

Mutual Boosting Effects of Sensitization with Timothy Grass Pollen and Latex Glove Extract on IgE Antibody Responses in a Mouse Model

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Type I allergy to natural rubber latex can be an important health problem for latex-exposed individuals (e.g., health care workers, spina bifida children). Also beyond these risk groups, a high sensitization rate of varying and partly unknown clinical relevance has been reported. Atopy represents a risk factor for latex allergy and recent studies indicate that patients suffering from pollen allergies may have pollen allergen-specific IgE antibodies which cross-react with latex allergens. In order to investigate whether sensitization to pollen allergens can have priming effects on the production of IgE antibodies against latex *in vivo*, a mouse model was established. Groups of 10 BALB/C mice were immunized with Al(OH)₃-adsorbed pollen extracts from timothy grass, ragweed, mugwort, or birch. For control purposes, one additional group received adjuvant only and another group was not immunized. Half of the mice of each group were subsequently immunized with Al(OH)₃-adsorbed latex

glove extract, the other half with adjuvant only. Pollen and latex-specific IgE- and IgG1-antibody responses were analyzed by enzyme-linked immunosorbent assay and statistically evaluated by analysis of variance. Antibody responses to cross-reactive antigens were investigated by immunoblotting. We found significantly increased IgE reactivities to latex after pollen sensitization and vice versa. Moreover, mice immunized with timothy grass pollen extract alone – without subsequent latex immunization – displayed IgE reactivity to latex. Cross-reactive antibodies were directed against pollen antigens of approximately 60 kDa molecular weight. Our results thus demonstrate a mutual boosting effect of pollen and latex sensitization *in vivo* which may be also operative in polysensitized plant allergic patients. **Key words:** atopy/boosting effect/cross-reactivity/IgE/latex allergy/mouse-model/pollen allergens. *J Invest Dermatol* 114:1039–1043, 2000

Type I allergy to natural rubber latex represents an important health problem in certain risk groups which are frequently exposed to latex products. Approximately 10% of health care workers (Liss *et al*, 1997) and 35% of children with spina bifida (Niggemann *et al*, 1998) suffer from latex allergy. Sensitization to latex, however, is also frequently observed beyond these risk groups. In a group of 1000 blood donors 6.5% were found to have IgE antibodies against latex (Ownby *et al*, 1996) and 25% of 493 patients consecutively attending an allergy clinic were sensitized to latex (Ruëff *et al*, 1998). A series of serologic studies indicates that tropical fruits, vegetables, and latex contain cross-reactive allergens (Alenius *et al*, 1996; Brehler *et al*, 1997; Breiteneder and Scheiner, 1998; Chen *et al*, 1998; Moller *et al*, 1998). Recently, it was also reported that cross-reactive allergens are present in latex products

and respiratory allergen sources (i.e., pollens of grasses and weeds) (Fuchs *et al*, 1997). Although the prevalence for pollen sensitization is high in the general population in industrialized countries (Nielsen *et al*, 1994; Storm van's Gravesande *et al*, 1997) and atopy has been described as risk factor for latex allergy (Liss *et al*, 1997; de Groot *et al*, 1998; Field, 1998; Niggemann *et al*, 1998; Rueff *et al*, 1998), little is known about the clinical relevance of IgE cross-reactivity between pollen and latex allergens. It is also unknown whether *in vivo* sensitization to pollen allergens may represent a priming factor for the development of IgE antibodies which cross-react with latex and *vice versa*. In order to examine the mutual influence of pollen and latex sensitization on IgE production *in vivo*, we established a mouse model. BALB/C mice were first immunized with Al(OH)₃-adsorbed pollen extracts (timothy grass, ragweed, mugwort, or birch) or for control purposes received adjuvant only or no immunization. Subsequently, half of the animals received Al(OH)₃-adsorbed latex glove extract, the other half received adjuvant only. IgE and IgG1 antibody reactivities to latex glove and pollen extracts were measured in all groups and statistically evaluated to determine whether latex and pollen sensitization can exert mutual boosting effects on antibody production. By immunoblotting we searched for cross-reactive allergens in latex and pollen extracts. Our finding

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that pollen and latex sensitization can act as mutual priming factors for the production of cross-reactive antibodies is discussed as a possible pathogenetic factor for combined pollen/latex allergy.

MATERIALS AND METHODS

Allergen extracts

Pollen Pollen from timothy grass (*Phleum pratense*), ragweed (*Ambrosia elatior*), mugwort (*Artemisia vulgaris*), and birch (*Betula verrucosa*) were purchased from Allergon AB (Välinge, Sweden). Aqueous pollen protein extracts were prepared by homogenization of 2 g of each pollen in 100 ml of distilled water using an Ultra-Turrax T25 (IKA Labortechnik, Heidelberg, Germany) for 45 s and subsequent shaking for 1 h at room temperature (Vrtala *et al*, 1993). Extracts were centrifuged at $10,000 \times g$ to remove insoluble debris and the supernatants were lyophilized in 1 ml aliquots. The lyophilized extracts were stored at -20°C until use.

Latex glove Latex examination gloves (Meditrade, Kufstein, Austria) previously shown to contain a variety of allergens (Mahler *et al*, 1999) were used to prepare latex allergen extracts. Gloves were cut into pieces of approximately 1 cm^2 and extracted in distilled water under continuous shaking (260 r.p.m.) at 37°C for 2 h. Extracts were centrifuged at $4000 \times g$ for 30 min at 4°C to remove insoluble particles. Aliquots of 30 ml glove extract were lyophilized and stored at -20°C until use. The protein content was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fling and Gregerson, 1986) and Coomassie Blue staining (Bradford, 1976). Protein determinations were performed using the Micro BCA assay (Pierce, Rockford, IL).

Immunization of mice Sixty female 8 wk old BALB/C mice were purchased from Charles River (Kislegg, Germany). The animals were maintained in the animal care unit of the Department of General and Experimental Pathology of the University of Vienna according to the local guidelines for animal welfare. Six groups consisting of 10 mice each were subcutaneously immunized in the neck twice in 4 wk intervals with either pollen allergen extract (timothy grass, ragweed, mugwort, or birch pollen), adjuvant only or did not receive any immunization (Fig 1) (Vrtala *et al*, 1996). Each injection contained $10\text{ }\mu\text{g}$ allergen extract dissolved in $20\text{ }\mu\text{l}$ phosphate-buffered saline (PBS) adsorbed to $200\text{ }\mu\text{l}$ 2% (i.e., $4\text{ }\mu\text{g}$) $\text{Al}(\text{OH})_3$ (AluGel-S^R, Serva, Heidelberg, Germany). In the adjuvant control group $200\text{ }\mu\text{l}$ $\text{Al}(\text{OH})_3$ containing $20\text{ }\mu\text{l}$ PBS was administered. After this pretreatment, each group of mice was split into two subgroups consisting of five animals each. In 4 wk intervals, subsequently the subgroups received four immunizations with either $\text{Al}(\text{OH})_3$ -adsorbed latex extract ($10\text{ }\mu\text{g}$ adsorbed to $200\text{ }\mu\text{l}$ AluGel-S^R for each injection) or adjuvant alone (Fig 1). Blood samples were obtained from tail veins at the time of immunization (Fig 1).

Detection antibodies Purified monoclonal anti-mouse IgE and anti-mouse IgG1 from rat were purchased from PharMingen (San Diego, CA). Horseradish peroxidase and ^{125}I -labeled anti-rat Ig antibodies from sheep were obtained from Amersham Pharmacia Biotech Europe GmbH (Vienna, Austria).

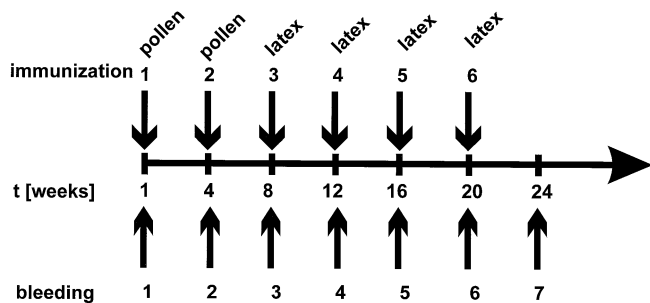


Figure 1. Scheme of the immunization and bleeding protocol. Four groups of 10 mice each were immunized in 4 wk intervals twice with pollen extracts from timothy grass, ragweed, mugwort, or birch, whereas one group received adjuvant only and one group remained untreated. Subsequently, half of each group was immunized four times in 4 wk intervals with latex extract or with adjuvant alone. Blood samples were obtained briefly before the first immunization (i.e., preimmune serum) and briefly before each consecutive treatment at weeks 4, 8, 12, 16, and 20 as well as at week 24.

Enzyme-linked immunosorbent assay (ELISA) measurements

Mouse IgE and IgG1 antibodies with specificity for pollen and latex antigens were detected by ELISA as previously described (Vrtala *et al*, 1996, 1998). In brief, ELISA plates (Nunc-Maxisorb, Nunc, Roskilde, Denmark) were coated with $100\text{ }\mu\text{l}$ per well protein extracts (PBS containing $10\text{ }\mu\text{g}$ timothy grass, ragweed, mugwort, birch-pollen or latex glove extract) overnight at 4°C . Plates were washed twice with $200\text{ }\mu\text{l}$ PBS, 0.05% vol/vol Tween and incubated with $200\text{ }\mu\text{l}$ blocking solution (PBS, 0.05% vol/vol Tween, 1% wt/vol bovine serum albumin) for 2.5 h at room temperature. Plates were then exposed to mouse sera ($100\text{ }\mu\text{l}$ per well diluted 1:20 for IgE measurements and 1:1000 for IgG1 measurements in PBS, 0.05% vol/vol Tween, 0.5% wt/vol bovine serum albumin) overnight at 4°C . After five washes with $200\text{ }\mu\text{l}$ PBS, 0.05% vol/vol Tween, bound mouse IgE and IgG1 antibodies were detected with monoclonal anti-mouse IgE or IgG1 antibodies from rat, which were diluted 1:1000 in PBS, 0.05% vol/vol Tween, and 0.5% wt/vol bovine serum albumin overnight at 4°C . Plates were washed five times with $200\text{ }\mu\text{l}$ PBS, 0.05% vol/vol Tween and bound rat antibodies were detected with a peroxidase-coupled anti-rat Ig anti-serum from sheep and visualized with ABTS substrate (Sigma, St Louis, MO). The optical densities (OD) corresponding to the amounts of bound antibodies were determined at 405 nm in an ELISA reader (Dynatech MR 7000, Guernsey, Channel Islands). OD values for the individual plates were determined after the same incubation periods. Quality controls included confirmation of specificity by determination of specific IgE for recombinant allergen molecules as described (Vrtala *et al*, 1996, 1998) using the same reagents and reference sera. Possible plate to plate variabilities were excluded by including reference sera on each of the different plates. All measurements were performed as duplicates which did not differ from each other more than 5%. The results analyzed represent means of duplicate determinations.

As reference for the baseline (cut off) for specific IgE production in Figs 2 and 3 served the adjuvant group which had not received latex.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Protein extracts ($10\text{ }\mu\text{g}$ per lane) were separated by analytical 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fling and Gregerson, 1986) and visualized by silver staining (Sammons *et al*, 1981) and Coomassie Blue staining (Bradford, 1976). A Rainbow Marker (Amersham, Buckinghamshire, U.K.) was used as molecular weight standard. Comparable amounts of each protein extract were blotted on to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) (Towbin *et al*, 1979). Nitrocellulose membranes were blocked twice for 30 min and once for 1 h in buffer A (50 mM Na phosphate, pH 7.5, 0.5% wt/vol bovine serum albumin, 0.5% vol/vol Tween, 0.05% wt/vol NaN_3). After blocking, sheets were incubated with mouse sera obtained at the seventh bleeding diluted 1:20 in buffer A and, for control purposes, with preimmune sera or buffer alone overnight at 4°C . Membranes were washed in buffer A and incubated with monoclonal anti-mouse IgE antibodies

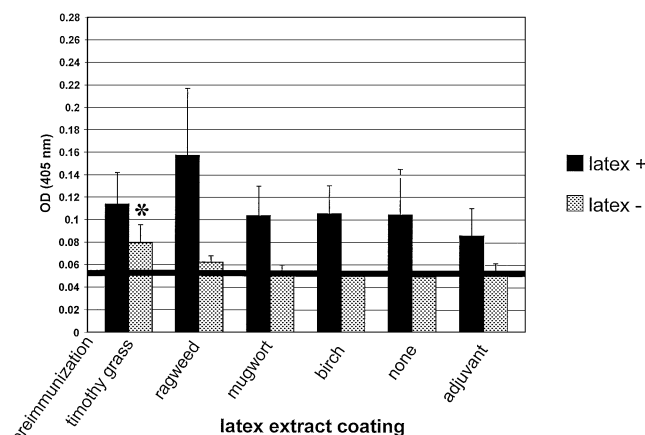


Figure 2. IgE responses to latex extract in the different mouse groups as determined by ELISA. The mean IgE-responses (y-axis: OD values) to ELISA-plate coated latex-extract were determined in blood samples collected from each mouse group (x-axis: pretreatment) at the fifth bleeding. Half of each group of mice received subsequent immunizations with latex extract (black bars), the other half received adjuvant alone (gray bars). Standard deviations are displayed for each group. The baseline for specific IgE production is represented by a horizontal black line.

from rat, diluted 1:1000 in buffer A overnight at 4°C. Bound rat antibodies were detected with 1:1000 diluted ^{125}I -labeled anti-rat antibodies from sheep and visualized by autoradiography (X-OMAT film, Kodak, Heidelberg, Germany).

Statistical analysis Results are presented as mean values and standard deviations. Significance of differences in multiple comparison of the ELISA results was calculated by adjusted four-factorial analysis of variance (ANOVA) unless otherwise stated. Adjusted factors were time of bleeding, allergen used for immunization, coated allergen and latex immunization.

RESULTS

IgE responses and time course of IgE reactivity to latex glove extract As previously reported for purified allergens (Vrtala *et al.*, 1998), pollen-specific IgE responses were detectable 4 wk after the first pollen immunization (i.e., second bleeding) and increased until the fifth bleeding (data not shown). Likewise, latex-specific IgE responses were detectable 4 wk after the first latex immunization (i.e., fourth bleeding) and continued to increase until the fifth bleeding. Therefore, latex- and pollen-specific IgE responses were analyzed from the fifth bleeding on.

In the ELISA measurements of mouse sera obtained at the time of the third latex immunization (i.e., fifth bleeding) we found that all groups of mice that had received latex extract (Fig 2, black bars) mounted IgE responses to latex extract which were significantly higher ($p < 0.0001$) than those of mice that instead had received adjuvant only (Fig 2, gray bars). Similar results were obtained when we analyzed mouse sera collected at the time of the fourth latex immunization (i.e., sixth bleeding) or 4 wk after the fourth latex immunization (i.e., seventh bleeding) (data not shown). The somewhat lower latex-specific IgE responses in the serum samples

obtained at the sixth and seventh bleeding were associated with an increase of latex-specific IgG1 reactivities (data not shown).

Timothy grass pollen presensitized mice without subsequent latex immunization exhibit significantly increased IgE responses to latex Analyzing the levels of latex-reactive IgE in groups of mice that had not received latex immunizations, we found that mice which were presensitized with timothy grass pollen extract consistently mounted higher latex-specific IgE responses than the other groups of mice (Fig 2): i.e., latex-reactive IgE levels of timothy grass pollen-pretreated mice measured in serum samples obtained during the fifth, sixth, and seventh bleeding were significantly higher than those of mice which had received other pollen extracts, adjuvant only or no preimmunization (Fig 2, gray bars, asterisks; sixth and seventh bleeding: data not shown). This finding was highly significant ($p < 0.0001$ in pairwise *t* tests with Bonferroni correction) and was equally present in the fifth, sixth, and seventh bleeding.

Latex-immunized mice mount higher IgE responses to pollen allergens compared with mice without latex immunization With exception of the timothy grass pollen-preimmunized group we found that almost all groups of mice which had received subsequent immunizations with latex extract (black bars) displayed a stronger IgE reactivity against pollen extracts than those which had not received latex (gray bars) (Fig 3a–d). This boosting effect was significant ($p < 0.043$) in all bleedings for the entity of all pollen-preimmunized mice.

The analysis of mouse sera (fifth bleeding) for IgE reactivity to natural pollen extracts showed that mice which were presensitized to timothy grass pollen extract, mounted the highest IgE responses

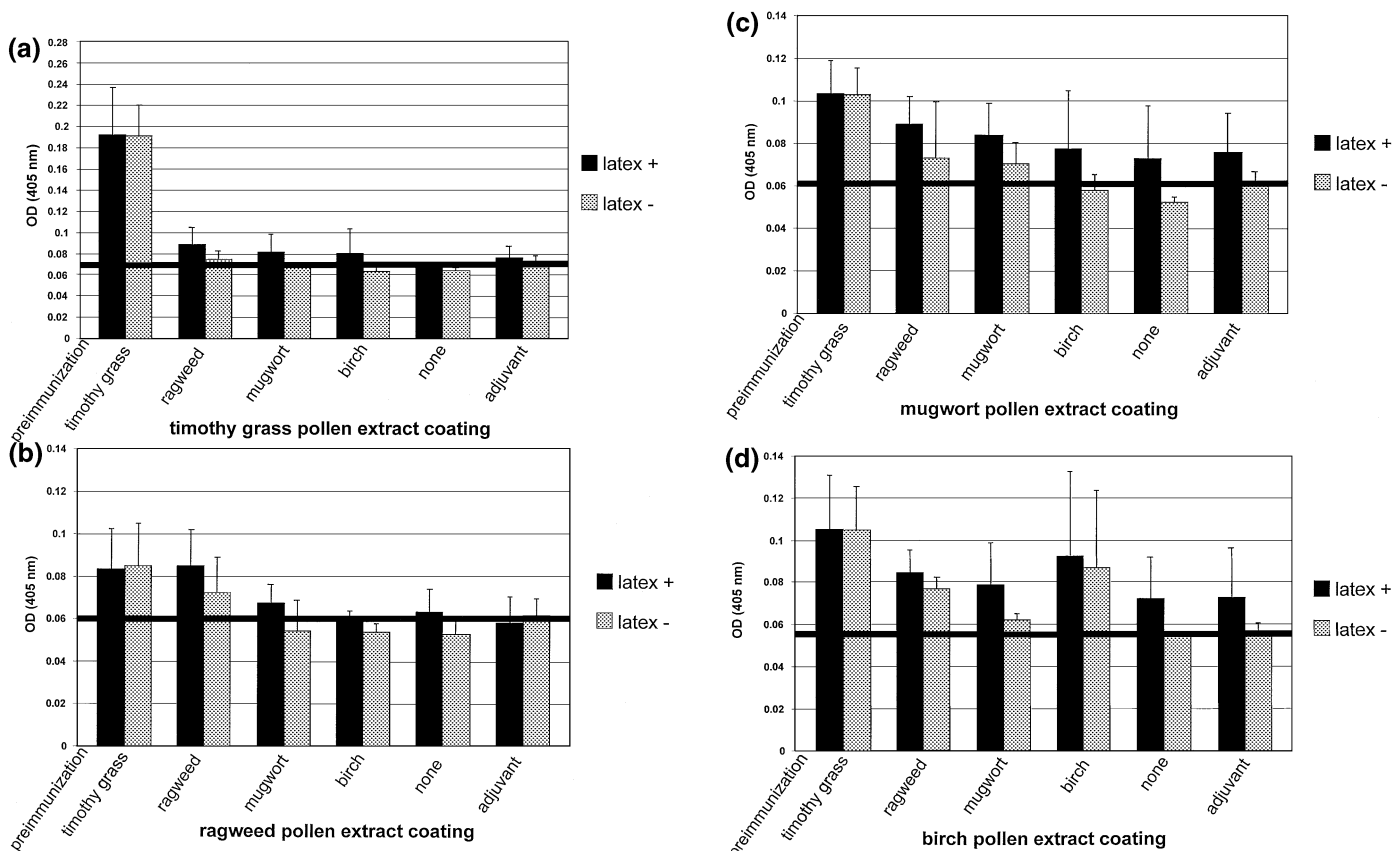


Figure 3. IgE responses to pollen extracts in the different mouse groups as determined by ELISA. The mean IgE-responses (y-axis: OD values) to ELISA-plate-coated timothy grass (a), ragweed (b), mugwort (c), and birch pollen extract (d) were determined in blood samples collected from each mouse group (x-axis: pretreatment) at the fifth bleeding. Half of each group of mice received subsequent immunizations with latex extract (black bars), the other half received adjuvant alone (gray bars). Standard deviations are displayed for each group. The baseline for specific IgE production is represented by a horizontal black line.

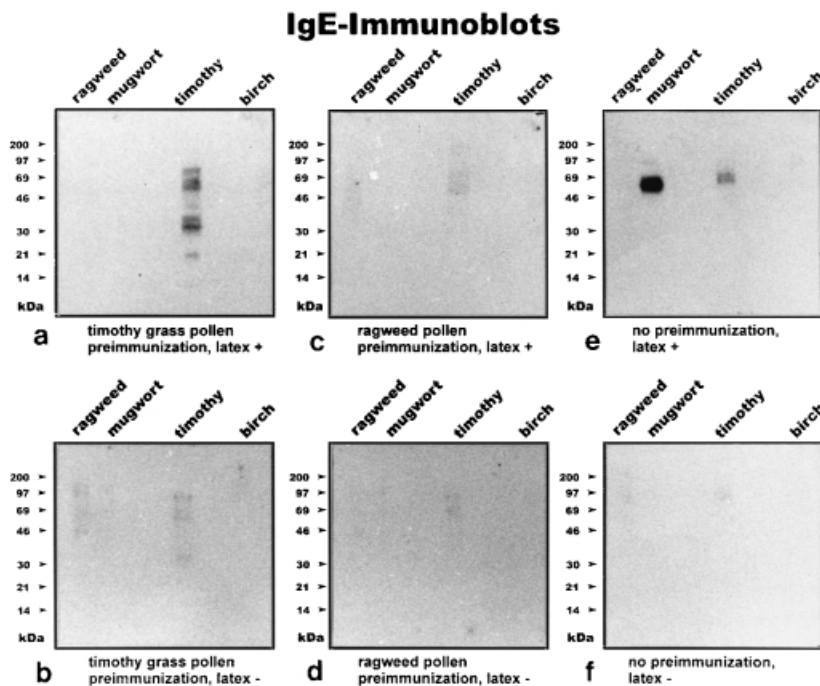


Figure 4. IgE reactivity to nitrocellulose-blotted pollen extracts. Sera from six representative mice who had received timothy grass pollen extract (a, b), ragweed pollen extract (c, d), or no preimmunization (e, f) with and without subsequent latex immunization were exposed to nitrocellulose-blotted pollen extracts from ragweed, mugwort, timothy grass, and birch. Bound IgE antibodies were detected and visualized by autoradiography. Molecular weights are displayed in kDa at the left margins of the nitrocellulose sheets.

against timothy grass pollen extract (Fig 3a, group 1). Interestingly, they also displayed the strongest IgE response against ragweed, mugwort, and birch pollen extract among all groups of mice (Fig 3b–d). The finding that timothy grass pollen preimmunization-induced IgE responses against ragweed, mugwort, or birch pollen extract and that ragweed preimmunization-induced IgE responses against mugwort pollen extract indicated the presence of cross-reactive epitopes in the various pollen extracts.

The statistical over-all analysis of the ELISA results of all groups, immunizations and bleedings regarding the potential of a given pollen extract combined with subsequent latex immunizations to induce any IgE response specific for the initial sensitizing allergen or cross-reacting allergens present in pollen from different allergen sources allowed to establish an order of allergenicity: Timothy grass and ragweed pollen extract induced the highest IgE responses, whereas mugwort and birch pollen extracts were weakly immunogenic.

Latex-immunized mice without pollen presensitization mount IgE responses to 60 kDa pollen allergens In order to characterize cross-reactive allergens in pollen and latex extracts, IgE immunoblot experiments were performed (Fig 4a–f). Results obtained with representative mouse sera exemplify the following. (i) Mice, which were not preimmunized with pollen extract but had received latex immunizations, contained IgE antibodies that reacted with pollen allergens (mugwort, timothy grass) of 60 kDa molecular weight (Fig 4e). No IgE reactivity was observed in mice that had received neither pollen nor latex extract (Fig 4f). (ii) Latex immunization lead from a subtle (cf. Fig 4c with Fig 4d) to a strong (cf. Fig 4a with Fig 4b) enhancement of IgE responses against the pollen extract used for preimmunization and to pollen extracts (timothy grass, ragweed) reportedly containing cross-reactive allergens (Fischer *et al*, 1996). (iii) The IgE immunoblot experiments further indicate that a high molecular weight (46–97 kDa) allergen complex may be important for the pollen–latex cross-reactivity in our mouse model.

DISCUSSION

Latex allergens are important elicitors of allergic skin manifestations, mucocutaneous and systemic anaphylactic reactions (Fuchs, 1994; Slater, 1994). Recently it was reported in latex-allergic

patients that timothy grass and weed (mugwort, ragweed) pollen extract *in vitro* inhibited IgE-binding to latex allergens suggesting the presence of cross-reactive allergens (Fuchs *et al*, 1997). Here we established a mouse model to investigate whether latex and pollen sensitization can exert mutual boosting effects on the production of IgE antibodies. We demonstrate that preimmunization with pollen extracts, in particular timothy grass pollen extract, can enhance the production of IgE anti-latex antibodies *in vivo*, as we found that mice which were immunized with timothy grass pollen extract only, mounted significant IgE responses to latex. Likewise we found that latex sensitization maintained higher levels of IgE antibodies against pollen allergens. The observed mutual boosting effects of latex and timothy grass pollen sensitization in our mouse model may represent a relevant mechanism explaining the clinical finding of atopy as risk factor for latex allergy in general (Liss *et al*, 1997; de Groot *et al*, 1998; Field, 1998; Niggemann *et al*, 1998; Rueff *et al*, 1998).

Whereas certain latex allergens represent genuine latex-specific proteins and thus are primarily recognized by certain risk groups of patients which are frequently exposed to latex, other latex allergens share IgE epitopes with antigens present in fruits and vegetables (Breiteneder and Scheiner, 1998). Putative plant-defense proteins, hevein, chitinases, and patatins were recently identified as widely distributed, and thus cross-reactive allergens in latex, tropical fruits, and potatoes (Chen *et al*, 1998; DiazPerales *et al*, 1998; Kostyal *et al*, 1998; Sowka *et al*, 1998a, b; Yagami *et al*, 1998; Blanco *et al*, 1999; Posch *et al*, 1999). Also plant pollens contain several highly cross-reactive allergens of which some are also expressed in somatic plant tissues. They include profilin, an actin-binding protein which is present in most eukaryotic cells and therefore represents a highly cross-reactive allergen (Valenta *et al*, 1991). Certain latex allergic individuals were found to contain IgE antibodies which cross-reacted with birch pollen and latex profilin (Vallier *et al*, 1995). In addition, a cluster of allergens of approximately 60 kDa molecular weight present in tree, grass, and weed pollens were found to share IgE epitopes that occur in somatic plant tissues (fruits, vegetables, and spices) (Bauer *et al*, 1996; Fischer *et al*, 1996; Heiss *et al*, 1996; Grote *et al*, 1998) and latex products (Fuchs *et al*, 1997). Patients containing IgE antibodies which cross-react between latex and plant food allergens may thus exhibit allergic reactions with both allergen sources (Beezhold *et al*, 1996). Whereas the clinical

relevance of cross-reactivity can be evaluated by provocation tests with the corresponding cross-reactive allergens, the effects of contact with a given allergen on the *in vivo* IgE production against the corresponding cross-reactive allergen require the establishment of an animal model. More specifically, our finding of induction of latex-specific IgE antibodies in timothy grass pollen-immunized mice may contribute to the elucidation of the as yet unknown way and relevance of sensitization in the peculiar group of latex-sensitized, but asymptomatic patients (Rueff *et al*, 1998; Branco Ferreira and Palma Carlos, 1999). Induction of cross-reacting antibodies due to the presence of common IgE-epitopes in latex and timothy grass pollen may explain the frequent finding of subclinical sensitization to latex, i.e., positive skin prick test without clinical symptoms and, sometimes, without history of previous latex exposure (Fuchs, 1994; Ownby *et al*, 1996; Rueff *et al*, 1998). This assumption is in agreement with the results of one clinical study on latex and pollinosis, reporting that patients who had a clinical history of latex allergy showed no pollen polysensitization, whereas individuals containing latex-specific IgE without clinical signs of latex allergy showed a broad profile of pollen polysensitization in particular to plantain and grass pollen (Drouet *et al*, 1994). We therefore suggest to evaluate the group of subclinically latex-sensitized individuals not only for so-called "latex-fruits", but also for sensitization against pollen allergens – especially grass and weed pollen. Monitoring of subclinically latex-sensitized individuals over a prolonged period may reveal whether increases of latex-specific IgE can be caused by pollen exposure and if so, whether such patients can then become symptomatic to latex allergens.

Immunoblot experiments performed in our study indicate that a previously identified allergen complex of approximately 60 kDa present in pollens of various plants, fruits, vegetables, and latex (Heiss *et al*, 1996; Fuchs *et al*, 1997) may be responsible for cross-reactivity also in pollen-latex-sensitized mice. Therefore, further molecular characterization of the 60 kDa cross-reactive allergen complex may not only give insight into the molecular basis of cross-reactivity, but may also provide diagnostic tools to identify patients containing cross-reactive antibodies and to monitor whether increases of cross-reactive antibody levels may be in parallel with the onset of clinical symptoms. The characterized cross-reactive marker allergens may then represent the basis for more precise therapeutic measures including avoidance of the cross-reactive allergen-containing sources and/or specific immunotherapy.

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