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Immunoblot Analysis of IgE and IgG Antibodies to Honey Bee Venom : Cross Sectional and Sequential Studies in Bee Sensitive Subjects.

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SUMMARY **To investigate the specific IgE and IgG immune response to honey bee venom (bv), we performed immunoblot analysis of sera from 47 bee sensitive subjects and followed the response during and after venom immunotherapy in 15 of these subjects. Fifteen venom proteins varying in molecular size from 20 to 105 kDa were identified as being antigenic and consisted of a high molecular weight (HMW) group (5 to 105 kDa, containing the previously identified allergens B and C) and a low molecular weight group (LMW) containing hyaluronidase and phospholipase A. In general for a given individual the anti-venom IgE and IgG response was qualitatively similar although some variation between individuals was apparent. Reactivity with hyaluronidase and phospholipase A appeared only in those subjects showing reactivity with HMW components. During immunotherapy specific anti-venom IgG and IgE responses tended to be linked. Increased responses being seen against all components in 4 of 12 subjects, reductions in 3 and unchanged responses in the remainder. Following immunotherapy (mean 4.0 years), spontaneous reduction of IgE and IgG was seen in 5 of 5 subjects. Loss of reactivity with the LMW components was prominent in these sera.**

Immediate hypersensitivity reactions to honey bee (Apis mellifera) stings are a common occurrence in South Australia and indeed this state has the highest fatality rate within the Australian Commonwealth. [1] We have previously reviewed the clinical manifestations and circumstances of our patients' allergic reactions to bee stings [2] and more recently have discussed the management. [3] During the workup of these patients at their first visit we routinely take blood to confirm the presence of specific anti-venom IgE antibodies and in many of these patients further blood specimens are taken during their bee venom immunotherapy. In the present study we have investigated the nature of the IgE and IgG immune response against bee venom using a sensitive immunoblot technique. The aims have been to determine if the anti-venom IgE and IgG responses in allergic subjects are against the same antigenic protein components and if variation occurs between allergic individuals. The responses during and after cessation of venom immunotherapy have also been studied in a smaller number of these patients and the immunoblot findings compared with the results obtained with commercial specific anti-venom immunoassays.

MATERIALS AND METHODS

Patients and Controls

Sera were obtained for 47 patients (32 male, 15 female, mean age 22.9 years) with recent generalized immediate hypersensitivity reactions to honey bee stings. Clinical details of these patients were obtained from an examination of the case records. Serum for analysis was obtained within 4 months of the subject having a systemic reaction. Sera were also obtained in 12 of these patients during venom immunotherapy and in 5 patients following the cessation of immunotherapy. Control sera were obtained from 6 healthy non bee allergic subjects, none of whom had had a recent sting, and from 12 patients with sys-

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temic autoimmune disorders (6 with systemic lupus erythematosus, 2 with rheumatoid arthritis, 2 with systemic sclerosis and 2 with primary biliary cirrhosis). All sera were stored in small aliquots at -20°C prior to study.

Immunotherapy

Venom immunotherapy was administered by the modified rush technique using Albay bee venom according to the manufacturer's instructions. Maintenance therapy was monthly by the subcutaneous route with 100 μg reconstituted venom protein.

Immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of bee venom was performed at neutral pH in 12% PA gel slabs with a 3.6% PA stacking gel. The conditions of the electrophoretic separation are described elsewhere.[4] A molecular marker solution (MW 14-100 kDa, Pharmacia) was applied to the first weli and 100μg of honey bee venom (Sigma Chemical Company, lot 87 FO763) was applied to each of the remaining wells. After electrophoresis the separated venom proteins were then transferred to nitrocellulose membranes (0.2μm, Bio-Rad Laboratories, Richmond USA) as previously described.[4] Following transfer the molecular marker strip was removed and stained with 0.1% amido black while the remaining membrane was 'blocked' in 7% bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, USA) and then cut into appropriate strips for incubation with the test sera. Test sera were diluted 1:200 for IgG analysis and 1:25 for IgE analysis in filtered phosphate buffered saline (PBS) containing 2% BSA. Each strip was incubated successively with biotinylated sheep anti-human IgG (Silenius Laboratories, Hawthorn, Victoria, Australia) at 1:200 or biotinylated rabbit antihuman IgE (Dakopatts Co, Copenhagen, Denmark) at 1:50, avidin D (Sigma Chemical Company Ltd) diluted to 2μg/ml and biotinylated alkaline phosphatase (Sigma Chemical Company Ltd) diluted to 40 μg/ml. Phosphatase activity was detected by adding substrate Fast Red dye (Sigma Chemical Company Ltd).

A positive control serum was included in each run and sequential sera from the same patient were analysed in adjacent wells to mitigate against interassay imprecision. Densitometry of stained strips was performed using a Camag electrophoresis scanner (Dynatech Instruments Inc, Torrance, California). Changes in staining intensity were also monitored visually.

Immunoassay

Specific anti-venom IgE and IgG were measured by radio immunosorbent assay (RAST Pharmacia). Results were expressed as a score 0 to 4+ or optical absorbance reading for IgE on a relative % binding as compared with a standard for IgG. These immunoassays are performed routinely in the diagnostic laboratory and their accuracy and precision are monitored by participation in the Quality Assurance Program of the Royal College of Pathologists of Australasia.

RESULTS

Cross-sectional study

Immunoblotting for venom-specific IgG

Sera from the 47 bee allergic subjects showed a consistent banding pattern with up to 15 individual bands being apparent in some sera **(Fig. 1-3).** These consisted of a high molecular weight (HMW) group varying in molecular size from 50-105 kDa. A prominent band (or in some blots a double band) was seen in most sera at 50 kDa with an additional less prominent band at 85 kDa and further less intensley staining bands between these two and in the heavier range. Prominent low molecular weight (LMW) bands were also evident in many sera in the region 35-40 kDa and 22-28 kDa. Because of the molecular size of these reactive proteins they were presumptively identified as hyaluronidase and phospholipase A respectively. When the LMW bands were present they were generally accompanied by prominent staining of the HMW components. The banding pattern observed among allergic subjects showed some variability in the intensity of staining of the bands, particularly the LMW components with some sera demonstrating dense hyaluronidase bands with little or no phospholipase reactivity whilst in other sera the reverse was seen **(Fig. 1 and 2).**

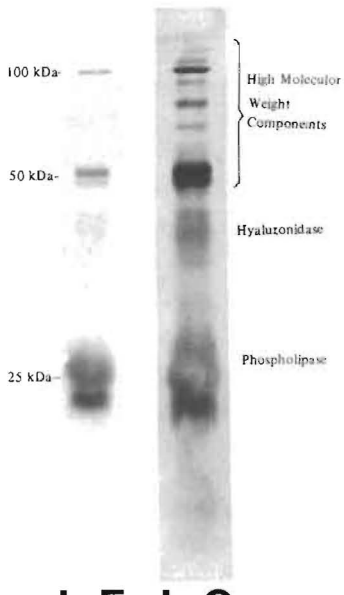

Fig. 1 IgE and IgG venom specific antibodies in a 7 year old boy who experienced on immediate generalized urticarial eruption and severe wheeze following a bee sting. RAST (bv) = 3+.

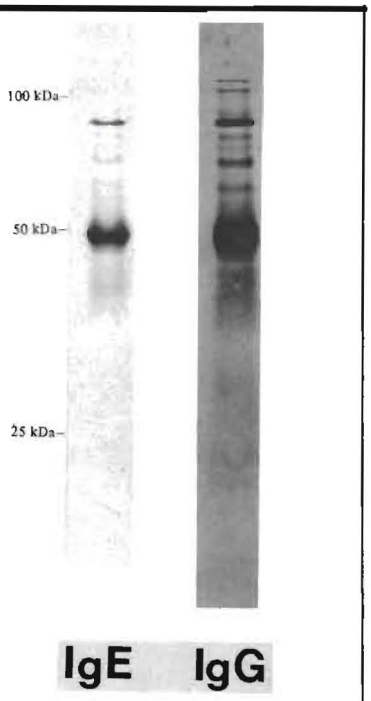

Fig. 2 IgE and IgG venom-specific antibodies in 42 year old male who exprienced a vascular collapse following a bee sting. Note relative absence of antibody to LMW components. RAST (bv) = 3+, IgG blocking = 66%.

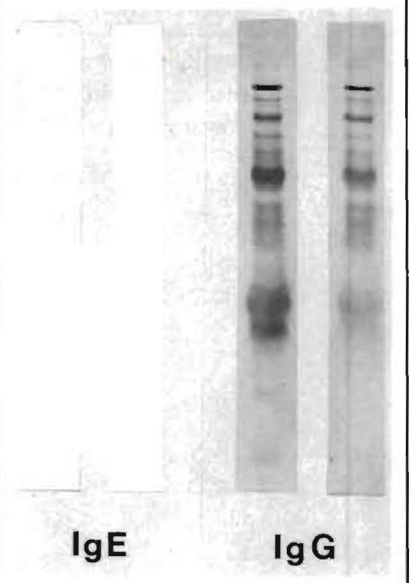

Fig. 3 IgE and IgG bee venom specific antibodies in a bee allergic subject during immunotherapy (left lane) and 42 months later following cessation of therapy (right lane). Note the decrease staining intensity of all components following cessation of immunotherapy. RAST (bv) optical absorbance decreased from 0.75 (3+) to 0.28 (2+) while IgG blocking decreased from 228% to 46%.

In the control sera weak reactivity with the HMW components was observed in some sera but no reactivity with the LMW components was observed for any of these sera.

Immunoblotting for venom-specific IgE

In the bee allergic subjects the IgE banding pattern was qualitatively very similar to that obtained from the immunoblotting for venom-specific IgG but the intensity of staining of individual bands was less strong and showed some variability compared with the intensity seen with the latter (Fig. 1, 2 and 3). In 18 of the bee allergic subjects no staining of any bands was detected despite these subjects having a positive RAST score to bee venom 1+ to 2+. No venom-specific IgE staining was detected in any of the control sera. In general those bee allergic subjects who showed strong venom-specific IgG reactivity also showed reactivity for the same bands in the IgE blots (Fig. 1&2). As with the IgG immunoblots, there was some variability between individuals for the IgE blots in that some subjects reacted strongly with one or both of the LMW components and generally these subjects also showed strong reactivity with the HMW components (Fig. 1&2). There was no apparent correlation between the banding pattern of the venom-specific IgE and IgG and the clinical features of the allergic response (data not shown). Those patients with prominent anti-venom IgE on immunoblot all had high RAST scores (>3+) to bee venom.

Sequential Studies

The results for the sequential venom-specific immunoblot and immunoassay studies obtained for 15 patients during and after venom immunotherapy are shown in Tables 1 and 2 and in Fig. 3. During immunotherapy the venom-specific IgE and IgG changes as reflected from an inspection of the immunoblots were quantitatively linked in all but one subject with increased staining intensity seen for both immunoglobulin classes in 3 subjects, reductions in 3 and no appreciable change in the remainder. These alterations were quantitatively linked in all but of the immunoassay results (Table 1). However there were some exceptions particularly for antivenom IgE e.g. venom specific IgE was found to increase by immunoblot analysis in three patients but with reduction in the RAST venom score. Inspection of the immunoblots did not reveal any additional bands occuring during immunotherapy as compared with the pattern prior to immunotherapy. Only changes in the intensity of the bands were observed.

Following the cessation of immunotherapy after a mean of 48 months, the venom-specific IgE and IgG declined substantially in all 5 subjects and agreement was observed between the immunoblot and immunoassay results (Table 2). A decrease in intensity of staining was observed for all bands with the greatest decline being seen for the LMW components (Fig. 3).

Table 1. Changes in specific IgE and IgG during immunotherapy.

Patient	Gender	Age + (years)	Duration of immunotherapy T_0 to T_1	Immunoblotting: change in staining intensity T_0 to T_1		Immunoassay: specific antivenom antibody IgE△		IgG#	
			(months)	IgE	IgG	T_0	T_1	T_0	T_1
1	M	18.8	6	↓	→	NT	4+	81	89
2	M	37.8	93	↓	↓	2+(0.23)	NT	66	31
3	F	17.7	46	NS	↓	3+(0.86)	2+(0.33)	30	40
4	M	9.5	5	↑	↑	3+(0.94)	3+(0.75)	159	228
5	M	33.5	5	NS	→	2+(0.16)	1+(0.13)	88	125
6	M	19.9	6	→	→	3+(0.59)	2+(0.20)	75	68
7	M	31.1	7	↑	↑	3+(1.10)	3+(0.91)	44	75
8	F	11.5	6	↓	↓	3+(0.58)	2+(0.33)	100	56
9	M	12.2	12	→	→	2+(0.33)	3+(0.53)	115	119
10	M	11.9	14	↑	↑	3+(0.73)	3+(0.66)	43	127
11	M	17.9	10	→	→	4+(1.44)	4+(1.30)	66	69
12	M	40.8	21	→	→	4+(1.59)	3+(0.68)	47	66

+Age at which patient commenced immunotherapy following sting anaphylaxis = time T_0
NS = No staining detected
NT = not tested
△RAST (bv) score (optical absorbance)
#% blocking

Table 2. Changes in specific anti–venom IgE and IgG after cessation of immunotherapy.

Patient	Gender	Age+ (years)	Duration of immunotherapy (months)	Time between cessation of immunotherapy and study period (T_2)	Immunoblotting: change in staining intensity T_0 to T_2		Immunoassay: Specific anti-venom antibody IgEΔ		IgG#	
				(months)	IgE	IgG	T0	T_2	T_0	T_2
3	F	17.7	47	35	NS	↓	3+(0.86)	2+(0.19)	30	22
4	M	9.5	61	42	↓	↓↓	3+(0.94)	2+(0.29)	159	48
13	M	8.5	38	47	↓	↓↓	NT	2+(0.15)	NT	30
14	M	20.6	12	84	↓	↓↓	2+(0.71)	2+(0.23)	177	52
15	M	54.4	60	30	↓	↓↓	3+1.02)	2+(0.22)	44	27

+ Age at which patient commenced immunotherapy following sting anaphylaxis = Time T_0
△ Score (optical absorbance)
# Blocking of standard
NT = not tested NS = No staining

DISCUSSION

The main findings for this immunoblot analysis of sera from bee allergic subjects include 1) identification of up to 15 bee venom antigenic proteins, varying in molecular size from 20 to 105 kDa, 2) the venom-specific IgE and IgG responses were qualitatively very similar within the group of allergic subjects with some variation between subjects, particularly with reactivity with the LMW components (MW 20-50 kDa), 3) there were variable quantitative changes of these venom-specific antibodies during immunotherapy with evidence of a linked response (i.e. the venom-specific IgE and IgG tended to change together in the same direction), 4) no new antigenic reactivity was detected during immu-

notherapy, and 5) there was substantial reduction of venom-specific IgE and IgG following cessation of immunotherapy.

A number of allergens in honey bee venom have been previously characterized.[5-10] These include the highly basic protein phospholipase A2 (MW approx. 20 kDa) which constitutes 12% by weight of the dried venom and tends to run irregularly in SDS-PAGE at neutral or basic pH.[10,11] They also include hyaluronidase (MW approx.38 kDa) which constitutes 2% of the dried venom, and the HMW components which constitute 2-4% of the dried venom.[5-8] The HMW components have been previously shown to contain 4 allergenic proteins including allergen B or acid phosphatase (MW 49 kDa)[9] and allergen C (MW 105 kDa). Hoffman and colleagues have shown that these HMW proteins contain considerable allergenic activity by RAST in bee allergic individuals.[10] In the present study we have observed up to 15 antigenic/allergenic proteins in honey bee venom with a substantial staining band, in reaction with some sera, in the molecular size range of approx. 25 kDa which we believed to be phospholipase. The slightly higher molecular weight obtained in our study as compared with previous studies may represent the anomalous migration of this highly charged basic protein at neutral pH[11] and indeed we noted that it rarely gave a sharp band in our blots. Likewise the bands obtained in the 35-40 kDa range were also diffuse and most likely represent hyaluronidase. The dense distinct band (or occasionally doublet band in some gels) noted at 50 kDa probably represents acid phosphatase and the distinct band at 85 kDa may represent allergen C although this could be placed in the reactive bands seen at 102 or 100 kDa which is closer to the published MW of allergen C (viz 105 kDa).

In the venom-specific IgG immunoblots reactivity with the HMW components was seen to some degree in all sera from bee allergic subjects and more weakly in some control sera from non allergic individuals. Reactivity with hyaluronidase and phospholipase or both was also found in a smaller number of allergic subjects and generally was associated with intense staining of the HMW components. Some variability between allergic subjects, particularly involving the intensity of staining of these LMW components was apparent. The venom-specific IgE blots gave a similar banding pattern to the IgG immunoblots but with less intense staining (with no bands being detected in those subjects with low bee venom RAST score).

Thus, it appears that for a given individual the same qualitative IgE and IgG antigenic response is generated following a bee sting with some allergic individuals showing stronger reactivity (more intense staining) with the LMW components. However it should be pointed out that many allergenic epitopes are conformational in nature rather than linear[12] and the former could easily be destroyed by the SDS used in the electrophoretic separation. Thus, the immunoblot analysis reflects the immune response to the more stable or linear epitopes and this explanation may be relevant in explaining the observed discrepancy noted in a few sera between the immunoblot and immunoassay (using non-denaturing buffers) found in the sequential studies.

In the sequential studies the venom specific IgE and IgG responses during immunotherapy appeared to be similar in a qualitative sense in a given individual and no new antigenic response against venom proteins was noted. Moreover it appeared that the quantitative changes occuring during immunotherapy involved both IgE and IgG in a linked response in the same direction, i.e. they increased or decreased together and this involved changes in reactivity to all the venom components, particularly the LMW ones. However the caveat should be added that the sequential studies involved only one serum sample during the immunotherapy period and this was taken at different periods. Hence more analysis on larger numbers and at more frequent intervals will be required to confirm this apparent linkage of venom-specific IgE and IgG responses. While examination of previous published data suggests complex alterations in absolute levels of venom-specific antibodies during immunotherapy, the overall data is consistent with both venom-specific IgE and IgG rising as a pair in the early stages of immunotherapy and then both declining, the decline being more marked for IgE than IgG.[13-16] However it is possible that the proportion of IgG_4 to the total IgG may rise during prolonged therapy.[17,18] It was not possible to study venom-specific IgG subclasses in the present study.

Following the cessation of immunotherapy after a mean of 4 years we observed a substantial decline in venom-specific IgE and IgG both from the immunoblot results and the specific immunoassays. These findings are in good agreement with other published studies[19,20] but it is still unclear why these previous sting allergic invidiuals loose their sensitivity despite continuing to have low levels of venom-specific IgE and low levels of the IgG 'blocking' antibody.

In summary this study, by using a sensitive immunoblotting technique to study the venom-specific IgE and IgG immune response in bee allergic individuals, has emphasized the similar qualitative nature of the IgE and IgG antibody response to a number of venom antigenic proteins and has noted an apparent quantitative 'linkage' of these response during immunotherapy. Some variation between allergic subjects in reactivity with the LMW components (phospholipase and hyaluronidase) was observed.

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