

Allergy to bumblebee venom. II. IgE cross-reactivity between bumblebee and honeybee venom

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To obtain more information on IgE cross-reactivity between bumblebee venom and honeybee venom, we tested sera from venom-sensitized patients for specific IgE against venoms from the European bumblebee (*Bombus terrestris*), the North American bumblebee (*Megabombus pennsylvanicus*), and the honeybee (*Apis mellifera*). RAST, RAST-inhibition, and immunoblotting experiments indicate that bumblebee venom and honeybee venom contain venom-specific IgE-binding epitopes. These results suggest that immunotherapy using honeybee venom may not be effective in all bumblebee venom-allergic patients. Our experiments also revealed differences in IgE binding for venom from European and American bumblebees.

**S. O. Stapel¹,
J. Waanders-Lijster de Raadt²,
A. W. van Toorenenbergen²,
H. de Groot³**

¹Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam;

²Laboratory of Allergology, Department of Clinical Chemistry, University Hospital Dijkzigt, Rotterdam; ³Department of Allergology, University Hospital Dijkzigt, Rotterdam, The Netherlands

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Dr S. O. Stapel
CLB, Department of Allergy
Plesmanlaan 125
1066 CX Amsterdam
The Netherlands

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Although bumblebees under natural conditions are usually not aggressive and seldom sting people, the use of these insects for pollination in greenhouses has increased the number of sting-induced anaphylactic reactions in The Netherlands (1). Since the presence of bumblebee venom-specific IgE has been shown in a number of patients, immunotherapy will probably be the treatment of choice. This has already been done by Kochuyt et al. (2), who claim that hyposensitization of bumblebee venom-allergic patients with the readily available honeybee venom leads to positive clinical results. Although various studies on the biochemical and immunochemical characteristics of Hymenopteran venoms have been published (3–8), reports on bumblebee venom allergens have been relatively few. Specific attention has been given to bumblebee venom peptides, designated bombolitins (bumblebee-venom counterparts of the honeybee mellitins), because of their physiologic activities (9, 10). An early paper showed that some proteins from honeybee and bumblebee venoms have different electrophoretic properties (11). Later reports stress the similarities of honeybee and bumblebee venoms (12, 13), and a recent paper concluded that honey-

bee and bumblebee venom allergens appear to be immunochemically and structurally closely related (14).

We have measured specific IgE to honeybee and bumblebee venom by RAST, and our findings seemed to conflict with the reported allergenic similarity of honeybee and bumblebee venom. Therefore, we also compared the immunochemical aspects of venom from the European honeybee and two bumblebee species, using RAST inhibition and immunoblotting.

Material and methods

Patients

Bumblebee venom-positive sera Kr, Ko, Hu, Sl, Ri, and Mi were obtained from patients who worked in a bumblebee farm and were frequently stung by bumblebees (1). Honeybee venom IgE-positive sera and yellow jacket venom IgE-positive sera were obtained during routine diagnostic screening in the Department of Allergy of the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, as was serum Ge, showing a

positive RAST for venoms from bumblebee, honeybee, and yellow jacket.

Venom preparations

Preparation of European bumblebee venom-sac extract was performed as follows: 25 freshly isolated venom sacs were frozen overnight in 2 ml phosphate-buffered saline (PBS). After the preparation was thawed, glass beads were added, followed by vigorous shaking on a vortex apparatus. After 2-h incubation on a vertical rotator and subsequent filtration (S&S 589⁵, Schleicher and Schuell, Dassel, Germany), protein content was measured (Pierce Chemicals, Rockford, IL, USA). For electrophoretic purposes, European bumblebee venom (obtained by capillary suction from venom sacs to keep contamination with venom-sac proteins as low as possible), as well as a commercially available preparation from VESPA Laboratories (Spring Mills, PA, USA), was used. American bumblebee venom was obtained from VESPA Laboratories, and honeybee venom from Sigma Chemicals Co. (St Louis, MO, USA). Honeybee venom was subjected to Sephadex G-100 gel filtration before immobilization to solid phase, in order to remove the major part of the mellitin peptide, since the abundance of this component in venom might interfere with the coupling of other polypeptides. All commercially available preparations were venom-sac extracts.

RAST and RAST inhibition

Specific IgE to venom from European bumblebee, North American bumblebee, and honeybee was determined by a RAST procedure, essentially according to the method of Aalberse et al. (15). An amount of 3 mg of European bumblebee venom protein (from freshly prepared sac extract) was coupled to 100 mg CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden), according to the manufacturer's instructions. For the American bumblebee venom allergosorbent, 1.6 mg of venom protein was immobilized on 100 mg of CNBr-Sepharose, and the same was done with 0.6 mg of honeybee venom protein.

For RAST testing, 0.5 mg allergosorbent and 50 µl of patient serum were used. After overnight incubation, unbound serum components were washed away, and ¹²⁵I-radiolabeled sheep anti-human IgE antibodies were added. After overnight incubation, excess radiolabeling was removed by washing, and the amount of allergosorbent-bound ¹²⁵I-labeled anti-IgE was determined. RAST results were expressed as international units per milliliter (IU/ml) on a calibration curve of chimeric IgE monoclonal

antibody (16). One unit of IgE represents approximately 2.4 ng of specific IgE. RAST values lower than 0.35 IU/ml were considered negative. Incubation of our allergosorbents with serum containing high total IgE (3000 IU/ml) did not result in nonspecific IgE binding. RAST-inhibition studies were performed by preincubating sera with the inhibitory component (in the presence of 6% bovine serum albumin [BSA]), for a 3-h period at 37°C, before incubation with allergosorbent. When sera contained high titers of venom-specific IgE, the amounts of serum and allergosorbent were adjusted in such a way that optimal inhibitory conditions were obtained (uninhibited RAST values: approximately 15% binding of added ¹²⁵I-labeled anti-IgE).

SDS-PAGE and immunoblotting

Venom samples for SDS-PAGE under reducing conditions were prepared by boiling for 5 min in SDS sample buffer in the presence of 80 mM of dithiothreitol. Approximately 30 µg of venom from honeybee, European bumblebee, and American bumblebee was subjected to electrophoresis, essentially according to the procedure of Laemmli (17), with 10–20% gradient gels. Staining of gels was performed with Coomassie brilliant blue R250. For immunoblotting, proteins were transferred to nitrocellulose paper (Schleicher & Schuell, Dassel, Germany). Before incubation with sera diluted 1:50 in PBS 0.3% (w/v) BSA/0.1% (v/v) Tween-20, blotting paper was preincubated with PBS/1.8% (w/v) BSA/0.1% (v/v) Tween-20 to prevent nonspecific binding. After removal of unbound compounds by washing (PBS/0.05% (v/v) Tween-20), radiolabeled sheep anti-human anti-IgE was added. After washing, the binding of anti-IgE was visualized by exposing immunoblots to radiographic film (Kodak X-Omat AR, Eastman Kodak Co, Rochester, NY, USA).

Venom-protein separation in the presence of SDS under nonreducing/nonboiling conditions was performed after incubation with nonreducing sample buffer for 10 min at 37°C. The samples applied were as follows: European bumblebee venom obtained by capillary suction from venom sacs, European and American bumblebee venom from Vespa Laboratories, and honeybee venom from Sigma Chemicals Co. The composition of the Tris-HCl Ready Gels used for this type of electrophoresis was 12% T, 2.6% C (resolving gel), and 4% T, 2.6% C (stacking gel) in 0.375 M Tris-HCl, pH 8.8. Gels were run in the Mini PROTEAN II cell (Biorad) using a Tris-glycine-SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). The best results were obtained when approximately 10 µg of venom

protein in sample buffer (62.5 mM Tris, 10% [v/v] glycerol, 2% [w/v] SDS, 0.025% [w/v] bromophenol blue, without reducing agent) per lane was applied. For preparation of venom blots, two-well prep comb gels were loaded with 65 µl diluted venom.

For molecular weight reference, Kaleidoscope Prestained Standard was used. Gels were run for 1 h at 20–25 mA per gel.

Protein staining was carried out with Coomassie brilliant blue R250 for electrophoresis (Merck, Darmstadt, Germany). Nitrocellulose membranes were stained with Bio-Rad Colloidal Gold Total Protein Stain. Proteins were transferred to 0.45 µm supported nitrocellulose membranes, and blots were washed three times with 1% BSA in PBS supplemented with 0.2% (v/v) Tween-20, pH 7.2.

Detection of serum IgE binding to blotted venom protein was carried out as follows. Sera from patients were diluted 1:2 with PBS/1% (w/v) BSA/0.2% (v/v) Tween-20. Membranes were placed in the Mini-PROTEAN II Multiscreen apparatus, and the channels were filled with 600 µl serum dilution. After shaking overnight and five washes with PBS/0.2% (v/v) Tween-20, 600 µl 1:2 diluted ¹²⁵I-labeled anti-IgE (Pharmacia, Uppsala, Sweden) was added. After a 4-h incubation, the membranes were washed five times with PBS/0.2% (v/v) Tween-20 and two times with distilled water. Membranes were air-dried, and loaded into a Kodak X-Omatic cassette (KP 68374) with a radiographic film (3M safety). The cassette was stored at –70°C for 1–4 days. Processing of the film was performed in the Kodak RPX-Omat processor.

Results

RAST and RAST inhibition

Hymenopteran venom RAST results obtained with sera Kr, Ko, Hu, Mi, Ri, and Sl (patients, sensitized to European bumblebee venom by occupational activities), as well as with serum Ge, are shown in Table 1. Also shown are the results obtained with five honeybee venom IgE-positive sera and five yellow jacket venom IgE-positive sera. These results show that a positive RAST for bumblebee venom did not imply that the RAST for honeybee venom (or yellow jacket venom) would also be positive in all cases. Conversely, the presence of high levels of anti-honeybee-venom (or anti-yellow-jacket-venom) IgE did not necessarily correlate with high titers of anti-bumblebee venom IgE. RAST-inhibition experiments, using sera Mi, Kr, and Ge, showed that the RAST for European bumblebee venom was, as expected, inhibited by European bumblebee venom (Figs. 1A, C, and D), partially by American bumblebee venom (Fig. 1C

Table 1. RAST results for bumblebee venom-sensitized sera and control sera. EB: European bumblebee venom, AB: American bumblebee venom, HB: honeybee venom, YJ: yellow jacket venom. Results are expressed in IU/ml of specific IgE. Total IgE expressed in IU/ml. nd: not determined

Patient	EB	AB	HB	YJ	IgE
Kr	29.3	25.2	2.6	1.1	101
Ko	28.3	14.1	23.9	14.6	402
Hu	26.2	23.1	2.0	0.5	517
Mi	46.3	36.1	16.1	nd	662
Ri	13.1	4.0	4.4	20.7	1400
Sl	12.9	2.8	1.6	1.1	299
Ge	27.0	11.9	18.0	19.4	1500
108	2.3	nd	20.0	0.1	69
371	0.6	nd	6.6	0.2	12
406	5.0	nd	28.3	0.1	40
1431	7.5	nd	34.8	0.1	240
1604	6.1	nd	20.4	0.2	395
186	0.5	nd	0.3	20.7	nd
370	0.5	nd	0.2	7.8	25
426	0.3	nd	0.2	32.6	33
460	0.6	nd	0.1	11.6	58
499	0.6	nd	0.0	5.5	190

and D), but not substantially by honeybee venom. The American bumblebee venom RAST was completely inhibited by venoms from American bumblebee and European bumblebee, but not by honeybee venom (Fig. 1F). The honeybee venom RAST was inhibited by European bumblebee venom for serum Mi (Fig. 1B), indicating that the positive honeybee venom RAST for this serum was the result of cross-reactivity by bumblebee venom-induced IgE. Serum Ge contained honeybee venom-specific IgE, as the honeybee venom RAST was not inhibited by European bumblebee venom (Fig. 1E). American bumblebee venom hardly inhibited the honeybee venom RAST for this serum (Fig. 1E). These results suggest that this patient was primarily sensitized to honeybee venom.

Our RAST-inhibition results indicate that honeybee venom and bumblebee venom contain venom-specific IgE-binding epitopes, and from the experiments with serum Kr it must be concluded that the venoms from European and American bumblebee also are not completely cross-reactive, since the RAST for American bumblebee venom was completely inhibited by European bumblebee venom (Fig. 1F), while the reverse was not true (Fig. 1C).

To further substantiate this latter conclusion, we performed inhibition experiments with a number of sera (Kr, Hu, Ko, Ri, Sl, and Ge): after preincubation with venom from either European bumblebee or American bumblebee (and with 6% BSA as control), incubates were tested by RAST

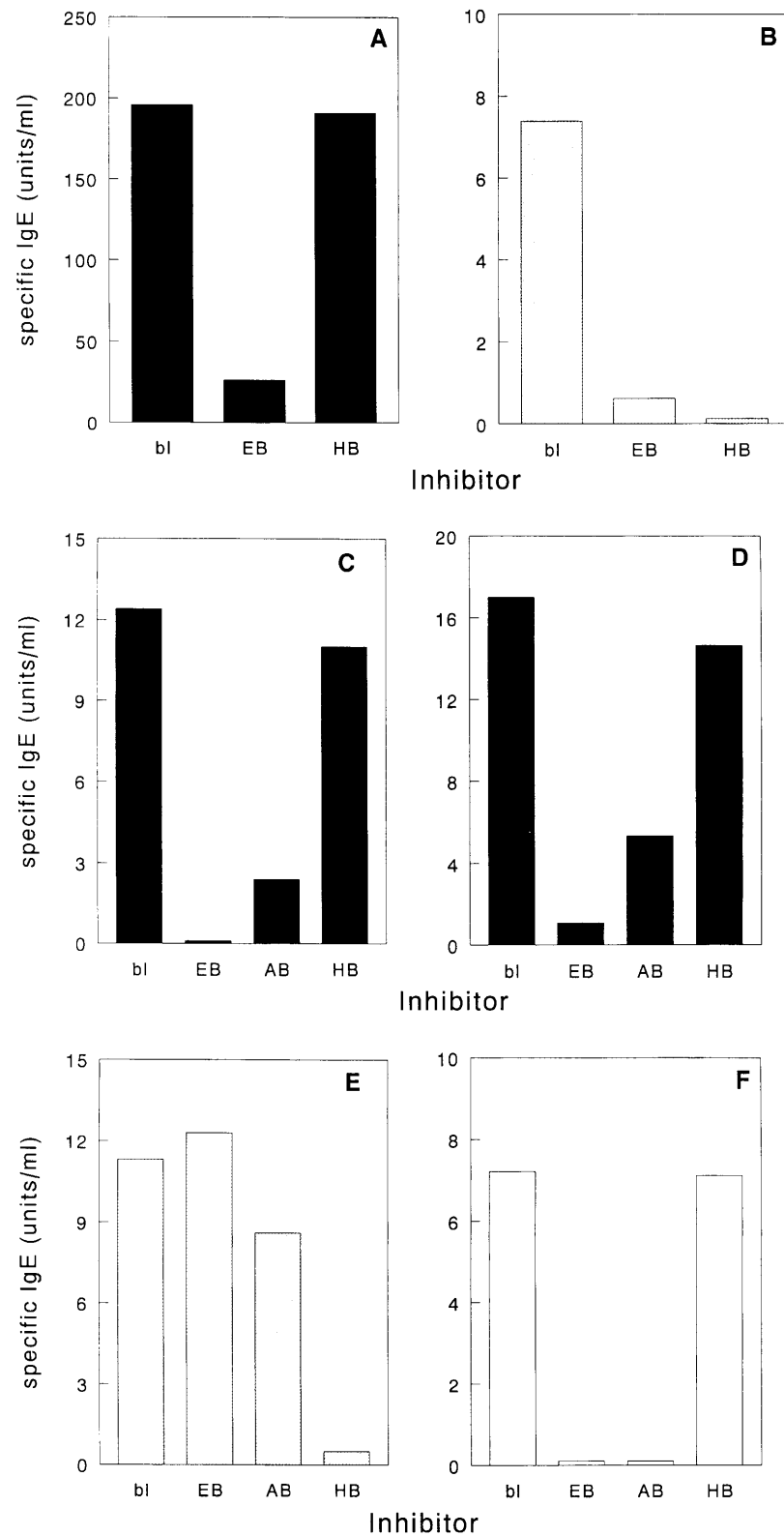


Fig. 1. Venoms from bumblebee and honeybee contain venom-specific IgE-binding epitopes. RAST-inhibition experiments with venoms from European bumblebee (EB), American bumblebee (AB), and European honeybee (HB); in all experiments, 6% BSA was used as control (bl). European bumblebee venom RAST indicated by black bars; American bumblebee venom RAST by open bars; honeybee venom RAST by gray bars. A) serum Mi and inhibition of European bumblebee RAST by venoms EB and HB; B) serum Mi and inhibition of honeybee venom RAST by venoms EB and HB; C) European bumblebee RAST for serum Kr, inhibited by venoms EB, AB, and HB; D) same experiments for serum Ge; E) honeybee venom RAST inhibition for serum Ge by venoms EB, AB, and HB; F) American bumblebee venom RAST inhibition for serum Kr by EB, AB, and HB.

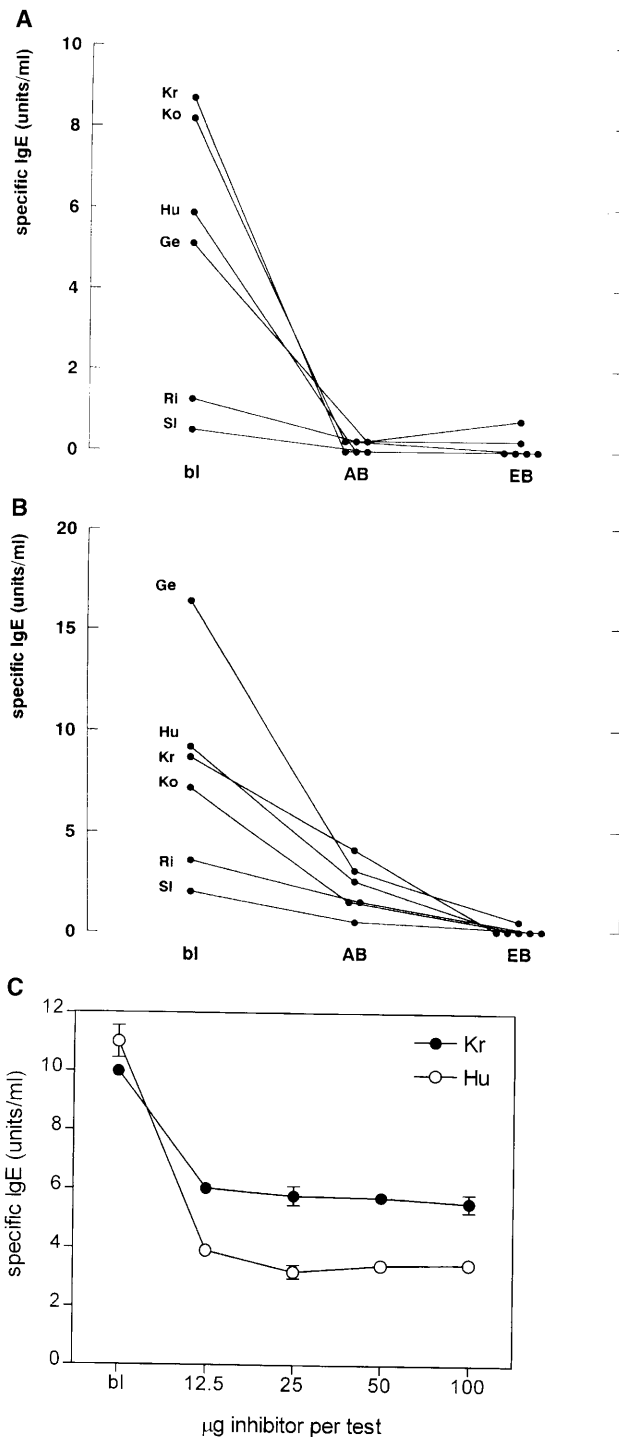


Fig. 2. Venoms from European bumblebee and American bumblebee contain venom-specific IgE-binding epitopes. A) inhibition of American bumblebee RAST for sera Kr, Ko, Hu, Ge, Ri, and Sl by venoms EB and AB; B) inhibition of European bumblebee RAST for same sera by venoms EB and AB; C) inhibition of RAST for European bumblebee venom for sera Kr and Hu by American bumblebee venom: increasing amount of inhibitory venom AB did not result in stronger inhibition of EB venom RAST.

procedure, with both bumblebee allersorbents. Results are shown in Fig. 2A and B.

It is clear that, for all sera tested, the RAST for American bumblebee venom was inhibited completely by European bumblebee venom, while the European bumblebee venom RAST was only partially inhibited by American bumblebee venom. Incomplete inhibition of the European bumblebee RAST was not due to insufficient levels of inhibitory American bumblebee venom, as we used at least 50 µg of the inhibitory component per test, representing at least a 10-fold excess of inhibitory venom over the immobilized venom. As can be seen in Fig. 2C, the use of more American bumblebee venom in this type of test would not result in increased inhibitory activity.

SDS-PAGE and immunoblotting

Fig. 3A shows the results of the Laemmli SDS-PAGE procedure, followed by Coomassie brilliant blue staining of honeybee, European bumblebee, and American bumblebee venom proteins. Information about the identity of most protein bands, although not acquired by measuring specific enzymatic activities, could be obtained by comparing our electrophoretic results with those presented by Hoffman & Jacobson (14).

All venoms tested showed a major polypeptide band in the 17-kDa region, presumably representing the allergenic component phospholipase A (PLA) (MW: 15 488, ref. 14). The venoms from European and American bumblebee venom, respectively, contained a polypeptide of 31–33 kDa absent from honeybee venom. This protein may be part of the fraction four proteins described by Hoffman & Jacobson (14). We also observed a weak 45-kDa polypeptide band, also present in honeybee venom, which probably represents the allergenic enzyme hyaluronidase (7, 14). The European bumblebee venom preparation contained an additional 95-kDa polypeptide which appeared to be absent in the venoms from honeybee and American bumblebee. Finally, the latter venom showed a component, apparently of about 28 kDa, which could not be seen in the venoms from honeybee and European bumblebee. This protein probably represents the 27-kDa venom protease (14). Low-molecular-weight bombolitin polypeptides, previously described in bumblebee venom (9, 10), were not observed.

Immunoblotting experiments, carried out with sera Kr, Ko, Hu, and Mi, revealed major differences in serum IgE specificities (Fig. 3B). Sera Kr and Hu reacted strongly only to bumblebee venom polypeptides: Kr reacted to the 17-kDa PLA component, while Hu IgE bound mainly to the

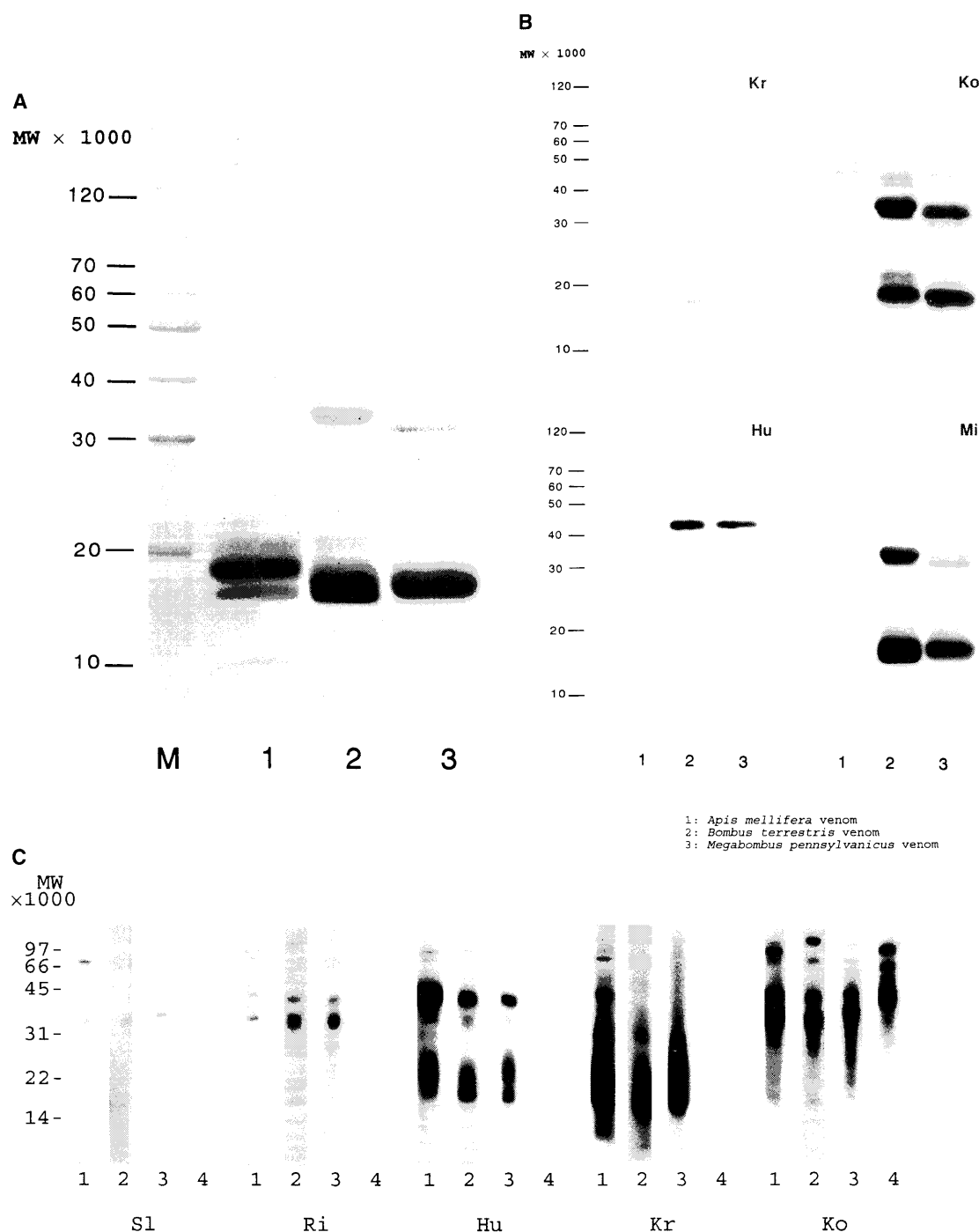


Fig. 3. SDS-PAGE and immunoblotting of bumblebee venoms and honeybee venom shows presence of venom-specific components and IgE-binding epitopes. **A)** SDS-PAGE according to Laemmli followed by Coomassie brilliant blue staining. M: molecular weight markers; venoms from honeybee (1), European bumblebee (2), and American bumblebee (3). **B)** Immunoblotting (SDS-PAGE according to Laemmli) with sera Kr, Ko, Hu, and Mi. Samples applied were honeybee venom (1), European bumblebee venom obtained by capillary suction (2), and American bumblebee venom (3). **C)** Immunoblotting (SDS-PAGE with mildly denaturated venom proteins) with sera Sl, Ri, Hu, Kr, and Ko. Samples applied were European bumblebee venom obtained by capillary suction (1), European bumblebee venom purchased from Vespa Laboratories (2), American bumblebee venom (3), and honeybee venom (4).

45-kDa bumblebee venom compound, presumed to represent hyaluronidase. Sera Ko and Mi contained IgE antibodies binding to many bumblebee polypeptides, and also, although to a significantly

lesser extent, to several honeybee venom components. IgE from serum Ko further bound to the 95-kDa polypeptide exclusively present in our European bumblebee venom preparation. It is

clear that there are major differences between IgE binding to blotted honeybee and bumblebee venoms.

When venoms from honeybee and both bumblebee species were subjected to the SDS electrophoretic procedure without reduction and boiling of the protein sample, satisfactory separations for these previously investigated venoms (14, 18, 19) were obtained. The IgE-binding patterns of sera Sl, Ri, Hu, Kr, and Ko to blotted bumblebee and honeybee venom are illustrated in Fig. 3C. All IgE-binding components corresponded to bands visible in the protein stain (not shown), and the intensity generally correlated with RAST scores. Apart from some differences in electrophoretic mobility and quantity for some of the proteins, venoms from the two bumblebee species tested showed comparable immunoblotting results. No significant differences were observed for the two different European bumblebee venom preparations in this procedure. Three components of approximately 90, 42, and 33 kDa bound IgE in most of the sera. Three sera with a strongly positive RAST for bumblebee venom also showed binding to components of about 22, 19, and 16 kDa. Sera Sl and Ri showed no IgE binding to honeybee venom, and only weak binding was observed with sera Hu and Kr. Serum Ko bound to components of about 93, 63, 53, 42, 18, and 16 kDa. Most of the IgE-binding activity was directed against 42- and 93-kDa polypeptides. When control sera were tested on these blotted venoms, no binding or only trace binding to a single protein was observed. This immunoblotting procedure also revealed considerable differences in IgE binding to Apidae venoms.

Discussion

We subjected sera from patients, sensitized to European bumblebee venom, to RAST tests with immobilized venom-sac extracts from European bumblebee, American bumblebee, and European honeybee. We found that two of these sera (Kr and Hu) with high IgE titers to bumblebee venom, showed very low IgE binding to honeybee venom (Table 1). Conversely, sera from five patients with high IgE titers to honeybee venom showed low IgE-binding activity to bumblebee venom. These results are remarkable, considering the previously reported extensive IgE cross-reactivity between the Apidae venoms (12–14). On the other hand, we did observe partial venom IgE cross-reactivity, as illustrated by the inhibition experiments with serum Mi. The observed positive RAST for honeybee venom for this serum turned out to be the result of IgE cross-reactivity: IgE, induced by bumblebee venom during the patient's occupa-

tional activities, bound to honeybee venom allergosorbent. This binding could be inhibited by bumblebee venom, while the positive RAST for bumblebee venom could not be inhibited by honeybee venom. For reasons unknown, we could not completely inhibit the European bumblebee venom RAST for patient Mi with our European bumblebee venom preparation (88% inhibition), and increasing the amount of inhibitory venom did not lead to complete inhibition; possibly, IgE from this patient bound more strongly to immobilized bumblebee venom than to venom in solution. Completely homologous inhibition was obtained with other sera, however. The presence of venom-specific IgE-binding epitopes, as already observed by Hoffman & Jacobson (14), and also indicated by our RAST-type experiments, was further substantiated by immunoblotting: sera Kr and Hu showed no reactivity with blotted honeybee venom (in agreement with the RAST results obtained with these sera), although the total amount of protein applied on the gel was the same for all venoms tested.

Serum Ko contained IgE which bound to a 95-kDa component in European bumblebee venom. The presence of a polypeptide with a similar MW was reported by Hoffman & Jacobson (14) in venom from *Bombus pennsylvanicus* (here designated *Megabombus pennsylvanicus*), but we could detect such a component only in our European bumblebee venom preparation.

We are aware that we cannot exclude the possibility that some of the observed differences in electrophoretic and IgE-binding patterns may have to be ascribed to different venom preparation procedures. Honeybee venom, for instance, was subjected to gel filtration, while the other preparations were not. This procedure, however, did not seem to result in a major loss of IgE-binding components (apart from mellitin), when we compared our bee venom electrophoretic pattern with the results shown by Nelson et al. (7). Possible differences in venom preparation, however, would not explain the different reactivity of sera Ko and Mi, which showed strong IgE reactivity to bumblebee 17-kDa protein, and weak binding to its electrophoretic counterpart in blotted honeybee venom (Fig. 3B). As shown in Fig. 3A, this allergen was prominent in the electrophoretic pattern of all three venoms tested, and there is no reason to assume that IgE-binding epitopes in honeybee venom PLA would have suffered more from the electrophoretic and blotting procedure than bumblebee PLA.

Since the dissociating conditions employed in the SDS-PAGE procedure described by Laemmli (17) can reduce, or even eliminate, the potency of antibodies to bind to blotted polypeptides, we also

performed immunoblotting experiments in which venoms were incubated with SDS at room temperature before electrophoresis, without reducing agent in the sample buffer, according to the procedure of Peltre et al. (20). We found in earlier experiments (data not shown) that this procedure revealed more IgE-binding components in yellow jacket venom. The destruction of conformational venom epitopes by SDS and boiling has been studied extensively and described for venoms from yellow jacket and honeybee by Hoffman (21).

Indeed, when we performed the alternate "non-reducing/nonboiling" procedure, we observed more IgE-binding polypeptides for sera Hu and Kr in bumblebee venom, an observation which underlines the fact that the Laemmli procedure can lead to denaturation of conformational epitopes. Comparable observations have been reported by Gall et al. (22), who found that boiling of mare's milk led to loss of IgE-binding capacity, and by Wüthrich et al. (23), who observed that the activity of celery extract in skin tests and RAST was strongly reduced after boiling. Immunoblotting of serum Kr, on venom proteins separated by Laemmli's procedure for SDS-PAGE, showed IgE binding to blotted European bumblebee venom 17-kDa protein, but not to American bumblebee venom. This is not in accordance with the positive RAST results for both venoms. On the other hand, immunoblotting after "nonreduced/nonboiling" electrophoresis showed IgE binding to both venoms, underlining the lability of certain phospholipase epitopes, as described by Gall et al. (21).

Be that as it may, results from both immunoblotting procedures confirmed our RAST results, indicating that honeybee and bumblebee venoms contain different IgE-binding epitopes. Unexpectedly, our results also showed that the venoms of the two bumblebee venoms differ in their IgE-binding epitopes. In all inhibition experiments performed (Fig. 2A and B), we found that European bumblebee venom completely inhibited the RAST for American bumblebee venom, but the reverse was not true. The incomplete inhibition by American bumblebee venom, obtained with all the sera tested, could not be ascribed to insufficient amounts of inhibitor venom (Fig. 2C). This means that RAST and blotting experiments, using appropriate sera, might be helpful in resolving problems (as mentioned by Hoffman & Jacobson [14]) concerning the determination of phylogenetic relationships of bumblebee species.

In conclusion, we detected, as expected, IgE cross-reactivity between bumblebee and honeybee venoms, but, in addition, we observed also the presence of venom-specific IgE-binding epitopes. As can be concluded from our immunoblotting

results, IgE in bumblebee venom-sensitized patients binds to components in bumblebee venom which are not recognized in honeybee venom. This raises the question of whether these patients can be successfully treated with honeybee venom immunotherapy. Kochuyt et al. (2) reported on a bumblebee venom-sensitized patient, without detectable cross-reactive antibodies to honeybee venom, who nevertheless experienced beneficial effects from such a treatment. Until more data on such cases are available, we propose to investigate the IgE-binding specificities of sera from bumblebee venom-sensitized patients, before immunotherapy with honeybee venom, since this may not be the optimal immunotherapy treatment.

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