

Honeybee venom allergy: immunoblot studies in allergic patients after immunotherapy and before sting challenge

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By immunoblot techniques, detailed antibody studies were performed with sera of 20 honeybee-venom-allergic patients during or at the end of specific immunotherapy (median duration: 3 years) and before honeybee sting challenge. Before immunotherapy, all patients had experienced systemic allergic reactions to a honeybee sting, with a mean severity of 3.5 ± 0.5 according to the Müller classification. After the sting challenge, 10 patients (reactors) reacted again with a systemic allergic reaction, whereas 10 patients (nonreactors) did not. No differences were observed between reactors and nonreactors in total serum IgE and specific IgE to honeybee venom at the time of challenge. For immunoblot, honeybee venom (RELESS) was separated on 7.5–20% SDS–PAGE. For detection of specific IgE, IgG, IgG1, IgG4, and IgM, an alkaline phosphatase-linked second antibody was used. Both groups showed 11 antibody-binding bands: at 52, 46, 40, 31, 18.7, 16.9, 13, 11, 10, 9, and 8 kDa; however, the antibody-binding pattern was individual. The reactors differed from nonreactors in showing intense IgE and less IgG4 binding to at least one single component of the venom extract. For nonreactors, the inverse relationship was observed. The hypothesis, “intensity of IgE $\geq$ IgG4 leads to allergic symptoms”, was highly significant ($P=0.00026$; chi-square). These immunoblot findings could offer predictive value in distinguishing reactors from nonreactors.

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Since the introduction of pure insect-venom-specific immunotherapy (IT) in 1974, its efficacy has been shown by a number of studies (1, 2). Nevertheless, the immunologic mechanisms underlying this treatment are still unclear. The fact that nonallergic beekeepers exhibit specific IgG4 antibodies in their sera most frequently and that specific IgG, especially IgG4 “blocking antibodies”, increase during IT led to the supposition that these antibodies could protect against immediate allergic reactions (3, 4). Thus far, several investigators have been unable to show a relationship between increased IgG and clinical protection (5–7). On the other hand, passive IT with hyperimmune sera from beekeepers increased the level of tolerance to a specific dose of honeybee venom (8–11). Venom-specific IT is considered a safe and effective treatment for patients with allergic systemic reactions after Hymenoptera stings. According to the literature and depending on the insect, the reported

efficacy range is 70–90% (12). Presently, only a sting challenge with a living insect can demonstrate the success of treatment. A more practical method would be preferable if it correlates closely to clinical protection as judged by the outcome of a sting challenge.

Allergen extracts consist of a complex mixture of proteins and, potentially, multiple epitopes, so that measurements of the absolute amount of specific IgE and IgG do not reveal the antibody pattern to the individual allergens in the extract. However, immunoblot methods separate the complex allergen mixture by molecular size on SDS–PAGE. After electrophoretic transfer of the separated proteins onto a nitrocellulose membrane and incubation of this matrix with patient sera, the specific antibody pattern of an individual can be evaluated. The most common allergens described in honeybee venom are phospholipase A₂ (PLA₂) (15–20 kDa), hyaluronidase (43 kDa), acid phosphatase (49 kDa),

Table 1. Demographic and immunologic data, Müller class of reaction of original sting, duration of immunotherapy (IT), time of reexposure to sting challenge (st.ch.) during (dur.) or at end (end) of IT, and reactions to sting challenge of all patients investigated, divided into nonreactors and reactors. (A = angioedema, D = dyspnea, E = erythema, F = flush, P = pruritis, U = urticaria, W = wheezing)

Nonreactors						CAP bee		kU _A /l	
Patient no.	Sex	Age (years)	Müller class	IT (years)	Reexposure st.ch.	kU _A /l	cl.	IgE kU/l	Reaction st.ch.
1	F	41	IV	6	end	4.9	3	60.7	0
2	F	55	IV	3	dur.	2.7	2	40.4	0
3	M	54	III	4	end	0.5	1	44.9	0
4	M	45	III	3	end	0.4	1	30.7	0
5	F	36	III	7	end	6.4	3	759.0	0
6	M	36	IV	4	dur.	0.8	2	13.4	0
7	M	51	IV	3	dur.	2.0	2	92.8	0
8	M	23	III	3	end	9.8	3	55.4	0
9	M	45	IV	3	dur.	7.9	3	194.0	0
10	F	17	III	3	dur.	2.3	2	132.0	0
Reactors									
11	F	60	IV	0.2	dur.	8.2	3	70.9	U, A, D
12	F	32	III	0.3	dur.	28.1	4	319.0	F, U
13	M	35	III	3	end	2.1	2	16.0	E, U, W
14	M	40	IV	7	dur.	29.5	4	187.0	F, D
15	M	48	III	3	end	0.3	0	6.3	A, U
16	M	20	III	4	dur.	10.5	3	115.0	U, D
17	F	72	IV	3	dur.	2.0	2	16.8	U
18	M	32	IV	3	dur.	2.6	2	38.6	P, U
19	F	49	IV	3	end	2.2	2	172.0	P, F, U
20	F	52	IV	14	dur.	0.3	0	91.4	A

melittin (2.8 kDa), and many other components of unknown allergenic activity (2).

The purpose of this study was to examine the individual isotype-specific immunoblot patterns of honeybee-venom-allergic patients during or after IT, in relation to clinical protection as judged by sting challenge.

Material and methods

We investigated 20 honeybee-venom-allergic patients (11 men, nine women; age range 17–72, $\bar{x} \pm s = 42 \pm 13$ years). All had a history of previous systemic allergic reactions caused by a honeybee sting, with a mean severity of 3.5 ± 0.5 according to the Müller classification (13). The patients received specific IT with honeybee venom and a maintenance dose of 100 µg, for a median duration of 3 years. All patients underwent sting challenge with a living honeybee (6) either during (12 patients) or 1 year after stopping (eight patients) IT. Ten patients who developed a systemic reaction to the challenge (reactors) were matched with 10 patients who tolerated the sting challenge well. The latter were selected from a much larger group of nonreactors on the basis of a similar distribution of age, sex, Müller grade of the original reaction, and duration of IT. The demographic data of the 20 honeybee-venom-allergic patients studied, grouped into reactors and nonreactors, are shown in Table 1.

Blood was drawn immediately before the sting challenge, and sera were kept frozen (–20°C) until analyzed. IgE and specific IgE were measured by the Pharmacia CAP System (Pharmacia Diagnostics, Sweden). The results were expressed in kU/l for IgE and kU_A/l, as well as CAP classes ranging 0–6 for specific IgE.

Immunoblot

RELESS honeybee venom (5 µg/cm gel) was separated on a 7.5–20% SDS–PAGE (14), and transferred to nitrocellulose (NC). The NC was blocked with 0.1% Tween 20 in 0.1 M Tris-HCl, pH 7.4, 0.1 M NaCl, 2.5 mM MgCl₂, cut into strips, and incubated overnight with patient sera in the following dilutions: IgE 1:4; IgG, IgG1, IgG4 1:80; IgM 1:40. For detection of specific IgE, IgG, and IgM, an alkaline phosphatase-linked second goat-antihuman antibody (Jackson Inc., DIANOVA, Hamburg, Germany) was used. For detection of the subclasses IgG1 and IgG4, mouse monoclonal antibodies (NL 16+GB 7B, Unipath, MEDAC, Germany) and a third alkaline phosphatase-linked goat-antimouse antibody (Jackson Inc., DIANOVA, Hamburg, Germany) were employed. Serum of a highly sensitized honeybee-venom-allergic patient served as positive control, and serum of a nonatopic person without history of insect sting, as negative control. A typical immunoblot is shown in Fig. 1.

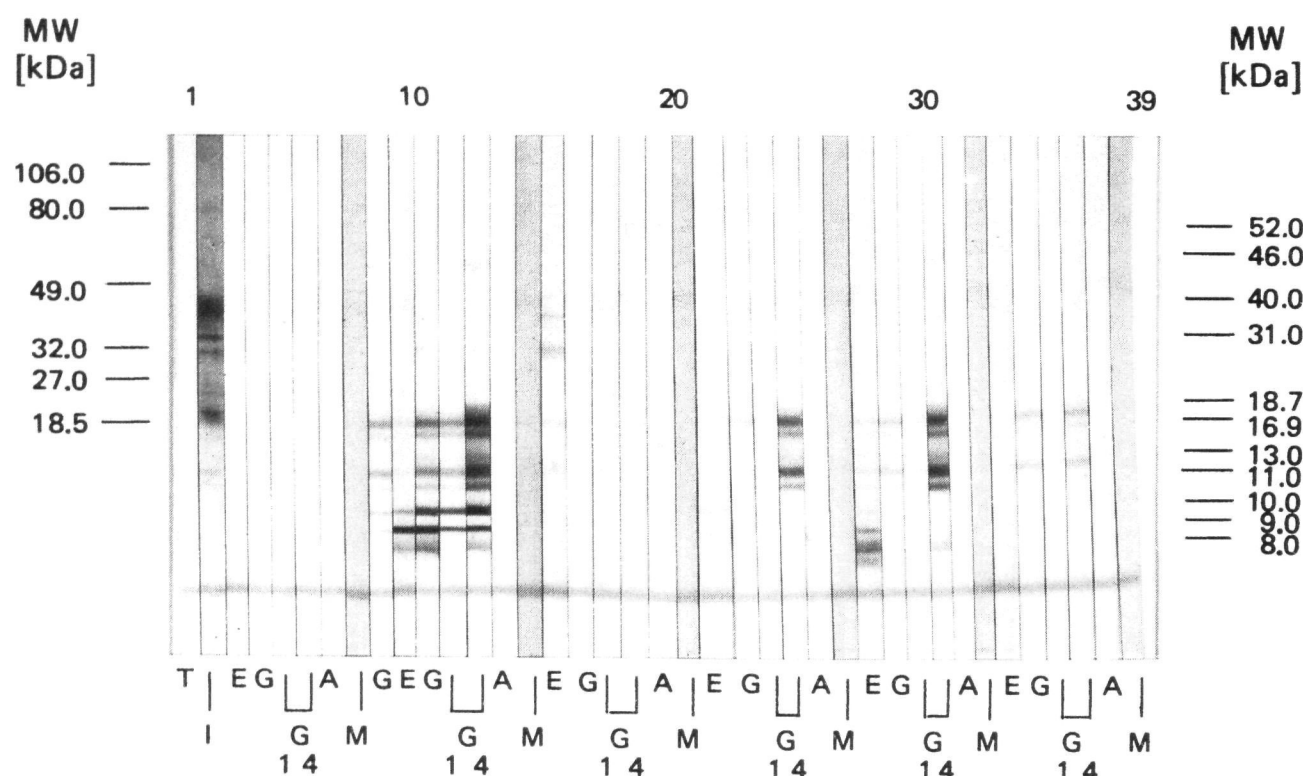


Fig. 1. Binding of IgE, IgG, IgG1, IgG4, IgM, and IgA to honeybee venom components in immunoblotting by sera from five allergic patients: molecular weights (MW) of marker proteins (left), honeybee venom components (right). Lane 1) negative buffer control; lane 2) India ink protein staining; lanes 3–8) serum of nonallergic person; lane 9) positive control; lanes 10–15, 16–21, 22–27, 28–33, 34–39) one patient each.

The frequency and intensity of antibody binding to each protein band was detected by a 12 cx XRS Omni Media Scanner (X-Ray Scanner Corporation, Torrance, CA, USA) and evaluated with a Bio Image Work Station (Millipore), as shown in Fig. 2. For statistical evaluation, the Mann–Whitney U test and chi-square test were used, and the intensity was expressed by a score from 0 to 6 only for graphical presentation of the individual values.

Results

The immunologic data evaluated in the sera before the sting challenge, grouped for reactors and non-reactors, are shown in Tables 1 and 2. In all but two patients of the reactor group, specific IgE to honeybee venom could be detected with the CAP system. There was a tendency toward higher specific honeybee-venom IgE and lower total IgE levels in the reactor group, although statistically significant differences were not observed.

Immunoblot

On immunoblot, 11 immunoglobulin-binding bands were detected in the regions of 52, 46, 40, 31, 18.7, 16.9, 13, 11, 10, 9, and 8 kDa in all patients. The regions of 18.7 and 16.9 kDa represent the molecular size of phospholipase A₂ in different glycosylated forms, and 10 kDa represents the tetramer of melittin.

Table 2. Clinical and immunologic data expressed in mean ($\bar{x} \pm s$), median, or geometric mean (g.x) of all patients and separately for nonreactors (NR) and reactors (R)

	Sex		Age (years) ($\bar{x} \pm s$)	Müller class ($\bar{x} \pm s$)	IT (years) $\bar{x}$	IgE kU/l g.x	CAP class ($\bar{x} \pm s$)	CAP kU _A /l g.x
	M	F						
All	10	10	42 ± 14	3.5 ± 0.5	3	65.7	2.2 ± 1.1	2.79
NR	6	4	40 ± 13	3.4 ± 0.5	3	73.4	2.2 ± 0.8	2.35
R	5	5	44 ± 15	3.6 ± 0.5	3	58.8	2.2 ± 1.4	3.30

IgG and IgG4 were found most frequently in this study and were detected in all of the above-mentioned regions. IgE was detected in all except the 11-kDa band; IgM, in all except the 11- and 8-kDa bands; and IgG1, in all except the 52-, 46-, and 40-kDa bands (Table 3).

Differences were noted in frequency and intensity of immunoglobulin binding between the reactors and nonreactors (Fig. 3a and b). As is apparent, the reactor group showed more frequent and intense immunoglobulin binding of all classes, but statistically significant differences were seen only with IgE ($P<0.005$). The immunoglobulin-binding pattern for each patient is shown in Fig. 4a and b. IgE binding was seen in all but two patients (nos. 15 and 20) in the reactor group. These two patients also had a negative CAP class to honeybee venom. In the nonreactor group, IgE binding was detected in two patients (nos. 5 and 8) only, although

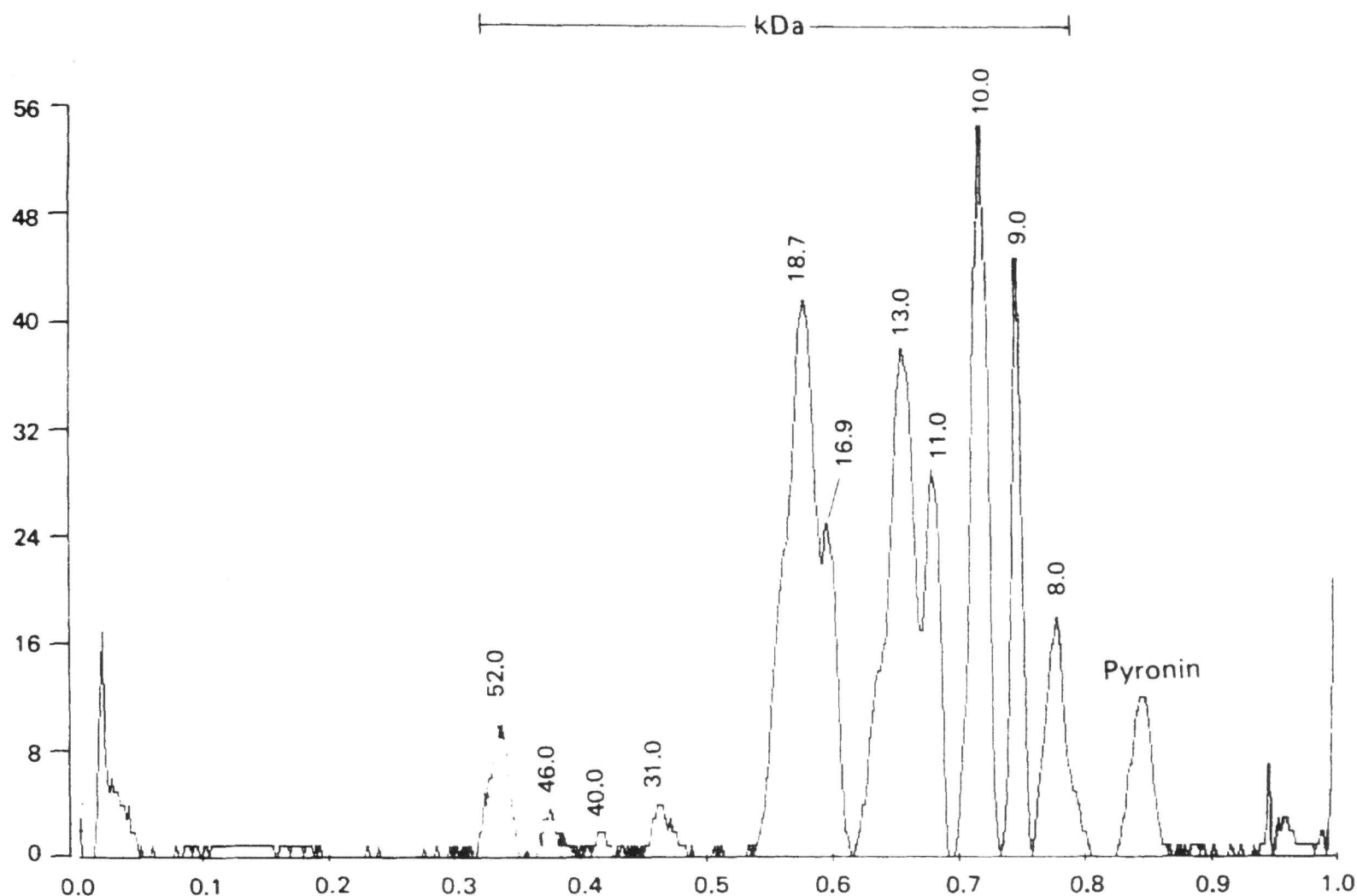


Fig. 2. Intensity of IgG4 binding (lane 13, Fig. 1) to components of honeybee venom quantitated by 12cx XRS Omni Media Scanner. Numbers at peaks indicate molecular weight regions.

all patients showed a positive CAP class to honeybee venom (Table 1). IgG4 could be detected in all patients except one of each group (nos. 4 and 20). In analysis of the intensity of binding, it was obvious that the ratios of specific IgE/IgG4 varied with different protein bands and between the two groups. Only the reactors expressed more specific IgE with less or equal intensity of specific IgG4 to at least one component of the venom extract, whereas the nonreactors did not (Table 4). The hypothesis, "the ratio of $\text{IgE} \geq \text{IgG4}$ to a single allergen causes allergic reactions, whereas $\text{IgG4} > \text{IgE}$ means protection", was highly significant ($P=0.00026$; chi-square).

Discussion

The differences noted between the reactors and nonreactors were marked in frequency and intensity of binding of the specific isotype antibodies (Fig. 3), as well as in the IgE/IgG4 ratio to a single protein band (Fig. 4), while the specific IgE determined by the CAP System showed no significant differences. As has been shown for other allergens, IgE binding to an allergen extract is highly individual, as is the antibody response induced by IT (15–18). It is well established that, up to a maximum

Table 3. Numbers of patients with immunoglobulin binding to 11 molecular-weight (MW) regions of honeybee venom in sera of 10 reactors (R) and 10 nonreactors (NR)

Antigen MW (kDa)	IgE R/NR		IgG R/NR		IgG1 R/NR		IgG4 R/NR		IgM R/NR	
52	1	–	5	–	–	–	7	5	1	–
46	1	–	5	–	–	–	3	–	4	–
40	1	–	4	2	–	–	2	1	1	–
31	1	–	3	2	1	1	1	1	1	–
18.7	7	1	9	8	8	4	9	9	2	3
16.9	2	–	8	5	7	–	8	8	1	1
13	2	1	8	6	6	4	8	7	–	1
11	–	–	7	4	3	1	8	5	–	–
10	6	1	5	3	3	3	6	6	1	–
9	6	–	2	2	1	1	2	1	–	–
8	2	–	3	1	1	1	2	–	–	–

level, the amount of IgG antibody produced during IT is a function of the cumulative therapeutic dose given, and the induction of IgG antibody depends on the concentration of proteins present in the therapeutic extract (4, 16). One may postulate that patients with a negative outcome of specific IT are more selectively sensitized to "minor allergens". These allergens may not be present in sufficient concentration in the therapeutic extract, or the

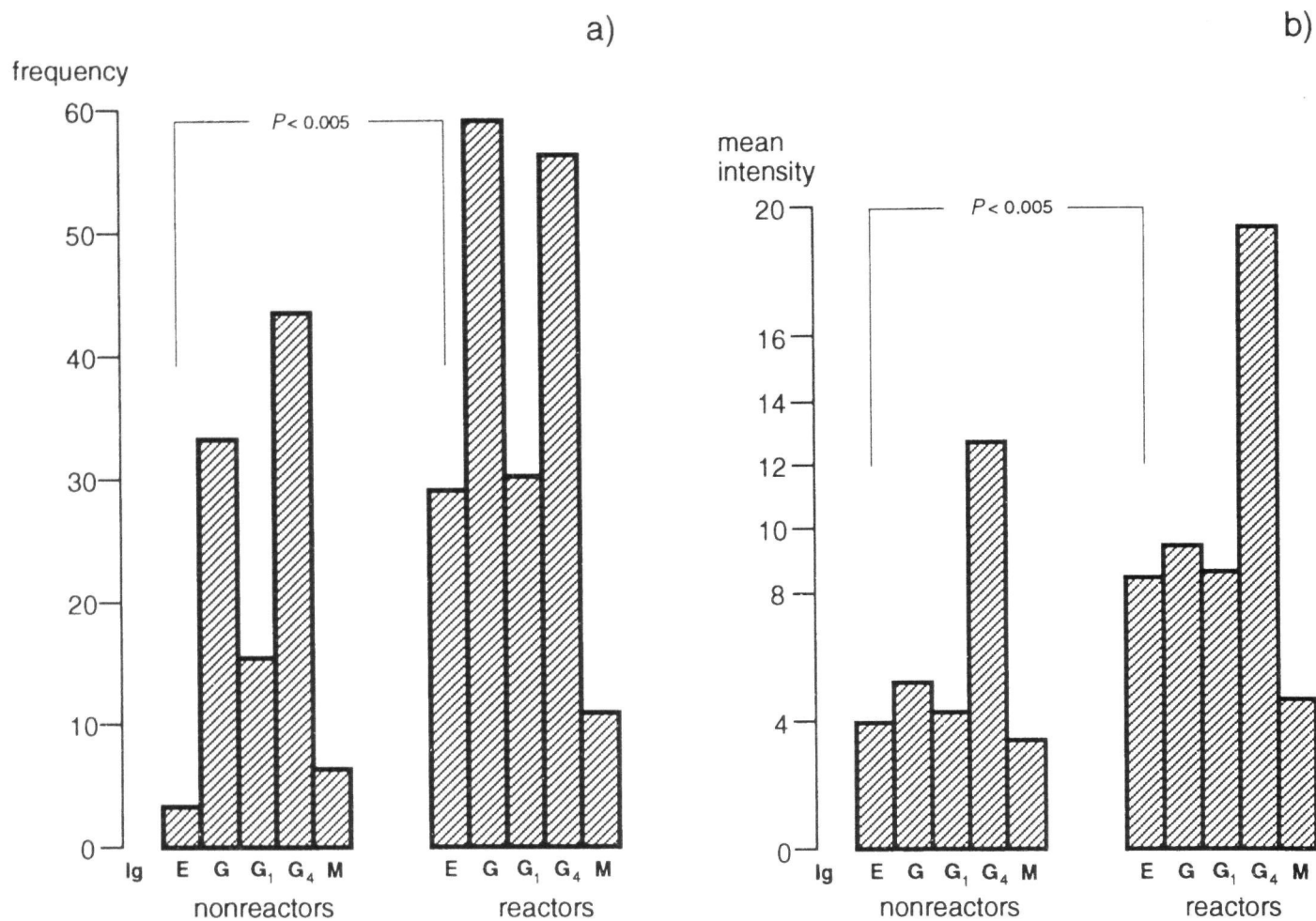


Fig. 3. **a)** Sum of frequency and **b)** mean intensity of IgE, IgG, IgG1, IgG4, and IgM binding to all 11 components of honeybee venom found by immunoblotting in sera of reactors and nonreactors.

patients may not be able to produce adequate amounts of specific IgG4 to these components. This assumption is supported by the data presented in Fig. 4 and Table 4.

We propose the following hypothesis: protection is indicated by the presence of significant amounts of specific IgG4 to all single allergenic components to which the patient has specific IgE, whereas absent or insufficient IgG4 to only one allergen to which IgE is present is associated with allergic symptoms. This hypothesis fits all our subjects except patients 15 and 20 of the reactor group. Interestingly, these two patients also had no detectable specific IgE (Cap system) before the sting challenge (Table 1), although an IgE-mediated allergic reaction was diagnosed before specific IT.

This hypothesis may also be relevant to the long-standing controversy in the literature on the protective function of specific IgG4. If we examine the absolute level of specific IgG4 to an allergen extract, the relationship with IgE ≥ IgG4 to a single component is not evident. The increase of specific IgG4 during IT is apparent, but no absolute value can be identified that would predict further allergic reactions (6, 19). Determination of a specific IgG4 response to single allergens (e.g., PLA₂) would fail to predict further reactions, because of

Table 4. Ratio of IgE/IgG4 binding to honeybee venom components (kDa) of patients with detectable IgE in immunoblot. Intensity expressed as scanner data (see also Fig. 4)

Nonreactors				
No. 5	13 kDa:	4/16		
No. 8	18 kDa:	4/34;	10 kDa:	4/20
Reactors				
No. 11	9 kDa:	45/44		
No. 12	40 kDa:	8/0;	31 kDa:	14/0;
No. 13	10 kDa:	7/5		
No. 14	10 kDa:	5/3;	9 kDa:	14/0;
No. 16	9 kDa:	4/4		
No. 17	52 kDa:	6/6		
No. 18	9 kDa:	5/0		
No. 19	46 kDa:	1/0;	9 kDa:	4/0

the patient's highly individual sensitization (6). The immune response to each individual allergenic component of an allergen extract may instead allow us to distinguish between reactors and non-reactors. The specific IgE/IgG4 antibody ratio reflects the allergic or protective state of a sensitized patient. This may be of predictive value in insect allergy, but more studies are needed to confirm our data.

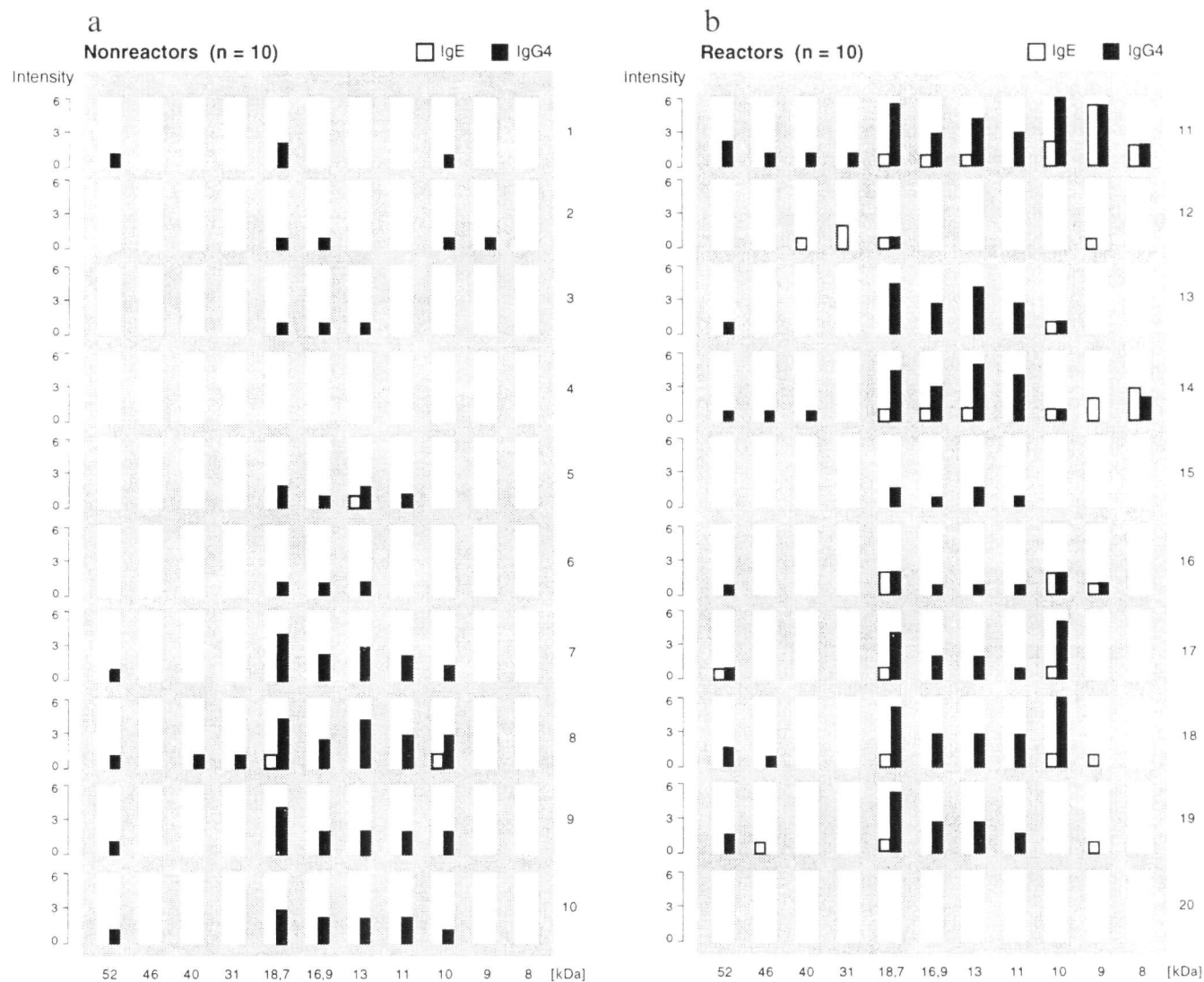


Fig. 4. Binding of IgE and IgG4 to 11 honeybee venom components (x-axis) in immunoblotting by sera from 20 allergic individuals. Nonreactors nos. 1–10, reactors nos. 11–20. For graphic presentation, intensity is expressed in classes (y-axis). Further details are presented in Table 4.

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