

Allergen Cross-Reactivity Between Proteins of the Latex from *Hevea brasiliensis*, Seeds and Pollen of *Ricinus communis*, and Pollen of *Mercurialis annua*, Members of the *Euphorbiaceae* Family

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ABSTRACT

Allergen cross-reactions among three strongly sensitizing Euphorbiaceae species, i.e., the rubber tree (*Hevea brasiliensis*), castor bean (*Ricinus communis*), and the Mediterranean weed *Mercurialis annua* were studied in Finnish patients (n = 25) allergic to natural rubber latex (NRL), but with no known exposure to castor bean or *M. annua*, and French patients allergic to castor bean (n = 26) or to *M. annua* (n = 9), but not to NRL. In immunoglobulin E (IgE)-immunoblotting, 28% of NRL-allergic patient sera recognized castor bean seed and 48% reacted to castor bean pollen proteins. Likewise, 35% of the NRL-allergic

patient sera bound to *M. annua* pollen allergens. Nineteen percent of castor bean-allergic patients showed IgE to NRL and 8% to *M. annua* proteins. Sera from patients allergic to *M. annua* reacted in 44% to NRL, in 56% to castor bean seed, and in 78% to castor bean pollen proteins. In immunoblotting, castor bean seed extract inhibited the binding of NRL-reactive IgE to 20 kDa, 30 kDa of NRL, and 55 kDa of proteins; NRL extract, in turn, inhibited the binding of castor bean-reactive IgE to 14, 21–22, 29, and 32–34 kDa of castor bean proteins. In ELISA inhibition, NRL extract inhibited 33% of the binding of *M. annua*-reactive IgE of pooled sera to *M. annua* pollen. In conclusion, allergen cross-reactivity in vitro was observed among three botanically related Euphorbiaceae members, *H. brasiliensis*, *R. communis*, and *M. annua*, but the molecular specificity of the observed cross-reactions as well as their clinical significance remains to be elucidated. Allergen cross-reactivity should be taken into account in diagnostic work. (Allergy and Asthma Proc 23:141–147, 2002)

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The *Euphorbiaceae* family (spurges) embodies members considered to contain strongly sensitizing allergens, such as the latex of *Hevea brasiliensis* and the pollen and seeds of *Ricinus communis*. During the last 15 years, immediate allergic reactions to natural rubber latex (NRL)

proteins have been increasingly reported and recognized as a major worldwide problem associated especially with health care workers using protective gloves and children with spina bifida or other congenital malformations requiring multiple surgical operations at an early age (reviewed in Turjanmaa *et al.*¹). Allergy to the castor bean has been observed in and around castor oil processing and whenever castor bean pomace, used as a fertilizer, has been used.²⁻⁵ Clinical allergy to *Mercurialis annua* pollen was first described in Spain⁶ and Italy⁷ and recently established by skin-prick testing (SPT) in almost half of 420 pollen-allergic patients in Spain.⁸

A multitude of purported cross-reactions are known to be associated with NRL allergy,^{1,9-12} which can interfere especially with the *in vitro* diagnostic tests. *M. annua* does not grow in northern Europe, implying that exposure to this plant is possible, *e.g.*, in Finnish people only when traveling to Mediterranean and other southern countries. *R. communis* does not grow wild in Finland and people are not exposed to castor bean pollens. Whether allergy to castor bean exists among Finnish subjects has not been assessed. Clinical allergy or finding *in vitro* reactive immunoglobulin E (IgE) antibodies to these plants then would most likely be caused by allergen cross-reactions. This study was undertaken to examine the extent of putative allergen cross-reactions within the three members of the *Euphorbiaceae* family. Specifically, we wanted to determine whether patients who are allergic to the latex of *H. brasiliensis* but, as far as we know, have not been exposed to castor bean or to *M. annua*, would have IgE binding to *R. communis* or *Mercurialis* proteins. In addition, we wanted to investigate whether any of the major or clinically important latex allergens, *i.e.*, prohevein (Hev b 6.01),¹³ mature hevein (Hev b 6.02),¹⁴ the 16-kDa acidic protein (Hev b 5),^{15,16} or the patatin-like allergen Hev b 7^{17,9} were involved in these alleged cross-reactions.

MATERIALS AND METHODS

Patients and Sera

Sera were available from 26 patients from southern Europe allergic to castor bean, 9 patients from southern Europe allergic to *M. annua* pollen, and 25 Finnish patients allergic to NRL. The diagnoses of all patients were based on clinical histories, positive SPT reactions to the relevant allergens, and positive radioallergosorbent test (RAST) results for the specific allergens. The mean age for the castor bean-allergic patients was 41 years (range, 18–68 years; 13 males and 13 females) and the mean age for *M. annua*-allergic patients was 36 years (range, 23–50 years; 2 females and 7 males). The mean age of the NRL-allergic patients was 40 years (range, 8–66 years; 17 females and 8 males). Eleven of the 23 NRL-allergic adults were health care workers. One of the patients, a boy 8 years of age, had spina bifida as an associated condition. Sera from 20 non-

allergic control subjects from France and 18 from Finland, respectively, served as controls.

Allergen Extracts

Castor Bean. A lyophilized extract of seeds of the castor bean was obtained from Centre for Biochemical Technology, Delhi University (Delhi, India). Ten milligrams of the extract was dissolved into 1 mL of distilled water and the solution was filtered through a 0.45- μ m Millex-HV filter (Millipore, Molsheim, France). Protein concentration of the solution, as measured by the Lowry's method, was 7.66 mg/mL.

An extract (1:20 wt/vol) of castor bean pollen (Lot 604 169) was obtained through the courtesy of Dr. Thomas W. Green (Berkeley Biologicals, Berkeley, CA).

M. annua. A freeze-dried extract of pollen of *M. annua* was obtained through the courtesy of Dr. Alberto Martinez (Ifidesa-Aristegui Co., Bilbao, Spain). Ten milligrams of the lyophilized material (L-930512) was dissolved into 1 mL of phosphate-buffered saline, pH 7.4, and the solution was filtered through a 0.45- μ m Millex-HV filter. Protein concentration of the solution was 4.64 mg/mL.

NRL. NRL from the rubber tree, *H. brasiliensis*, was collected in Malaysia, deep-frozen immediately, shipped to Finland in dry ice, and stored at -70°C . For the extraction of soluble proteins, NRL was thawed, the coagulated solids were removed, and the liquid was centrifuged at $100,000 \times g$ for 90 minutes, after which the supernatant was collected and stored at -70°C . Total protein concentration of the NRL extract was 13 mg/mL.

Purified NRL Proteins. Purified natural prohevein (Hev b 6.01) and hevein (Hev b 6.02) were available from a previous study.¹⁴ Hev b 7 (patatin-like NRL allergen) was extracted from freeze-dried NRL and purified as described elsewhere,¹⁸ and recombinant Hev b 5, produced as a maltose binding protein fusion protein in *Escherichia coli* was a generous gift of Prof. Markku Kulomaa (University of Jyväskylä, Finland).

IgE Immunoblotting

Immunoblotting was performed as described earlier^{19,20} to verify the ability of the proteins to bind relevant IgE. Briefly, for electrophoresis, the allergen preparation was diluted 1:3 into an electrophoresis sample buffer containing 2% sodium dodecyl sulfate and 5% 2- β -mercaptoethanol and kept in boiling water for 2 minutes. The dissolved preparation was applied to 12.5% acrylamide gels containing 0.1% sodium dodecyl sulfate, using a 4% stacking gel. Electrophoresis was carried out for 45 minutes at a constant voltage of 200 V (Mini Protean II Cell; Bio-Rad, Richmond, CA). Total protein staining was performed with a commercial kit (Blotting Detection Kit for Total Protein; Amer-

sham, Amersham, UK) using molecular weight protein markers of 14.3–200 kDa (Amersham). The protein concentration of the preparations was adjusted to 450 µg/mL before total protein staining and immunoblotting.

After electrophoresis, the proteins were transferred from the gel to a 0.2-µm pore-size nitrocellulose filter (Trans-Blot; Bio-Rad) for 1 hour at 100 V (Mini Trans-Blot Cell, Bio-Rad). After blotting, the membrane was shortly stained with Ponceau-S red to show a successful transfer of proteins. Then, the membrane was cut into strips, washed, and incubated for 45 minutes in a blocking buffer containing 5% nonfat milk powder in Tris-buffered saline (TBS). Sera diluted 1:5 in the blocking buffer were added, and the strips were incubated overnight at +4°C with continuous shaking. After appropriate washing, biotinylated goat anti-human IgE antibody (Vector, Burlingame, CA) was added and incubated for 30 minutes at room temperature. Thereafter, streptavidin-conjugated alkaline phosphatase (Bio-Rad) was added and incubated for 30 minutes at room temperature. After washing, the strips were placed in a color development solution (Alkaline Phosphatase Conjugate Substrate Kit; Bio-Rad). The reaction was stopped after 8 minutes by rinsing the strips in tap water.

IgE Immunoblot Inhibition

The antigen preparations were electrophoresed and the proteins were transferred to nitrocellulose membrane as described previously. Then 150 µL of serum was incubated for 2 hours at room temperature with an equal volume of inhibitor solution. Four different concentrations of inhibitor, containing (in a 1-ml final volume) 0.1, 1, 10, and 100 µg of inhibitory proteins, were used for each serum. The inhibitor solution was replaced with an equal volume of TBS in control experiments. The mixture then was added to the strips and blocking buffer containing 5% nonfat milk powder in TBS was added until the final dilution of the sera was 1:5. Then, strips were incubated overnight at +4°C,

after which the immunologic detection of IgE binding was performed as described previously.

SPT with NRL Proteins and Extracts of Castor Bean and *M. annua* Pollen

An extract of latex gloves (Exona; Semperit, Vienna, Austria) was prepared and the SPT was performed as described earlier.²¹ To test Finnish NRL-allergic patients, extracts of castor bean seeds (10, 1, 0.1 and 0.01 mg/mL) and of *M. annua* pollen (2, 1, 0.1, and 0.01 mg/mL) were made in phosphate-buffered saline and the SPT performed as described previously.

RAST

Commercially available reagents and kits (Pharmacia, Uppsala, Sweden) were used to measure specific IgE to allergens of NRL, castor bean and *M. annua*. ELISA for IgE antibodies to allergen extracts and to purified allergens were performed essentially as described earlier.¹⁴

RESULTS

IgE Antibodies to NRL, Castor Bean, and *M. annua* Allergens Detected by Immunoblotting

The results of the immunoblot analyses are compiled in Table I. In each patient category, several sera from the patients reacted in IgE immunoblotting, in addition to the presumably causative allergens (frequency ranging from 44 to 92%), to allergen preparations of the various *Euphorbiaceae* extracts (frequency ranging from 8 to 78%), suggesting allergen cross-reactivity. Interestingly, *M. annua*-allergic patients recognized allergens in castor bean seed extract even more often (7/9) than they did in the homologous *M. annua* pollen (4/9). Castor bean-allergic patients recognized castor bean pollen allergens clearly less frequently (15%) than castor bean seed allergens (73%) and almost one-half of NRL-allergic patients reacted to one or several castor bean pollen allergens.

TABLE I				
Correlation Between the Occurrence of Clinical Allergy and Specific Serum IgE Among Three Members of the <i>Euphorbiaceae</i> Family				
Patient Categories	Number of Positive Reactions/Total Tested (%) in IgE Immunoblotting* to			
	NRL	CB Seed	CB Pollen	<i>M. annua</i> Pollen
NRL-allergic patients	22/24 (92%)	7/25 (28%)	12/24 (48%)	7/24 (29%)
CB-allergic patients	5/26 (19%)	19/26 (73%)	4/26 (15.9%)	2/26 (8%)
<i>M. annua</i> -allergic patients	4/9 (44%)	7/9 (78%)	5/9 (56%)	4/9 (44%)
Nonallergic controls, France (n = 20)	0	0	0	0
Nonallergic controls, Finland (n = 18)	0	0	0	0

*At least one clear-cut band.
CB, castor bean.

Characteristic immunoblot binding profiles of IgE binding to NRL or to castor bean seed extract from selected NRL-allergic and castor bean-allergic patients are depicted in Fig. 1. It can be seen that castor bean-allergic patient sera showed IgE that bound to different NRL antigens than IgE from NRL-allergic patient sera. A characteristic doublet or triplet band at ~32–34 kDa in the castor bean seed preparation was recognized frequently by IgE from both castor bean-allergic patients and NRL-allergic patients. Bands were detected often also at ~14-, 22-, 28-, and 36-kDa regions of the castor bean seed extract. Typical *M. annua* pollen antigens were seen at 45, 35, and 18 kDa (data not shown). All control sera gave negative results.

IgE Antibodies Detected by ELISA

Sera from 21/23 (91%) NRL-allergic patients and 6/26 (23%) of castor bean-allergic patients, but none of the 9 *M. annua*-allergic patients, reacted against the NRL ex-

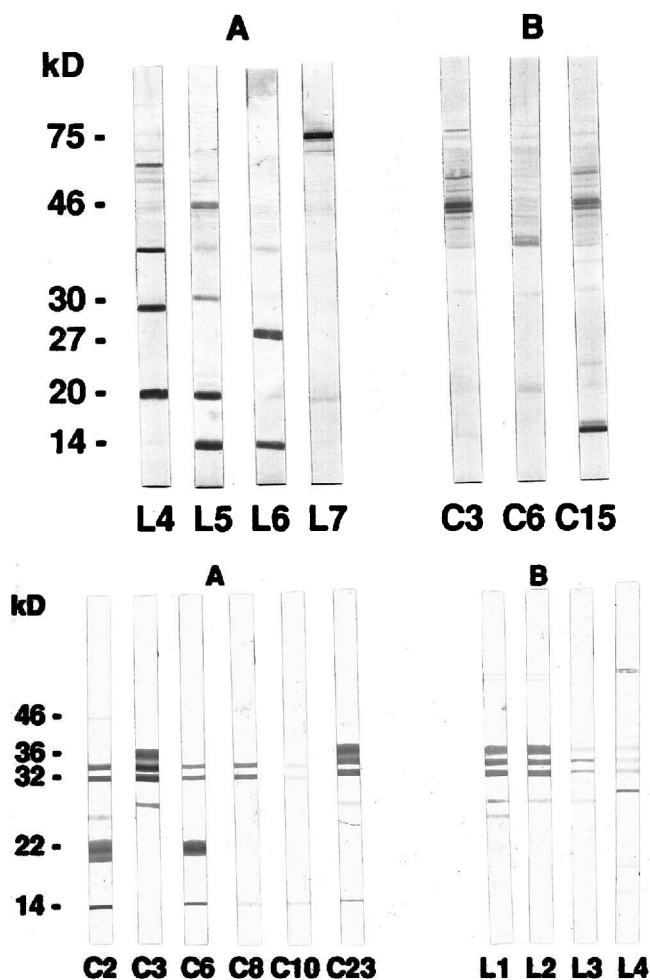


Figure 1. (A) IgE immunoblot profiles for sera from four NRL-allergic and three castor bean-allergic patients binding to NRL extract. Sera were diluted 1:5. kD denotes the location of molecular weight markers. (B) IgE immunoblot profiles for sera from six castor bean-allergic and four NRL-allergic patients binding to castor bean seed extract.

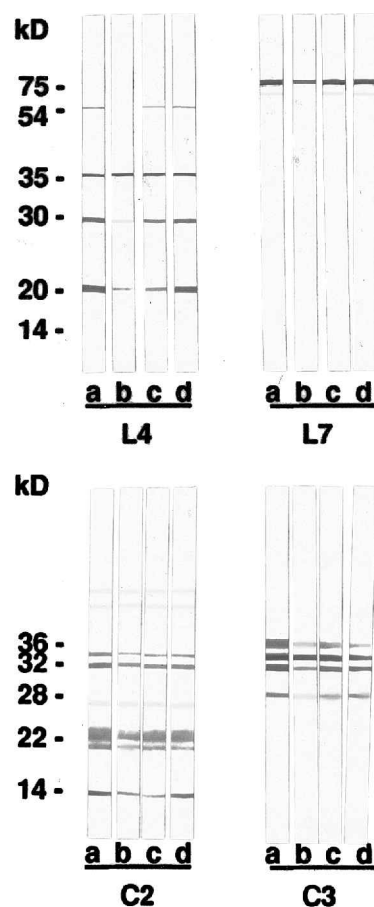


Figure 2. (A) Inhibition of binding of NRL-specific IgE to NRL extract in immunoblotting with castor bean seed extract. L4 and L7 denote sera of two NRL-allergic patients. Concentration of inhibitor: a, 0; b, 100 μ g/mL; c, 10 μ g/mL; d, 1 μ g/mL. (B) Inhibition of binding of castor bean-specific IgE to castor bean seed extract in immunoblotting with NRL extract. C2 and C3 denote sera of two castor bean-allergic patients. Concentration of inhibitor: a, 0; b, 100 μ g/mL; c, 10 μ g/mL; d, 1 μ g/mL.

tract (data not shown). Against castor bean seed extract, 24/26 (92%) castor bean-allergic patients, 17/25 (68%) NRL-allergic patients, but none of the 9 *M. annua*-allergic patients had IgE antibodies. Sera from the 26 castor bean-allergic patients and 9 *M. annua*-allergic patients also were tested in ELISA for IgE antibodies against NRL-specific Hev b 5, Hev b 6.01 (prohevein), Hev b 6.02 (hevein), and Hev b 7 (patatin-like protein). All results were negative (data not shown).

IgE Inhibition Results

Crosswise IgE immunoblot inhibitions were performed with sera from two castor bean-allergic patients and two NRL-allergic patients against both castor bean and NRL extracts (Fig. 2). Castor bean extract inhibited in a dose-dependent manner the binding of IgE from one patient (L4) to 20, 30, and 55 kDa of NRL proteins. No inhibition was observed with regard to 36 and 75 kDa of NRL protein

bands. NRL protein inhibited, in turn, the binding of castor bean-allergic patients' IgE to immobilized castor bean proteins with molecular weights of ~14, 21–22, 29, and 32–34 kDa.

IgE inhibition experiments were performed also with ELISA using pools of five to eight patients' sera from each category (NRL-allergic, castor bean-allergic, and *M. annua*-

a-allergic) and each of the same allergens immobilized on microtiter wells. As summarized in Fig. 3, the highest cross-inhibition (~35%) was seen when NRL proteins inhibited the binding of *M. annua*-specific IgE to solid phase *M. annua* pollen extract (Fig. 3 A). Both castor bean extract and *M. annua* extract inhibited the binding of NRL-specific IgE to solid-phase NRL by ~20% (Fig. 3 B). The binding of

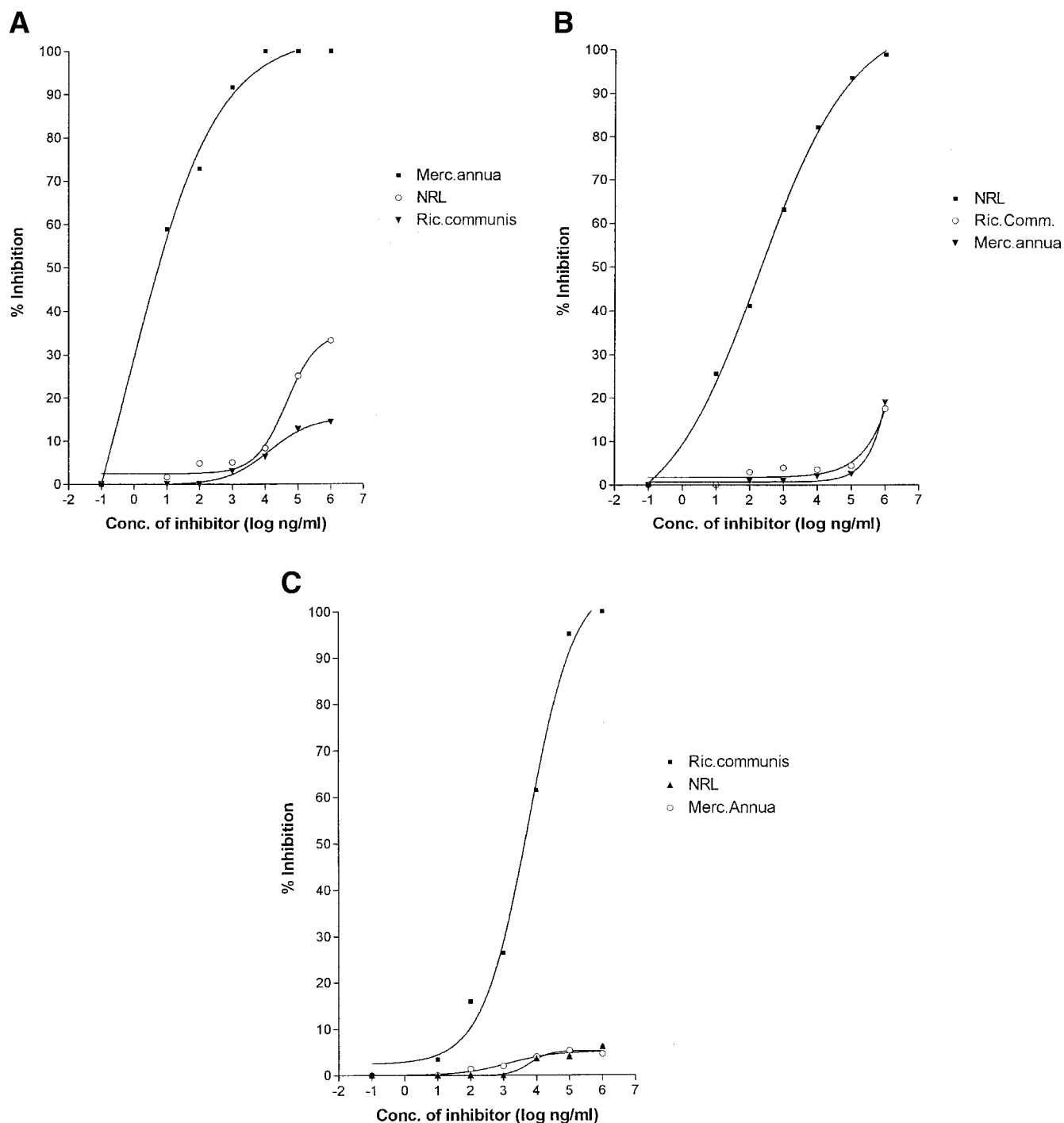


Figure 3. IgE ELISA inhibition. (A) *M. annua*, NRL, and *R. communis* extracts were used to inhibit the binding of *M. annua*-specific IgE to *M. annua* pollen extract. (B) Same extracts as in panel A were used to inhibit the binding of NRL-specific IgE to NRL extract. (C) Same extracts as in panel A were used to inhibit the binding of castor bean-specific IgE to castor bean seed extract.

castor bean-specific IgE to castor bean seed extract was inhibited only by the homologous allergen preparation (Fig. 3 C).

SPT Results with Castor Bean and *M. annua* Extracts

SPTs, performed on six randomly picked Finnish NRL-allergic patients who were not known to have been exposed to castor bean or *M. annua* gave negative results to castor bean seed extract. Six other Finnish NRL-allergic patients, three with positive and three with negative IgE immunoblot reaction to *M. annua* pollen extract were later given the SPT against the same extract. All the immunoblot-positive patients tested positive in the SPT (at a concentration of 1 mg/mL [wt/vol]) and the immunoblot-negative subjects showed negative test results.

DISCUSSION

Allergen cross-reactions, occurring *in vivo* or *in vitro*, in recent years have received increasing attention. It could be anticipated that allergen cross-reactions are especially common among members belonging to the same botanical family and that such cross-reactions may have relevance in particular to diagnosis of allergies. Yet, allergen cross-reactions frequently have been observed also between species that are botanically unrelated, perhaps best exemplified in the so-called latex-fruit syndrome.^{22,23} In this syndrome hevein-like domains, representing the N-terminal region of prohevein molecules and N-terminal regions also of banana, avocado, and chestnut endochitinases^{11,12,24–26} have been shown to be responsible for at least part of the cross-reactivity. When IgE class antibodies to several allergens are detected at the same time, the possibility of cosensitization, not necessarily implying cross-reactivity, also should be considered. Carbohydrate determinants in IgE-mediated *in vitro* reactions also have been proposed to induce nonspecific cross-reactions, exemplified by reported cross-reactivity between grass and weed pollen and NRL¹⁰; however, little is known of the molecular specificity of such reactions.

This study shows that, alleged allergen cross-reactivity *in vitro* is not uncommon between the three members of the *Euphorbiaceae* family. A considerable proportion (28–48%) of Finnish NRL-allergic subjects who, to the best of our knowledge, have not been exposed to pollen of the *M. annua* weed or to castor bean had strongly reacting IgE antibodies binding to these allergens. The reason why *M. annua*-allergic patients seemed to react, unexpectedly, even more often to castor bean than to *M. annua* itself remains unknown; however, the possibility exists that some of the homologous allergens had degraded during preparation of the extracts. Because as many as 68% of the Finnish NRL-allergic patients reacted to castor bean in the IgE ELISA, the possibility of primary sensitization to castor bean may not be ruled out. Certain immunoblot bands (at ~14, 26, 28, 32, and 34 kDa; Fig. 2 A) were dose-dependently inhibited by

the putative cross-reacting NRL allergens. Similarly, castor bean extract inhibited the binding of NRL-specific IgE to NRL allergens (at ~20, 30, and 55 kDa; Fig. 2 B). We also found that rabbits immunized with NRL developed IgG antibodies that recognized a large variety of castor bean proteins, many of which seemed to be the same or similar proteins that bound IgE from patients sera (data not shown). However, the molecular specificity of these cross-reactions remains to be elucidated. ELISA inhibition results showed that ~30% cross-inhibition occurred when NRL extract was used to inhibit the binding of pooled *M. annua* IgE to solid-phase *M. annua* pollen extract (Fig. 3 A). It was of interest that the source for cassava (*Manihot ultissima*), a food used worldwide (obtained from a local grocery store in Marseilles, and extracted as described for castor bean seeds), also belonging to the *Euphorbiaceae* family, did not reveal cross-allergenicity, *i.e.*, IgE from sera of patients allergic to NRL, castor bean, or *M. annua* did not bind to preparations made of cassava (data not shown).

We also wanted to determine whether some of the important NRL allergens could explain any or some of these observed cross-reactions, in analogy to the latex-fruit syndrome. However, neither castor bean-allergic nor *M. annua*-allergic patients had IgE to purified Hev b 6.01, Hev b 6.02, Hev b 7, or to recombinant Hev b5 in levels exceeding those of nonallergic control subjects. Furthermore, neither of these NRL allergens showed significant inhibition of binding of castor bean-specific or *M. annua*-specific IgE to their homologous target allergen preparations. We did not have the opportunity to study *Hevea* profilin (Hev b 8) in this context, but, recently, it was reported that *M. annua* profilin (Mer a 1), a major *M. annua* allergen, and *Hevea* profilin did not show cross-reactivity.²⁷ Thus, it seems plausible that the aforementioned NRL allergens do not explain the *Euphorbiaceae*-associated cross-reactions observed in this study. In addition, it would have been interesting to study whether the recently characterized *Hevea* enolase (Hev b9) and manganese superoxide dismutase (Hev b10), additional minor NRL allergens that have been suggested to be responsible for cross-reactivity between molds and NRL, play a role in the observed *in vitro* reactions.^{28,29}

We performed SPTs primarily for the purpose of diagnosing the actual allergies and the patients were not invited to participate in extended studies on allergen cross-reactions. However, we did carry out some preliminary SPTs on Finnish NRL-allergic patients against castor bean and *M. annua*, allergens to which these patients were not known to have been exposed. Because none of the six Finnish NRL-allergic patients prick-tested against castor bean showed positive reactions, although many patients had IgE to castor bean seed in ELISA, one may assume that such *in vivo* cross-reactions are uncommon between castor bean and NRL. Interestingly, however, all three NRL-allergic patients who had positive immunoblot results against *M. annua* also had positive SPT against the same *M. annua* pollen

extract. These three patients had positive SPTs, in addition to NRL, also to some common pollen or food allergens, but similar reactivity also was seen in SPT-negative patients. Obviously, these preliminary results have to be confirmed in a larger patient series. In France, castor bean-allergic and *M. annua*-allergic patients also were tested occasionally against NRL in SPT with negative results. Evidence for NRL allergy among the 35 French patients was documented only in one instance; one of the *M. annua*-allergic patients had a positive latex RAST. The negative SPT results with castor bean support the contention that at least NRL-allergic patients' IgE cross-reactive *in vitro* with castor bean do not seem to result in clinical symptoms. However, in *M. annua* pollen, there could be cross-reactive allergen structures that can elicit hypersensitivity reactions also *in vivo*. Clearly, SPTs with purified NRL, castor bean, and *M. annua* allergens and/or appropriate provocation tests are needed for conclusions that are more definite.

In summary, allergen cross-reactivity *in vitro* was observed among three botanically related *Euphorbiaceae* members, *H. brasiliensis*, *R. communis*, and *M. annua*. Possible cross-reactivity between NRL and *M. annua* allergens was observed to occur *in vivo*. Allergen cross-reactivity may be increasingly important to be considered in diagnostic work.

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