

Allergens, IgE, mediators, inflammatory mechanisms

Hornet venom allergen antigen 5, Dol m 5: Its T-cell epitopes in mice and its antigenic cross-reactivity with a mammalian testis protein

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Background: A major venom allergen of white-faced hornet (*Dolichovespula maculata*) is antigen 5, Dol m 5. It is a 204-residue protein having 23% to 35% sequence identity with several proteins from diverse sources. The biologic function of antigen 5 and its sequence-related proteins is not known.

Objective: This study was done to delineate the T-cell epitopes of Dol m 5 and to test their cross-reactivity with a sequence-related mouse testis protein, tpx.

Methods: T-cell epitope mapping and cross-reactivity were studied with 15- or 20-residue peptides by their stimulation of spleen cells from mice immunized with recombinant Dol m 5 or tpx fragments.

Results: Three of 20 peptides studied were found to represent major T-cell epitopes of Dol m 5, being recognized by five or all of six mouse strains tested. One major epitope peptide, residue 176-195, showed cross-reactivity in BALB/c mice with the homologous antigen 5s from yellow jackets and wasps, as well as with mouse tpx. The cross-reactivity of Dol m 5 and mouse tpx is not reciprocal because spleen cells from tpx-immunized mice were stimulated by the hornet peptide, but cells from Dol m 5-immunized mice were not stimulated by the corresponding tpx peptides.

Conclusion: Cross-reactivity of vespid antigen 5 and mouse tpx may be of importance in insect allergy because human and mouse testis proteins are highly homologous. (*J Allergy Clin Immunol* 1997;99:630-9.)

Key words: Allergen, antigen 5, hornet, yellow jacket, wasp

Hornets contain three venom allergens of known sequence.¹⁻³ They are antigen 5, hyaluronidase, and phospholipase A₁. Homologs of these venom allergens are present in other vespids, wasps, and yellow jackets.⁴⁻⁷ The distantly related insects, fire ant has homologs of vespid antigen 5 and phospholipase A₁,^{8,9} and honeybee has a homolog of vespid hyaluronidase.¹⁰

A common feature of vespid venom allergens is their partial sequence identity with other proteins in our environment. Antigen 5 has 23% to 35% sequence identity with proteins from mammalian testis,¹¹ human brain,¹² hook worm,¹³ lizard venom,¹⁴ and plants.¹⁵ The

Abbreviations used

MHC: Major histocompatibility complex
tpx: A mouse testis protein

biologic function of antigen 5 is not known. Its homologs from human brain and plants are induced after infection or trauma, but their functions are not known. Vespid hyaluronidase and phospholipase have partial sequence identity with mammalian sperm hyaluronidase^{10, 16} and mammalian lipase, respectively.¹⁷

T helper cells of different types have a decisive role in the regulation and synthesis of antibodies of different isotypes by B cells. Because of these important functions, the T-cell epitopes of several major allergens have been mapped in human beings and in mice. The major allergens include those from cats,¹⁸ honeybee venom,¹⁹ mites,^{20, 21} and birch tree and ryegrass pollens.^{22, 23} Tests with T-cell epitope peptides of bee venom and birch allergens^{19, 22} or with mite or ryegrass allergens²⁴ have shown that T-cell responses to allergens are prevalent in normal and allergic individuals; this has also been demonstrated with IgG antibody responses.

This study presents our findings on the mapping of T-cell epitopes of hornet antigen 5 and on its antigenic cross-reactivity with yellow jacket and wasp antigen 5s, as well as with a mouse testis protein (tpx). The sequences of these proteins are shown in Fig. 1 together with those of a human testis protein. There are three isoforms of antigen 5 from white-faced hornet (*Dolichovespula maculata*); that shown in Fig. 1 is form 2 and is designated as Dol m 5.02 according to an accepted system of allergen nomenclature.²⁵ The yellow jacket (*Vespula vulgaris*) and the wasp (*Polistes annularis*) are designated as Ves v 5 and Pol a 5, respectively.

It has been reported that subcutaneous or intranasal treatment of mice with T-cell epitope peptides of the major cat or mite allergen^{18, 26} leads to T-cell anergy and reduced antibody responses on subsequent immunization with allergen. T-cell epitope peptides of the major cat allergen Fel d 1 are currently being evaluated for clinical efficacy as immunotherapeutic reagents in patients,²⁷ and the peptides being tested in clinical trials are the same ones found to represent T-cell epitopes in

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Supported in part by National Institutes of Health grant AI-17021.

Received for publication Sept. 18, 1996; revised Nov. 8, 1996; accepted for publication Nov. 12, 1996.

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0091-6749/97 \$5.00 + 0 1/179223

Hum		KDPAFTALLTTQLQVQREIQVQREIVNKHNE	1- 31
Mou		KDPDFTSLLTNQLQVQREIQVQREIVNKHNE	1- 31
Dol	NNYCKIKCRKG	IHTLCKFGTSMKP NCGRNVVKAYGLTNDKNEILKRHND	1- 50
Ves	NNYCKIKCLKGGVHTACKYG	SLKP NCGNKVVVS YGLTKQEKQDILKEHND	1- 50
Pol	VDYCKIKCPSG	IHTVCQYGESTKPSKNCAGKVIKSVGPTEEEKKLIVSEHNR	1- 50
Hum	LKRAVS	PPASNMLKMEWSREVTTNQQRWANKCTLQ HSDP	32- 70
Mou	LRRSVN	PTGSDILKMEWSIQATTNAQKWANKCILE HSSK	32- 70
Dol	FRQNVAKGLETRGKPGPQPPAKNMNVLVWNDELAKIAQTWANQCDEFN	HDD	51-100
Ves	FRQKIARGLETRGNPGPQPPAKNMKNLVWNDELAYVAQVWANCQCYG	HDT	51-100
Pol	FRQKVAQGLETRGNPGPQPAASDMNDLVWNDELAHIAQVWASQCQFLVEDK		51-101
Hum	EDRKTSTRCGENL YMSS	DPTSWSSAIQSWYDEILDVYGVGPKSPN	71-116
Mou	DDRKINIRCGENL YMST	DPTLWSTVIQSWYNENEDFVYGVGAK PN	71-115
Dol	CRNTAKYQVGQNIASSTTATQFDRPSKLIKQWEDEVTEFNKVGQLQNSN		101-150
Ves	CRDVAKYQVGQNVALTGSTAAKYDDPVKLVKMWEDEVKDYNPKKKFSGND		101-150
Pol	CRNTAKYPVGQNIASSTTATQFDRPSKLIKQWEDEVTEFNKVGQLQNSN		102-151
hum	AVVGHYTQLVWYSTYQVCGIAYCPNQDSLKYVVCYCPAGNNMNRKNTPYQQ		117-170
mou	SAVGHYTQLVWYSSFKIGCGIAYCPNQDNLKYFYVCHYCPMGNNVMKKSTPYQQ		116-169
Dol	FRKVGHYTQMVGKTKKEIGCGSIKYIEDNWYTHYLVVCNYGPGGNDFNQPIYERK		151-204
Ves	FLKTGHYTQMVGWANTKEVCGSIKYIQEKWHKHLYLVVCNYGPGSGNFMNEELYQTK		151-204
Pol	FAKIGHYTQMVGKTKKEIGCGSLKYMENNMQNHLYLCNYGPAGNYLGQLPYTKK		152-205

FIG. 1. Amino acid sequences of human and mouse testis-specific proteins (tpx) and hornet, yellow jacket, and wasp antigen 5s. These proteins are designated as *Hum*, *Mou*, *Dol*, *Ves*, and *Pol*, respectively.^{4,11} Residues common to tpxs and antigen 5s are represented by bold-faced characters. Underlined region contains the hornet T-cell peptide cross-reacting with homologous regions of the other proteins.

mice. Thus the present T-cell epitope data of Dol m 5 in mice may also be applicable in human beings.

METHODS

Natural vespid antigen 5s and recombinant Dol m 5.02 and its recombinant fragments

The natural proteins were isolated as described previously.^{28,29} The recombinant protein and fragments were expressed in bacteria by using a pQE12 vector (QIAGEN, Chatsworth, Calif.) as reported previously.³⁰ The recombinant protein differs from the natural protein by having the additional peptide sequences of MRGS and RSH₆ at its N- and C-terminal ends, respectively.

Synthetic peptides of Dol m 5.02

These peptides of 15 to 20 residues in length are listed in Table I; they were synthesized by BioSynthesis, Inc. (Lewisville, Texas). They were purified by high-performance liquid chromatography to greater than 85% purity and characterized by matrix-assisted laser desorption mass spectrometry by the manufacturer. They were further characterized by amino acid analysis³¹ in this laboratory. Concentrations of all peptide solutions were determined by amino acid analysis.

Recombinant fragments N and C of murine tpx

These two recombinant fragments correspond to residues 14-112 and 107-169, respectively (Fig. 1). They were expressed in bacteria by using a pQE8 vector. The required complementary DNAs were obtained by polymerase chain reaction with the primers listed in Table II and the template plasmid, clone B4(1)4, which encodes mouse tpx.³² Recombinant plasmids, resulting from ligation of the appropriately cut cDNAs and pQE8, were verified by DNA sequencing of the fragment-

encoding regions. Recombinant tpx N and C fragments were isolated from bacterial lysate by metal chelation chromatography, followed by reverse-phase chromatography. Their yields were about 10 mg per liter of culture. On sodium dodecylsulfate-polyacrylamide gel electrophoresis, they each showed one main band of the expected size. The recombinant fragments have the additional peptide sequences of MRGS₆GS and KLN at their N- and C-terminal ends, respectively. All experimental manipulations are the same as those we had used for Dol m 5.02.³⁰

Immunization of mice

The following strains of mice were tested: ASW/sn, BALB/c byJ, C3H/He, C57BL/6, P/J, and RIII. Their H-2 haplotypes are s, d, k, b, p, and r, respectively. They were obtained at 8 to 10 weeks of age (The Jackson Laboratory, Bar Harbor, Maine). Unless stated otherwise, only female mice were used.

Groups of four mice were each immunized intraperitoneally with 0.2 ml of 10 µg/ml immunogen + 5 mg/ml alum in 0.005 mol/L sodium phosphate (pH 6.4) at weeks 0, 2, 4, 6, and 8. Sera were collected by retro-orbital puncture 1 week after immunization.

ELISA

Wells of MicroTest III flexible assay plates (Becton Dickinson, Oxnard, Calif.) were coated overnight with 5 µg/ml antigen in 0.05 mol/L Tris-HCl (pH 8.0), then treated for 1 hour with diluent buffer to block the remaining reactive sites. Diluent buffer contained 0.1 mg/ml bovine serum albumin, 0.5 mg/ml Tween-20, 0.5 mol/L NaCl, and 0.05 mol/L Tris-HCl (pH 8.0). For colorimetric assay, the wells were incubated for 1 hour in succession with varying concentrations of mouse sera to be tested, 10 µg/ml rabbit antibody specific for mouse IgGs and

TABLE I. Synthetic peptides of Dol m 5.02

Sequence	Residues	Amino acid analysis
NNYCKIKCRKGIHTLCKFGT	1-20	+++
GIHTLCKFGTSMKPNCGRNV	11-30	+++
SMKPNCGRNVVKAYGLTNDE	21-40	+
VKAYGLTNDEKNEILKRHND	31-50	+++
KNEILKRHNDFRONVAKGLE	41-60	+++
FRONVAKGLETRGKPGPQPP	51-70	+++
TRGKPGPQPPAKNMNVLVWN	61-80	+++
AKNMNVLVWNDELAKIAQW	71-90	+++
DELAKIAQWANCDFNHDD	81-100	+++
ANQCDFNHDDCRNTAKYQVG	91-110	++
CRNTAKYQVGQNIASSTTA	101-120	++
QNIASSTTATQFDRPSKLI	111-130	++
TQFDRPSKLIQWEDEVTEF	121-140	+++
KQWEDEVTEFNYKVGLQNSN	131-150	+++
NYKVGLQNSNFRKVGHYTQM	141-160	+++
FRKVGHYTQMVWGKT	151-165	+++
HYTQMVWGKTKEIGCGSIKY	156-175	+++
KEIGCGSIKYIEDNWTYHYL	166-185	+++
IEDNWTYHYLVCNYGPGGND	176-195	+++
VCNYGPGGNDFNQPIYERK	186-204	+++

+++ and ++ denote that all residues are within 20% and 30% of the expected values, respectively; + denotes that all residues are within 20% of the expected values, except for one residue, which is within 50% of the expected value.

TABLE II. Primers for polymerase chain reaction amplification of tpx fragments N and C

No.	Oligonucleotide
	Q ¹⁴ V Q R E I ¹⁹
1	CGT GGA TCC CAA GTT CAA AGA GAG ATC A ¹¹² G V G Y V ¹⁰⁷
2	CGT AAG CTT AGC TCC TAC ACC ATA AAC V ¹⁰⁷ Y G V G A ¹¹²
3	CGT GGA TCC GTT TAT GGT GTA GGA GCT Q ¹⁶⁹ Q Y P T S ¹⁶⁴
4	CGT AAG CTT TTG TTG ATA TGG GGT GCT

Oligonucleotides 1 and 3 are sense primers, each containing a *Bam*HI restriction site at the 5' end. Oligonucleotides 2 and 4 are anti-sense primers, each containing a *Hind*III restriction site at the 5' end. Oligonucleotides 1 and 2 are for amplification of cDNA encoding tpxN, and oligonucleotides 3 and 4 are for cDNA encoding tpxC.

1:100 diluted peroxidase conjugate of goat antibody specific for rabbit IgG. The bound peroxidase was detected by absorbance at 490 nm after incubation with a substrate solution of 16 mg/ml phenol, 0.5 mg/ml 4-aminoantipyrine, and 0.005% H₂O₂ in 0.1 mol/L sodium phosphate (pH 7).

For fluorometric assay, the wells were incubated in succession with mouse sera and alkaline phosphatase conjugate of goat antibody specific for mouse IgGs. The bound phosphatase was detected by fluorescence after incubation with 0.2 mmol/L 4-methylumbelliferyl phosphate in 0.05 mol/L sodium carbonate buffer (pH 9.8) + 1 mmol/L MgSO₄. (All reagents used in these assays were obtained from Sigma Biosciences, St. Louis, Mo.)

Antibody titers represent reciprocal sera dilutions to provide half maximal changes in absorbance or fluorescence.

TABLE III. Antibody titer for sera from mice of different strains immunized with rDol m 5

Mice		Half maximal antibody titer	
Strain	Haplotype	rDol m 5	nDol m 5
C57Bl/6	b	2 × 10 ¹	6 × 10 ¹
BALB/c byj	d	3 × 10 ⁴	1 × 10 ⁴
C3H/He	k	3 × 10 ³	8 × 10 ²
P/J	p	4 × 10 ⁴	1 × 10 ⁴
RIII	r	8 × 10 ⁴	2 × 10 ⁴
ASW/Sn	s	2 × 10 ⁵	5 × 10 ⁴

Sera were from week 5 bleedings after three biweekly injections.

Proliferation assay

Spleen cells from two mice were pooled 8 to 10 days after the fifth or sixth biweekly immunization as described previously. These cells (4 × 10⁵) were cultured in triplicate with three different concentrations of each test antigen or peptide in 0.2 ml of culture medium at 37° C in 5% CO₂. Tritiated thymidine (1 μCi, 2 Ci/mmol) was added on day 3, and the cells were collected on day 4 to determine the uptake of tritiated thymidine. The culture medium contained RPMI-1640 (Rockefeller University Cell Culture Service), 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer (pH 7.3), and 54 μmol/L 2-mercaptoethanol. Proliferation data are expressed as stimulation indices, which represent the ratio of tritiated thymidine incorporations in the presence and absence of testing antigen or peptide. Reproducibility of triplicate measurements of tritiated thymidine incorporation was ±15%.

RESULTS

Murine antibody responses to Dol m 5.02

Six mouse strains, each of a different major histocompatibility complex (MHC) haplotype, were immunized with recombinant (r)Dol m 5.02 with alum as adjuvant. Only one strain, C57Bl/6, was a poor responder with antibody titer of less than 10²; and the other five strains ASW/sn, BALB/c, C3H/He, P/J, and RIII were high responders with antibody titers of 10³ to 10⁵ (Table III). These sera showed slightly lower titers for natural (n)Dol m 5 than for rDol m 5. One strain, BALB/c, was also immunized with nDol m 5, and it showed higher antibody titers with nDol m 5 than with rDol m 5. We have shown previously that nDol m 5.02 induced mainly murine antibodies specific for its discontinuous B-cell epitopes, whereas the recombinant protein induced antibodies specific for continuous epitopes that are common with those of natural protein.³⁰

Murine T-cell responses to Dol m 5.02 and its peptides

Proliferation assays were done with spleen cells from groups of two recombinant protein-immunized mice, which are described above, for antibody determination. These spleen cells were stimulated with rDol m 5.02 or

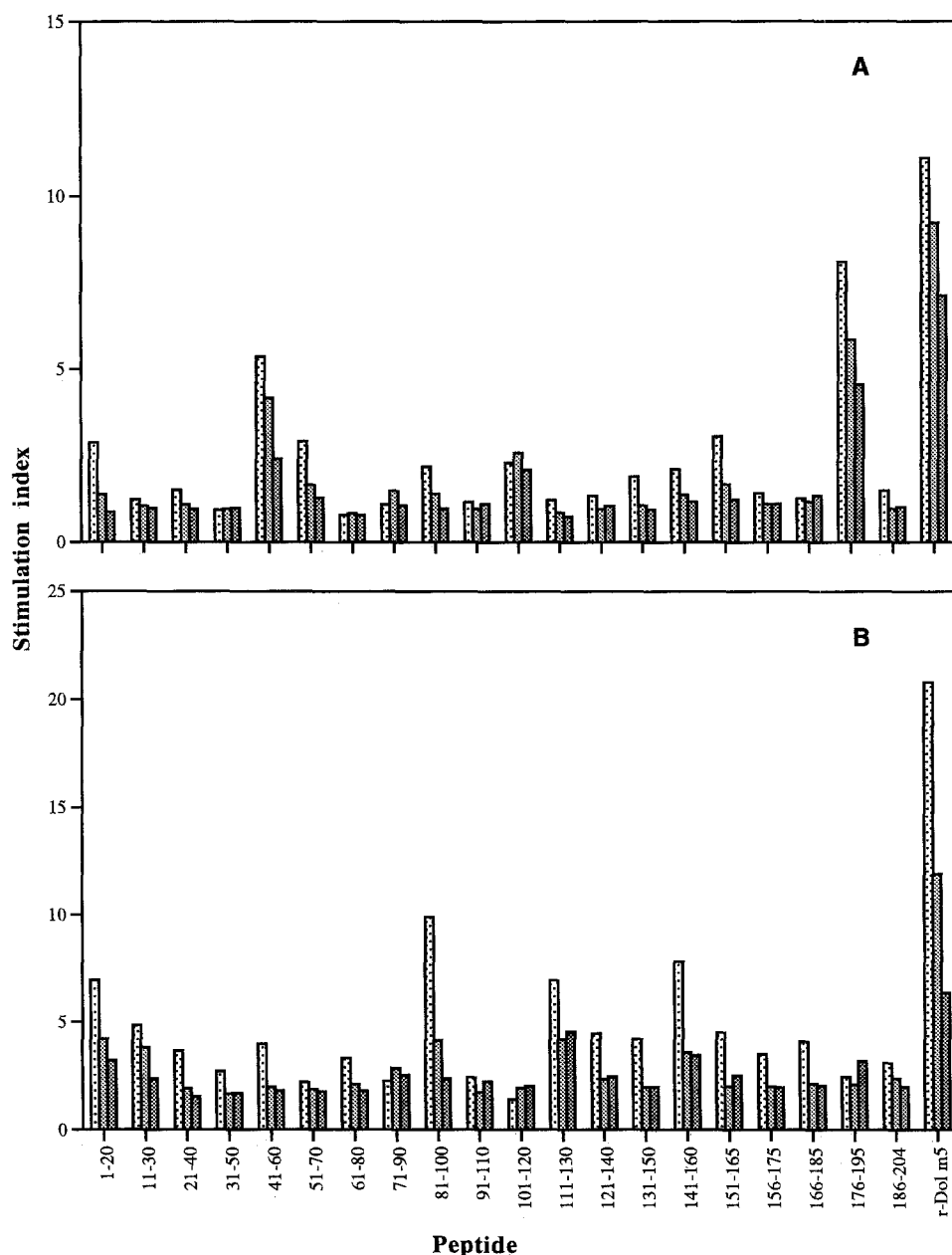


FIG. 2. Proliferation of spleen cells from mice immunized with rDol m 5. In **panels A and B**, mice were of BALB/c byj and P/J strains, respectively. The three bars indicate stimulation index values at peptide concentrations of 1×10^{-5} , 1.25×10^{-6} , and 1.56×10^{-7} mol/L, and rDol m 5 was tested at concentrations 10-fold lower than those of peptides. Background tritiated thymidine incorporations were 4700 and 1730 cpm $\pm$ 15% for triplicate measurements in **panels A and B**, respectively.

a series of 15- to 20-residue peptides with 10-residue overlaps; these peptides together encompass the length of Dol m 5.02 (Table I). Cell proliferations expressed as stimulation indices for BALB/c and P/J mice are shown in the upper and lower panels of Fig. 2, respectively; and those for ASW/sn and C3H/He mice are shown in Fig. 3. As shown in the figures, each stimulating antigen or peptide was tested at three concentrations. All 20 peptides tested showed stimulation indices of greater than 2 at the maximal concentration of 1×10^{-5} mol/L in at

least one of the four mouse strains. If an arbitrary cutoff value of 2 is used, these data would suggest that the T-cell epitopes are distributed throughout the entire molecule of Dol m 5. The peptide mapping experiments with the four strains of mice shown in Figs. 2 and 3 were carried out twice, each time with groups of two mice after five or six immunizations. Closely similar results were obtained, and only the data for one of these two sets of experiments are shown in these figures.

In Table IV the stimulation index data of the peptides

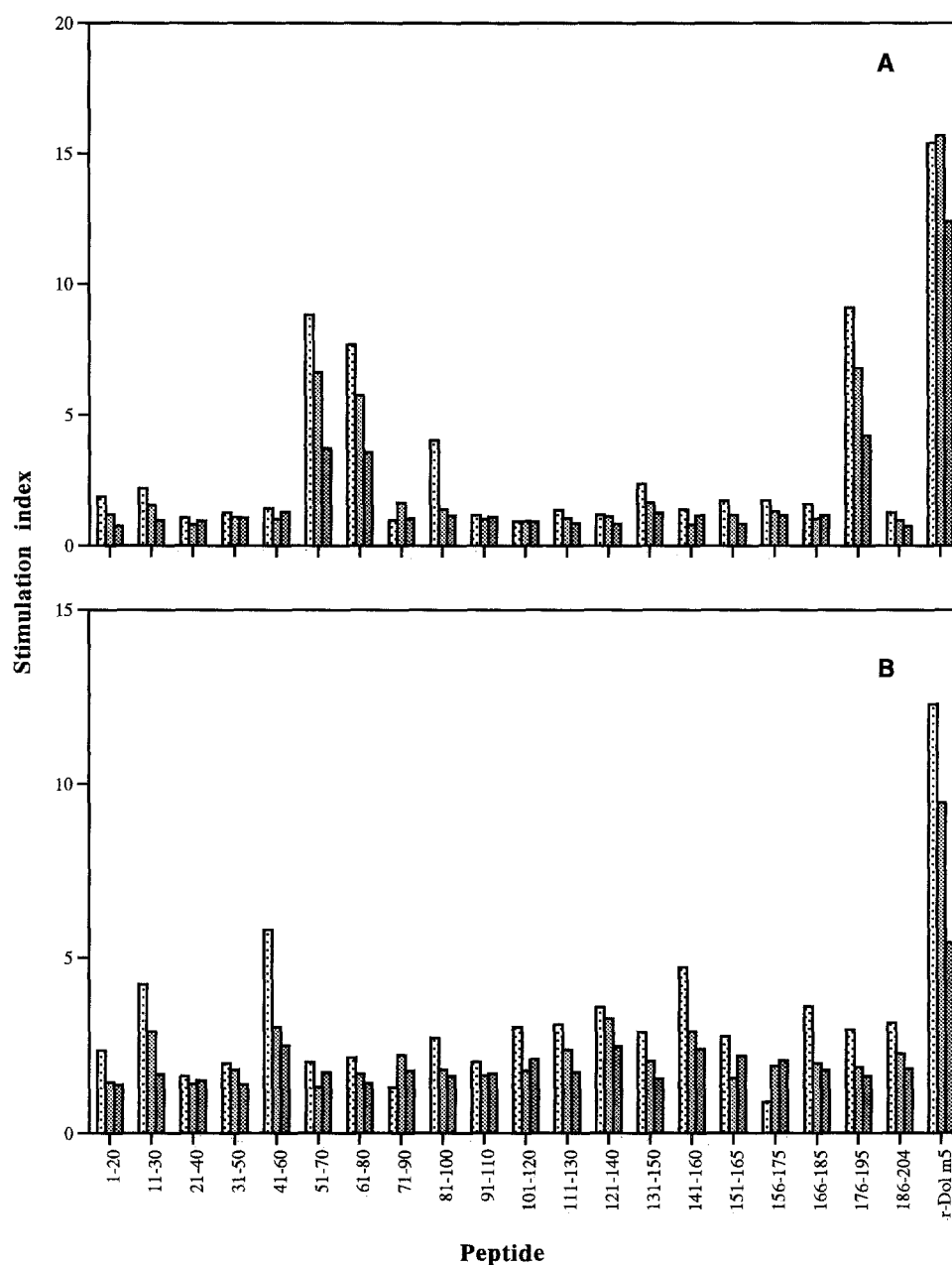


FIG. 3. Proliferation of spleen cells from mice immunized with rDol m 5. In **panels A and B**, mice of ASW/sn and C3H/He strains were used, and background incorporations of radioactivity were 5640 and 3130 cpm $\pm$ 15% for triplicate measurements, respectively. Peptide and rDol m 5 concentrations were the same as those in Fig. 2.

and rDol m 5.02 for the four mouse strains (shown in Figs. 2 and 3) together with data from RIII and C57Bl/6 mice are summarized. The data for BALB/c mice (shown in parentheses in Table IV) are from an experiment separate from that in Fig. 2, and reasonable agreement of the data from these two experiments is seen. The stimulation indices of rDol m 5.02 at 1×10^{-6} mol/L ranged from 7 to 35 for the six mouse strains tested, and there is a rough correlation of the stimulation index in Table IV and the antibody titer in Table III. The stimulation indices of the peptides at 1×10^{-5} mol/L

ranged from 2 to 18, depending on the peptide and the mouse strain; values of less than 2 were omitted in Table IV. With one exception, the high antibody responder mice in Table III each responded to three or more peptides with high stimulation index values of greater than 4. The exception is the mouse strain RIII, which responded to only two peptides with stimulation indices of about 2. The poor antibody responder C57Bl/6 mice responded to eight peptides, but only one peptide had a high stimulation index of 3.9.

The overlapping peptides in Table I cover all possible

TABLE IV. Proliferation of rDol m 5-specific spleen cells from mice of different strains on peptide stimulation

Stimulating peptide or antigen	Stimulation index					
	BALB/c	ASW/Sn	C3H/He	P/J	RIII	C57Bl/6
1-20	2.9	(8.6)		2.4	6.9	2.5
11-30		(2.9)	2.2	4.3	4.8	
21-40					3.7	2.1
31-50				2.0	2.7	2.4
41-60	5.4	(9.3)		5.8	4.0	
51-70	2.9	(5.8)	8.8		2.2	
61-80			7.7	2.2	3.3	2.1
71-90					2.2	
81-100	2.2	(3.8)	4.0	2.7	9.9	2.1
91-110					2.4	2.2
101-120	2.3	(5.8)		3.0		2.7
111-130		(2.5)		3.3	4.0	2.5
121-140				3.6	4.5	2.5
131-150		(2.3)	2.3	2.9	4.2	2.0
141-160	2.1	(5.6)		4.7	7.8	2.8
151-165	3.1	(6.5)		2.8	4.5	2.1
156-175					3.5	2.6
166-185				3.6	4.1	2.4
176-195	8.1	(17.7)	9.1	2.9	2.5	2.3
186-204		(2.0)		3.1	3.1	3.1
rDol m 5	11.1	(20.9)	15.4	12.3	20.8	35.2

Stimulation index values for BALB/c, ASW/sn, C3H/He, and P/J mice are taken from Figs. 2 and 3 at peptide and rDol m 5 concentrations of 1×10^{-5} and 1×10^{-6} mol/L, respectively. Values in parentheses for BALB/c mice and those for RIII and C57Bl/6 are from other experiments. Values of less than 2 are not given. Background tritiated thymidine incorporation values were 4700 (3970), 5640, 3130, 1730, 1760, and 1050 cpm, respectively, for the six mouse strains; reproducibility of triplicate measurements was $\pm 15\%$.

sequences of Dol m 5 of 11 residues or less. Sequences of more than 11 residues in length, which span the overlap regions of two adjoining peptides, are not covered. The small number of peptides that stimulated spleen cells of RIII mice may be a consequence of their T-cell epitope peptides of more than 11 residues in length. The minimal size of a T-cell epitope is a nonapeptide, and its upper size limit is variable, as suggested by various studies.³³ Recent crystallographic studies of a human class II MHC molecule indicated its binding site to be of a 13-mer peptide,³⁴ and naturally processed peptides eluted from class II MHC molecules ranged in size from 13 to 17 residues.³⁵

Proliferation assays were done with spleen cells of BALB/c mice immunized with nDol m 5.02. Results identical to those given in Fig. 2, A, were observed (data not shown). Assays were also done with spleen cells of BALB/c mice immunized with recombinant fragments 1-114 and 151-204 of Dol m 5.02, and positive responses were obtained only with synthetic peptides, which correspond to those present in the fragments (data not shown).

Antigenic cross-reactivity of vespid antigen 5s

Sequence homologies of hornet, yellow jacket, and wasp antigen 5s are shown in Fig. 1 and designated as Dol, Ves, and Pol, respectively. Vespids antigen 5s are known to have varying extents of antigenic cross-reactivity at both T-cell and B-cell levels.⁴ Their cross-reactivity

was tested with selected hornet T-cell peptides of Table IV by their stimulation of spleen cells from BALB/c mice immunized with Ves v 5 or Pol a 5. The results summarized in Table V indicate that only one of the eight hornet peptides, residue 176-195, has significant cross-reactivity with both yellow jacket- and wasp-specific spleen cells.

Antigenic cross-reactivity of Dol m 5 and mouse tpx

Antigenic cross-reactivity of Dol m 5 and mouse tpx is shown by tests with their recombinant fragments. Recombinant fragments IN and C contain residues 1-114 and 151-204 of Dol m 5, and recombinant fragments tpx-N and tpx-C contain residues 14-112 and 107-169 of the mouse protein. Sequence data for the parent proteins of these fragments are given in Fig. 1.

Sera from BALB/c mice immunized with recombinant fragment C of Dol m 5 showed a cross-reaction with the corresponding recombinant fragment tpx-C. A similar reciprocal cross-reaction was detected with sera from BALB/c mice immunized with recombinant fragment tpx-C. In addition, the tpx-C-specific sera showed a weak cross-reaction with nDol m 5. These cross-reactions were detected by ELISA, and the results are given in Table VI. Similar studies were done with recombinant fragments IN and tpx-N, and no cross-reaction was detected.

Cross-reaction at the T-cell level was detected by

TABLE V. Stimulation of spleen cells from vespid antigen 5-immunized BALB/c mice by hornet peptides

Stimulating peptide or antigen	Stimulation index for spleen cells specific for		
	rDol m 5	nVes v 5	nPol a 5
1-20	2.9	2.2	1.3
41-60	5.4	2.2	1.6
51-70	2.9	1.4	1.3
81-100	2.2	1.6	1.2
141-160	2.1	1.9	2.1
151-165	3.1	1.2	1.8
176-195	8.1	8.1	2.8
rDol m 5	11.1	9.0	6.3
nVes v 5	ND	31.7	ND
nPol a 5	ND	ND	17.5

Stimulation index values for rDol m 5 are taken from Fig. 2. Values given are for maximal concentrations tested; 1×10^{-5} mol/L peptide and 1×10^{-6} mol/L antigen. Background tritiated thymidine incorporation values were 4700, 1670, and 4130 cpm, respectively; reproducibility of triplicate measurements was $\pm 15\%$.

ND, Not done.

proliferation assay with spleen cells from tpx-N- and tpx-C-immunized BALB/c mice. The results are given in Fig. 4, A and B, respectively. Similar stimulation index values were observed for the cross-reacting fragments. Reciprocal cross-reactions were also detected with BALB/c spleen cells specific for recombinant fragments IN and C or for rDol m 5 (data not shown).

In Fig. 4, A, tpx-N-specific spleen cells were tested with the overlapping peptides of Dol m 5, which are homologous with tpx-N. The results in Fig. 4, A suggest that the peptides in the regions of residues 41-100 and 131-150 of Dol m 5 contain T-cell epitopes cross-reacting with tpx-N with stimulation index values of 2 to 3.

The results in Fig. 4, B, with tpx-C-specific spleen cells suggest that only one peptide region of Dol m 5, residue 176-195, contains T-cell epitope(s) cross-reacting with tpx-C with a stimulation index of 6 as compared with a value of 8 for fragment C. Additional tests with peptides 178-195 or 176-193 showed findings similar to those of peptide 176-195 (data not shown). Thus the T-cell epitope of Dol m 5 cross-reacting with tpx-C is in the region of residue 178-193. However, the cross-reaction was not found to be reciprocal because tpx peptide 140-159, which corresponds to Dol m 5 peptide 176-195, did not stimulate the proliferation of Dol m 5-specific spleen cells from BALB/c mice (data not shown).

Cross-reactivity of Dol m 5 and tpx was also studied in P/J mice, and negative results were obtained (data not shown).

DISCUSSION

We have mapped the T-cell epitopes of Dol m 5 with a series of 20 overlapping peptides (Table I). A majority of these peptides was found to stimulate the prolifera-

TABLE VI. Antibody titers of sera from BALB/c mice immunized with recombinant fragments of Dol m 5 or tpx

Immunogen	Antibody titer specific for		
	Natural Dol m 5	Recombinant fragment IN	Recombinant tpx-N
Recombinant fragment IN	4×10^3	5×10^4	$\ll 1 \times 10^1$
Recombinant tpx-N	$\ll 1 \times 10^1$	$< 1 \times 10^1$	7×10^2
Immunogen	Antibody titer specific for		
	Natural Dol m 5	Recombinant fragment C	Recombinant tpx-C
Recombinant fragment C	2×10^4	6×10^4	1×10^2
Recombinant tpx-C	3×10^1	7×10^2	6×10^4

Sera were from week 5 bleedings after three biweekly immunizations.

Reproducibility of antibody titers on duplicate assay was $\pm 30\%$.

The symbol $\ll$ designates "much less than."

tion of spleen cells from rDol m 5- or nDol m 5-immunized mice. Because of MHC restriction in peptide recognition and possibly different T-cell receptor usage, varying patterns of peptide stimulation were observed in the six mouse strains tested. In this study we do not provide any proof that the peptide-induced proliferation of Dol m 5-specific spleen cells reflects that of CD4⁺ Th2 cells with MHC class II restriction, as we had done with bee venom allergen melittin-specific spleen cells.³⁶ This was demonstrated by isolation of CD4⁺ T-cell clones, followed by tests of their lymphokine release and MHC restriction.

Of the 20 peptides in Table IV, three are T-cell epitopes in five or all of six mouse strains tested, 13 in three or four mouse strains, and four in one or two mouse strains. Thus only three peptides (81-100, 131-150, and 176-195) represent "universal" T-cell epitopes recognized by nearly all mouse strains tested; but their stimulation indices vary with mouse strain. The stimulation index value is a reflection of the size of T-cell population specific for the antigen or peptide. Therefore the peptides with high stimulation indices likely represent the dominant epitopes. Two of the dominant epitopes with a broad MHC restriction pattern are peptides 141-160 and 176-195. These hornet peptides show cross-reactivity with yellow jacket and wasp antigen 5s in BALB/c mice (Table IV), as well as in P/J mice (data not shown).

In model experiments on peptide therapy to induce immune unresponsiveness in mice (unpublished data), we had sought to circumvent the problem of MHC restriction and T-cell epitope dominance by using fragments of 50-100 residue size, because such fragments could be prepared efficiently by recombinant technology. But this approach was hampered by the limited solubility of these fragments (unpublished observation). The T-cell epitope peptides described above showed greater solubility than the larger fragments. They will be useful

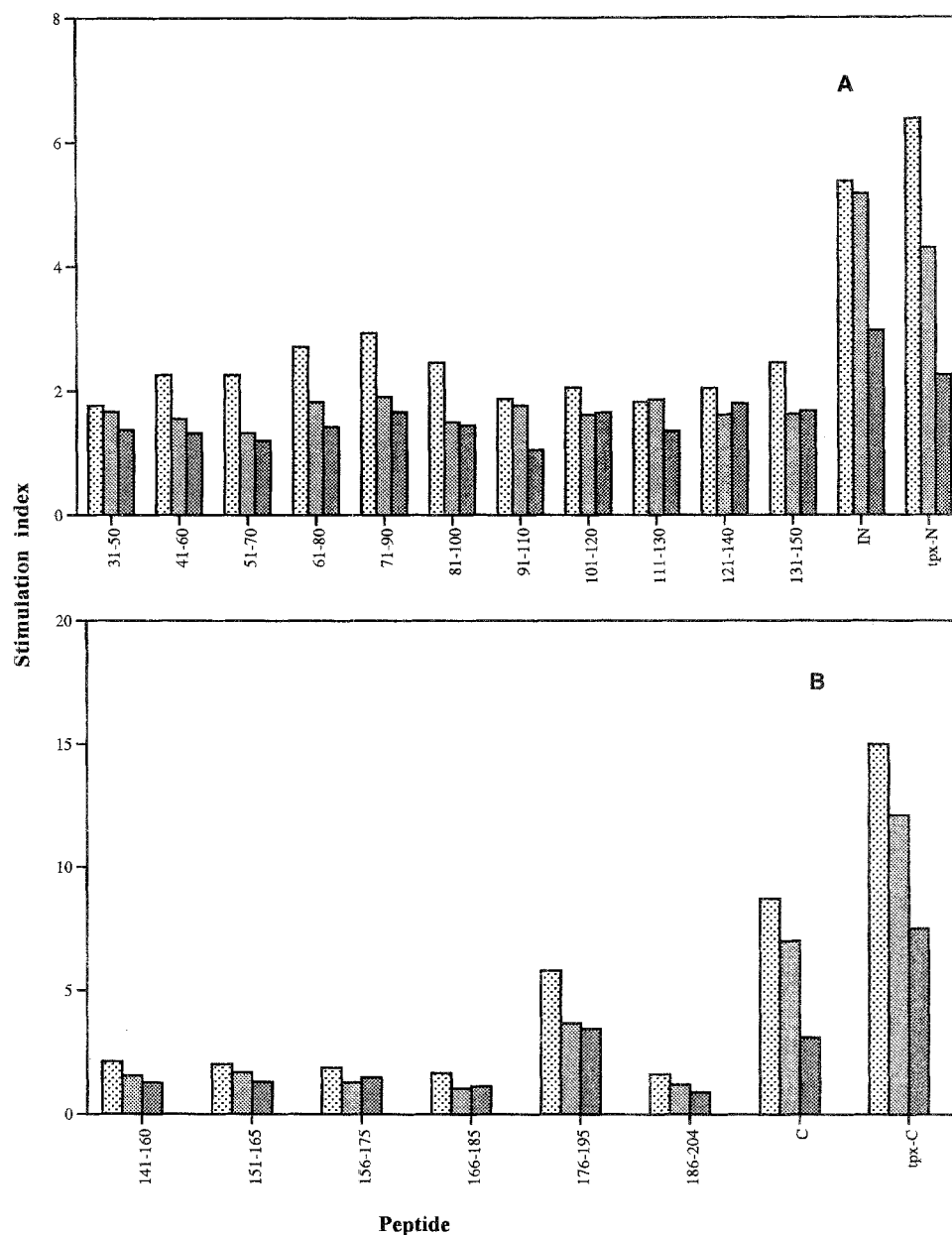


FIG. 4. Proliferation of spleen cells from BALB/c byj mice immunized with rtpx-N and rtpx-C (**panels A and B**). Peptide concentrations were the same as those in Fig. 2, and concentrations of tpx and Dol m 5 fragments IN and C were the same as those of Dol m 5 in Fig. 2. Background incorporations of radioactivity were 7270 and 5110 cpm $\pm$ 15% for triplicate measurements in **panels A and B**, respectively.

reagents for studies on peptide therapy, so that we can determine, for a multivalent immunogen such as Dol m 5, how many T-cell epitope peptides are required for efficient suppression of immune responses. These findings will be compared with those of melittin, which contains only one major T-cell epitope.³⁶

We carried out studies on antigenic cross-reactivity of Dol m 5 and a mouse testis protein, tpx, because of their sequence similarity. Cross-reactivity was detected at both T-cell and B-cell levels in BALB/c mice. One of the cross-reactive T-cell epitopes was identified as peptide

176-195 (Fig. 1). However, the cross-reactivity is not reciprocal because spleen cells from Dol m 5-immunized mice did not respond to stimulation by tpx peptide 140-159, which shares sequence similarity with Dol m 5 peptide 176-195. When male or female mice were immunized with Dol m 5, indistinguishable antibody titers were obtained (unpublished observation).

Several groups of investigators have observed that more men than women, in a ratio of about 2 to 1, had insect allergy as judged by their systemic and large local reactions or by their death statistics.³⁷ It has been

assumed that these results are primarily due to greater exposure because men work outdoors more often than women do. Clinical data are often combined for patients with bee and vespid allergies,³⁸ but only vespid venoms contain antigen 5. It remains to be established whether antigenic cross-reactivity of vespid antigen 5 and tpx is of clinical relevance.

Sequence similarity of other allergens with mammalian proteins is known. A dominant T-cell epitope peptide of the mite allergen Der p 1 was shown to cross-react with the invariant chain-derived CLIP peptide in mice of H-2^b haplotype.³⁹ Similar to Dol m 5, the cross-reactivity of Der p 1 and CLIP peptides is not reciprocal. The degree of sequence identity of Der p 1 and CLIP peptides is about the same as that of Dol m 5 and tpx peptides. Bee venom allergen phospholipase A₂ has sequence similarity with the human enzyme but no antigenic cross-reactivity at the T-cell level was detected.¹⁹

The biologic activity of a T-cell epitope peptide depends on its binding to the class II MHC and T-cell receptor molecules. Several authors have reported that substitution of the major contact residues of a T cell epitope for T-cell receptor binding may lead to increased or decreased activity relative to that of the natural T-cell epitope.^{40, 41} This is also suggested by the data in Table V for hornet peptides 81-100 and 176-195. These two peptides have about the same degree of sequence homology with their yellow jacket homologs, but peptide 176-195 retains the activity to stimulate yellow jacket-specific T cells, whereas peptide 81-100 does not.

Because of their different T-cell activities, it is possible that a cross-reacting T-cell epitope of an allergen may activate the proliferation of host-specific memory cells, although the natural T-cell epitopes of the host cannot. This would help to explain why susceptible persons can become readily sensitized to minute amounts of allergen and why the allergic response is long-lasting. To date, the only common feature known for allergens from different sources is their varying extents of sequence similarity with other proteins in our environment. The proposed role of antigenic cross-reactivity in allergic diseases is in addition to the reported findings that susceptible persons are genetically predisposed to mount allergen-specific immune responses and also have genes that influence the propensity of IgE synthesis.⁴²

We thank Dr. Masanori Kasahara of Hokkeido University School of Medicine, Sapporo, Japan, for a gift of plasmid DNA encoding mouse tpx, clone B4(1)4; Mr. Miles Guralnick of Vespa Laboratory, Spring Mills, Pa., for gifts of vespid venoms; and Dr. Sheena Mische and the staff of the Protein Sequence Facility of Rockefeller University, New York, for oligonucleotide synthesis and DNA sequencing.

REFERENCES

- Fang KSY, Vitale M, Fehlner P, King TP. cDNA cloning and primary structure of a white-face hornet venom allergen, antigen 5. *Proc Natl Acad Sci USA* 1988;85:895-9.
- Soldatova L, Kochoumian L, King TP. Sequence similarity of a hornet (*D. maculata*) venom allergen phospholipase A1 with mammalian lipases. *FEBS Lett* 1993;320:145-9.
- Lu G, Kochoumian L, King TP. Sequence identity and antigenic cross reactivity of white face hornet venom allergen, also a hyaluronidase, with other proteins. *J Biol Chem* 1995;270:4457-65.
- Lu G, Villalba M, Coscia MR, Hoffman DR, King TP. Sequence analysis and antigen cross reactivity of a venom allergen antigen 5 from hornets, wasps and yellowjackets. *J Immunol* 1993;150:2823-30.
- King TP, Lu G, Gonzalez M, Qian N, Soldatova L. Yellow jacket venom allergens, hyaluronidase and phospholipase: sequence similarity and antigenic cross-reactivity with their hornet and wasp analogs and possible implications for clinical allergy. *J Allergy Clin Immunol* 1996;98:588-600.
- Hoffman DR. Allergens in Hymenoptera venom XXV: the amino acid sequences of antigen 5 molecules and the structural basis of antigenic cross-reactivity. *J Allergy Clin Immunol* 1993;92:707-16.
- Hoffman DR. Allergens in Hymenoptera venom XXVI: the complete amino acid sequences of two vespid venom phospholipases. *Int Arch Allergy Immunol* 1994;104:184-90.
- Hoffman DR. Allergens in Hymenoptera venom XXIV: the amino acid sequences of imported fire ant venom allergens Sol i II, Sol i III, and Sol i IV. *J Allergy Clin Immunol* 1993;91:71-8.
- Schmidt M, McConnell TJ, Hoffman DR. Identification of nucleic acid sequence of two proteins from the fire ant venom, Sol i III and Sol i I [abstract]. *J Allergy Clin Immunol* 1994;93:223.
- Gmachl M, Kreil G. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. *Proc Natl Acad Sci USA* 1993;90:3569-73.
- Mizuki N, Sarapata DE, Garcia-Sanz JA, Kasahara M. The mouse male germ cell-specific gene Tpx-1: molecular structure, mode of expression in spermatogenesis, and sequence similarity to two non-mammalian genes. *Mamm Genome* 1992;3:274-80.
- Murphy EV, Zhang Y, Zhu W, Biggs J. The human glioma pathogenesis-related protein is structurally related to plant pathogenesis-related proteins and its gene is expressed specifically in brain tumors. *Gene* 1995;159:131-5.
- Hawdon JM, Jones BF, Hoffmann DR, Hotez PJ. Cloning and characterization of ancylostoma-secreted protein. *J Biol Chem* 1996;271:6672-8.
- Morrisette J, Kratzschmar J, Haendler B, El-Hayek R, Mocha-Morales V, Martin BM, et al. Primary structure and properties of helothermine, a peptide toxin that blocks ryanodine receptors. *Biophys J* 1995;68:2280-8.
- Cornelissen B, Hooft von Huijsduijnen R, Van Loon L, Bol J. Molecular characterization of messenger RNAs for pathogenesis related proteins 1a, 1b and 1c produced by TMV infection of tobacco. *EMBO J* 1986;5:37-40.
- Lin Y, Kimmel LH, Myles DG, Primakoff P. Molecular cloning of the human and monkey sperm surface protein PH-20. *Proc Natl Acad Sci USA* 1993;90:10071-5.
- Oka K, Nakano T, Tkalecic GT, Scow RO, Brown WV. Molecular cloning of mouse hepatic triacylglycerol lipase: gene expression in combined lipase-deficient (cld/cld) mice. *Biochim Biophys Acta* 1991;1089:13-20.
- Briner TJ, Kuo M, Keating KM, Rogers BL, Greenstein JL. Peripheral T-cell tolerance induced in naive and primed mice by subcutaneous injection of peptides from the major cat allergen Fel d 1. *Proc Natl Acad Sci USA* 1993;90:7608-12.
- Carballido JM, Carballido-Perrig N, Kagi MK, Meloen MH, Wuthrich B, Heusser CH, et al. T cell epitope specificity in human allergic and non-allergic subjects to bee venom phospholipase A2. *J Immunol* 1993;150:3582-91.
- O'Brien RM, Thomas WR, Nicholson I, Lamb JR, Tait BD. An immunogenetic analysis of the T-cell recognition of the major house dust mite allergen Der p 2: identification of high- and low-responder HLA-DQ alleles and localization of T cell epitopes. *Immunology* 1995;86:176-82.
- O'Hehir RE, Hoyne GF, Thomas WR, Lamb JR. House dust mite allergy from T cell epitopes to immunotherapy. *Eur J Clin Invest* 1993;23:763-72.
- Ebner C, Schenk S, Najafian N, Siemann U, Steiner R, Fischer GW,

- et al. Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. *J Immunol* 1995;154:1932-40.
23. Blaher B, Suphioglu C, Knox RB, Singh MB, McCluskey J, Rolland JM. Identification of T-cell epitopes of Lol p 9, a major allergen of ryegrass (*Lolium perenne*) pollen. *J Allergy Clin Immunol* 1996;98:124-32.
24. Upham JW, Holt B, Baron-Hay M, Yabuhara A, Hales BJ, Thomas WR, et al. Inhalant allergen-specific T-cell reactivity is detectable in close to 100% of atopic and normal individuals: covert responses are unmasked by serum-free medium. *Clin Exp Allergy* 1995;25:634-42.
25. King TP, Hoffman DR, Lowenstein H, Marsh DG, Platts-Mills TAE, Thomas WR. Allergen nomenclature. *WHO Bull* 1994;72:797-806.
26. Hoyne GF, O'Hehir RE, Wraith DC, Thomas WR, Lamb JR. Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J Exp Med* 1993;178:1783-8.
27. Norman PS, Ohman JL, Long AA, Creticos PS, Geftler ML, Shaked Z, et al. Follow on study of the first clinical trial with T cell defined peptides from cat allergen Fel d 1 [abstract]. *J Allergy Clin Immunol* 1995;95:259.
28. King TP, Sobotka A, Alagon A, Kochoumian L, Lichtenstein LM. Protein allergens of white-faced hornet, yellow hornet and yellow-jacket venoms. *Biochemistry* 1978;17:5165-74.
29. King TP, Kochoumian L, Lam T. Immunochemical observations of antigen 5, a major venom allergen of hornets, yellowjackets and wasps. *Mol Immunol* 1987;24:857-64.
30. King TP, Kochoumian L, Lu G. Murine T and B cell responses to natural and recombinant hornet venom allergen, Dol m 5.02 and its recombinant fragments. *J Immunol* 1995;154:577-84.
31. Chang J, Knecht R, Braun D. A complete separation of dimethylaminoazobenzene-sulphonyl-amino acids. Amino acid analysis with low nanogram amounts of polypeptide with dimethylaminoazobenzene-sulphonyl chloride. *Biochem J* 1982;203:803-6.
32. Kasahara M, Gutnecht J, Brew K, Spur N, Goodfellow PN. Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. *Genomics* 1989;5:527-34.
33. Wucherpfennig KW, Sette A, Southwood S, Oseroff C, Matsui M, Strominger JL, et al. Structural requirements for binding of an immunodominant myelin basic protein peptide to DR2 isotypes and its recognition by human T cell clones. *J Exp Med* 1994;179:279-90.
34. Stern LJ, Brown JH, Jardetzky TS, Gorga JC, Urban RG, Strominger JL, et al. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* 1994;368:215-21.
35. Rudensky AY, Preston-Hurlburt P, Hong S, Barlow A, Janeway CA Jr. Sequence analysis of peptides bound to MHC class II molecules. *Nature* 1991;353:622-7.
36. Fehlnner PF, Berg R, Tam JP, King TP. Murine T cell responses to melittin and its analogs. *J Immunol* 1991;146:799-806.
37. Muller UR. Insect sting allergy. Stuttgart: Gustav Fisher, 1990:54.
38. Settiple G, Chafee F, Klein D, Boyd G, Sturam J, Freye H. Anaphylactic reactions to Hymenoptera stings in asthmatic patients. *Clin Allergy* 1980;10:659-65.
39. Kristensen NM, Hoyne GF, Hayball JD, Hetzel C, Bourne T, Lamb JR. Induction of T cell responses to the invariant chain derived CLIP peptide in mice immunized with group 1 allergen of house dust mite allergen. Submitted for publication.
40. Sloan-Lancaster J, Allen PM. Significance of T-cell stimulation by altered peptide ligands in T cell biology. *Curr Opin Immunol* 1995;7:103-9.
41. Wucherpfennig KW, Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 1995;80:695-705.
42. Marsh DG, Neely JD, Breazeale DR, Ghosh B, Freidhoff LR, Ehrlich-Kautzky E, et al. Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. *Science* 1994;264:1152-6.