

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

Manuscript received July 8, 2002; revised October 8, 2002; accepted for publication November 11, 2002

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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 oxacalcitriol 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 Labortechnik, 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 (eotaxin-2), CCL17 (TARC), CCL27 (CTACK)]. **Table 1** 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 $\rightarrow$ 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

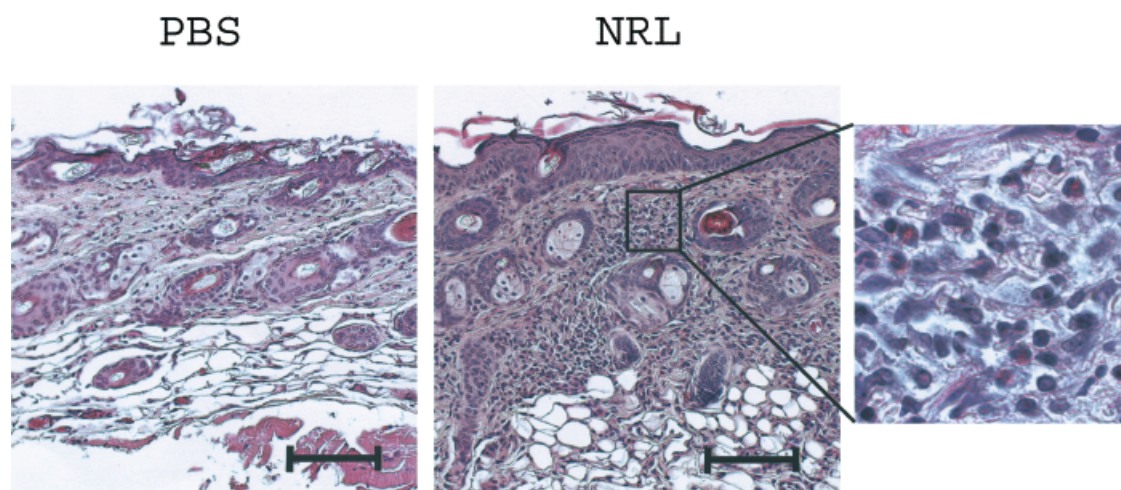


Figure 1. Histologic features of epicutaneous sensitized skin sites in BALB/C mice. Marked infiltration of inflammatory cells can be seen in NRL-sensitized skin sites compared with PBS-treated sites. Scale bar = 100 μ m.

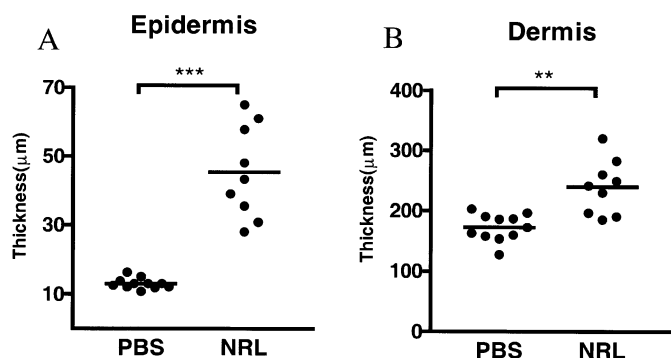


Figure 2. Thickness of epidermal and dermal layers of mice skin after epicutaneous sensitization with NRL and PBS. A significant 3.5-fold thickening of epidermis (A) and a 1.5-fold thickening of dermis (B) were observed in NRL-sensitized sites. ** $p < 0.01$, *** $p < 0.001$.

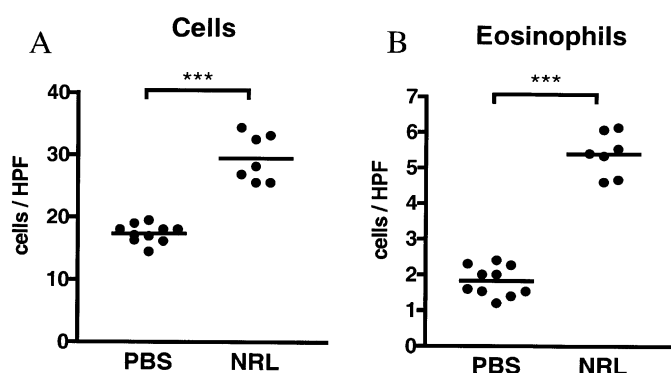


Figure 3. Infiltration of total inflammatory cells (A) and eosinophils (B) in skin after epicutaneous sensitization with NRL and treatment with PBS. The skin infiltrating cells were counted in 15–20 HPF at $\times 1000$ and expressed as cells per HPF. *** $p < 0.001$.

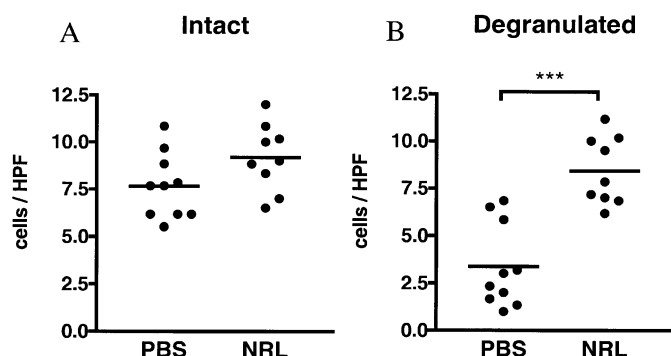


Figure 4. The number of mast cells in epicutaneous sensitized skin sites. (A) The number of intact mast cells and (B) degranulated mast cells in NRL-sensitized and PBS-treated skin sites. The skin infiltrating mast cells were counted in 15–20 HPF at $\times 400$ and expressed as cells per HPF. *** $p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

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

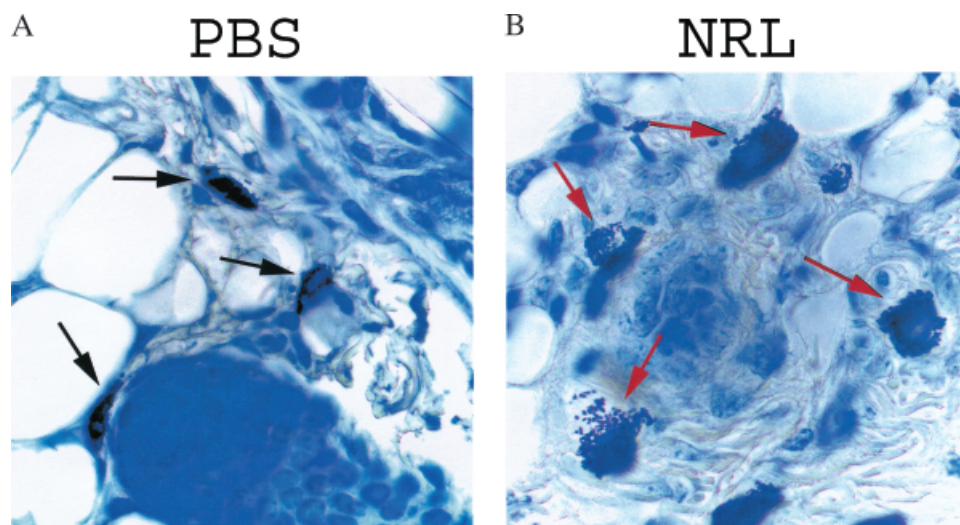


Figure 5. Morphologic features of mast cells after epicutaneous sensitization with NRL and PBS. (A) Intact mast cells (black arrows) in the PBS-treated skin site. Densely stained granules are seen within the cell membrane. (B) Degranulated mast cells (red arrows) in the NRL-treated skin site. Some granules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

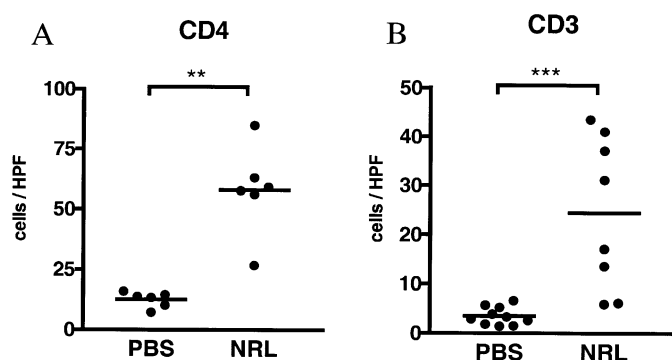


Figure 6. The number of CD4-positive cells (A) and CD3-positive cells (B) in mice skin sensitized with NRL and treated with PBS. The CD3- and CD4-positive cells were counted in 10 HPF at $\times 1000$ and expressed as cells per HPF. ** $p < 0.01$, *** $p < 0.001$.

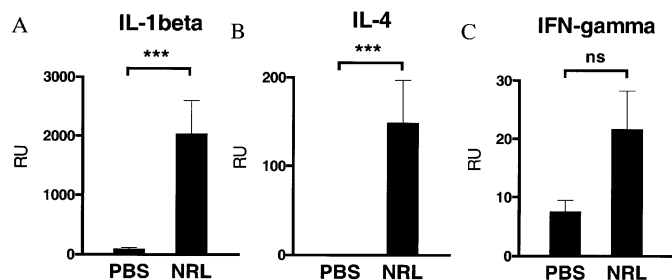


Figure 7. Real-time, quantitative reverse transcription-PCR analysis of IL-1 β (A), IL-4 (B), and IFN- γ (C) mRNA expression levels in skin biopsies of BALB/C mice after epicutaneous sensitization with NRL or PBS. RU are expressed as fold differences relative to the calibrator. The columns and error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

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

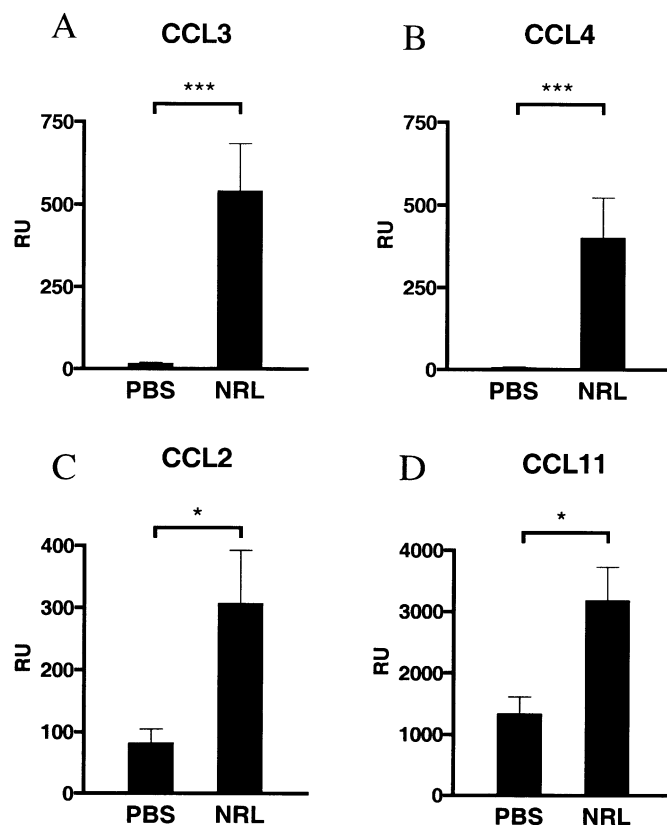


Figure 8. Real-time, quantitative reverse transcription-PCR analysis of CCL3 (A), CCL4 (B), CCL2 (C), and CCL11 (D) mRNA expression levels in skin biopsies from BALB/C mice after epicutaneous sensitization with NRL or PBS. RU are expressed as fold differences relative to the calibrator. The columns and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

(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

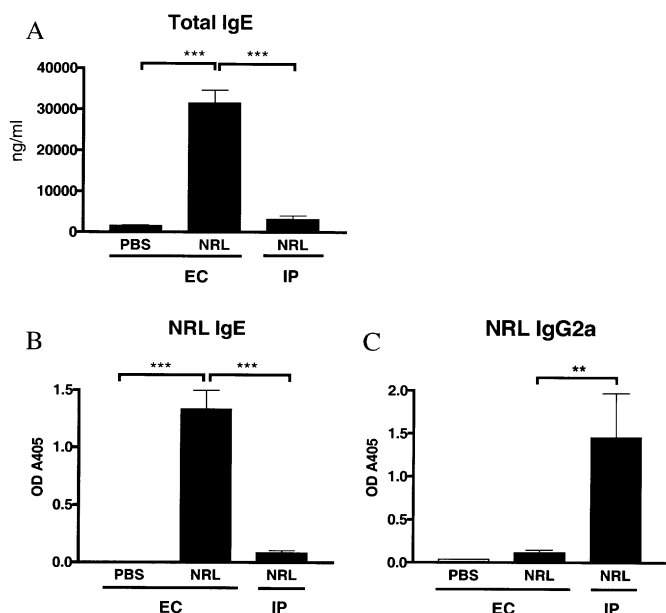


Figure 9. Total IgE and NRL-specific antibody responses in BALB/C mice after epicutaneous or IP sensitization with NRL or treatment with PBS. (A) Total serum IgE levels. (B) NRL-specific IgE serum levels. (C) NRL-specific IgG2a serum levels. Specific IgE and IgG2a antibody levels of the sample are expressed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

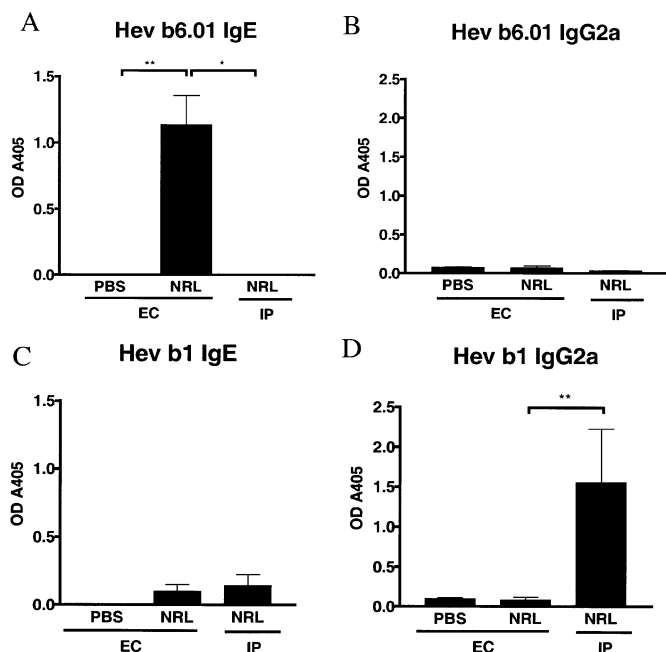


Figure 10. Hev b6.01 and Hev b1 specific antibody responses in BALB/C mice after epicutaneous or IP sensitization with NRL or PBS. (A) Hev b6.01-specific IgE serum levels. (B) Hev b6.01-specific IgG2a serum levels. (C) Hev b1-specific IgE serum levels. (D) Hev b1-specific IgG2a serum levels. Specific 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.

This study was supported by grants from the Academy of Finland (grant numbers 37852 and 50809). We thank Sari Tillander, Helena Honkasalo, and Tiula Stjernvall for their expert and excellent technical assistance.

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