

IgE, IgG, IgG1 and IgG4 patterns in yellow jacket allergic patients during immunotherapy with a venom depot extract

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Summary

Using immunoblot studies, detailed antibody patterns were performed with the sera of 10 yellow jacket allergic patients undergoing specific immunotherapy with a yellow jacket venom SQ-depot extract (ALK, Denmark). Five males and five females (age range 10–65, mean 48 years) were investigated. All patients had a history of systemic reactions after an insect sting and a positive skin-prick test at a dose of at least 100 µg/ml yellow jacket venom.

Yellow jacket venom (ALK) was separated on a 7.5–20% SDS-PAGE, transferred to nitrocellulose (NC), and then the NC-strips were incubated with the patients' sera. For detection of IgE, IgG, IgG1 and IgG4, an alkaline phosphatase linked 2nd antibody was used. Prior to immunotherapy, a strong IgE binding was detected at 25 kD in nine of 10 patients, representing Antigen-5 (Ag-5) as major allergen. Reactivity to this antigen was also present with the other immunoglobulin classes, although not as marked. In addition, a second frequent IgG and IgG1-binding band was seen at 35 kD (phospholipase A). Only weak or no binding to this band was found with IgE and IgG4. Binding to hyaluronidase (43 kD) was seen only in three cases.

During immunotherapy, a significant increase in IgG and particularly IgG4 staining was found with Ag-5, whereas hyaluronidase induces mainly an IgG1 response and phospholipase A showed only a weak IgG response. In addition, the formation of new IgG4 binding to proteins in the region of about 70–90 kD was found in most patients. The dose necessary for the induction of this antibody formation was ≥ 150.00 SQ yellow jacket venom. We conclude that the increase in the total amount of specific IgG4 to an allergen extract (protein mixture) during immunotherapy gives no information about the increase of IgG4 to the patient's special major allergen.

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Introduction

Immunotherapy (IT) with pure insect venom is an established method for treating insect venom allergic patients with systemic reactions to insect stings. This treatment has been demonstrated to be effective in reducing the risk of life-threatening reactions in $\geq 90\%$ of the patients [1,2]. During IT, specific IgG and especially IgG4 ('blocking antibodies') increase, and a controversial

discussion has been raised as to whether these antibodies could have protective functions in preventing immediate allergic reactions. Several investigators were so far unable to show a relationship between increased IgG and a clinical protection, whereas, on the other hand, a passive IT with hyperimmune sera from beekeepers increase the level of tolerance to a specific dose of bee venom [3–6].

Allergen extracts consist of a mixture of different proteins so that the total amount of specific IgE and IgG does not reveal the individual antibody pattern to the major and/or minor allergens. SDS-PAGE separates the

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Table 1. Demographic and immunological data of all 10 patients before immunotherapy. Data show lower and upper quartile with median in parentheses and mean $\pm$ s.d.

	Sex		Age (years)		IgE (IU/l)		RAST (PRU)		IgG (%)	
	m	f	mean $\pm$ s.d.	($\bar{x}$)	Quartile	($\bar{x}$)	Quartile	($\bar{x}$)	Quartile	($\bar{x}$)
Patients	5	5	46.6 $\pm$ 17	(48)	43–276	(49)	0.7–12.8	(0.82)	5.8–24	(7.0)
Atopic	4	1	40.8 $\pm$ 18	(48)	43–505	(45)	0.82–3.5	(0.82)	5.8–47	(7.0)
Non-atopic	1	4	51.5 $\pm$ 15	(56)	43–73	(58)	0.7–12.8	(0.89)	7.0–15	(8.5)

complex allergen mixture with respect to the molecular size, followed by electrophoretic transfer of the separated proteins onto a nitrocellulose membrane. By incubation of this matrix with patients' sera, the specific individual antibody pattern can be evaluated. The purpose of this study was to examine the individual specific IgE and specific IgG patterns and their changes during IT, using the immunoblot technique.

Material and methods

Patients

We included 10 allergic patients (demographic data, Table 1) with previous systemic reactions caused by the sting of a yellow jacket (YJ). Systemic manifestations included cutaneous (urticaria and angioedema), respiratory, intestinal and vascular symptoms. The allergic reactions were classified according to Müller [7] and ranged from class 2–4 (mean $\pm$ s.d. = 3.0–0.9). All patients had a positive skin test and a positive RAST with yellow jacket venom. Five patients had in addition a positive skin-prick test to at least one of 12 common inhalant allergens tested and were therefore called atopics.

Treatment

All patients were treated with yellow jacket venom SQ-depot extract (ALK, Denmark). Weekly injections with increasing doses were followed according to the schedule recommended by the manufacturer until a maintenance dose (MD) of 100 000 SQ was reached. The maintenance dose was given once per month. Blood samples were drawn initially at regular intervals during IT until the maintenance dose was reached, and again 1 year later. Sera were kept frozen (-18°C) until analysed. IgE, specific IgE and specific IgG were measured by Pharmacia PRIST- and RAST-kits (Pharmacia-Diagnostics, Sweden). The results were expressed in Phadebas RAST units (PRU) for specific IgE and in per cent for specific IgG.

Immunoblot

ALK yellow jacket venom (11 000 SQ/cm gel) was separated on a 7.5–20% SDS-PAGE, modified after Neville [8], and transferred to nitrocellulose (NC). The NC was blocked with 0.1% Tween, cut in strips and incubated with patient sera as described by Westphal [9]. For detection of specific IgE and IgG, an alkaline phosphatase linked 2nd goat-anti-human antibody (DIANOVA) was used. For detection of IgG1 and IgG4, mouse monoclonal antibodies (MEDAC) were chosen and detected by a 3rd alkaline phosphatase linked goat-anti-mouse antibody (DIANOVA). Serum of a highly sensitized YJ allergic patient served as positive control and sera from non-atopic individuals without a history of adverse reactions to an insect sting and a negative RAST as negative controls. For a semiquantitative calculation, the intensity of antibody binding to each protein band was optically determined and expressed by a score from 0–4. The scores of each protein band were summarized and divided by the total group showing the relative intensity of antibody binding to a single protein.

Results

The maintenance dose of 100 000 SQ yellow jacket venom was reached between 4 and 11 months in all patients (mean $\pm$ s.d. 7.7 $\pm$ 2.5). The mean cumulative dose until maintenance dose was about 350 000 SQ, and 1.350 000 SQ after 1 year at maintenance dose. No systemic side-effects were seen. Only one patient had large local reactions, and his dose therefore had to be increased slowly. For personal reasons, two other patients interrupted the therapy for a few weeks. This explains the relatively long time for reaching maintenance dose in the present patient population. Table 1 shows the demographic and immunological data of the total group, divided into atopics and non-atopics before IT. There were more male patients in the atopic group, and their first systemic reaction to an insect sting occurred at a younger age. The other parameters showed no significant differences between atopics and non-atopics.

Table 2. IgE, specific IgE and specific IgG levels of all 10 patients during immunotherapy (IT). Data show range, lower and upper quartile with median in parentheses

	IgE (IU/l)			Specific IgE (PRU)			Specific IgG (%)		
	Range	Quartile	($\bar{x}$)	Range	Quartile	($\bar{x}$)	Range	Quartile	($\bar{x}$)
Before IT	832	43-276	(49)	17.3	0.7-12.8	(0.8)	254	6-24	(7)
MD	513	21-222	(33)	12.8	0.2-3.5	(0.7)	269	20-71	(46)
1 year at MD	795	22-216	(47)	17.5	0.2-2.9	(0.7)	245	37-102	(59)

MD, maintenance dose. * $P=0.01$; ** $P=0.002$.

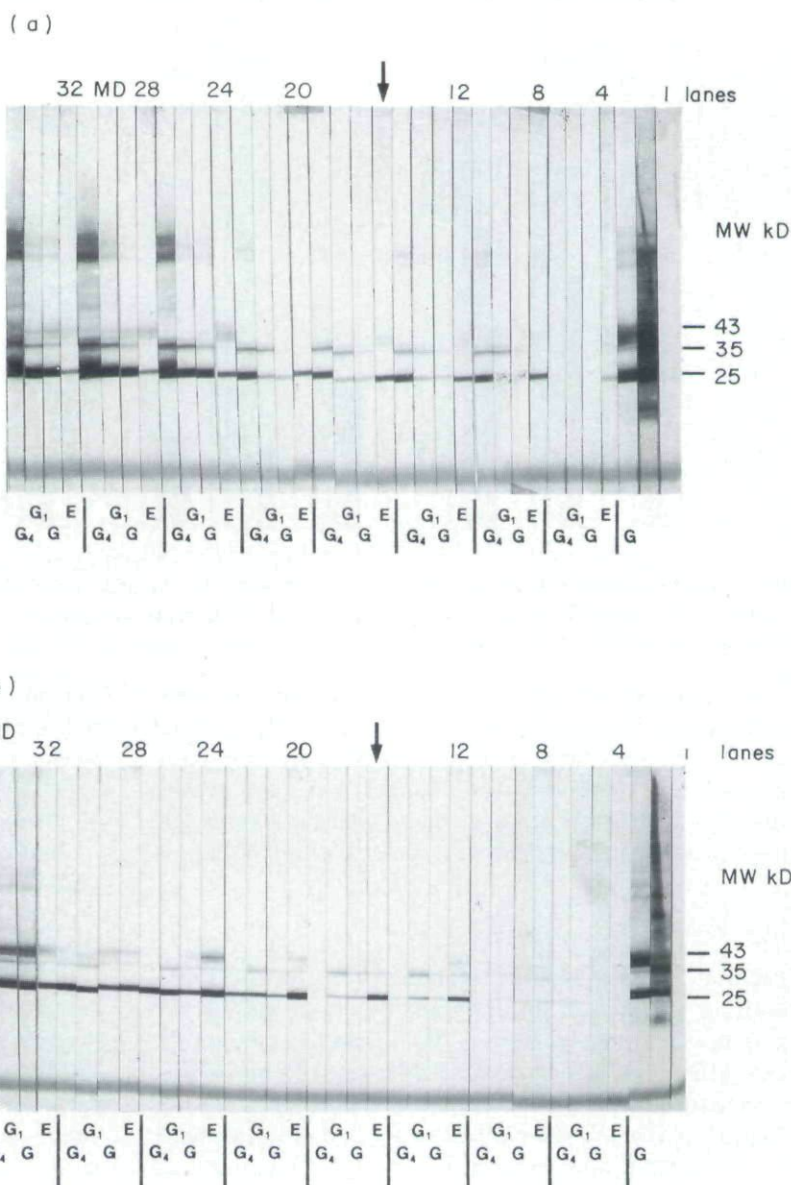


Fig. 1. Antibody pattern of (a) a 48-year-old non-atopic man with an anaphylactic reaction and (b) a 49-year-old atopic woman with angioedema and dyspnoea after a yellow jacket sting before and during immunotherapy. Lane 1, buffer control. Lane 2, indian ink protein staining. Lane 3, positive control serum (IgG). Lanes 4–7, negative RAST serum with all antibody classes tested. Lanes 8–35, patient sera drawn at different time intervals after a YJ sting. The arrow indicates the beginning, MD start of maintenance dose of IT. Note the marked increase of IgG4. For further explanations, see text.

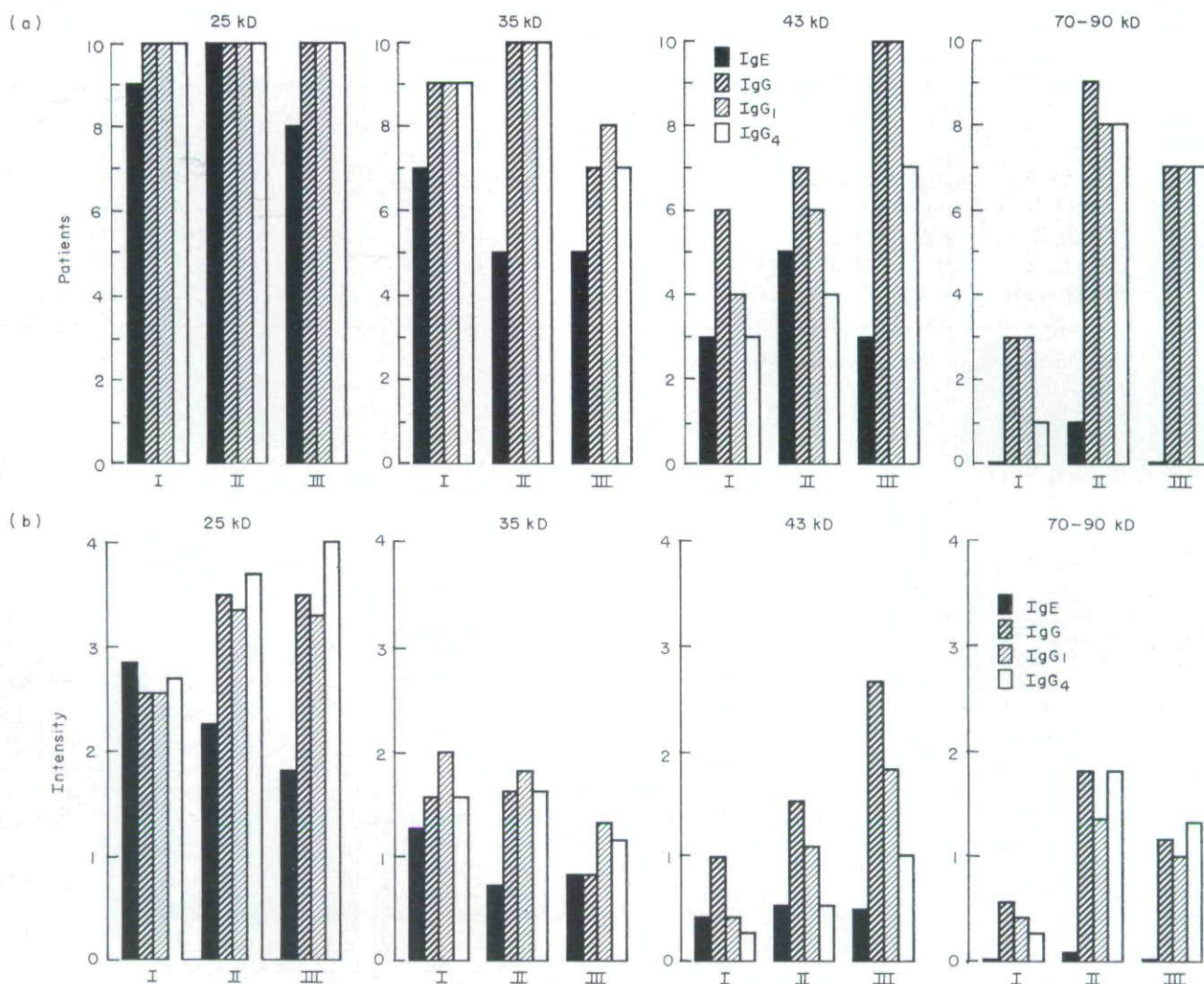


Fig. 2. (a) Frequency distribution and (b) relative intensity of immunoglobulin binding to vespid venom proteins in 10 YJ allergic patients, (I) before, (II) at the start of maintenance dose, and (III) after one year at maintenance dose of IT. The region between 70–90 kD was evaluated together, although it resolved in some patients into six different bands.

The antibody changes during IT are presented in Table 2. Specific IgE showed a slight decrease, total IgE did not change and specific IgG increased. With the non-parametric Mann–Whitney U-test, changes of total and specific IgE were not statistically significant, whereas specific IgG showed a statistically significant increase ($P=0.002$).

Immunoblot

Figures 1a and b show examples for the individual antibody pattern of all immunoglobulin classes in two patients before and during IT. Figure 1a shows the individual antibody pattern of a 48-year-old non-atopic man with an anaphylactic reaction to a yellow jacket sting. The first two blood samples, taken at 6 and 12 weeks (lanes 8–15) after the sting showed IgE and IgG₄ binding at 25

kD, and IgG₂ and IgG₄ binding at 35 kD. No changes in the antibody pattern could be detected during these 6 weeks. After starting IT, an increase in IgG, particularly IgG₄ to all protein components and a weak IgE binding at 43 kD was seen, which was more pronounced at MD.

Figure 1b shows the individual antibody pattern of a 49-year-old atopic woman with angioedema and dyspnoea after a yellow jacket sting. With the first blood sample (lanes 8–11), drawn 1 week after the sting, only very weak IgE, IgG and IgG₁ binding at 25 and 35 kD could be detected. With the second blood sample (lanes 12–15), drawn 5 months after the sting, antibody binding (IgE and IgG₁) increased to all three antigens, but only very weak IgG₄ binding was found. With increasing dosages of the venom depot extract, an increasing intensity was observed with all antibody classes, mainly at 25 kD.

The frequency (Fig. 2a) and relative intensity (Fig. 2b) of antibody binding to each protein band were evaluated in all patients. The region above 70 kD was regarded as one band, although some patients had up to six different bands at the same time period. Prior to IT, IgE binding was detected at 25, 35 and 43 kD, representing Ag-5, phospholipase A and hyaluronidase respectively. Nine of the 10 patients showed IgE-binding at 25 kD, seven at 35 kD and only three patients at 43 kD. No IgE-binding was seen above 43 kD. Two of the three patients with antibody binding at 43 kD (hyaluronidase) had in addition a positive RAST (class ≥ 2) for honeybee venom.

All patients produced IgG, IgG1 and IgG4 binding at 25 kD and nine patients at 35 kD. Binding at 43 kD was noted in six patients with IgG, in four patients additionally with IgG1 and in three patients with IgG4. In the region between 70–90 kD, only three patients had very weak IgG and IgG1 binding and only one patient showed additionally IgG4 binding. In the beginning, IgG classes showed the same distribution between the different proteins as IgE. Until maintenance dose, the frequency distribution increased in all IgG classes and showed a small decrease after 1 year of MD, except at 43 kD, indicating the induction of new antibody formation.

Figure 2b shows the relative intensity binding of antibodies for each protein band detected, before IT, at maintenance dosage and after 1 year at MD. The ratio between IgE:IgG was about 1:1 in the beginning and increased to about 1:2 during therapy, except for the 35 kD band.

There were, however, differences in the immunoglobulin responses for the different protein components in the venom. At 25 kD, a slight decrease in relative intensity was seen with IgE and an increase of all IgG classes investigated, mainly IgG4. The 35 kD band showed no major changes in antibody binding, whereas the 43 kD band showed a pronounced increase with IgG, followed by IgG1 and IgG4. In the region between 70–90 kD, no IgE binding could be detected, and only three patients showed weak IgG binding which increased during therapy in all patients. At MD dose, only one patient developed a weak IgE band in this region. After 1 year at maintenance dose, seven patients showed still all IgG classes in this region.

Discussion

The aim of immunotherapy is clinical protection, but no *in vitro* parameter for assessing successful treatment is established as yet. The rise of specific IgG and a decreased or unchanging specific IgE during IT is well documented [5,6]. Hoffman *et al.* [10] described five major allergenic proteins in yellow jacket venom: Vmacl (97 kD), hyaluronidase (46 kD), Vmac3 (39 kD), phospholipase A and B

(34 kD) and antigen-5 (22 kD). In an IgE-RAST with purified protein fraction, he observed binding mostly with phospholipase (46%), hyaluronidase (26%), followed by Vmacl and Vmac3 (10%) and antigen-5 (8%) in 39 sera tested. In the present study prior to active treatment, the patients' sera had the most frequent IgE binding at 25 kD (90%), representing most likely Ag-5 as the major allergen with the method employed, followed by phospholipase (60%) and hyaluronidase (30%). No IgE binding was seen with other proteins. Einarsson [11], studying 35 sera of yellow jacket allergic patients by crossed radioimmuno-electrophoresis (CRIE), found an IgE binding frequency to Ag-5 of 100%, to phospholipase A of 80% and 17% to hyaluronidase, in agreement with our results. These three proteins are the main components in the venom used in this study, as could be shown by others [12,13]. Sensitization (IgE-binding) to these allergens differs however in the individual patient's sera, as does the antibody response during IT (Fig. 2).

The data described here differ from those of Hoffmann [14] who found IgE-binding to phospholipase A to be much more prevalent and of greater intensity on acid gel blots. This could be due to the method of analysis because, as also shown by him, *in vitro* treatment of the yellow jacket phospholipase A in preparation for SDS-PAGE analysis, caused a 700-fold decrease in RAST inhibitory activity. He thus concluded that for antibody binding, the conformational structure of phospholipase A is more important than for Ag-5.

During IT, Ag-5 (25 kD) showed a decrease in IgE binding, while IgG, IgG1 and especially IgG4 showed an increase. With phospholipase A, only weak changes were seen in IgE and IgG intensity. Even if the conformational structure of this allergenic protein is important, one would expect a weak binding of all antibody classes, with an increase during IT. Golden *et al.* [15] reported that up to a maximum level, the amount of IgG antibody produced during IT is a function of the cumulative dose given, as is also evident from our data at 25 kD, 43 kD and 70–90 kD, but not at 35 kD.

Müller *et al.* [16], in a sting challenge study after IT and while looking at specific honeybee phospholipase A₂ antibodies, showed no significant changes in specific IgG, a fall in IgG1 and a slight, statistically significant increase in IgG4 in patients with systemic reactions to the sting challenge, whereas patients with no reactions showed a highly statistically significant increase in IgG and IgG4 and no fall in IgG1.

Litwin *et al.* [17], using isolated honeybee venom phospholipase A₂ and a pepsin hydrolysate of phospholipase A₂ for IT, found no changes with the pepsin fragment, a decline or an increase of allergen specific IgG antibody, always in three out of nine patients tested, whereas

treatment with the phospholipase A₂ showed a small increase. These observations, together with our results, could lead to the suggestion that phospholipase A in insect venom is not highly immunogenic for IgG responses with the therapeutic extracts used.

Only three patients showed IgE binding to hyaluronidase (43 kD), indicating that this protein is only a minor allergen in this group, detected by immunoblot. It is notable that in these three patients, a positive RAST with honeybee venom was seen in addition, supporting the theory of cross-reactivity of hyaluronidase in honeybee and vespid venoms, as shown by others [10,18,19]. During IT, an increase of IgG and IgG1 was seen, whereas IgG4 showed only a slight increase. The IgG binding to the region between 70–90 kD and its marked increase during IT with all IgG classes tested cannot be explained. Possibly, these proteins represent Vmacl (97 kD), although no sensitization (IgE-binding) was seen in this region, except for one patient. If one would assume an aggregation of the other venom proteins and their precipitation in this region, there should be an IgE binding as well. Whatever their nature is, these proteins show a high immunogenic activity for the IgG and especially the IgG4 response, resulting in an increase of total allergen specific IgG, without a relationship to the major allergens.

In conclusion, we have shown that Ag-5 is the major allergen in all 10 yellow jacket allergic patients evaluated by immunoblotting and that this protein induces a good IgG and especially an IgG4 response during IT, whereas hyaluronidase showed mainly an IgG1 response and phospholipase A only a weak IgG response during IT. In most patients, new IgG4 binding to proteins in the region of ≥ 70 kD was found. The data also show that in individual patients, the determination of the total amount of allergen-specific IgG4 during IT gives no explanation about the increase of IgG4 to the patients' major allergen. In order to determine the possible role of IgG4 for immune protection, more detailed information about the individual IgG response to the major allergens is needed.

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