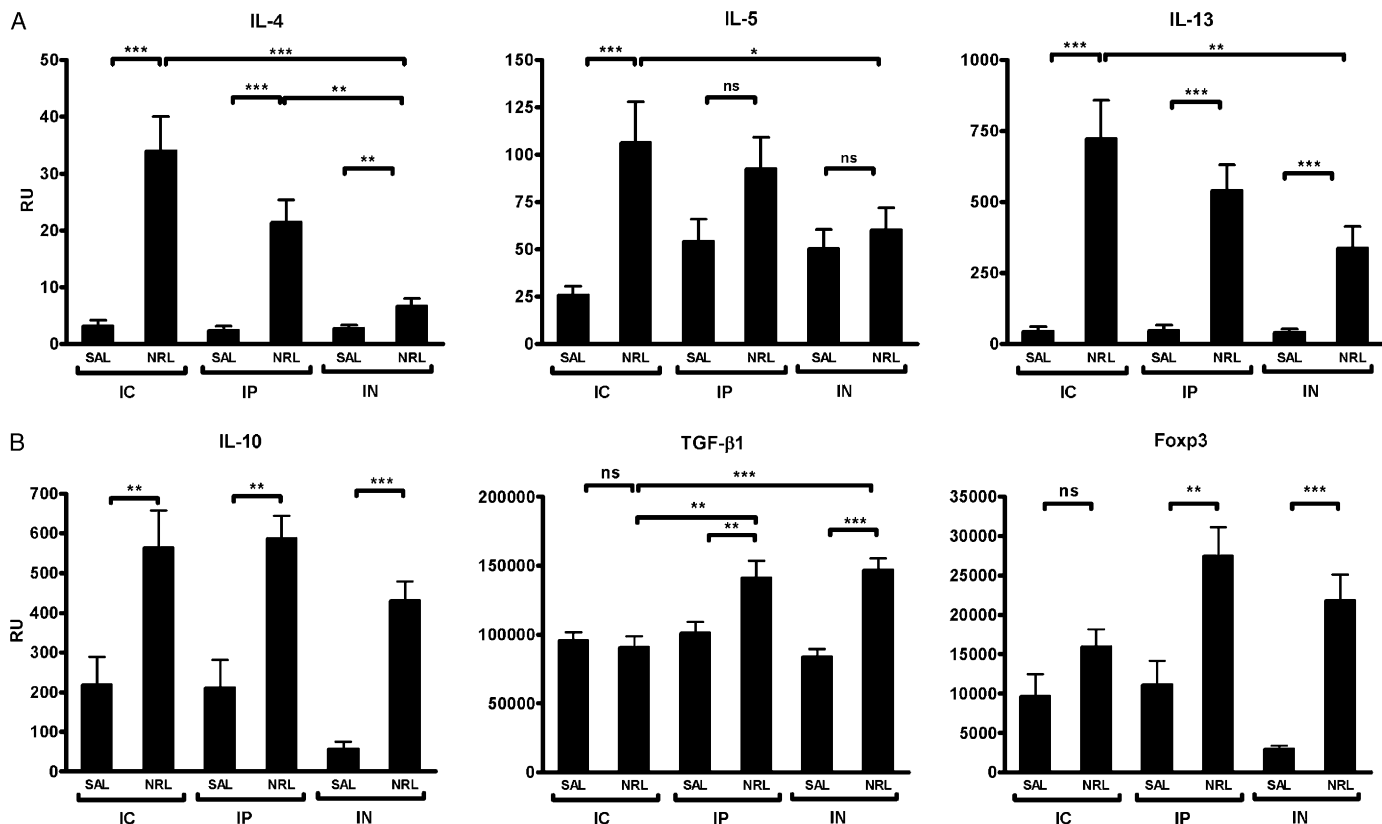


Figure 4
Expression levels of cytokines and Foxp3 mRNAs. T-helper type 2 cytokine mRNA (A) and regulatory cytokine and forkhead box 3 (Foxp3) mRNA (B) expression in lung samples after exposures to vehicle (saline (SAL)) or natural rubber latex (NRL). The columns and error bars represent mean \pm SEM; ns, non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 12-15$ mice per group.

NRL allergen, Hev b 6.01 (Fig 5). In contrast, IN NRL exposure did not induce detectable levels of prohevein (Hev b 6.01)-specific IgE antibodies.

Discussion

Sensitization to NRL products has been one of the leading causes of occupational asthma during the last several years (Fish, 2002). The sensitization routes and pathomechanisms

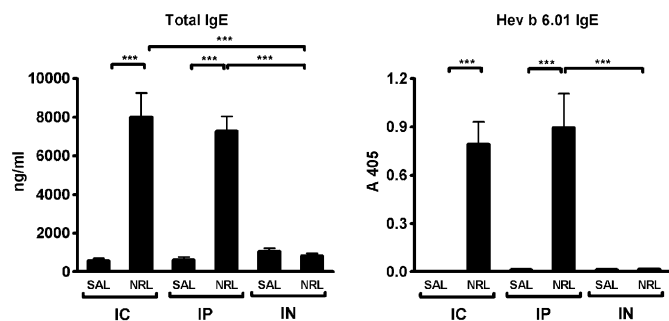


Figure 5
Immunoglobulin E (IgE) levels. Total serum and prohevein (Hev b 6.01)-specific IgE levels in mice after exposures to vehicle (saline (SAL)) or natural rubber latex (NRL) were measured by ELISA. Specific IgE levels of the serum are expressed as absorbance units (405 nm). The columns and error bars represent mean \pm SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 12-15$ mice per group.

of NRL-induced rhinitis and asthma are, however, not fully understood. As sensitization to NRL proteins may take place through various exposure routes, we investigated their significance in the development of allergic asthma in mouse models. Our findings demonstrate that cutaneous, but not airway, NRL administration in the absence of adjuvant induces a local Th2-dominated lung inflammation and an intense AHR after airway NRL challenge.

Chronic airway inflammation, an integral feature of allergic asthma, is characterized by the accumulation of inflammatory cells, such as eosinophils (Herrick and Bottomly, 2003). In this study, lung eosinophilia was significantly stronger after IC exposure compared with IN or IP exposures, demonstrating that repeated cutaneous NRL exposure without an external adjuvant is highly efficient in inducing eosinophilic lung inflammation after airway NRL challenge. AHR and airway mucus overproduction contribute significantly to the pathophysiological events in allergic asthma (Herrick and Bottomly, 2003). Both IC and IP exposure, but not IN exposure, followed by NRL airway challenge induced a major increase in AHR to inhaled MCh. Intracutaneously and intraperitoneally exposed mice also showed a significant increase in mucus production. Only a minor increase in mucus production was seen in the airways after IN NRL exposure. In agreement with these findings, Howell *et al* (2002) recently reported that repeated topical NRL exposure to tape-stripped skin elicited an increase in AHR to inhaled MCh, but repeated intratracheal exposure of NRL was unable to induce AHR to inhaled MCh (Howell

et al, 2004). On the other hand, Woolhiser *et al* (2000) previously demonstrated IgE response and AHR following IN exposure of 50 mg of NRL administered 5 d per wk for 10 wk. Our results, however, clearly demonstrate that dermal route exposure using much lower allergen dosage and length of exposure leads to vigorous IgE response, AHR, and airway inflammation after airway challenge.

In order to investigate the mechanisms of leukocyte recruitment to the airways, in detail we studied several CC chemokines in lung tissue. Eosinophils are known to respond to CCL3, CCL5, CCL8, CCL11, and CCL24 (Zimmermann *et al*, 2003). In this study, expressions of CCL8, CCL11, and CCL24 mRNA were significantly higher in intracutaneously exposed mice compared with intranasally exposed mice with marked airway eosinophilia after cutaneous exposure. These chemokines were, however, equally expressed in intraperitoneally and intracutaneously exposed groups although lung eosinophilia was significantly more intense in the intracutaneously exposed mice. Expression levels of CCL1 and CCL17, which are known to attract Th2 cells (Panina-Bordignon *et al*, 2001), were also higher after IC and IP exposure compared with IN exposure. Taken together, our results demonstrate that cutaneous and IP NRL exposure elicits induction of various chemokines in lung tissue, which in turn may recruit Th2-type inflammatory cells, a characteristic of allergic airway inflammation.

A consensus exists that IL-4 and IL-13 are key regulators of IgE class switching (Geha *et al*, 2003). In addition, it has been shown that IL-13 contributes to AHR and mucus overproduction (Kuperman *et al*, 2002). Expression levels of IL-4 and IL-13 mRNA were significantly enhanced after all routes of exposure with NRL in lung tissue in this study. But increases were markedly higher after IC exposure and to a lesser extent after IP exposure, in comparison with IN exposure. The expression level of IL-5, a key cytokine regulating eosinophil recruitment and survival (Hamelmann and Gelfand, 2001), was significantly elevated only after IC NRL exposure. Thus, the greater number of eosinophils in the lungs of intracutaneously exposed mice may be because of an increased eosinophil survival rate induced by increased levels of IL-5 in the airways. On the other hand, CCL24 and IL-5 have been shown to cooperatively promote eosinophil accumulation into airways and to increase AHR to inhaled MCh (Yang *et al*, 2003). Only a few studies exist in the literature describing cytokine expression in lungs after NRL administration. Hardy *et al* (2003) recently reported that mice sensitized intraperitoneally with NRL allergens, and with NRL glove extract demonstrated elevated levels of IL-5 protein in the BAL fluid. They found no significant differences in the levels of IL-4 protein between controls and latex-sensitized groups. Our results clearly indicate that repeated cutaneous NRL exposure induces Th2-dominated cytokine expression in the lungs after airway challenge.

Increased levels of total and allergen-specific IgE antibodies in the patient serum are characteristic of allergic asthma (Busse and Lemanske, 2001; Lemanske and Busse, 2003). In this study, both IC and IP NRL exposure elicited a significant elevation of total serum IgE levels. As an example of allergen-specific IgE response, we investigated antibody responses against Hev b 6.01, which is a major NRL allergen (Alenius *et al*, 1996; Wagner and Breiteneder, 2005).

The sera from intracutaneously or intraperitoneally exposed mice contained high levels of IgE antibodies to Hev b 6.01. On the contrary, IN exposure failed to induce elevation of total and specific IgE antibodies. In agreement with these findings, Hufnagl *et al* (2003) also reported increased NRL allergen-specific IgE levels after IP immunization, and a recent study by Woolhiser *et al* (2000) demonstrated that different exposure routes can induce differences in NRL-specific IgE profiles in mice.

It is of interest that airway inflammation, AHR, and IgE levels were substantially lower after IN exposure compared with IC exposure. To address the role of regulatory cytokines in the induction of airway inflammation, we analyzed the mRNA levels of IL-10 and TGF- β 1, both of which are known to play important roles in the downregulation of immune responses (Terui *et al*, 2001; Akbari *et al*, 2003; Nagler-Anderson *et al*, 2004). Proportional increase (allergen-exposed mice vs PBS-exposed control) in the expression of IL-10 mRNA was markedly more prominent in intranasally exposed mice (7.4-fold increase) compared with intracutaneously (2.6-fold increase) and intraperitoneally exposed (2.8-fold increase) mice. In line with these results, Akbari *et al* (2001) have reported that respiratory exposure to allergens can induce T cell tolerance, which appears to be mediated by IL-10 production. On the other hand, we found significant induction of TGF- β 1 mRNA only after IN and IP NRL exposure but not after IC exposure. Furthermore, proportional expression of transcription factor Foxp3, which is expressed predominantly by CD4+CD25+ T regulatory cells (Fontenot *et al*, 2003; Khattri *et al*, 2003), was significantly upregulated after IN exposure (7.3-fold) but to a lesser extent after IP exposure (2.5-fold) and IC exposure (1.6-fold). Thus, it is possible that increased levels of IL-10 and TGF- β 1 as well as Foxp3+ regulatory T cells in the lungs of intranasally exposed mice suppress the inflammatory responses, resulting in diminished airway inflammation. The link between TGF- β and regulatory T cells is supported by the finding that TGF- β is able to promote differentiation of T cells into Foxp3+ regulatory T cells (Chen *et al*, 2003; Fantini *et al*, 2004).

These findings underline the role of cutaneous route allergen exposure in the elicitation of allergic airway inflammation and AHR. Instead of epicutaneous (Spergel *et al*, 1998; Lehto *et al*, 2003) allergen application, we used IC allergen exposure assuring that the actual allergen dose entering the body was the same in all exposure routes. The present observations are of importance when considering prevention of airway hypersensitivity to NRL allergens. Glove powder is the most important carrier of NRL allergens to airways, and thus primary prevention has mainly focused on the use of non-powdered latex gloves (Charous *et al*, 2002). As the exposure via the cutaneous route could be an important way for sensitizing airways, attention will need to be paid to the use of low- or non-allergen NRL gloves instead of using only non-powdered gloves.

Materials and Methods

Allergens NRL and Hev b 6.01 were purified as earlier described (Lehto *et al*, 2003).

Mice exposure protocols and AHR Six- to eight-week-old female BALB/cJbom mice were obtained from Taconic M&B A/S (Ry, Denmark), Social and Health Services of Finland; Provincial Office of Southern Finland approved all experimental protocols.

Exposure protocols are shown in Fig 6. Mice were exposed for 4 wk before airway challenge. In saline (SAL) groups exposures were made with PBS and with NRL in NRL groups. Mice were anesthetized with isoflurane (Abbott Laboratories Ltd, Queenborough, UK) for IC and IN exposure. The backs of the mice were shaved and tape-stripped (Tegaderm, 3M Health Care, St Paul, Minnesota) four times before IC exposure to remove loose hair and to induce standardized skin injury mimicking the scratching that is a characteristic feature in patients with atopic dermatitis. In IC exposure, mice were injected once a week with 40 μ g of NRL in 100 μ L of PBS or with 100 μ L PBS as control. IN exposure was also made once a week with the same amounts of NRL in 50 μ L PBS. In IP exposure, mice were injected on days 1 and 14 with 80 μ g NRL together with 2 mg alum (Pierce, Rockford, Illinois) in 100 μ L PBS. Mice were challenged after exposures via the respiratory route using an ultrasonic nebulizer (Aerogen Ltd, Galway, Ireland) with 0.5% NRL (in PBS) for 20 min on days 28, 29, and 30. On day 31, AHR to MCh (Sigma-Aldrich Co, St Louis, Missouri) was measured by using whole-body plethysmography (Buxco Electronics Inc., Sharon, Connecticut) as previously described (Hamelmann *et al*, 1997) and monitored by average Penh (enhanced pause = pause \times (peak inspiratory box flow/peak expiratory box flow)) values. MCh concentrations were 3, 10, 30, and 100 mg per mL. The mice were then sacrificed with carbon dioxide, and samples (BAL fluids, lung biopsies, and blood) were taken for subsequent analysis. The experiment was repeated two times, and the combined results are presented.

BAL and lung histology Lungs were lavaged with 0.8 mL of PBS, and BAL specimens were cytocentrifuged (Shandon Scientific Ltd, Runcorn, UK) at 150 \times *g* for 5 min. Cells were stained with May-Grünwald-Giemsa and counted blinded in 15–20 high-power fields at \times 1000 under light microscopy.

Formalin-fixed lungs were embedded in paraffin, and lung sections (4 μ m) were stained with H&E or with PAS solution. Inflammation in different lung areas was expressed after H&E staining as score values 1–5 (1, slightly inflamed tissue; 2, inflammation of the area over 20%; 3, inflammation of the area over 50%; 4, inflammation of the area over 75%; and 5, entire tissue inflamed). The number of mucus-producing cells was determined after PAS staining by choosing three bronchial sections randomly and counting PAS-positive cells per 100 μ m. The slides were analyzed blinded with a Nikon EclipseE800 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

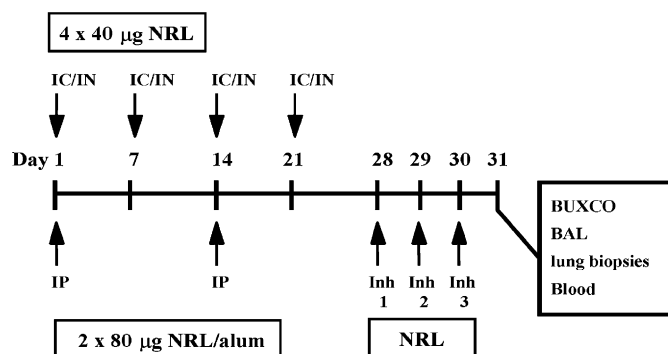


Figure 6 Intra-cutaneous (IC), intraperitoneal (IP), and intranasal (IN) exposure schedules. Mice were exposed for 4 wk before airway challenge. In saline (SAL) groups exposures were made with phosphate-buffered saline and with natural rubber latex (NRL) in NRL groups. Airway challenges were made by NRL. Airway hyperreactivity to methacholine was measured 24 h after airway challenges and different samples were taken for subsequent analysis.

Total and specific IgE antibodies in serum Total IgE levels were measured as previously described (Lehto *et al*, 2003). A specific ELISA for purified Hev b 6.01 allergen was slightly modified. Plates were first coated for 3 h at +20°C and then overnight at +4°C. The blocking time was 1 h at +20°C and 1:10 diluted samples were incubated for 2 h at +20°C. Biotin-labeled anti-mouse isotype-specific antibody was added for 2 h at +20°C. Streptavidin-conjugated alkaline phosphatase (Zymed, San Francisco, California, diluted 1:1000) was incubated for 30 min at +20°C, color substrate (*p*-nitrophenyl phosphate, Sigma-Aldrich Co) was added, and absorption at 405 nm was read. The sera, biotinylated anti-mouse antibodies, and streptavidin-conjugated phosphatase were diluted in 0.2% BSA/0.05% Tween/PBS.

Expression of mRNA in lungs RNA extraction and synthesis of cDNA was carried out as described earlier (Lehto *et al*, 2003). Real-time quantitative PCR was performed with an AbiPrism 7700 Sequence Detector System (SDS) (Applied Biosystems, Foster City, California) as previously described (Lehto *et al*, 2003). PCR primers and probes were from Applied Biosystems as predeveloped reagents (18S ribosomal RNA, IL-4, IL-5, IL-10, IL-13, CCL1, CCL3, and Foxp3) or were self-designed. The self-designed sequences of CCL11, CCL17, and CCL24 have been described earlier (Lehto *et al*, 2003), and others are as follows: TGF- β 1 (forward 5'-CAA GGG CTA CCA TGC CAA CTT-3', probe 5'-CAC ACA GTA CAG CAA GGT CCT TGC CCT CT-3', and reverse 5'-ATG GGC AGT GGC TCC AAA G-3'), CCL8 (forward 5'-CCC TTC GGG TGC TGA AAA G-3', probe 5'-TAC GAG AGA ATC AAC AAT ATC CAG TGC CCC-3', and reverse 5'-TCT GGA AAA CCA CAG CTT CCA-3'). The results are expressed as relative units (RU), which were calculated by the comparative C_T method (Lehto *et al*, 2003).

Statistics Statistical tests were performed using GraphPad Prism Version 4 (GraphPad Software Inc.). Single group comparisons were conducted by the non-parametric Mann-Whitney *U* test. A *p*-value of less than 0.05 was considered to be statistically significant.

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Supplementary Material

The following material is available online for this article.

Figure S1 Histology of H & E stained perivascular using tissues.

Figure S2 Histology of PAS-stained peribronchial lung sections.

Figure S3 Chemokine receptor mRNA expression.

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Supplementary Figures

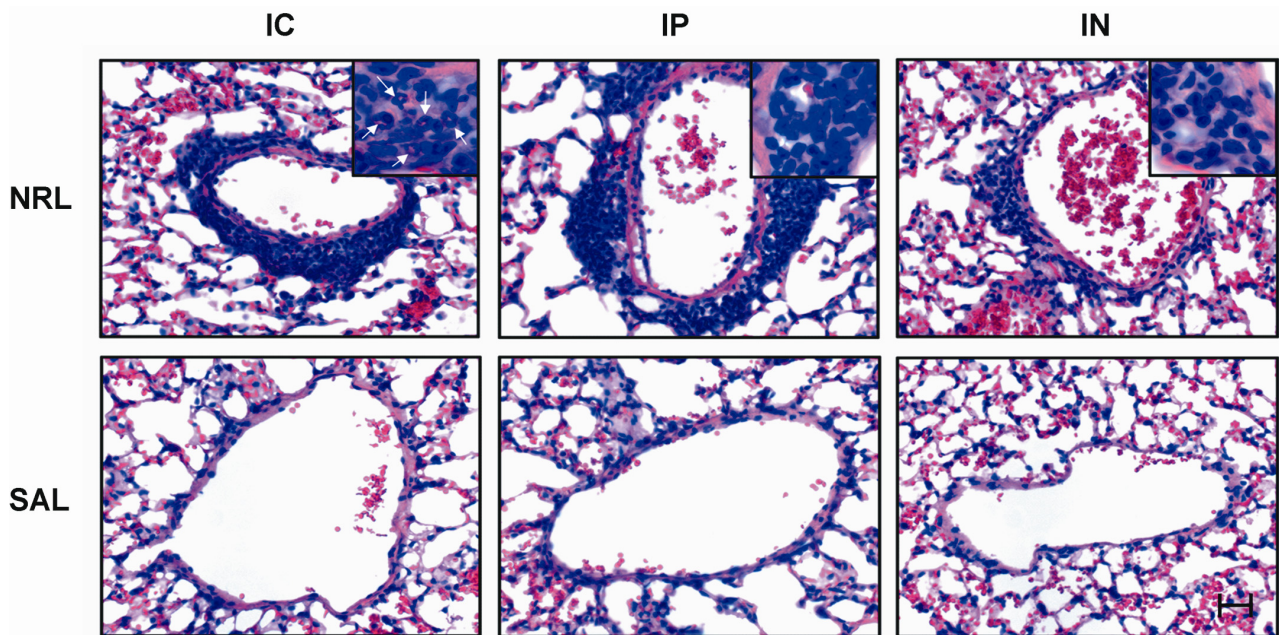


Figure S1. Representative micrographs from perivascular lung tissues are shown after IC, IN or IP exposures (H&E staining, magnification x400 and x1000 in inserts). Arrows in the insert of IC NRL exposure photograph point to eosinophils. *Scale bar*= 20 μ m.

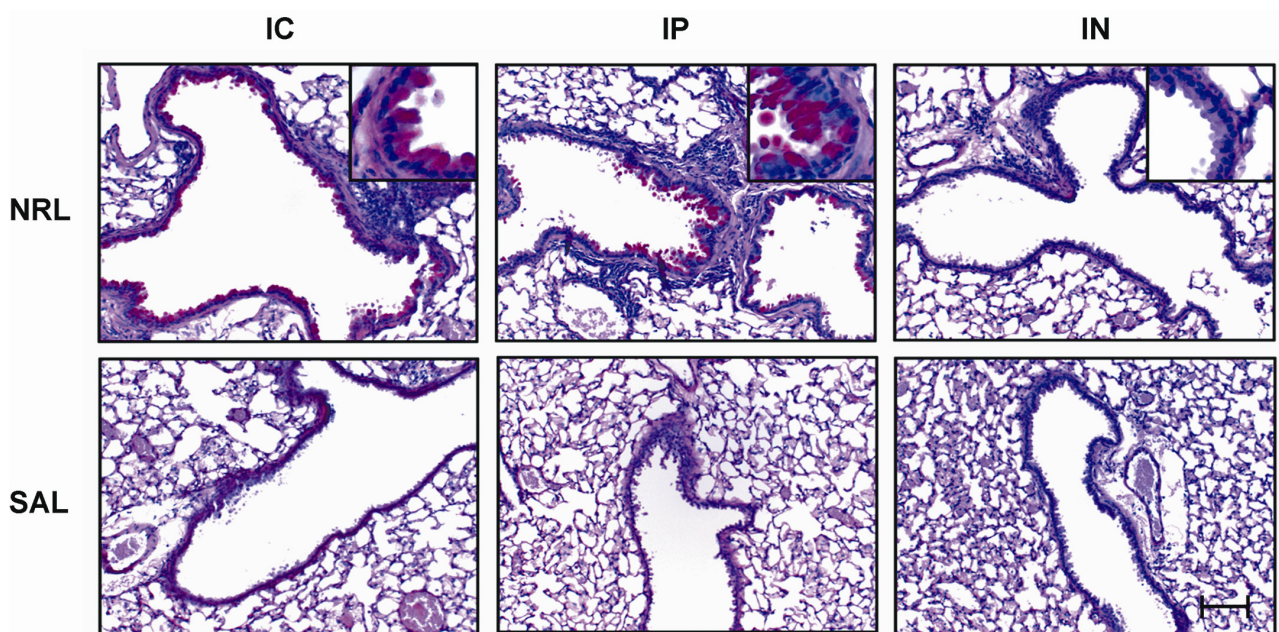


Figure S2. Histological features of PAS-stained peribronchial lung sections (magnification x200 and x400 in inserts). *Scale bar*= 100 μ m.

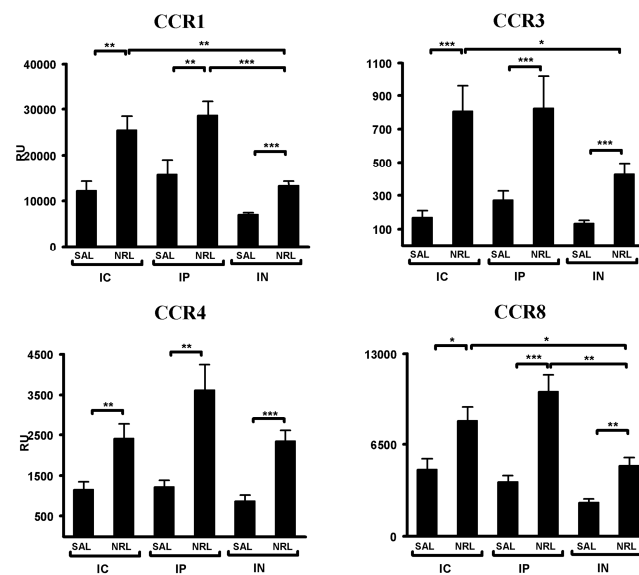


Figure S3. Chemokine receptor mRNA expression levels in lung samples after exposures to vehicle (SAL) or NRL. RU values are expressed as relative differences to the calibrator. The columns and error bars represent mean \pm SEM; * p <0.05, ** p <0.01, *** p <0.001; n = 12-15 mice per group.

ORIGINAL PAPER

Hev b 6.01 and Hev b 5 induce pro-inflammatory cytokines and chemokines from peripheral blood mononuclear cells in latex allergy

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Clinical and Experimental Allergy

Summary

Background Hev b 6.01 (prohevein) and Hev b 5 [acidic natural rubber latex (NRL) protein] are major IgE-binding allergens in NRL allergy.

Objective To examine allergen-specific cytokine and chemokine responses in NRL-allergic patients.

Methods Fourteen NRL-allergic patients and 10 healthy controls participated in the study. Hev b 6.01 and Hev b 5 were purified under non-denaturing conditions by chromatographic methods. Specific IgE antibodies were measured by ELISA and proliferation of peripheral blood mononuclear cells (PBMC) by ³H-thymidine incorporation assay. Allergen-specific induction of cytokine and chemokine mRNA in PBMC was measured by real-time PCR and protein levels by ELISA. Surface expression of chemokine receptors was analysed by flow cytometry.

Results Twelve (86%) NRL-allergic patients had positive skin prick test reactions and IgE antibodies against Hev b 6.01, but less than 30% responded to Hev b 5. Cell proliferation against Hev b 6.01, but not against Hev b 5, was significantly increased. Both allergens elicited significantly higher expression of pro-inflammatory and T-helper type 2 cytokines (TNF, IL-12p40, IL-13) and chemokines (CCL3, CCL4, CCL20) in the NRL-allergic patients than in controls. Interestingly, mRNA expression of the regulatory cytokine TGF-β1 was reduced, whereas IL-10 expression was enhanced after allergen stimulations in patients with NRL allergy. Finally, the NRL-allergic patients showed increased CCR4 expression on CD3⁺CD8⁻ T cells and decreased CXCR3 expression on CD3⁺CD8⁺ T cells.

Conclusion Allergen-specific induction of cytokines and chemokines in PBMC and chemokine receptor expression on circulating T cells may contribute to the pathogenesis of NRL allergy.

Keywords chemokine receptor, chemokines, cytokines, latex hypersensitivity, PBMC

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Introduction

Natural rubber latex (NRL) allergy has been one of the most frequently encountered occupational diseases especially among the health care workers (HCW) [1]. The prevalence of NRL allergy in HCW has been reported to be between 0.6% and 17% [2]. However, recent studies show that incidence decreases when attention is paid to the quality of NRL gloves [3, 4]. Symptoms of NRL allergy vary from mild localized reactions, e.g. contact urticaria and rhinitis, to more severe symptoms, e.g. generalized urticaria, asthma and anaphylaxis [2, 5]. In addition to

type I symptoms, NRL allergy may present with eczema on the hands [6]. This finding is further supported by experiments in mice showing that cutaneous NRL exposure causes T-helper type 2 dermatitis [7].

In contrast to IgE-mediated reactions, knowledge of NRL-induced cell-mediated immunity is scanty. Researchers have mainly studied proliferation of peripheral blood mononuclear cells (PBMC) against NRL allergens [8–11]. Evidence exists that the major IgE-binding NRL allergen Hev b 6.02 (hevein domain of prohevein) [12, 13] contains dominant T cell epitopes [14]. In addition, Hev b 6.03 [12]

(C domain of prohevein) has HLA-DR-binding motifs and induces T cell proliferation [10]. Certain Hev b 5 peptides have been shown to induce proliferation of peripheral T cell lines of patients with NRL allergy [15]. However, only little is known about cytokine and chemokine responses to NRL allergens.

In the present study, we examined responses of PBMC from NRL-allergic patients against the two major NRL allergens, i.e. Hev b 6.01 and Hev b 5 [12]. Our results suggest that allergen-induced production of pro-inflammatory and regulatory cytokines and chemokines may play an important role in the pathogenesis of NRL allergy.

Materials and methods

Patients

Fourteen patients with NRL allergy (13 women and one man; mean age 46 years), who were diagnosed at the Skin and Allergy Hospital, Helsinki University Central Hospital, in 2003 participated in the study. Six of them were HCW and seven were other occupational NRL-glove users. Three patients had asthma, five allergic rhinitis and three atopic eczema. All patients had a positive skin prick test (SPT; weal diameter at least 3 mm and half of histamine control, when negative control was 0) to a commercial latex extract (Stallergenes, Antony Cedex, France) and a positive NRL-glove challenge test [6]. Ten healthy subjects (nine women and one man; mean age 42 years), seven of whom were HCW, served as controls. The study was approved by the ethics committee of Helsinki University Central Hospital.

Natural rubber latex allergens

NRL [13, 16] was subjected to buffer exchange by using a BioGelP6 desalting gel column (1.6 × 14 cm; Bio-Rad Laboratories, Hercules, CA, USA) in PBS.

Hev b 6.01 was purified from the B-serum of latex [17] by gel filtration and anion exchange chromatography without reversed phase chromatography [18]. Briefly, gel filtration was performed on a Superdex 75 HR 16/60 column (Amersham Biosciences, Uppsala, Sweden) in PBS. Prohevein-containing fractions were concentrated and the buffer was changed to 20 mM Tris-HCl, pH 8.5, by using a BioGelP6 desalting column. Anion-exchange chromatography was performed on a MonoQ column (HR5/5, 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.

Hev b 5 was purified from the C-serum of latex [17] by buffer exchange and cation exchange chromatography. C-serum was first subjected to buffer exchange by using a

BioGelP6 desalting gel column in 20 mM Na citrate, pH 3.8. Cation exchange chromatography was performed next on a MonoS 4.6/100 PE (Tricorn, Amersham Biosciences) column 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.

Commercial ELISA kits utilizing monoclonal antibodies to Hev b 5 and Hev b 6.02 (Fit Biotech Ltd, Tampere, Finland) were used to analyse the molecular identity of the purified NRL proteins. For purity check and quantification, the allergens were first subjected to reversed phase chromatography. For further confirmation of their identity and for additional molecular characterization, the allergens were subjected to molecular mass determination and peptide mass fingerprinting by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry [19, 20]. N-terminal sequencing was performed essentially as previously described [20].

Skin prick test and immunoglobulin E enzyme-linked immunosorbent assay

SPT was performed with a purified NRL extract (500, 50, 5 and 0.5 µg/mL in PBS), Hev b 6.01 (50, 5 and 0.5 µg/mL in PBS) and Hev b 5 (50, 5 and 0.5 µg/mL). Histamine (10 mg/mL; ALK, Copenhagen, Denmark) was used as a positive control, and PBS as a negative control.

IgE ELISA was performed as previously described [21]. Briefly, the concentration of Hev b 6.01 and Hev b 5 was adjusted to 2 µg/mL and that of NRL to 20 µg/mL for coating microtitre plates (100 µL/well). The wells were then post-coated with 1% human serum albumin (Red Cross Finland Blood Service, Helsinki, Finland) in 50 mM sodium carbonate buffer, and 100 µL of patient or control serum (diluted 1 : 10) was added. Biotinylated goat anti-human IgE (Vector Laboratories Inc., Burlingame, CA, USA; diluted 1 : 1000) was added, followed by streptavidin-conjugated alkaline phosphatase (Bio-Rad, Hercules, CA, USA; diluted 1 : 3000) and colour substrate (p-nitrophenyl phosphate, ICN Biomedicals Inc., Aurora, OH, USA). The optical density was measured at 405 nm with an automated ELISA reader (Titertek Multiscan, Eflab, Turku, Finland).

Proliferation of peripheral blood mononuclear cells

Proliferation assays with PBMC were performed in complete RPMI 1640 containing 5% heat-inactivated human AB serum as described earlier [22]. PBMC were isolated from heparinized venous blood by using Ficoll density gradient centrifugation and washed twice with PBS. PBMC proliferation was determined by stimulation of cells in triplicate (10⁵ cells/200 µL/well) alone or together allergens (NRL 100, 50, 10, 5, 1 µg/mL, Hev b 6.01 10, 5, 1 and 0.1 µg/mL, Hev b 5 10, 5, 1 and 0.1 µg/mL). Phyto-

haemagglutinin (PHA, 45 µg/mL; Murex Biotech Ltd, Dartford, UK) was used as a positive control. After 6 days of culturing, proliferation was measured by tritiated methyl-thymidine ([methyl-³H]-TdR, Amersham Biosciences, Little Chalfont, UK) incorporation. The results were expressed as stimulation indexes (SI; uptake of isotope in stimulated culture/uptake of isotope in non-stimulated control culture).

Cytokine and chemokine measurements

Induction of cytokines, chemokines and chemokine receptors was investigated in 10 NRL-allergic patients with high levels of antigen-specific IgE, as well as positive SPT and PBMC proliferative responses to NRL allergens. Of these 10 patients, nine had positive SPT responses to Hev b 6.01 and four to Hev b 5 (weal area > 9 mm²). Besides, two additional patients showed borderline SPT reactivity to Hev b 5 (weal area 7–9 mm²). Isolated and washed PBMC were stimulated in complete RPMI 1640 medium on 24-well plates (Costar, Corning Incorporated, Corning, NY, USA) in the presence or absence of Hev b 6.01 (10 µg/mL) or Hev b 5 (10 µg/mL). PHA (45 µg/mL) and *Staphylococcus aureus* enterotoxin B (superantigen, SEB; 1 µg/mL; Sigma-Aldrich Co. Ltd, Gillingham, UK) were used as polyclonal activators of PBMC. Each 1.5 mL well contained 3 × 10⁶ cells, the total number of cells per stimulation being 6 × 10⁶. Cell pellets were collected after 6 h of incubation and used for RNA isolation or flow cytometry analysis. Cell culture supernatants were obtained after 24 h of incubation and stored at –70 °C before measurement of cytokine or chemokine protein levels.

RNA extraction, synthesis of cDNA and real-time quantitative PCR with an AbiPrism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) were performed as described earlier [7, 16, 22]. Applied Biosystems, from where PCR primers and probes were purchased, also designed them. The results were expressed as relative units (RU), which were calculated by the comparative CT method [7].

Protein levels of TNF, IL-10 and CCL3 were assayed by commercial ELISA kits. TNF and IL-10 kits were from eBioscience Inc. (San Diego, CA, USA) and their sensitivity was 4 and 2 pg/mL, respectively. CCL3 kit was from R&D Systems (Minneapolis, MN, USA) and its lower detection limit was 8 pg/mL.

Flow cytometry

Three-colour flow cytometric analysis on T cell surface markers was performed with FACSCalibur (BD Biosciences, San Jose, CA, USA) using FACSComp software version 4.01 (BD Biosciences). Monoclonal antibodies (phycoerythrin-Cy5 (PE-Cy5)-conjugated anti-CD3, clone UCHT1; fluorescein isothiocyanate (FITC)-conjugated

anti-CD8, clone SK1; PE-conjugated anti-CXCR3, clone 1C6/CXCR3; and PE-conjugated anti-CCR4, clone 1G1) and corresponding isotype controls were purchased from BD Biosciences. We used CD8 expression to discriminate between T cell subtypes, as activation of human peripheral blood T cells can result in a virtually complete loss of the surface CD4 marker, but only in a partial loss of CD8 surface expression [23]. Furthermore, the vast majority of CD3⁺CD8[–] T cells are CD3⁺CD4⁺ T cells. In brief, 2 × 10⁵ PBMC were incubated with the fluorochrome-conjugated mAbs at +4 °C in the dark for 30 min; washed with staining buffer (0.45 µm filter-sterilized PBS with 0.1% bovine serum albumin and 0.1% Na-azide); and fixed in 1% paraformaldehyde (BD Biosciences). 20 000 events within a lymphocyte gate were collected. CD3⁺ cells within the lymphocyte gate were further gated to determine the number of chemokine receptor-positive cells within CD3⁺, CD3⁺CD8⁺ and CD3⁺CD8[–] populations. Data were subsequently analysed with BD CELLQuestPro software (BD Biosciences).

Statistics

A non-parametric Mann–Whitney *U*-test was used to compare the two groups using the GraphPadPrism Version 4 (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as means ± SEMs unless otherwise specified. A *P*-value < 0.05 was considered to be statistically significant.

Results

Analysis of the purity and identity of the allergens used for the studies

Both purified allergens Hev b 6.01 and Hev b 5 gave single peaks in reversed phase chromatography when monitored at the wavelength 214 nm. The integrated peak areas were also utilized to determine the allergen concentrations for the experiments. MALDI-TOF mass spectrometric analysis of Hev b 6.01 gave a single mass of 18 517 Da (and its double charged form), which is within the mass accuracy of the used instrument, in agreement with the molecular mass calculated from the sequence (O49860, residues 1–173) present in the databases. The identity of Hev b 6.01 was further confirmed by peptide mass fingerprinting after digestion with trypsin as well as with sequence analysis where it gave a pure N-terminal sequence EQCGRQAGGK. Analysis of Hev b 5 by MALDI-TOF mass spectrometry gave a single molecular mass of 15 952 Da (and its double-charged form), which corresponds to the molecular mass calculated from the sequence (Q39967) present in the databases. The identity of Hev b 5 was further confirmed by peptide mass fingerprinting after cleavage with trypsin. According to the database

information (Q39967), the N-terminal residue in Hev b 5 is acetylated, which prevents its Edman degradation. Our purified Hev b 5 preparation gave no signals in Edman degradation for 10 cycles, indicating that its N-terminus is blocked.

Skin prick test and immunoglobulin E enzyme-linked immunosorbent assay

All 14 patients showed positive (weal area ≥ 9 mm²) SPT to NRL (Fig. 1a), 12 (86%) to Hev b 6.01 and four (29%) to Hev b 5. In addition, two patients showed borderline positive SPT responses (weal area 7–9 mm²) to Hev b 5. In ELISA, 13 (93%) patients demonstrated increased IgE to NRL, 12 (86%) to Hev b 6.01 and three (21%) to Hev b 5 (Fig. 1b). All control sera were negative against all NRL allergens.

Proliferation of peripheral blood mononuclear cells

Proliferative responses of PBMC to Hev b 6.01 (10 μ g/mL) were significantly higher ($P < 0.01$) in NRL-allergic patients than in controls, whereas stimulation indexes against NRL and Hev b 5 showed no differences between the two groups (Fig. 1c). There was a strong proliferative response against PHA (patients: mean SI 82.674 + 34.58, $n = 14$; controls mean SI 79.40 + 13.76, $n = 10$) but no differences between responses in patients and controls were seen. Baseline levels of CPM varied from 32 to 1142.

Hev b 6.01 induces tumour necrosis factor, interleukin-13, interleukin-12p40 and interleukin-10 production but down-regulates transforming growth factor- β 1 expression

Stimulation of PBMC with Hev b 6.01, and to a lesser extent with Hev b 5, elicited a significantly stronger induction of TNF mRNA and protein in the NRL-allergic patients than in the non-allergic controls (Figs 2a and b). Both allergens also induced marked expression of IL-12p40

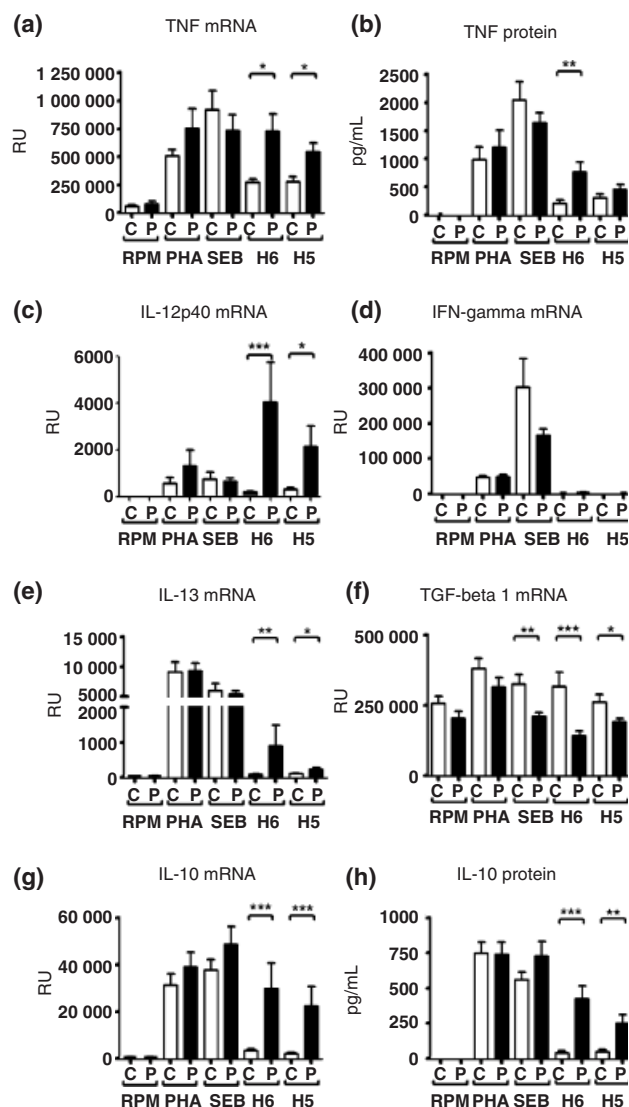


Fig. 2. Cytokine mRNA and protein expression in 10 NRL-allergic patients (P) and 10 control subjects (C). PBMC were cultured with medium (RPM), phytohaemagglutinin (PHA), *Staphylococcus aureus* enterotoxin B (SEB), Hev b 6.01 (H6) or Hev b 5 (H5). Relative units (RU) are relative differences compared with the calibrator. Results are expressed as mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

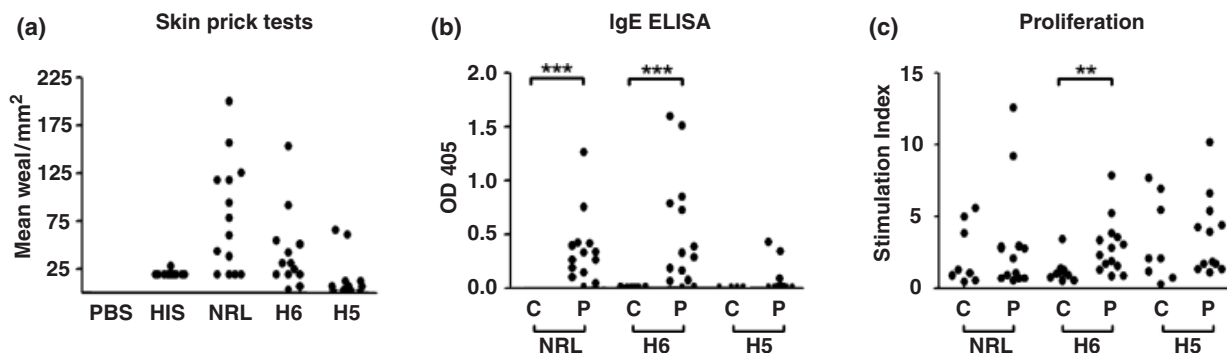


Fig. 1. (a) Skin prick test results with 50 μ g/mL of natural rubber latex (NRL), Hev b 6.01 (H6) and Hev b 5 (H5) among 14 NRL-allergic patients. Histamine (10 mg/mL, HIS) served as a positive control. (b) IgE antibodies against NRL, Hev b 6.01 and Hev b 5 in the sera of 14 patients (P) and 10 control subjects (C). (c) Proliferation of PBMC after stimulation with NRL (50 μ g/mL), H6 (10 μ g/mL) and H5 (10 μ g/mL); ** $P < 0.01$, *** $P < 0.001$.

mRNA in NRL-allergic patients (Fig. 2c). Hev b 6.01 elicited significant expression of the T-helper type 2 (Th2) cytokine IL-13 mRNA (Fig. 2e), whereas expression of the Th1 cytokine IFN- γ (Fig. 2d) remained at the baseline level. Interestingly, PBMC from NRL-allergic patients expressed significantly lower amounts of the regulatory cytokine TGF- β 1 mRNA (Fig. 2f) after allergen stimulation compared with the controls, whereas expression IL-10 mRNA and protein (Figs 2g and h) was up-regulated.

Polyclonal stimulators (PHA and SEB) elicited marked induction of several cytokines (i.e. TNF, IFN- γ , IL-13 and IL-10) in NRL-allergic patients as well as in non-allergic controls (Figs 2a, b, d, e, g and h). The only significant finding was that SEB stimulation down-regulated the expression of TGF- β 1 mRNA in the patients' PBMC (Fig. 2f).

Hev b 6.01 and Hev b 5 induce the expression of CCL3, CCL4 and CCL20 chemokines

Stimulation of PBMC with Hev b 6.01 and Hev b 5 elicited significantly stronger mRNA expression of the pro-inflammatory chemokines CCL3 (both mRNA and protein), CCL4 and CCL20 in NRL-allergic patients than in controls (Fig. 3). In contrast, expression of the Th1-type chemokine

CXCL10 mRNA was significantly induced only after polyclonal stimulation (PHA and SEB), and there were no differences in the expression levels between patients and the controls (Fig. 3e). Expression of the Th2-type chemokine CCL17 mRNA was enhanced to a lesser degree after Hev b 6.01 and Hev b 5 stimulation than after PHA or SEB stimulation (Fig. 3f). Levels of CCL17 mRNA were comparable in the patients and the controls (Fig. 3f).

Chemokine receptor expression on T cells

Surface expression of the chemokine receptors CCR4 and CXCR3 on CD3⁺CD8⁻ and CD3⁺CD8⁺ T cells did not change after different stimulations (Figs 4a and b). However, baseline expression of CCR4 on CD3⁺CD8⁻ T cells was significantly higher and that of CXCR3 on

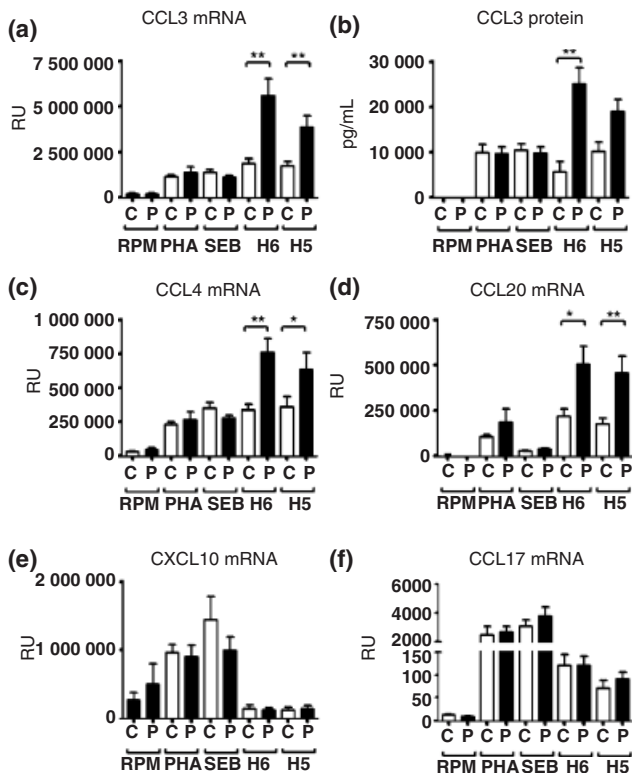


Fig. 3. Chemokine mRNA and protein expression in 10 NRL-allergic patients (P) and 10 control subjects (C). The symbols are the same as in Fig. 2.

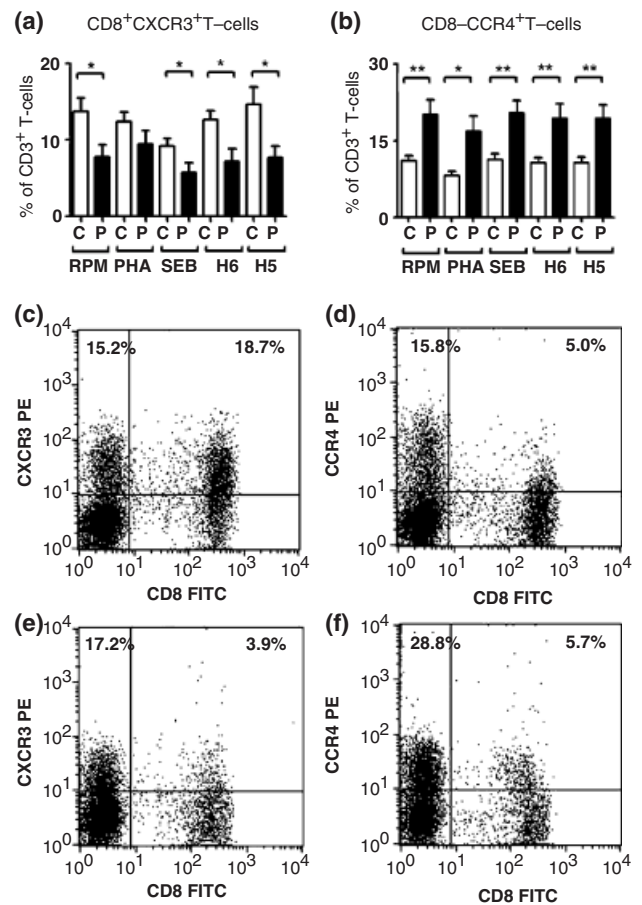


Fig. 4. Flow cytometric analysis of CXCR3 and CCR4 expression on T cells in 10 NRL-allergic patients (P) and 10 control subjects (C). Results are expressed as mean \pm SEM; * P < 0.05, ** P < 0.01, *** P < 0.001 (n = 10) (a, b). Representative plots of one control subject (c, d) and one NRL-allergic patient (e, f) after PBMC stimulation with Hev b 6.01. Lymphocytes were gated on the CD3⁺ cells and the percentage indicates the number of cells within the quadrant.

CD3⁺CD8⁺ T cells was significantly lower in the NRL-allergic patients than in the controls (Fig. 4).

Discussion

During the last few years, considerable progress has been made in the identification and molecular characterization of NRL allergens [12, 14, 15, 17, 18, 21]. A number of studies show that Hev b 6.01 (prohevein), Hev b 6.02 (4.7 kDa hevein domain of prohevein) together with Hev b 5, are major NRL allergens especially in HCW [13, 24]. In agreement with this, most of our patients (86%) showed positive SPT and IgE ELISA to Hev b 6.01. However, less than 30% of the patients were positive to Hev b 5. Similarly, proliferative PBMC responses to Hev b 6.01, but not to Hev b 5, were significantly greater in NRL-allergic patients than in non-allergic controls. Thus, our findings show that Hev b 6.01 is a more important NRL allergen than Hev b 5 in our NRL-allergic patients. It should be noted, however, that sensitization profiles against NRL allergens may vary between patient populations from different countries. In agreement with the present low Hev b 5 SPT and IgE positivity rate, our recently submitted paper (Palosuo *et al.* unpublished data) showed a low frequency of IgE to Hev b 5 (28%) in Finland as compared with Spain (49%) and USA (71%).

In order to examine allergen-specific cytokine profiles, PBMC were stimulated with NRL allergens and polyclonal stimulators. In general, there were no differences in the cytokine and chemokine profiles after polyclonal stimulation between NRL-allergic patients and normal subjects. However, cytokine and chemokine responses to allergen-specific PBMC stimulation differed significantly between these study groups. Hev b 6.01 and to a lesser extent Hev b 5 induced increased mRNA expression of the Th2 cytokine IL-13 [25].

The expression of pro-inflammatory cytokines TNF [26] (both in mRNA and protein level) and IL-12p40 [26, 27] was increased in the NRL-allergic patients compared with the controls. Induction of IL-12p40 mRNA was markedly higher after allergen specific than polyclonal stimulation. Interestingly, there were no detectable levels of IFN- γ mRNA after allergen stimulation, although it has been convincingly demonstrated that IL-12 is the major inducer of this Th1 cytokine [27]. Recent studies suggest that atopic patients produce less IFN- γ after IL-12 stimulation as compared with non-atopic subjects [28], and other studies show that Th2 cell differentiation occurs in the presence of IL-12 signalling [29]. Thus, many studies support the concept that while IL-12-dependent activation is required for Th1 responses, activation of this pathway alone is not sufficient to repress or reverse Th2 responses [29–31]. It is therefore possible that due to the allergic status of the patients, IFN- γ production is inhibited, although significant amounts of IL-12p40 are

expressed in response to NRL allergen activation. These results emphasize the importance of TNF and IL-12/IL-23 in the induction of NRL-specific immune responses in addition to Th2-skewed responses.

To address the role of regulatory cytokines in the induction of NR-specific immune responses, we analysed the expression of IL-10 and TGF- β 1, both of which are important suppressors of immune responses [32–34]. Decreased mRNA expression of TGF- β 1 in NRL-allergic patients after allergen and SEB stimulation suggests that this cytokine participates in the cellular responses in NRL allergy. On the contrary, allergen-induced expression of IL-10 (both in mRNA and protein level) was significantly increased. Increased levels of IL-10 in NRL-allergic patients may suppress the development of a Th1 response and thus facilitate the development of a Th2-dominated-allergic response. In line with this hypothesis, we demonstrated recently using IL-10-deficient mice that both dendritic cell-derived and T cell-derived IL-10 plays a critical role in the promotion of a Th2 response and cutaneous eosinophilia in a murine model of allergic dermatitis [35].

We also studied the induction of several CC chemokines, which critically regulate the recruitment of lymphocytes to inflammation sites [36, 37]. Expression of pro-inflammatory chemokines CCL3, CCL4 and CCL20 was enhanced after Hev b 6.01 and Hev b 5 stimulation. Polyclonal activation of PBMC induced much lower production of these chemokines, and there were no differences between patients and controls. Recent findings show that CCL3 and CCL4 also have other roles than to attract inflammatory cells. It has been suggested that CCL3 is needed for optimal mast cell degranulation [38] and both CCL3 and CCL4 can co-stimulate T cell responses [39, 40]. Interestingly, there were no significant differences in the mRNA expression of either the Th2 type chemokine CCL17 or the Th1 type chemokine CXCL10 between patients and controls after allergen stimulation. Together, these results show that pro-inflammatory chemokines, rather than Th1 or Th2 chemokines, are differentially expressed in allergen-stimulated PBMC of patients and controls, and may therefore play a role in the pathogenesis of NRL allergy.

It is of interest that both NRL allergens used in the present study induced clear-cut cytokine and chemokine responses in the patients' PBMC, although SPT and IgE reactivity were lower against Hev b 5 than Hev b 6.01. It is well known that cell-mediated responses are not always directly correlated with IgE-mediated responses. Therefore, patients who do not exhibit detectable IgE reactivity against a particular allergen may still demonstrate cell-mediated reactivity to the same allergen as seen in the present study. It should be noted, however, that reactivity against Hev b 6.01 was in general stronger compared with the reactivity against Hev b 5.

Th1 and Th2 cells differ in their chemokine receptor expression and their responsiveness to various chemokines [36]. We therefore studied the expression of Th1 (CXCR3) and Th2 (CCR4) type chemokine receptors on the surface of circulating T lymphocytes. No changes in the amounts of these receptors were found after any stimulation. However, CCR4 expression was constitutively higher on the patients' CD3⁺CD8⁻ T cells (most of which are CD3⁺CD4⁺ T cells), whereas CXCR3 expression was constitutively lower on their CD3⁺CD8⁺ T cells. In line with these results, it has been observed that CCR4 expression on peripheral blood CD4⁺ T cells is enhanced especially in severe atopic dermatitis [41], and that levels of CXCR3 transcripts are decreased in PBMC from such patients [42]. These results suggest that differences in the expression of CCR4 and CXCR3 on circulating T cells may have an impact on the development and maintenance of NRL allergy.

In conclusion, the present results demonstrate that allergen-specific stimulation of PBMC induces clear-cut differences in the cytokine and chemokine profiles of NRL-allergic patients and non-allergic control subjects. It is of interest that pro-inflammatory cytokines and chemokines in particular are predominantly expressed after Hev b 6.01 and Hev b 5 stimulation and that regulatory cytokines also seem to be involved in NRL-specific immune responses. These findings may reflect the molecular mechanisms involved in the development and maintenance of NRL allergy and may open new avenues for disease prevention.

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Latex allergy: low prevalence of IgE to highly purified Hev b 2 and Hev b 13

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Running head: Low prevalence of IgE to Hev b 2 and Hev b 13

Abstract

Background: Hev b 2 and Hev b 13 have been recently identified as major latex allergens by detecting specific IgE antibodies in >50% of sera from *Hevea brasiliensis* 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 used in ELISAs to assess sera from 215 latex-allergic patients and 172 atopic non-sensitized controls from Finland, Spain and USA 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 to 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. 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

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 [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 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 (Table I). For the Finnish patients, the diagnosis of NRL allergy was based on a compatible clinical history, positive SPT (Stallergenes, Antony Cedex, France, and/ or high-allergen glove extracts), and a positive IgE antibody serology (latex- ImmunoCAP, Phadia, Uppsala, Sweden). Descriptions of patients and control subjects from Spain and the USA have been reported in detail elsewhere [8, 19]. This study was approved by local ethics committees in Finland (Tampere University Hospital and National Public Health Institute), Spain (Hospital Universitario de Gran Canaria Dr. Negrín) and USA (the Research and Ethics Committees of the Johns Hopkins Medical Institutions).

Purification of NRL 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, 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].

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 1, A). Elution was performed using a linear gradient of NaCl (0 to 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 1, A) were pooled and 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 1, B). 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 1, B) 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 to 1 M in 30 minutes) in the equilibration buffer.

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 phosphate buffered saline. Prohevein-containing fractions were concentrated and the buffer was changed to 20 mM Tris-Cl, 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-Cl, pH 8.5. Elution was performed with a linear gradient of NaCl (0 to 1 M in 30 minutes) 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 1, D). 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 BioGelP6DG column.

Hev b 13 containing fractions were applied to a cation exchange column, MonoS (HR 5/5, Amersham Biosciences) (Fig 1, *E*). Elution was performed using a linear gradient of NaCl (0 to 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 1, *E*).

The purity of all four allergen preparations was ascertained by reversed-phase chromatography on a C1 column (2x20mm, 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 (TFA). 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 Fig. 1 *C* and *F*, respectively.

Identification and characterization of the purified allergens

Monoclonal antibodies to the four allergens and commercial ELISA kits 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 masses and peptide mass fingerprint analyses were performed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and electrospray quadrupole time-of-flight (Q-TOF) mass spectrometric analyses [21]. N-terminal sequencing was performed as previously described [22].

IgE ELISA

IgE antibodies to Hev b 5 and Hev b 6.01 were measured by ELISA as previously described [23]. Briefly, Hev b 6.01 and Hev b 5 were separately coated on microtiter 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; diluted 1:1000) was added, followed sequentially by streptavidin-conjugated alkaline phosphatase (Bio-Rad, Hercules, CA; diluted 1:3000) and substrate (p-nitrophenyl phosphate, ICN Biomedicals Inc., Aurora, Ohio). When internal pooled control sera had reached predetermined OD-values, the optical density 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 solid-phase antigens.

Data analysis

Nonparametric statistics (Mann-Whitney U-test) were used to perform statistical tests because the majority of OD values were not normally distributed. A *P* value of 0.05 or less was considered a statistically significant difference between groups. Correlation between individual serum IgE antibody levels to the various allergens was assessed by a nonparametric Spearman *r* test.

Results

Identification and assessment of the purity of Hev b 2 and Hev b 13 preparations

The Hev b 2 preparation used in this study for serological evaluation was shown to be homogeneous by reversed-phase chromatography (Fig 1, C).. 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 analyzed by reversed-phase chromatography or peptide mass fingerprint analyses in this final 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 ETCDFPA, 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 40313.2 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 3 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 analyzed due to its low recovery and large size.

Prevalence of IgE antibodies to four purified NRL allergens in three different 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 USA (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 USA (11%). The prevalence of IgE to Hev b 13 ranged between 18 to 30% in all three geographic regions, suggesting a relatively low overall frequency of sensitization to this allergen. Prevalence of IgE antibodies to Hev b 5 was unexpectedly low in Finland (28%), which contrasted with 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 optical density 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 highest mean anti-Hev b 6.01 level was present in the sera from the Spanish patients. 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 lowest mean anti-Hev b 6.01 level was present in American sera.

IgE binding to solid-phase Hev b 13 can be inhibited by adding soluble Hev b 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. Fig 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 carried out for 8 sera chosen for their moderate 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 µg/ml produced a 2-4% inhibition of the binding of IgE to solid-phase Hev b 13. Two other sera mimicked Figure 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 as 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. 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 [8]. Kurup et al. used similar allergen preparations in serological assays and reported that ~70 to 80% of NRL allergic adults had IgE antibodies reactive with Hev b 2 and Hev b 13 [10]. Importantly, the Hev b 13 that had been produced in *E.coli* by recombinant DNA technology did not bind human IgE [18]. Since 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 healthcare workers [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 skin prick tests 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 prior to 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 analyzed using a commercial capture enzyme immunoassay (FIT kit, FIT Biotech Ltd).

One may argue that the extensively purified allergen preparations were devoid of *in vivo* reactivity and therefore unsuitable for IgE prevalence assessment. This, however, was shown to not be the case since skin prick tests carried out to eight Finnish NRL-allergic individuals (at 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 wheal at least 3 mm). Three of the eight patients were positive for Hev b 5 and six for Hev b 6.01. Among the four Hev b 13-positive patients, one was negative to Hev b 6.01 and to other allergens tested, indicating the presence of a pure Hev b 13-specific reaction.

During the purification process of Hev b 13, 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 the final 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 other structures 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 significant 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 chose to use the 98th percentile of local atopic controls to define positive threshold for the presence of Hev b allergen reactive IgE antibody. This approach enables one to control for possible differences associated with variability resulting from the local geographic background 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 for 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 total inhibition, it is likely that the serum does not contain IgE antibodies that are specific anti-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 suggested 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 analyzed by immunoblotting after two-dimensional gel electrophoresis [30].

Since 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 number of anti-Hev b 13 positive sera was low, this was indeed the case. In the whole patient series (n=215), the correlation coefficient (r) between the presence of Hev b 6.01 and Hev b 13 reactive antibodies as assessed by the Spearman rank test was 0.517 ($p < 0.0001$). Alternatively, no correlation was observed between 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 as an ELISA coating antigen in the present study and previously with immunoblotting [31], we have been able to verify markedly lower prevalence rates for 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 in across three different geographic areas. Among the Spanish patients, the prevalence of IgE to Hev b 2 and Hev b 13 was low (5% and 30%, respectively).

While the present study focused on the significance of Hev b 2 and Hev b 13 as NRL allergens in three geographical areas, we also included a comparable analysis of prevalence rates for Hev b 6.01 and Hev b 5 which are known to be major NRL allergens. Prevalence of IgE to Hev b 5 was unexpectedly low (28%) in Finland while it was higher in Spain (49%) and the USA (71%). In contrast, IgE to Hev b 6.01 was high (54-74%) across all three geographic areas. The reasons for the different sensitization rates may be due to different properties of NRL gloves used in these countries, and differences in glove usage habits. We noted significant differences also in the relative levels of IgE antibodies specific for the four Hev b allergens (Fig 4). These may reflect local differences in exposure to these and other possibly cross-reactive allergens. Differences may exist in patient selection and demographics in the three series of specimens.

In conclusion, the low frequency of IgE to exhaustively purified Hev b 2 and Hev b 13 in NRL-allergic patients from three different geographic areas suggests that the rates of sensitization to these allergens are relatively low in comparison to previous reports. This observation implies that 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, production of these allergens in immunologically active form by recombinant DNA technology is needed to ultimately assess their clinical significance in NRL allergy.

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Legends for the Figures and the Table

Table 1. Demographic properties of NRL-allergic patients and control subjects (NRL, natural rubber latex; HCW, healthcare workers).

Figure 1. Purification steps and confirmation of purity by reversed-phase chromatography of Hev b 2 (A, B and C) and Hev b 13 (D, E and F) proteins.

Figure 2. Dose-dependent binding of a murine monoclonal anti-Hev b 6.02 antibody (diluted 1:1000) onto Hev b 13 coated (1 µg/ml) microtiter wells. The binding of the antibody to wells coated with purified Hev b 6.02, (1µg/ml) is shown for comparison.

Figure 3. Distribution of IgE antibodies to Hev b 2, Hev b 5, Hev b 6.01 and Hev b 13 in three patient populations. 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.

Figure 4. Comparison of mean IgE antibody levels to Hev b 2, Hev b 5, Hev b 6.01 and Hev b 13 in three patient populations. Number of patients in Finland, Spain and USA were 79, 74 and 62, respectively. The columns and the error bars represent means ± SEMs. * p<0.05, ** p<0.01, *** p<0.001, ns = not significant.

Figure 5. Illustration of patterns of competitive inhibition of IgE binding to solid-phase Hev b 13 (1 µg/ml) with soluble Hev b 6.01 and Hev b 13. Sera from three anti-Hev b

13- positive NRL-allergic patients were analyzed. Panel A denotes no inhibition, Panel B, partial inhibition and, Panel C, total inhibition by the presence of soluble Hev b 6.01.

	Finland		Spain		USA	
	Patients	Controls	Patients	Controls	Patients	Controls
No. 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

Table 1.

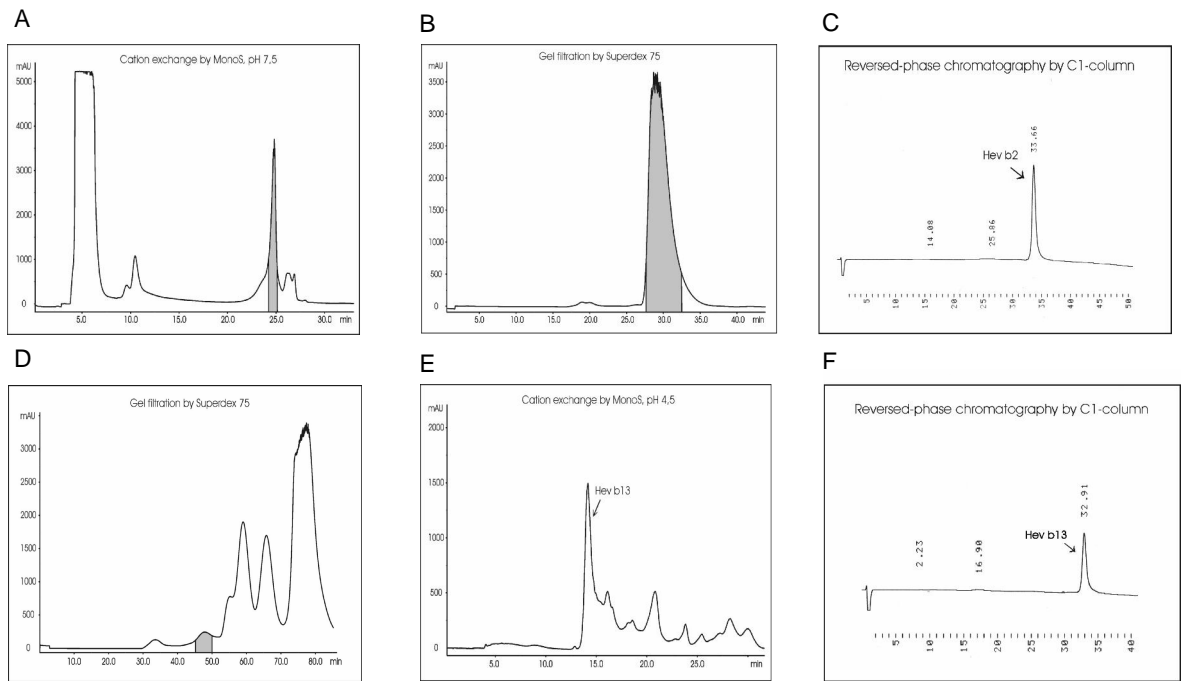


Figure 1.

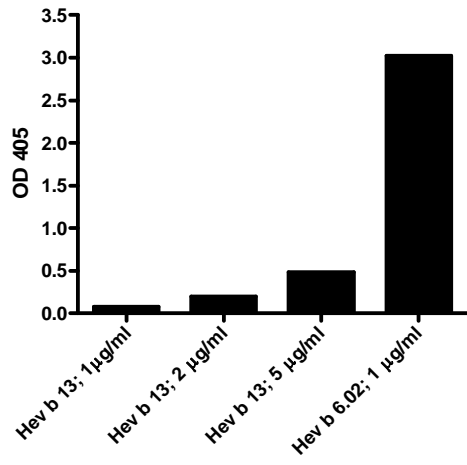


Figure 2.

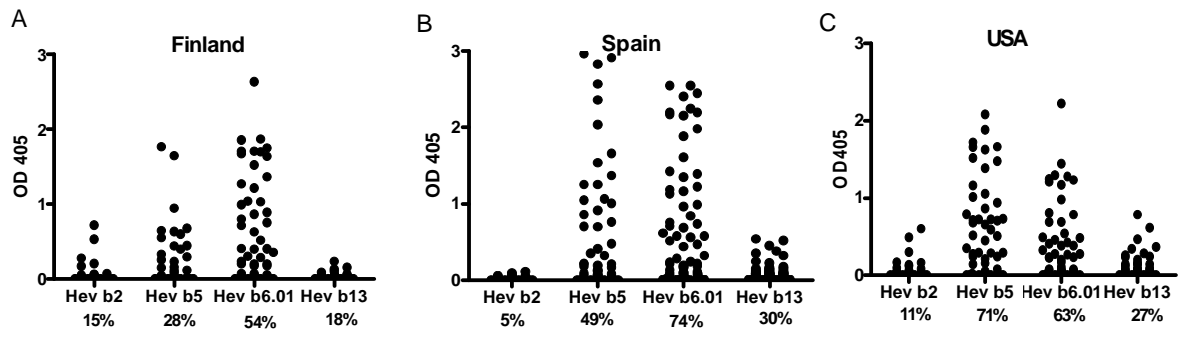


Figure 3.

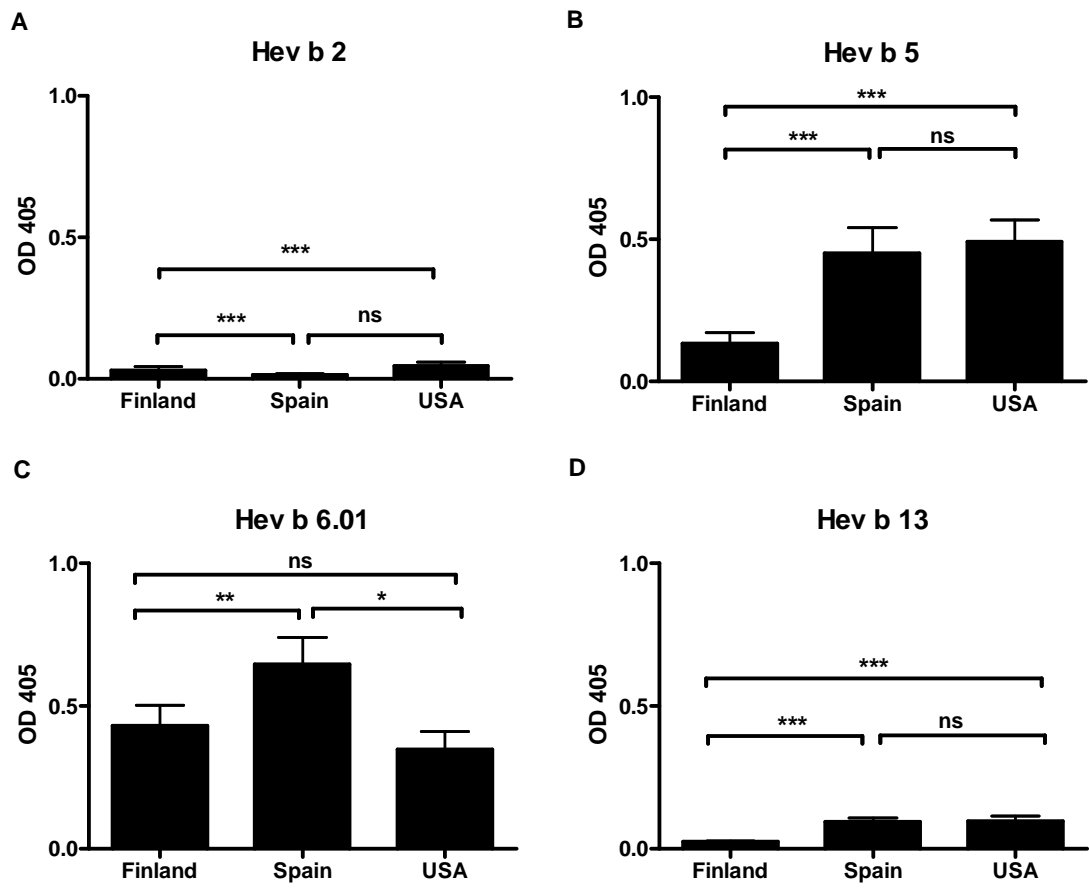


Figure 4.

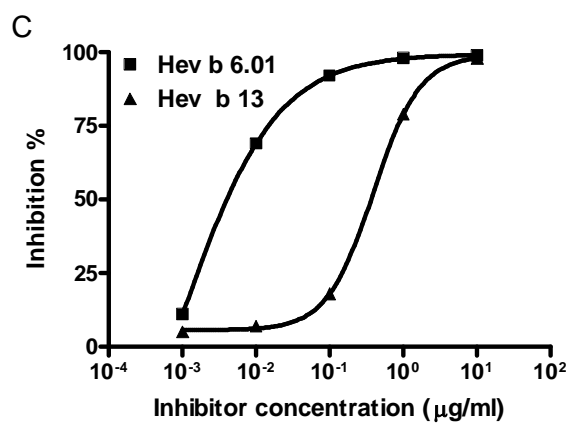
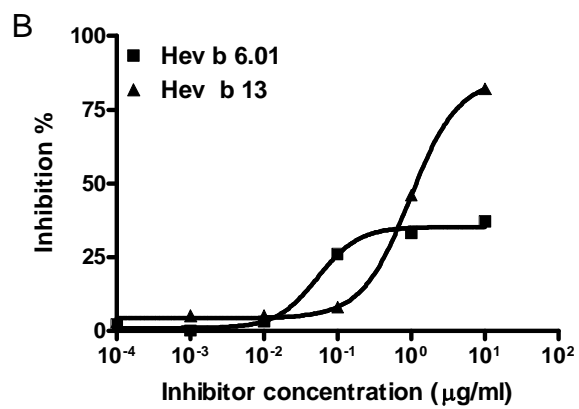
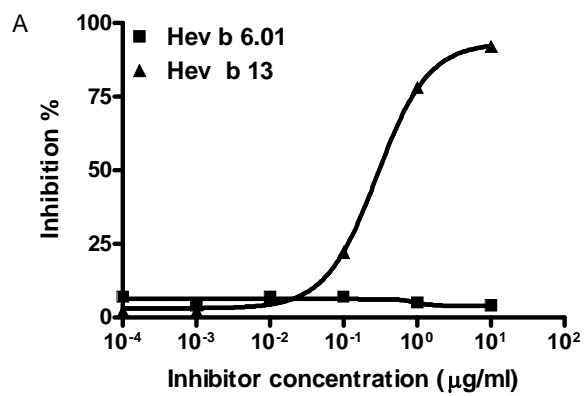


Figure 5.