

An HLA-associated nonresponsiveness to melittin: A component of bee venom

Penny Lympny, BSc, D. Mike Kemeny, PhD, Ken I. Welsh, PhD,* and Tak H. Lee, MD, FRCP London, England

Previous work has demonstrated a close association between certain histocompatibility antigens and the gene that controls the IgE response to certain ragweed allergens. For example, there is a 90% association between IgE production to the short ragweed allergen, Amb a V, and an HLA class II allele. To assess whether these HLA linkages are specific for ragweed, we have investigated the association between HLA antigens and the capacity of individuals to mount a specific IgE response to melittin in patients with bee-venom allergy. Twenty-two subjects with bee-venom sensitivity, 22 healthy beekeepers without bee-venom allergy, and a normal population of 149 unselected individuals were studied. With serologic tissue typing and restriction fragment length polymorphism analysis, we have demonstrated a significant decrease in the HLA-DR4 and DQw3 alleles in subjects who are allergic to melittin compared to the control populations. There was also a negative association between the presence of HLA-DR4 and DQw3 alleles with the capacity of the individuals to mount an IgE response to phospholipase A₂ (PLA₂). The bee-venom sensitive subjects had a slightly lower titer of anti-PLA₂ IgG when these subjects were compared to the bee-venom insensitive beekeepers. These results support the view that either HLA-DR or HLA-DQ has a protective role in controlling the IgE immune response. Lack of an IgE response to melittin or PLA₂ is unlikely to be due to a failure to recognize allergen. (J ALLERGY CLIN IMMUNOL 1990;86:160-70.)

Associations between histocompatibility antigens and specific diseases have been recorded many times, both for specific responsiveness to an individual antigen^{1,2} and in terms of immune responses within a particular immunoglobulin class.³⁻⁶ Exposure toward most inhaled allergens is at an immunogenetically limiting dose of a few nanograms per year. Under such circumstances, significant associations between particular HLA types and specific immune responsiveness to a particular allergen has been demonstrated.^{1,2} It has previously been demonstrated that there is a close association between the gene that controls the IgE response to a minor ragweed allergen *Amb a* III and HLA-A2 and HLA-A28,¹ although confirmatory studies have yet to be performed. There is

Abbreviations used

PLA ₂ :	Phospholipase A ₂
PBS:	Phosphate-buffered saline
EDTA:	Ethylenediaminetetraacetic acid
SDS:	Sodium dodecyl sulfate
RFLP:	Restriction fragment length polymorphism
SSC:	Sodium citrate
³² P:	Radioactive phosphorus
dCTP:	Deoxycytidine triphosphate

also a 90% association between HLA-Dw2 and the IgE response to the minor ragweed allergen *Amb a* V.² The association is most striking with pure allergens and is usually much more pronounced for specific IgE antibody than for IgG antibody.

Antibody responses (IgG and IgE) against ragweed pollen have been reported to be weakly associated with HLA-B7.² It has also been suggested that a gene that controls response to *Amb a* III maps very closely to the class I locus, and Marsh et al.¹ have postulated that an IR *Amb a* III locus is associated with HLA-A2 and HLA-A28. Blumenthal et al.,⁷ however, failed to confirm this finding. More recently, Marsh et al.² have reported a 90% association between HLA-Dw2

From the Department of Allergy and Allied Respiratory Disorders and *Department of Tissue Typing, United Medical and Dental Schools, Guy's Hospital, London, England.

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Reprint requests: T. H. Lee, MD, Department of Allergy and Allied Respiratory Disorders, 4th Floor, Hunt's House, Guy's Hospital, London SE1 9RT, England.

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and IgE antibody production to *Amb a* V. Blumenthal et al.⁷ studied two families and postulated that the gene that regulates the response to ragweed was separate from HLA-D and was near GLO. There is no similar data on bee-venom hypersensitivity, although Hecht⁸ has studied two families with bee-venom allergy and has suggested that familial, probably genetic factors, predispose an individual to Hymenoptera sensitivity.

In studying the genetic control of atopic diseases, definition of the population, purity of the antigens under study, and assessment of a positive response to the allergen are critical to the successful identification of the molecular conditions that determine a defined specificity in immune responsiveness toward well-characterized antigenic molecules. In the present study, we have evaluated the HLA associations with the capacity of individuals to mount an IgE response to melittin, the principal component of honey-bee venom. Melittin is a well-characterized peptide of molecular weight 2840 daltons. Its amino acid sequence has been determined, and the gene has been cloned. The patient population under study is well defined and was well motivated to participate in this study. We were therefore in an excellent position to define the association of HLA phenotypes with the immune response to melittin in this defined population.

METHODS

Subjects

Twenty-two subjects with bee-venom sensitivity, 22 healthy beekeepers without bee-venom allergy, and a normal population of 149 unselected individuals were studied. All study subjects and control subjects were mainland white subjects of the United Kingdom. Subjects with bee-venom allergy had positive skin prick tests to honey-bee venom, as defined by a wheal >3 mm of the size elicited by diluent control, a previous history of local (defined as swelling of >2 inches) or systemic reactions after a bee sting, and a RAST score to melittin of >1+. Control subjects were beekeepers who were skin prick-test negative, gave no history of any allergic reaction to honeybee venom, and in addition, they had a negative RAST to melittin. The control population of 149 individuals were not skin prick tested.

RAST

Sera from all subjects were assayed for specific IgE to melittin, PLA₂, cat fur, *Dermatophagoides pteronyssinus*, and mixed grass pollen by RAST, as previously described.^{9, 10} Cyanogen bromide-activated disks were coated with allergen solution at an appropriate concentration (0.2 mg/ml of melittin, 0.1 mg/ml of PLA₂, and 20 mg/ml of cat, grass, and *D. pteronyssinus* extract). The disks were washed as described and freeze-dried. After the disks were freeze-dried, they were washed with PBS and 0.05% polysorbate 20 (Tween 20) (Sigma Ltd., Poole, Dorset, En-

gland) in a constant flow-washing device,¹¹ dried, and placed in microtiter plates (Nunc, Gibco/Biocult, Hounslow, Middlesex, England). Undiluted serum samples or whole bee venom standard (from a pool of serum with a known RAST score of 4+) diluted in horse serum (Sera Laboratories, Crawley, Sussex, England) were added to each well. The plates were incubated overnight at room temperature and then washed as before, dried, and transferred to new microtiter plates. Finally, ¹²⁵I-labeled anti-IgE (diluted in horse serum to elicit 100,000 cpm/well) was added to each well. The plates were incubated overnight at room temperature, washed, dried, transferred to 5 ml tubes (Starstedt, Beaumont Leys, Leicester, England), and counted in a gamma counter for 1 minute. A positive score was recorded when the counts per minute bound were 3 SD above the mean of six normal human sera. RAST scores were measured with specific Phadebas (Pharmacia Ltd., Milton Keynes, England) reference disks. A score of 1+ or higher was accepted as being elevated.

ELISA for PLA₂

IgG antibodies to PLA₂ were measured with a sandwich ELISA.¹² Briefly, rabbit anti-PLA₂ IgG (10 µg/ml) was diluted in a coating buffer (0.1 mol/L of sodium bicarbonate in PBS/azide), added to each well of a microtiter plate (Nunc), and incubated overnight at 4° C. The plate was washed three times with PBS containing 0.05% Tween 20 and dried. A solution containing 10 µg/ml of PLA₂ in ELISA buffer (500 µl of horse serum [Sera Laboratories] and 500 µl of Tween 20 in 99 ml of PBS) was added to each well, and the plate was incubated for 1 hour at 4° C before being washed three times and dried. The test sera or serially diluted standard serum (from a pool of sera with known levels of IgG to PLA₂) in ELISA buffer was added to each well and incubated for 2 hours at 4° C. The plate was then washed and dried as before.

A monoclonal anti-IgG clone 8a4 (Unipath, Inc./Oxoid Ltd., Basingstoke, Hants, England) was diluted in ELISA buffer, added to each well, and incubated for 1 hour at 4° C. The plate was then washed as previously described. Rabbit antimouse IgG alkaline phosphatase conjugate (Sigma Ltd.) was diluted in ELISA buffer, added to each well, and incubated for 1 hour at 4° C. The plate was washed three times as before.

A 1 mg/ml solution of *p*-nitrophenyl phosphate (Sigma Ltd.) was added to each well. The plate was incubated at 37° C for 40 minutes. The absorbance of the individual wells was measured at 405 nm with a Titertek microtiter-plate reader (Flow Laboratories, Rickmansworth, Herts, England), and the concentrations of the test sera were calculated from a standard curve. The minimum level of detection was 3 µm/ml.

Tissue typing

All subjects were tissue typed for class I histocompatibility antigens with a two-stage microcytotoxicity test against a panel of 120 specific antisera with a standard National Institute of Health microcytotoxicity method, as previously described.¹³ Citrated whole venous blood, 20 ml,

was defibrinated by shaking with 10% calcium gluconate, thrombin (50 IU/ml in PBS), and defibrinating beads (BDH Chemicals Ltd., Poole, Dorset, England). The defibrinated blood was layered onto Lymphoprep (Nycomed, Oslo, Norway) and centrifuged. The lymphocytes were removed from the interface, and the cells were washed twice in complement fixation test buffer (Flow Laboratories) by centrifugation. The resultant cell pellet was resuspended in complement fixation test buffer, and the suspension was added to undiluted defined specific antisera, obtained from multiparous female subjects or from patients who had been transfused, in 60-well Terasaki trays (Nunc) and incubated at room temperature for 30 minutes. Undiluted rabbit complement (Buxton, Derbyshire, England) was then added to each well, and the mixture was incubated for an additional 60 minutes at room temperature. Excess complement was removed by inverting the plate briskly two or three times. Trypan blue dye (Sigma Ltd.), diluted 1:1 with normal saline, was added to each well as a stain to identify nonviable cells under phase-contrast microscopy.

Class II histocompatibility antigens (specifically HLA-DR, HLA-DQw1, and DQw2 specificities) were determined on purified B-lymphocytes that were prepared from a mixed lymphocyte suspension with Dynal beads (Dynal Ltd., Wirral, Merseyside, England) according to the manufacturers instructions. The prepared B cells were tested in 60-well Terasaki trays (Nunc) against a panel of 50 specific antisera and 10 control antisera (United Kingdom Transplant Service, Bristol, U.K.) in a two-stage microcytotoxicity test, as already described for the class I antigens. The cells were stained with acridine orange/ethidium bromide. (Fifteen milligrams of acridine orange and 50 mg of ethidium bromide in 1 ml of 95% ethanol and 49 ml of distilled water was diluted to 1 L with PBS.) The plates were examined under fluorescence microscopy. The viable cells fluoresced green and nonviable cells appeared red.

DNA extraction

Twenty-five milliliters of peripheral venous blood was withdrawn into 5% EDTA (BDH Chemicals) in PBS and stored at -20°C until it was processed to lyse the red blood cells. DNA was extracted with a modified proteinase K and phenol/chloroform extraction method. The whole blood was thawed and washed with a 10 mmol/L of Tris (Sigma Ltd.) and 1 mol/L of EDTA (BDH) ($1 \times \text{TE}$) solution and centrifuged. The supernatant was decanted and discarded. The cell pellet was resuspended in swelling buffer consisting of 10 mmol/L of Tris, 20 mmol/L of NaCl (BDH), 5 mmol/L of MgCl_2 (BDH), and 10% Nonidet P-40 (NP40, Sigma Ltd.), left on ice for 15 minutes, and then pelleted by centrifugation. The pellet was resuspended in 100 mmol/L of NaCl, 50 mmol/L of EDTA with 0.07% SDS (BDH), and 500 μg of proteinase K (Gibco-BRL, Uxbridge, Middlesex, England) at 37°C overnight; 5 mol/L of sodium perchlorate (BDH) and 20% SDS were then added to the extract, and the solution was mixed with Tris-buffered phenol and chloroform/isoamyl alcohol (mixed 24:1 vol/vol). The solution was mixed for 30 minutes at room temperature

on a rocking mixer and then centrifuged. The aqueous layer was removed into another tube, and the phenol/chloroform extraction was repeated as already described. Two subsequent extractions were performed by adding chloroform only to the aqueous layer and mixing for 5 minutes at room temperature before centrifugation. Finally, the DNA was precipitated from the aqueous layer with 2 vol of chilled absolute ethanol and dialyzed against a 10 mmol/L of Tris and 1 mmol/L of EDTA solution for 1 hour in open Eppendorf tubes (Eppendorf, Hamburg, West Germany) that were sealed with boiled dialysis membrane (BRL). The DNA was then dissolved by heating at 65°C for 20 minutes. The concentration of DNA was determined by measuring the ultraviolet absorption of 260 nm. An optical density reading of 1 corresponded to approximately 50 $\mu\text{g}/\text{ml}$ of double stranded DNA.

RFLP

Eight to ten micrograms of prepared DNA was digested with Taq 1 or Msp 1 restriction endonuclease enzyme according to the manufacturer's instructions (Anglian Biotech Ltd., Colchester, Essex, England). Resultant fragments were fractionated by size on a 0.6% agarose gel (Sigma Ltd.), which was electrophoresed at 50 V for 19 hours in 0.07 mol/L of Tris, 0.06 mol/L of boric acid (BDH), and 0.02 mol/L of EDTA. The DNA from the gel was denatured and transferred onto Hybond-N filters (Amersham, Buckinghamshire, England) with the method described by Southern.¹⁴ Filters were washed once in a saline solution containing SSC (0.75 mol/L of NaCl and 0.1 mol/L of SSC) (BDH) and exposed to ultraviolet light for 5 minutes to cross-link DNA to the filter. Filters were then prehybridized at 65°C for 2 hours in a prehybridization buffer consisting of 1 mol/L of NaCl, 0.12 mol/L of SSC, 4% of dextran sulfate, 0.1% of Ficoll, 0.1% of polyvinylpyrrolidone, 0.1% of bovine serum albumin, fraction V, and 0.5% of SDS, also containing 1 mg of single-stranded sonicated salmon sperm DNA (Sigma Ltd.).

The cDNA probes used were DR β , DQA, DQ β , DP α , and DP β , prepared as described.¹⁵⁻¹⁸ Each probe was labeled with ^{32}P -dCTP (Amersham) triethylammonium salt according to the method described by Feinberg and Vogelstein.¹⁹ Each cDNA probe was boiled with water and cooled on ice to separate the strands of DNA. To this was added oligolabeling buffer prepared according to the method previously described.¹⁹ Finally, ^{32}P -dCTP and Taq 1 polymerase were added, and the probe was incubated at room temperature for 4 hours. After 4 hours, an equal volume of 0.01 mmol/L of Tris and 0.01 of EDTA was added to the oligolabeled probe before being loaded onto a spin column of Sephadex G-50, which had been preequilibrated by centrifuging the column twice with 0.01 mol/L of Tris and 0.01 mol/L of EDTA. The oligolabeled probe was added to the column and centrifuged. The eluent was collected in an Eppendorf tube. An aliquot of the labeled probe was counted in a β -scintillation counter. A volume of labeled probe sufficient to produce 1×10^7 cpm was added to the prehybridized filters and buffer. The filters were then hybridized

TABLE I. Bee-venom sensitive subjects

Subject No.	Age (yr)	Sex	Atopic status	RAST score to melittin	Clinical reactions to bee sting
1	57	M	A(m)	1	Rhinitis, large local swelling
2	47	M	A(m,g)	2	Urticaria, asthma
3	33	F	N/A	1	Anaphylaxis
4	49	F	N/A	1	Rhinitis, asthma
5	40	M	A(m,g)	2	Local swelling, convulsions
6	67	F	N/A	1	Anaphylaxis
7	51	F	A(g)	2	Urticaria, angioedema
8	19	F	N/A	1	Urticaria, asthma, angioedema
9	44	M	A(g)	1	Urticaria, asthma
10	58	F	A(g)	1	Anaphylaxis
11	62	F	N/A	1	Asthma, angioedema
12	13	M	N/A	1	Anaphylaxis
13	27	F	N/A	2	Anaphylaxis
14	22	F	A(m)	1	Anaphylaxis
15	21	M	N/A	2	Asthma, angioedema
16	67	M	N/A	1	Angioedema
17	43	M	A(g)	1	Angioedema
18	63	M	A(g)	4	Asthma, urticaria
19	23	M	N/A	2	Angioedema
20	20	M	A(g)	1	Angioedema, asthma, urticaria
21	66	F	A(m,g)	2	Urticaria, asthma
22	63	M	A(g)	2	Anaphylaxis

A, Atopic; m, mite (*D. pteronyssinus*); g, grass; N/A, nonatopic.

to the ^{32}P -oligolabeled class II probe for 16 hours. The filters were then washed twice in solution of 0.3 mol/L of NaCl and 0.04 mol/L of SSC containing 0.2% SDS at 65°C for 5 and 15 minutes, respectively, and then twice in a solution of 30 mmol/L of NaCl and 4 mmol/L of SSC containing 0.2% SDS for 15 minutes in a shaking water bath. The filters were wrapped in Saran wrap (Dow Corning Genetic Research Instrumentation Ltd., Dunmow, Essex, England) and then exposed to Kodak XAR-5 film (Sigma Ltd.) with intensifying screens at -70°C for 4 days. Autoradiographs were analyzed with the data published previously.²⁰⁻²²

Statistical analysis

Statistical analysis of the class I and II specificities was performed initially with a population genetics (POPGEN, designed by Dr. J. d'Amaro, University Hospital, Leiden, The Netherlands) analytical program to identify significant differences between the normal population, bee-venom insensitive subject, and bee-venom allergic individuals. These data were then analyzed with a two-tailed chi-squared analysis to determine the significance of the differences between the incidence of the HLA alleles in the melittin-sensitive and melittin-insensitive subjects. Similarly, a chi-squared test was used to determine the significance of the incidence of anti-PLA₂ IgE antibodies in both the melittin-sensitive

and the melittin-insensitive subjects. The *p* values were corrected for multiple comparisons.

RESULTS

Serum IgE to melittin and inhalant allergens

The characteristics of the bee-venom sensitive patients who were studied are presented in Table I. Patients who were allergic to honeybee venom had RAST scores of 1 to 4. Five bee-venom allergic subjects had a raised IgE to mite allergen, and 10 subjects had a raised IgE to grass pollen. None of these individuals had evidence of IgE to cat dander.

In the beekeepers who were not sensitive to bee venom, the RAST scores were <1 when the IgE to melittin was measured (Table II). One subject had a raised IgE to cat dander, four had a raised IgE to mite allergen, and three subjects had a raised IgE to grass pollen.

Tissue typing

There was no significant difference in the frequency of class I or class II histocompatibility antigens between the beekeepers who were not allergic to bee

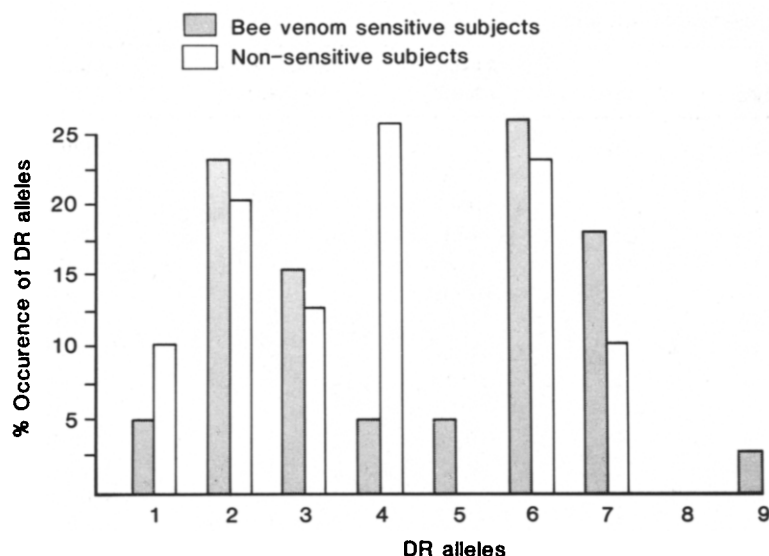


FIG. 1. Percentage occurrence of major histocompatibility complex class II DR alleles in subjects who were allergic to bee venom (■) and in beekeepers who were not (□). The DR specificities were investigated with serologic tissue typing.

TABLE II. Bee-venom insensitive subjects

Subject No.	Age (yr)	Sex	Atopic status	Clinical reactions to bee sting
23	59	F	N/A	Local swelling
24	63	M	N/A	Local swelling
25	48	M	A(m)	No reaction
26	45	M	A(m)	Local swelling
27	63	M	N/A	Local swelling
28	52	F	N/A	Local swelling
29	55	M	A(m)	Local swelling
30	69	M	N/A	No reaction
31	32	M	N/A	Local swelling
32	60	F	A(c,m)	Local swelling
33	46	M	A(g)	No reaction
34	68	M	N/A	Local swelling
35	76	M	N/A	No reaction
36	67	M	N/A	Local swelling
37	72	M	N/A	Local swelling
38	55	M	A(g)	Local swelling
39	67	M	A(g)	No reaction
40	83	M	N/A	No reaction
41	66	F	N/A	Local swelling
42	66	M	N/A	Local swelling
43	36	F	N/A	Local swelling
44	67	F	N/A	Local swelling

N/A, Non-atopic; A, atopic; m, mite (*D. pteronyssinus*); c, cat; g, grass.

venom and a normal population of 149 unselected individuals (data not presented). In contrast, comparison of the bee-venom sensitive population with the beekeepers who were not allergic to bee venom indicated that there was a significant decrease

($p < 0.01$) in HLA-DR4 in the bee-venom sensitive patients (Tables III and IV; Fig. 1); 5% of the total possible alleles (44) in the bee-venom sensitive subjects were of the HLA-DR4 specificity, whereas 25% of the total alleles in the beekeepers who were not

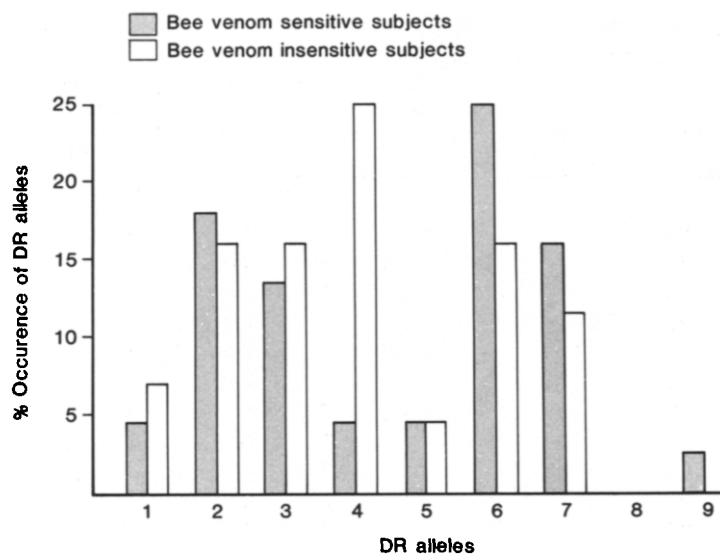


FIG. 2. Percentage occurrence of major histocompatibility complex II DR alleles in subjects who were allergic to bee venom (■) and in beekeepers who were not (□). The DR specificities were investigated with RFLP analysis.

TABLE III. Individual class I and class II specificities determined by tissue typing of lymphocytes obtained from bee-venom sensitive subjects

Subject No.	Class I							Class II			
	A		B		C		BW	DR		DQw	
1	2	3	18	60	5	*	6	6	7	1.2	2
2	2	29	44	62	3	*	4/6	2	7	1.1	2
3	2	3	27	17	1	6	4	1	7	1	2
4	2	19	44	*	5	*	4	4	7	3.1	2
5	1	28	51	17	7	*	4	2	6	1.1	1.2
6	3	30	7	13	7	*	4/6	2	7	1.2	2
7	1	3	35	51	4	*	4/6	3	(9)	2	3
8	2	24	18	39	7	*	6	2	5	1.2	3
9	3	11	7	14	7	*	6	2	6	1.2	1.3
10	3	24	8	62	3	7	6	3	6	1.3	2
11	2	28	7	62	3	7	6	2	6	1.1	1.2
12	24	29	7	27	1	7	4/6	6	*	1.1	*
13	2	11	18	44	5	7	4/6	3	6	1.2	2
14	2	11	18	44	7	*	4/6	3	6	1.2	2
15	2	24	49	60	3	*	4/6	2	6	1.2	1.3
16	2	*	39	44	5	7	4/6	4	*	3.2	*
17	1	3	7	*	2	7	6	3	*	2	*
18	3	24	7	14	7	*	6	2	*	1.1	2
19	2	*	60	62	3	*	6	1	6	1.2	1
20	1	25	8	18	7	*	4/6	3	6	1	2
21	11	32	14	62	3	*	6	5	7	2	3
22	1	3	7	8	7	*	6	2	*	1	*

*Only one allele detected.

allergic to bee venom demonstrated this specificity. Comparison of the bee-venom sensitive subjects with the control population of 149 unselected individuals also demonstrated a significant difference ($p < 0.01$)

in the incidence of HLA-DR4 between the groups. (Twenty-seven percent of the total possible alleles in the normal population were of this specificity compared to 5% in the bee-venom sensitive population.)

TABLE IV. Individual class I and class II specificities determined by tissue typing of lymphocytes obtained from bee-venom nonsensitive subjects

Subject No.	Class I				Class II			
	A	B	C	BW	DR	DQw		
23	2	*	8	56	1	3	1	2
24	25	29	7	17	7	*	4/6	2
25	3	11	7	51	7	*	4/6	1.2
26	2	3	7	60	3	7	6	3.2
27	1	24	27	39	1	7	4/6	3.2
28	11	28	27	35	*	*	4/6	3
29	3	32	7	60	7	*	4/6	3
30	1	*	8	55	3	7	6	2
31	1	25	8	39	7	*	6	2
32	2	28	35	*	4	7	6	3.1
33	11	29	35	44	3	7	6	2
34	2	32	27	62	1	3	4/6	3.1
35	2	29	44	51	7	*	4/6	*
36	2	3	35	44	4	*	4/6	2
37	3	24	7	62	1	7	6	*
38	2	3	7	44	7	*	4/6	1.3
39	11	25	7	62	3	7	6	*
40	3	29	17	44	6	*	4	3.1
41	1	2	7	8	7	*	6	2
42	2	31	14	62	3	*	6	3.2
43	2	32	61	51	8	*	4/6	1.2
44	3	31	7	27	2	7	4/6	3

*Only one allele detected.

There were no other significant differences between venom-sensitive subjects and the beekeepers not allergic to bee venom in the remaining HLA-DR alleles as determined by the serologic tissue-typing technique.

RFLP analysis

Analysis of the HLA-DR alleles of all the subjects with RFLP analysis confirmed the results of the serologic tissue-typing studies. There was a statistically significant decrease ($p < 0.01$) in the incidence of the HLA-DR4 allele in the bee-venom sensitive subjects compared to the subjects who were beekeepers but who were not allergic to bee venom (Fig. 2); 5% of the total alleles in the bee-venom sensitive subjects exhibited HLA-DR4, whereas 25% of the total alleles in the beekeepers who were not allergic to bee venom demonstrated the presence of HLA-DR4. There was also a statistically significant decrease ($p < 0.01$) in the frequency of HLA-DR4 in the bee-venom sensitive subjects compared to normal individuals.

A representative example of a hybridization experiment with a cDNA probe for DR β to detect DR alleles is illustrated in Fig. 3. In the venom-sensitive subjects, there was a decrease in the incidence of the 14 kb, 6.4 kb, 6 kb, and 2.7 kb phenotype corresponding to HLA-DR4.

The cDNA probes DQA and DQ β were used to establish the specific subtypes of the class II HLA-DQw alleles (Fig. 4). The only statistically significant difference ($p < 0.05$) in the DQw subtypes was a decrease in the incidence of the 4.0 kb phenotype in the bee-venom allergic subjects corresponding to the DQw3 allele. With a DQ β probe, the reduction in DQw3 allele was demonstrated to involve both DQw3.1 and DQw3.2 specificities (data not presented). The beekeepers who were not allergic to bee venom demonstrated no significant differences in their DR and DQw phenotypes from differences demonstrated by normal populations (data not presented).

The cDNA probes DP α and DP β were used to establish the class II HLA-DP alleles (Fig. 5). There were no significant differences between the bee-venom sensitive and venom-insensitive beekeepers (Fig. 5).

Serum IgE and IgG to PLA₂

To assess whether the negative association between DR4 and DQw3 and an IgE antibody response to melittin was antigen specific, the levels of IgE and IgG antibodies to bee-venom PLA₂ were compared. The concentration of IgE to PLA₂ in the subjects who were allergic to melittin as measured by RAST ranged

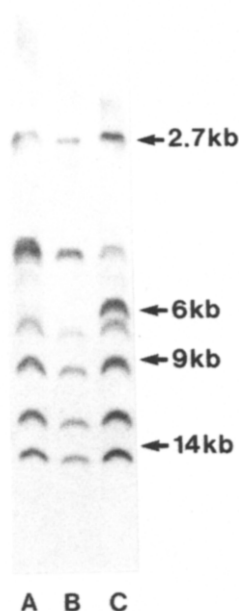


FIG. 3. A representative example of an experiment analyzing the DR alleles with RFLP. Kilobase markers (Kb) are indicated by the arrows and were obtained from λ -phage DNA digested with Hind III restriction endonuclease (BRL Chemicals). DNA samples from bee-venom allergic subjects (tracks A and B) and bee-venom insensitive subjects (track C) were digested with specific restriction enzyme Taq I, and the fragments were separated by agarose gel electrophoresis. The DNA was then blotted onto a nylon membrane with the Southern¹⁴ blot technique, and the filter was hybridized with the ³²P-labeled probe DR β .

from 0.8 to 780 ng/ml (mean, 93 ng/ml) (Fig. 6), whereas the concentration of serum IgE in the beekeepers who were not allergic to bee venom ranged from 0.4 to 1.4 ng/ml (mean, 0.76 ng/ml) (Fig. 6).

The subjects who had allergy to melittin had concentrations of IgG to PLA₂ that ranged from 1.9 to 34 μ g/ml (mean, 6.8 μ g/ml) (Fig. 7). The control subjects who were beekeepers, but who were not allergic to melittin, had serum concentrations of anti-PLA₂ IgG that ranged from 1.8 μ g/ml to 78 μ g/ml (mean, 28 μ g/ml) (Fig. 7).

DISCUSSION

The major allergen found in bee venom, PLA₂,²³⁻²⁵ is a protein of 20,000 daltons. Melittin, the major component by weight, produces an IgE response in approximately 30% of patients and is the

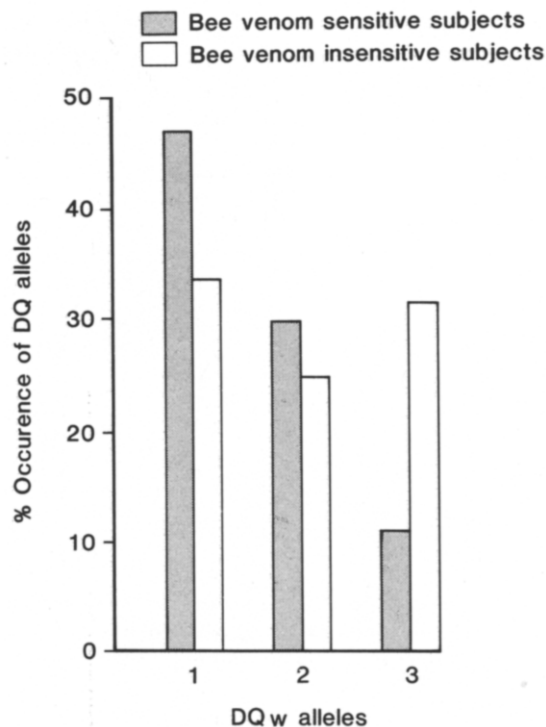


FIG. 4. Percentage occurrence of major histocompatibility class II DQw alleles in subjects who were allergic to bee venom (■) and in beekeepers who were not (□). The DQw specificities were investigated with RFLP analysis.

major allergen in 6% of bee-venom allergic individuals.^{26, 27} Melittin has a molecular weight of 2840 daltons. It possesses surfactant and anticoagulant properties²⁸ and is reported to be capable of generating eosinophil chemotactic factors from human polymorphonuclear leukocytes, rat mast cells, and rat mast cell-depleted peritoneal cells.²⁹ Monoclonal antibodies to melittin have been produced in mice.³⁰ A fragment of melittin (amino acids, 20 to 26) was devoid of hemolytic activity and lymphocyte migration inhibitory activity,³¹ suggesting that the peptide fragment necessary for expression of lytic and T cell stimulatory activity was similar, if it were not identical. This 20 to 26 amino acid portion of the molecule also appears to be critical for binding of IgE and IgG murine monoclonal antibodies.³²

We have demonstrated a significant negative association between human IgE immune response to bee-venom allergy and possession of HLA-DR4 and DQw3. This association could be with a haplotype containing both DR4 and DQw3. The association was true for both the IgE response to melittin and PLA₂; therefore, the negative association was not antigen specific. The negative association was only observed for the IgE immune responsiveness and not for the IgG responsiveness, since the IgG responses to PLA₂

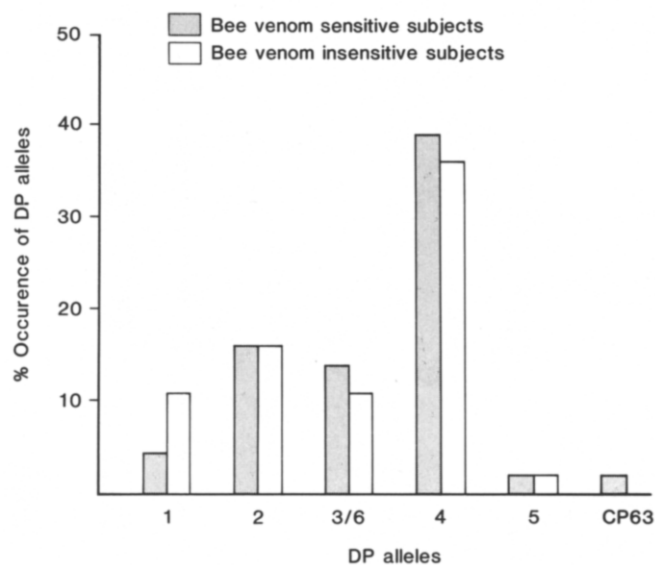


FIG. 5. Percentage occurrence of major histocompatibility complex class II DP alleles in subjects who were allergic to bee venom (■) and in beekeepers who were not (□). The DP specificities were investigated with RFLP analysis.

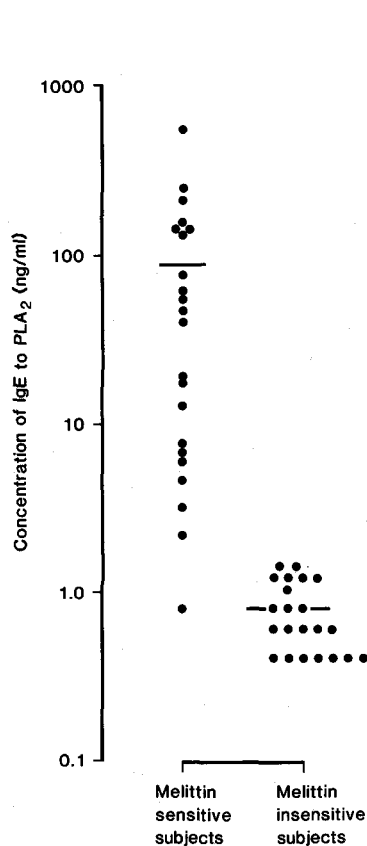
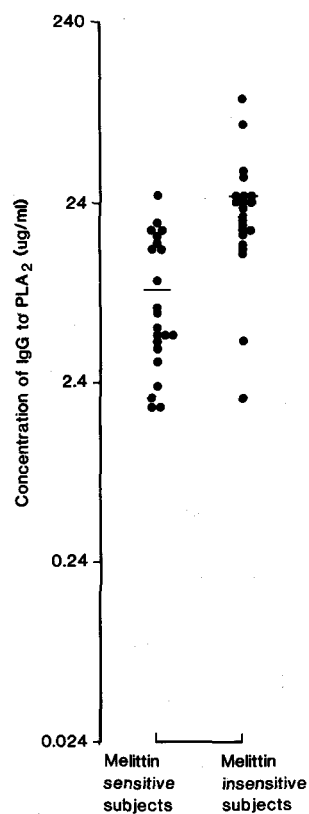


FIG. 6. The concentration of anti-PLA₂ IgE (nanograms per milliliter) was measured by RAST in melittin-sensitive and melittin-insensitive subjects.



were not linked to the HLA region markers studied. This is in contrast to the studies on human immune responsiveness to short ragweed allergen *Amb a V* and possession of HLA-Dw2, since both the IgE and IgG responses to *Amb a V* were found to be strongly associated to HLA-Dw2.

The study contained two groups of subjects as controls. The most appropriate control group consisted of beekeepers who were not allergic to melittin. The second group of control subjects consisted of a large number of unselected subjects in whom the prevalence of melittin sensitivity is unknown. Settipane and Boyd³³ have previously demonstrated that 0.4% of an unselected population of children in the United States reported bee-sting allergy. Formal studies of the prevalence of bee-venom allergy in an unselected population in the United Kingdom has not been performed, but the prevalence is likely to be very low. There were no significant differences in the prevalence of HLA class II alleles between the beekeepers who were not allergic to melittin and the unselected population. Irrespective of the control population that was used for the comparisons, the bee-venom allergic patients possessed a significantly reduced frequency of HLA-DR4, and the conclusions of this study remain unaltered.

Our conclusions were obtained by using both serologic tissue typing and RFLP analytic techniques. The critical elements in the success of these studies were the use of a defined polypeptide antigen of relatively simple structure and the availability of suitable procedures to measure serum IgE and IgG antibody responses. It is likely that the association between the IgE response to DR4 and DQw3 results from the linkage disequilibrium between DR4 and DQw3 in European white subjects.

There is a precedent for our observations; resistance to *Cryptomeira* pollinosis in Japan appears to be HLA linked.³⁴ The mechanism for the HLA-linked IgE non-response to *Cryptomeria japonica* may be mediated by allergen-specific suppressor T cells, as suggested by Matsushita et al.³⁵ Thus, it is possible that HLA-DQ defines a suppressor T cell population in our bee-venom subjects. This finding would support a protective role of the HLA-DQ allele, as suggested by Hirayama et al.³⁶ Alternatively, the bee-venom allergy may be associated with all class II DR and DQ alleles except HLA-DR4 and DQw3. Finally, it may be related to a nonrecognition of the relevant antigens in subjects with the HLA-DR4 and DQw3 haplotype. The observation that both bee-venom sensitive and control subjects produced a similar and measureable titer of IgG to PLA₂ suggests that, if nonrecognition

is the explanation, it is restricted to a particular immunoglobulin class IgE.

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