

Murine T and B Cell Responses to Natural and Recombinant Hornet Venom Allergen Dol m 5.02 and Its Recombinant Fragments¹

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White-face hornet venom allergen, Dol m 5.02, is a protein of 204 amino acid residues. This protein and its overlapping fragments, of 53–114 residues in size, containing the N-terminal, middle, and C-terminal regions of the molecule, can be expressed in high yield in bacteria by using the plasmid vector pQE12. Natural (n) and recombinant (r) Dol m 5.02s and the r-fragments are about equally immunogenic for IgG Ab response in BALB/c mice. n-Dol m 5.02 induces mainly murine IgG Abs specific for its discontinuous B cell epitopes and, to a lesser extent, Abs specific for its continuous epitopes. r-Dol m 5.02 and the r-fragments induce only Abs specific for continuous B cell epitopes that are common with those of the n-protein. Abs specific for the discontinuous epitopes show higher affinity than those specific for the continuous epitopes. r-Dol m 5.02 and the r-fragments are as efficient as n-Dol m 5.02 in inducing murine T cell responses specific for the n-protein. The differences in the immunogenicity of n- and r-proteins or r-peptide fragments for B and T cell responses are related to their conformations, inasmuch as only the n-protein is cross-linked by four disulfide bonds. These findings are relevant to the potential use of r-fragments as immunotherapeutic reagents in humans. *The Journal of Immunology*, 1995, 154: 577–584.

There is interest in the development of modified allergens for immunotherapy in patients, because modified allergens with reduced allergenicity can be used in higher doses with greater safety. Immunotherapy by repeated s.c. injections of allergens leads to an initial increase in IgE Abs followed by a long-term slow decrease and increased levels of IgG Abs. Recent findings suggest that immunotherapy also down-regulates allergen-specific T cell responses. The late-phase allergic reaction is believed to result from the inflammation-promoting cytokines that are released from allergen-specific T cells (1).

Recently, the use of T cell epitope peptides of allergens for immunotherapy has been suggested. Model experiments showed that treatment of mice with dominant T cell epitope peptides of either the major cat allergen Fel d 1 by the s.c. route (2) or the major mite allergen Der p 1 by intranasal instillation (3) induced suppression of allergen-specific immune response. Although the exact mechanism

of the reported immunosuppression is still under study (4, 5), these findings are of interest. Because T cell epitopes are small peptides, they are less likely to induce systemic reactions in patients than are the multivalent allergens. This feature is supported by the recent clinical trial with T cell epitope peptides of Fel d 1 (6).

The dominant T cell epitopes of an allergen may vary with individual patients because of MHC restriction; therefore, mixtures of T cell epitopes may have to be used for immunotherapy of patients. However, it is not a simple task to map all T cell epitopes recognized by different individuals. An alternative approach is to use a set of overlapping fragments of approximately 50 residues in size, which together would constitute the T cell epitopes of an allergen recognized by all individuals. One possible problem of this approach is the residual allergenicity of fragments, which would limit their usefulness. It is with this objective that we have examined the immunologic properties of recombinant (r)-fragments of an insect allergen in mice.

White-face hornet (*Dolichovespula maculata*) contains three known venom allergens. They are Ag 5 of unknown biologic function, hyaluronidase, and phospholipase A₁ (7). They are also known as Dol m 5, Dol m 2, and Dol m 1,

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³ Abbreviations used in this paper: r, recombinant; n, natural.

Table I. *r*-proteins of Dol m 5.02

Proteins	Amino Acid Residues	Caled m.w. ^a	A ₂₈₀ for 1 mg/ml Solution ^b	Yield/Liter of Culture
r-Dol m 5.02	1–204	24,992	2.26	5.9 mg
r-frag IN	1–114	14,446	1.05	9.2
r-frag N	15–93	10,376	1.21	4.5
r-frag M	102–168	9,066	1.64	6.4
r-frag C	151–204	7,903	2.36	4.4

^a All recombinant proteins have the additional peptide sequences of MRGS- and -RSH₆ at their N- and C-terminal regions, respectively.

^b Values given were calculated from their expected tyrosine and tryptophan content by using ϵ_{280} values of 1280 and 5690, respectively. Weight recoveries by the spectrophotometric methods are in good agreement with those from amino acid analysis.

according to an accepted allergen nomenclature (8). Homologues of the hornet proteins are present in other vespids, yellowjackets, and wasps (7). These proteins have been cloned and sequenced. A general feature of the sequence data is that these venom proteins have varying extents of sequence identity with other proteins in our environment.

Vespid Ag 5s have partial sequence identity with an androgen-dependent mammalian testis protein and with a plant-leaf pathogenesis-related protein (9). Vespid phospholipase A₁ has partial sequence identity with mammalian lipases (10). Vespid hyaluronidase (11) has partial sequence identity with bee venom hyaluronidase (12) and with a mammalian testis protein. This testis protein has hyaluronidase activity (13) and is involved in the sperm-to-egg binding in the early stages of fertilization (14).

There are three isoforms of Dol m 5: 5.01, 5.02, and 5.03 (15). Dol m 5.02 and 5.03 have been expressed previously in bacteria by using the pKK233–2 plasmid vector (15) and in insect cells using a *Baculovirus* vector (16). In this study, the *r*-proteins representing the full-length Dol m 5.02 and its fragments (Table I) were expressed in bacteria with the pQE12 plasmid vector. This expression system has the advantage that the *r*-protein contains a hexa-histidine sequence at its C-terminus so that it can be isolated by metal ion chelation chromatography. The antigenic and immunogenic properties of the natural (n)- and the *r*-Dol m 5.02 proteins in the murine system are described below. For convenience, the terms Dol m 5.02 and Dol m 5 will be used interchangeably in this paper.

Materials and Methods

Expression of *r*-Dol m 5 and its fragments

The necessary cDNAs were obtained by PCR amplification of a modified pUC8 plasmid template, p10 containing Dol m 5.02 cDNA (15). The necessary primers are listed in Table II. The sense and anti-sense primers were designed to contain respectively *Bam*HI and *Bgl*II restriction sites at their 5' ends. PCR was conducted for 25 cycles as follows: 10 ng template, 2 μ M each sense and anti-sense primers, 400 μ M each dNTPs, and 5 U Taq polymerase (Perkin-Elmer, Norwalk, CT) in 50 μ l of 100 mM Tris-HCl (pH 8.4) + 50 mM KCl + 1.5 mM MgCl₂. Each cycle consisted of 1.5 min at 90°, 1 min at 55° and 3 min at 72°.

After phenol-chloroform extraction and ethanol precipitation, the amplified cDNA (about 2 μ g) was digested in 60 μ l of 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 10 mM MgCl₂ with 20 U each of *Bam*HI and

*Bgl*II (Life Technologies, Gaithersburg, MD) at 37° overnight. The product was isolated by electrophoresis in 1.4% LGT agarose (FMC Bio-products, Rockland, ME). The gel slice containing the cDNA of interest was cut out, and 2 to 4 μ l of the melted gel (40 to 80 fm cDNA) was used for ligation with 18 ng (8 fm) of appropriately cut and dephosphorylated pQE-12 vector (QIAGEN, Chatsworth, CA). Ligation was conducted overnight at 15° in the presence of 2 U of T4 DNA ligase in 60 μ l of buffer as supplied by the manufacturer (Life Technologies).

Competent M15 (pREP4) cells were transformed with the above ligation mix then grown on agar plates as described by the manufacturer (QIAGEN). Selected colonies were screened by SDS gel electrophoresis for expression of the expected protein after induction with 1 mM IPTG for 1.5 h.

Positive colonies were grown in a culture of 400-ml vol. The collected bacteria pellet was washed free of culture with buffer, then dissolved in 6 ml of 6 M guanidine hydrochloride + 0.10 M Tris-HCl (pH 8.0) + 20 mM 2-ME, and heated to 100° for 5 min. After clarification by high-speed centrifugation, the solution was applied to a 0.8 $\times$ 3.5-cm column of Ni-NTA agarose (QIAGEN). The column was washed in succession with 6-ml portions of 6 M urea + 20 mM 2-ME buffered at pH 8.0, 6.3, 5.9, and 4.5. *r*-Dol m 5 and its fragments were eluted with 6 M urea buffered with 0.14 M NH₄Ac at pH 4.5.

r-Proteins were freed of urea and buffer salts by chromatography on a 8 $\times$ 0.9-cm column of C18 silica (Separations Group, Hesperia, CA) with a linear 2-propanol gradient of 0.5%/ml in 0.1% TFA. The *r*-proteins were eluted at 32 to 43% 2-propanol. After lyophilization they were found to be soluble in 0.02 N HOAc and their concentrations were determined by absorbance at 280 nm using conversion factors given in Table I.

Lyophilized *r*-proteins were found to be easily soluble in 0.02 N HOAc and practically insoluble in buffer of neutral pH. Dilute solutions of *r*-proteins in neutral buffer were prepared by dilution of a 0.02 N HOAc solution of the *r*-protein.

Characterization of *r*-Dol m 5 and its *r*-fragments

SDS slab gel electrophoresis was performed in 12.5% polyacrylamide gel (17). Samples were reduced by boiling in sample buffer + 1% 2-ME for 5 min before electrophoresis. For staining with Coomassie blue R-250, 2 μ g of each protein sample was used. For immunoblotting, 100 to 200 ng of each sample was used. The proteins in polyacrylamide gel were electroblotted onto a nitrocellulose paper in a Model TE 70 Semi-Phor transfer unit (Hoeffer Scientific Instruments, San Francisco, CA) following manufacturer's directions. The nitrocellulose paper was kept for 0.5 h in diluent buffer (see below), then in succession for 1 h periods with 1/300 diluted mouse anti-sera specific for n- or *r*-Dol m 5.02 and 1/100 diluted sheep anti-mouse IgG conjugated with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). The mouse sera were diluted in diluent buffer containing bacterial lysate (see below). The blots were stained in a substrate solution of 1.5 mg/ml 4-chloronaphthol and 0.01% H₂O₂ in 0.05 M Tris-HCl (pH 8.0).

For comparison with the *r*-protein, n-Dol m 5.02 was isolated as described previously (7, 18).

Immunization of mice

Groups of four female BALB/c mice at 8 to 10 wk of age (The Jackson Laboratory, Bar Harbor, ME) were immunized i.p. each with 0.2 ml of 10 μ g/ml immunogen + 5 mg/ml alum in 0.05 M sodium phosphate (pH 6.4) at wk 2, 4, 6 and 8. Sera were collected by retro-orbital puncture 1 wk after each immunization.

ELISA

Microtiter wells were coated overnight with 5 μ g/ml Ag in 0.05 M Tris-HCl (pH 8.0). Any remaining reactive sites of wells were blocked with diluent buffer, which contained 0.1 mg/ml BSA, 0.5 mg/ml Tween 20, 0.5 M NaCl, and 0.05 M Tris-HCl (pH 8.0). The wells were then incubated for 1 h periods in succession for varying concentrations of mouse sera to be tested, 10 μ g/ml rabbit Ab specific for mouse IgGs, and 1/100 diluted goat Ab specific for rabbit IgG conjugated with horseradish peroxidase. Finally, the bound peroxidase was detected by absorbance at 490 nm after incubation with a substrate solution of 16 mg/ml phenol, 0.5

Table II. Primers for PCR amplification of Dol m 5.02 and its fragments^{a,b}

No.	Oligonucleotide										Protein Encoded by Amplified cDNA
1	CGT	GGA	TCC	N ¹	N	Y	C	K	I ⁵		primer 1 + 8-Dol m 5.02, residue 1-204
				AAT	AAT	TAT	TGT	AAG	ATA		
2	CGT	GGA	TCC	L ¹⁵	S	K	F	G	T ²⁰		primer 2 + 5-frag N, residue 15-93
				CTC	AGC	AAA	TTT	GGA	ACT		
3	CGT	GGA	TCC	R ¹⁰²	N	T	A	K	Y ¹⁰⁶		primer 3 + 7-frag M, residue 102-168
				AGG	AAC	ACG	GCA	AAA	TAT		
4	CGT	GGA	TCC	F ¹⁵¹	R	K	V	G	H ¹⁵⁶		primer 4 + 8-frag C, residue 151-204
				TTC	CGT	AAA	GTC	GGC	CAT		
5	CGT	AGA	TCT	Q ⁹³	N	A	W	T	Q ⁸⁸		primer 1 + 6-frag IN, residue 1-114
				TTG	ATT	GGC	CCA	CGT	TTG		
6	CGT	AGA	TCT	M	A ¹¹⁴	I	N	Q	G	C ¹⁰⁸	primer 1 + 6-frag IN, residue 1-114
				CAT	GGC	TAT	ATT	TTG	TCC	AAC	
7	CGT	AGA	TCT	F ¹⁶⁸	K	T	K	G	W ¹⁶³		primer 1 + 6-frag IN, residue 1-114
				TTC	CTT	AGT	TTT	ACC	CCA		
8	CGT	AGA	TCT	K ²⁰⁴	R	E	Y	I	P	Q ¹⁹⁸	
				TTT	TCT	TTC	ATA	AAT	TGG	CTG	

^a Oligonucleotides 1 to 4 are sense primers, and they each contain a *Bam*HI restriction site at their 5' end. Oligonucleotides 5 to 8 are anti-sense primers, and they each contain a *Bgl*II site at their 5' end.

^b Oligonucleotide 6 contains a triplet codon for Met that is not present in Dol m 5.02.

mg/ml 4-aminoantipyrine, and 0.005% of H₂O₂ in 0.1 M sodium phosphate (pH 7). Ab titers were expressed as reciprocal dilutions required to give absorbance change of 1 in 30 min.

Ag and Ab solutions used above were prepared in diluent buffer. For ELISA on solid-phase r-Dol m 5, the diluent buffer contained, in addition to the ingredients given earlier, 10% bacterial lysate. This lysate was prepared by sonication of bacteria in PBS in 1/10 of the original culture volume and the suspension was clarified by centrifugation before use. Addition of bacterial lysate was necessary to reduce background color development, which might be due to the presence of Abs specific for bacterial proteins in the reagents used.

Inhibition ELISA

This ELISA was performed at a constant dilution of antiserum with increasing concentrations of inhibiting Ag. Antiserum dilution (7,000- to 50,000-fold) was chosen so that the maximal absorbance change was 0.4 to 0.9 in the absence of inhibitor.

The dissociation constant K_d of Ag-Ab reaction was calculated from the inhibition ELISA data according to the method of Friguet et al. (19) as modified by Seligman (20) using the equation:

$$K_d = \frac{af}{(1-f)}$$

where a is the total inhibitor concentration and f is the square root of the fraction of Ab bound to inhibitor. The calculations were made with total inhibitor concentrations in the range of f values of 0.45 to 0.84, corresponding to 20 to 70% inhibition.

Proliferation assay

Spleen cells were collected from BALB/c mice 9 to 10 days after the fifth or sixth biweekly immunization as described above. These cells (4×10^5) were cultured with varying concentrations of test Ag in 0.2 ml of culture medium at 37° and 5% CO₂. [³H]thymidine (1 μ Ci; 2 Ci/mmol) was added on day 3, and the cells were collected on day 4 to determine the uptake of [³H]thymidine. The culture medium consisted of RPMI 1640 (Life Technologies, Inc.), 10% fetal calf serum, 100 U/ml penicillin, 10 μ g/ml streptomycin, 10 mM HEPES buffer (pH 7.3), and 54 μ M 2-ME.

Results

Characterization of r-Dol m 5 and its r-fragments

These proteins or peptides were isolated in satisfactory yields from bacterial cultures (Table I). On SDS gel electrophoresis,

they migrated with the expected mobilities, and the bands were identified by dye-staining (Fig. 1A) and by immunoblot probed with antisera specific for n- and r-Dol m 5 (Figs. 1, B and C). These results indicate that the r-proteins are of >90% purity. Amino acid analysis of the r-proteins showed that they have the expected composition. Their amino acid sequences were confirmed by DNA sequencing of the entire regions of interest of the r-plasmids (data not shown). The r-proteins differ from the n-proteins by having the additional peptide sequences of MRGS and RSH₆ at their N- and C-terminal ends, respectively.

r-Proteins after 3 mo or longer storage at 4°C in 0.02 N acetic acid gave essentially the same SDS gel pattern with or without 2-ME reduction of sample before electrophoresis (results not shown). There was no change in the mobility of the main band, and only small increases in the intensity of the oligomer bands were observed. These results suggest that there is negligible air oxidation of the cysteinyl residues of r-proteins to form intramolecular or intermolecular disulfide bonds in the acid pH used for storage.

Murine Ab responses to n- and r-Dol m 5 and its r-fragments

The ELISA data in Table III show that both n- and r-proteins are about equally immunogenic for IgG Ab response in BALB/c mice; near maximal titers were obtained with both proteins at wk 5 after three i.p. injections of immunogen with alum as adjuvant. The data in Table III also indicate that IgG Abs specific for these two proteins cross-react with each other. IgG Abs specific for the n-protein gave a higher titer on solid-phase n-protein than on solid-phase r-protein. This is also the case for IgG Abs specific for the r-protein, which gave a higher titer on solid-phase r-protein than on solid-phase n-protein.

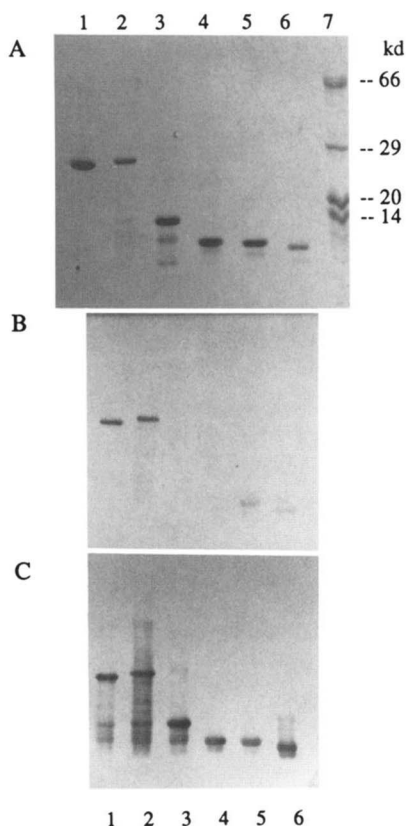


FIGURE 1. SDS gel electrophoresis and immunoblot of n- and r-Dol m 5.02, and r-fragments IN, N, M, and C (lanes 1 to 6, respectively). Two micrograms of each reduced protein was applied to SDS gel in A; lane 7 contains m.w. standards. Immunoblots in B and C were probed with murine Abs specific for n- and r-Dol m 5.02, respectively; and 100 ng of n-protein and 200 ng each of r-proteins were used for electrophoresis.

The data in Table III also indicate that r-fragments IN and C are almost as immunogenic as the r-protein but r-fragment M is not. The varying degree of immunogenicity of r-fragments is not simply a consequence of their size. r-fragment IN represents about one-half of the r-protein, but r-fragments M and C each represent about one-fourth of the r-protein. Abs specific for the r-fragments cross-react with the n-protein, as indicated by their similar titers on solid-phase r-fragments or n-protein.

Specificity of murine Ab responses to n- and r-Dol m 5 and its r-fragments

The specificity of Ab responses was analyzed by inhibition ELISA. In Figure 2A, n- and r-proteins inhibited the binding of n-protein specific Abs to solid-phase n-protein to maximal extents of approximately 90% and 10%, respectively; the estimated K_d values for the binding of n-protein-specific Abs with n- and r-proteins were 6 ± 4.3 nM and 1 ± 0.8 μ M, respectively. In Figure 2B, n- and r-proteins inhibited the binding of r-protein specific Abs to solid-phase r-protein to the extent of approximately 50%

and 70%, respectively; the estimated K_d values for the binding of r-protein-specific Abs for n- and r-proteins were 0.65 ± 0.39 and 1.9 ± 0.7 μ M, respectively. Because of solubility limits, the maximum concentration of r-protein that could be tested for inhibition was <30 μ M.

The inhibition ELISA data for Abs specific for r-fragments IN, M, and C are given in Figure 2, C to E, respectively. Maximal inhibition of Ab binding to solid-phase r-fragment was obtained with r-fragments but not with n-protein. The data suggest that these Abs bound r-fragments more tightly than n-protein. Their estimated K_d values, respectively, are 44 ± 10 nM and 8 to 50 μ M.

Murine T cell responses to n- and r-Dol m 5 and the r-fragments

T cell response was shown by proliferation assays with spleen cells from immunized mice, which were used for Ab response as described above. n- and r-proteins were equally active as Ags in stimulating proliferation of spleen cells from mice immunized with n- and r-proteins, as shown in Figures 3, A and B, respectively. Similar results were also obtained with enriched T cells, which were prepared by separation of spleen cells on nylon wool columns (data not shown). Other studies showed that r-fragments IN, N, M, and C stimulated proliferation of spleen cells from n- or r-protein-immunized mice (results not shown). These results together suggest that the T cell epitopes of Dol m 5 are present in these r-fragments.

Proliferative responses of spleen cells from mice immunized with r-fragments IN, M, and C are given in Figure 3, C to E, respectively. These spleen cells were tested with the appropriate r-fragment and the n- and r-proteins as the stimulating Ags. The proliferation data with n- and r-proteins are almost identical, and only the data for the r-protein are given in the figures. The spleen cells from mice immunized with r-fragments IN and C were also tested with r-fragments of another hornet allergen hyaluronidase (our unpublished observations) as stimulating Ags, and negligible proliferation was observed (data not shown).

In Figure 3C, r-fragment IN was a more effective Ag than the r-protein in stimulating the proliferative response. However, nearly the same proliferative responses were observed with r-fragment M and r-protein in Figure 3D and with r-fragment C and r-protein in E.

Discussion

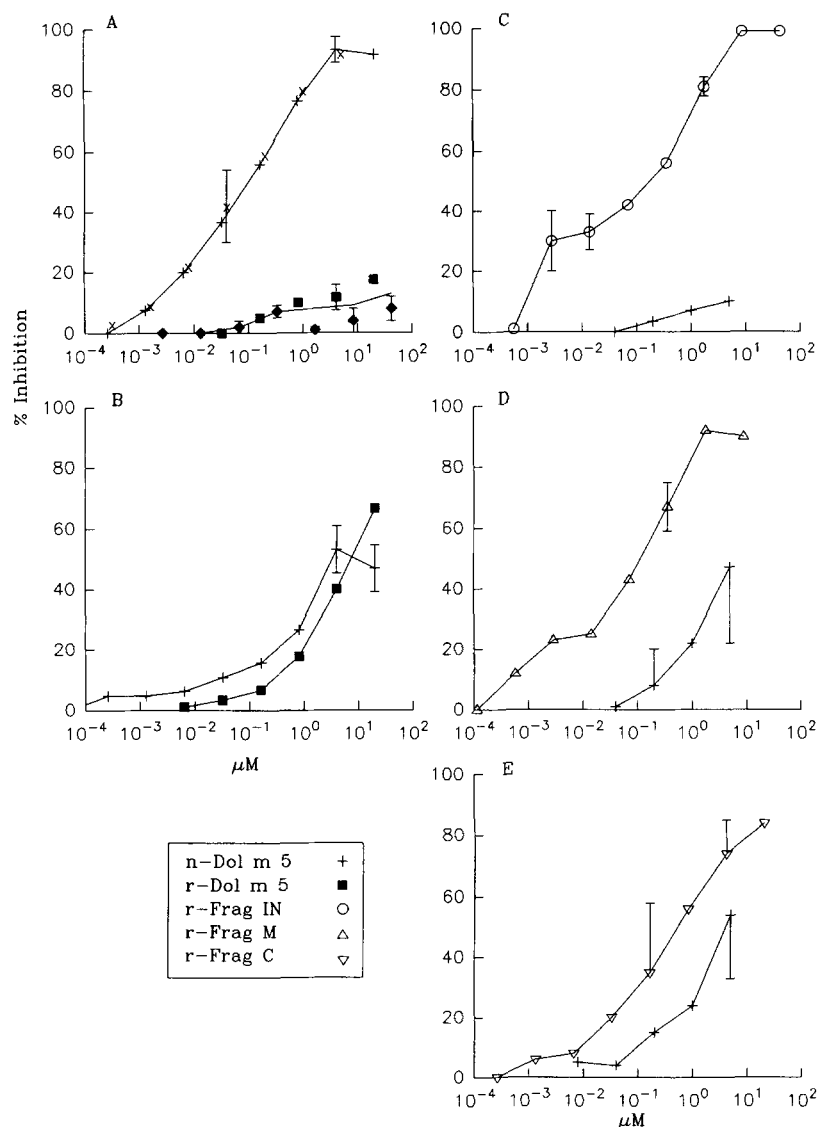
r-Dol m 5 and the r-fragments (Table I) are almost as immunogenic as n-Dol m 5 for B and T cell responses in mice. They differ with n-Dol m 5 in their specificity for Ab response, but they are similar to n-Dol m 5 in their specificity for T cell response. The proliferation data in Figure 3 indicate that the T cell epitopes of n-Dol m 5 are present in the r-fragments, and that these fragments are as immunogenic as the n-protein to induce T cell responses. This is to be expected because T cell epitopes of protein Ags are

Table III. Ab titers of sera from mice immunized with n- or r-Dol m 5 or r-fragments

Immunogen	Ab Titers ^a at Week		
	3	5	7
n-Dol m 5	4.4×10^4 (2.7×10^2)	7.7×10^4 (1.9×10^3)	7.7×10^4 (3.2×10^3)
r-Dol m 5	5.1×10^3 (4.6×10^3)	1.1×10^4 (1.6×10^4)	4.0×10^4 (1.6×10^4)
r-frag IN	1.0×10^2 (2.2×10^2)	9.0×10^3 (6.2×10^3)	2.6×10^4 (8.2×10^3)
r-frag M	$<1.0 \times 10^2$ (<10)	4.8×10^2 (5.0×10^2)	7.0×10^3 (7.0×10^3)
r-frag C	1.8×10^3 (7.0×10^3)	7.0×10^3 (4.5×10^3)	1.2×10^4 (1.2×10^4)

^a Ab titers were determined by ELISA on solid-phase Ag (the immunogen). The titers in parentheses were determined on solid-phase n-Dol m 5. The exceptions are those values for n-Dol m 5 as the immunogen, where the values in parentheses were determined on solid-phase r-Dol m 5. Sera from a pool of four mice from each group were used for these assays.

FIGURE 2. Inhibition ELISA of sera from mice immunized with n- and r-Dol m 5 and r-fragments IN, M, and C in panels A to E, respectively. Sera from wk 7 bleeding were used, and their dilutions were chosen so that the maximal absorbance changes were 0.4 to 0.9 in the absence of any inhibitor. The inhibitors were n- and r-Dol m 5.02 (+ and ■) and r-fragments IN, M, and C (○, △, and ▽, respectively). In A, the x and ◆ symbols correspond to + and ■, and represent the same experiment performed at a later date. Bars represent the range of duplicate or triplicate experiments performed on different dates.

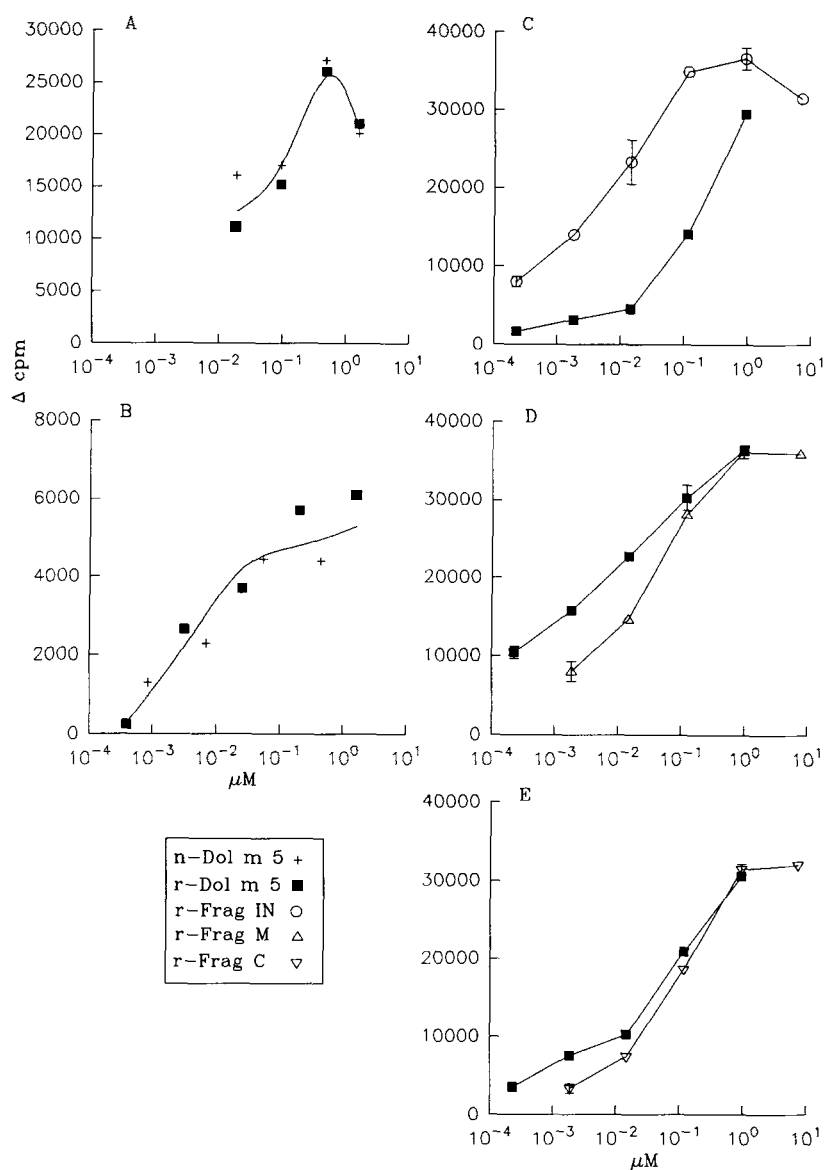


known to consist of only sequentially adjacent amino acid residues (21).

r-Dol m 5 and the r-fragments differ from the native protein by the presence of the additional peptide sequences of MRGS and SRH₆ at the N- and C-terminal regions, respectively, and they lack the four disulfide bonds that are

present in the native protein (22). Although n- and r-Dol m 5 both bind murine Abs specific for the n- or r-protein on immunoblots (Fig. 1), they have different conformations, as indicated by their different solubilities in buffers of neutral pH. It follows then, that n- and r-Dol m 5 can have different B cell epitopes of the discontinuous (topographic)

FIGURE 3. Ag-stimulated proliferative responses of spleen cells from mice immunized with n- and r-Dol m 5 and r-fragments IN, M, and C in panels A to E, respectively. The Ags are n- and r-Dol m 5.02 (+ and ■), and r-fragments IN, M, and C (○, △, and ▽, respectively). Spleen cell concentration was 2×10^6 cells/ml. [^{3} H]thymidine uptakes in absence of any Ag were in the range of 6000 to 9000 cpm, with the exception of B, which was 2200 cpm. Bars represent average deviation of triplicate measurements.



type, but that they may have common B cell epitopes of the continuous (sequential) type (23, 24).

The inhibition ELISA data in Figure 2A suggest the presence of two populations of Abs specific for n-Dol m 5; the majority ($\geq 90\%$) binds the discontinuous epitopes of the n-protein with high affinity, and the minority binds the continuous epitopes of both n- and r-proteins with low affinity. In Figure 2B, almost the same extents of maximal inhibition of binding of r-protein specific Abs to solid-phase r-protein by n- or r-protein, with approximately the same affinity, were observed. In Figure 2, C to E, the binding of r-fragment-specific Abs to solid-phase r-fragments was inhibited by r-fragments, but this binding was weakly inhibited by the n-protein. These findings together suggest that r-Dol m 5 and r-fragments induced Abs recognizing

continuous epitopes that are common with those of n-protein, and that they apparently did not induce Abs specific for discontinuous epitopes of the r-protein or fragments.

The inhibition ELISA data (Fig. 2) suggest that Abs specific for the discontinuous B cell epitopes of n-Dol m 5 have higher affinities than those specific for the continuous B cell epitopes. However, on direct ELISA (Table III) and immunoblot (Fig. 1), Abs specific for r-protein or fragments show almost the same degree of binding on solid-phase n- or r-protein; a similar finding is also made with Abs specific for n-protein. ELISA and immunoblot are performed with solid-phase Ag and solution-phase Ab, whereas inhibition ELISA is performed with Ag and Ab both in solution phase. The different physical states of the Ag in different assays may account for these results. It is

known that there is an enhancement of Ab affinity in solid-phase immunoassay because the high effective local concentration of Ag on solid phase suppresses the dissociation of Ag-Ab complex (25). This enhancement of Ab affinity can be greater for low-affinity Abs than it is for high-affinity Abs (26, 27).

In recent years, a number of allergens from different sources have been cloned and expressed. The n-allergens can be divided into two groups. In one group, the n-allergens, as well as the r-allergens, have no disulfide bonds and each r-allergen retains essentially all of the human IgE binding activities of the n-allergen by solid or solution-phase immunoassay, and this group includes the birch allergen Bet v 1 (28), the mite allergen Der p 2 (29, 30), and the group 5 grass allergen (31). In another group, n-allergens have disulfide bonds but their r-proteins do not. In these cases, each r-allergen has partial human IgE binding activities of the n-allergen only by solid-phase immunoassay, and this group includes the ragweed allergens Amb a 1 and 2 (32, 33), the grass allergen Lol p 1 (34), the mite allergen Der p 1 (35, 36), and the cat allergen Fel d 1 (37). These data for allergens with disulfide bonds are similar to the present findings for murine IgG response to n- and r-Dol m 5. Therefore, the data suggest that the IgEs produced in patients are mainly specific for the discontinuous epitopes of allergens.

In this study, mice were immunized i.p. with r-fragments in presence of alum as an adjuvant to induce B and T cell responses. Changing the route of immunization and deleting the use of adjuvant can alter the immune response. As noted above, treatment of mice with the dominant T cell epitope peptides of the major cat allergen Fel d 1 by the s.c. route (2) and of the major mite allergen Der p 1 by intranasal instillation (3), has been reported to suppress allergen-specific immune response. In addition, it has been reported that the nature of APCs can influence the Th cell differentiation, which in turn regulates the Ig isotypes (38).

These findings with the r-fragments of Dol m 5 in mice indicate that overlapping peptides of approximately 50 residues in size, although retaining all the T cell epitopes, do not contain the discontinuous and high-affinity B cell epitopes of the n-allergen. As noted in the preceding paragraph, the data suggest that the IgE Abs in patients are mainly specific for the discontinuous epitopes of allergens. Hence, it is likely that the fragments will have reduced allergenicity. Therefore, a mixture of overlapping r-fragments might be useful as an immunotherapeutic reagent if additional studies can establish whether treatment of mice with fragment mixtures by appropriate route can lead to T cell anergy with accompanying decrease in allergen-specific Abs. A similar approach has been reported with the use of protease digests of allergens (39, 40). However, most allergens are not readily available in quantity, and it

can be difficult to standardize mixtures of protease digests of proteins.

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