

Recombinant Allergens with Reduced Allergenicity but Retaining Immunogenicity of the Natural Allergens: Hybrids of Yellow Jacket and Paper Wasp Venom Allergen Antigen 5s¹

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The homologous venom allergen Ag 5s from the yellow jacket (*Vespula vulgaris*) and paper wasp (*Polistes annularis*) have 59% sequence identity of their respective 204 and 205 amino acid residues, and they have low degrees of antigenic cross-reactivity in insect allergic patients and in animal models. Hybrids containing different segments of these two vespid Ag 5s were expressed in yeast. Circular dichroism spectroscopy suggests the hybrids to have the secondary structure of natural Ag 5. Inhibition ELISA with human and murine Abs suggests the hybrids to have the discontinuous B cell epitopes of the natural Ag 5 but with an altered epitope density. The hybrids were immunogenic in mice for B and T cell responses to both Ag 5s. The N-terminal region of Ag 5 was found to contain its dominant B cell epitope(s). Hybrids containing 10–49 residues of yellow jacket Ag 5 showed 100- to 3000-fold reduction in allergenicity when tested by histamine release assay with basophils of yellow jacket-sensitive patients. Our findings suggest that hybrids represent a useful approach to map the discontinuous B cell epitope-containing regions of proteins. They also suggest that Ag 5 hybrids may be useful immunotherapeutic reagents in man. *The Journal of Immunology*, 2001, 166: 6057–6065.

Immunotherapy by repeated s.c. injections of increasing doses of allergens into patients is one way to reduce allergic symptoms (1). Treated patients showed rapid increases in their allergen-specific IgGs and slow decreases in their specific IgEs (2). Treated patients also showed changes in their T cell cytokine profile; their IL-4 and IL-5 levels decreased and their IFN- γ level increased (3). Studies have shown that immunotherapy with high doses of allergens was more effective than that with low doses for symptom reduction (1). However, effective dosages are limited by potential systemic reactions caused by the allergens.

Allergenicity depends on the interaction of a multivalent allergen with basophil or mast cell-bound IgE Abs. Therefore, allergenicity can be reduced by decreasing its B cell epitope density. Reduction of B cell epitope density of a protein can be accomplished by several approaches. One approach is by partial or complete denaturation of allergens on chemical modification (4–8), because the majority of B cell epitopes are of the discontinuous type, being dependent on the native conformation of proteins.

A second approach to reduce the accessibility of B cell epitopes of allergen is by polymerization on formaldehyde or glutaraldehyde treatment (9–11) or by attachment of nonimmunogenic polymers (12). One limitation of this approach is that near-complete loss of the discontinuous B cell epitopes usually occurred when allergens were modified with >100-fold reduction in allergenicity.

A third approach is by site-directed mutagenesis to alter selectively the contact amino acid residues of B cell epitopes of allergens. If the key contact residues of B cell epitopes are known, this can be a useful approach (13).

T cell peptide treatment of naive or primed mice was found to down-regulate T cell responses for cat and mite allergens (14, 15). Thus, a fourth approach is the use of T cell peptides as immunotherapeutic reagents, because T cell peptides lack the discontinuous B cell epitopes of native allergens.

We report here a new approach to prepare modified allergens. It is to prepare hybrids consisting of a small portion of the guest allergen of interest and a large portion of a homologous but poorly cross-reacting host protein. The homologous host protein functions as a scaffold to maintain the native structure of the guest allergen of interest so that the conformation-dependent B cell epitopes of the guest allergen of interest are preserved in the hybrid, but at a reduced density. Homologous proteins of >30% sequence identity and of similar functions are known to have closely similar three-dimensional structures (16, 17).

Clinical studies in patients and tests with experimental animals have shown that there is limited cross-reactivity of Abs specific for the yellow jacket and paper wasp venom proteins (18, 19). These observations form the basis of the present studies. Our model guest allergen Ag 5 is Ves v 5, a yellow jacket venom protein of 23 kDa, and our homologous host allergen is Pol a 5, a paper wasp venom protein of similar size. Ves v 5 and Pol a 5 have a 59% sequence identity, as shown in the upper portion of Fig. 1. Both can be expressed in yeast, and the recombinant proteins were shown to have the native conformation of the natural proteins (20). The structure of Ves v 5 was solved recently by x-ray crystallography.³ The darkened regions of the Ves v 5 structure in Fig. 2, A and B,

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³ A. Henriksen, T. P. King, O. Mirza, K. Meno, H. Ipsen, M. Gajhede, and M. Spangfort. Structure of a vespid allergen, Ves v 5. Submitted for publication.

V NNYCKIKCLK GGVHTACKYG .SLKP..NCG NKVVVSYGLT KOEKQDILKE
P VDYCKIKC.P SGIHTVCQYG ESTKPSKNCA GKVIKSVGPT EEEKKLIVSE

V HNDFRQKIAR GLETRGNPGP QPPAKNMKNL VWNDELAHYVA QVWANQCQY.
P HNRFRQKVAQ GLETRGNPGP QPAASDMNDL VWNDELAHIA QVWASQCQFL

V GHDTCRDVAK YQVGQNVALT GSTAAKYDDP VKLVKMWEDE VKDYNPKKKF
P VHDKCRNTAK YPVGQNIAYA G..GSNLPDV VSLIKLWENE VKDFNYNTGI

V SGNDFLKTGH YTMVWANTK EVGCGSIKYI QEKWHKHLYV CNYGPGSNFK
P TKQNFARKGH YTMVWGKTK EIGCGSLKYM ENNMQNHLYI CNYGPAGNYL

V NEELYQTK 204
P GQLPYTKK 205

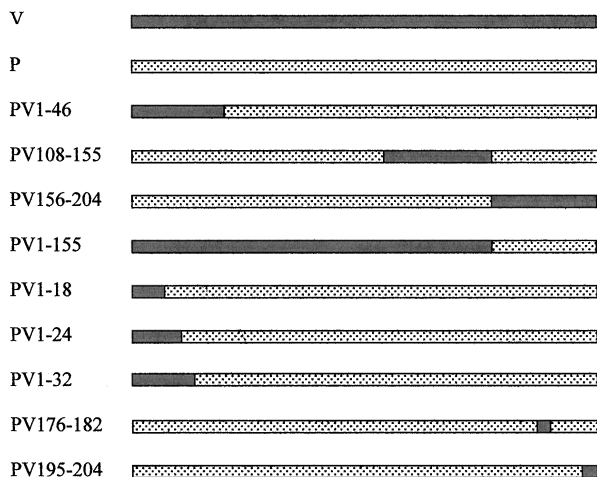


FIGURE 1. Upper portion, Amino acid sequences of Ves v 5 (V) and Pol a 5 (P). Lower portion, Schematic sequence representations of Ag 5s and hybrids. Residue numbers given for hybrids refer to those of Ves v 5.

represent portions of the molecule chosen for preparation of hybrids.

These hybrids are shown schematically in the lower portion of Fig. 1, and they are designated by the residue numbers of the Ves v 5 portion of the molecule. Hybrids PV₁₋₄₆, PV₁₀₈₋₁₅₅, and PV₁₅₆₋₂₀₄ contain, respectively, the first, the third, and the last quarter of the guest Ves v 5 molecule together with portions of the host Pol a 5 molecule to complete the Ag 5 molecule. A hybrid containing the second quarter of the Ves v 5 molecule was not prepared, as this is a region of high sequence identity of Ves v 5 and Pol a 5. Hybrid PV₁₋₁₅₅ has the opposite arrangements of the Ves v 5 and Pol a 5 fragments when compared with PV₁₅₆₋₂₀₄. The aforementioned four hybrids were designed without considering the structural features of Ves v 5. The other five hybrids, shown in Fig. 1, were designed subsequently to contain segments that are located on the surface of Ves v 5, and they are of 7–32 residues in length. We report below our immunochemical findings with these hybrids of Ves v 5 and Pol a 5.

Materials and Methods

The cDNAs encoding Ag 5 hybrids

The cDNA encoding Ves v 5 of the EA series was prepared by PCR amplification of the appropriate template (19) with primers 1 and 3, found in Table I, and that encoding Ves v 5 of the KR series with primers 2 and 3. The cDNA encoding Pol a 5 of the EA series was similarly prepared with primers 4 and 6 of Table I, and that encoding Pol a 5 of the KR series with primers 5 and 6.

PV₁₋₄₆ Ves v 5 and Pol a 5 have a common peptide sequence of EH at positions 47–48 and 49–50, respectively. The nucleotide sequence encoding peptide EH in Ves v 5 is GAG CAC, which is a *Bsi* HKA I restriction site. That of Pol a 5 is GAG CAT, and it was mutated to a *Bsi* HKA I site by the PCR overlap extension method (21) using primers 7 and 8, found in

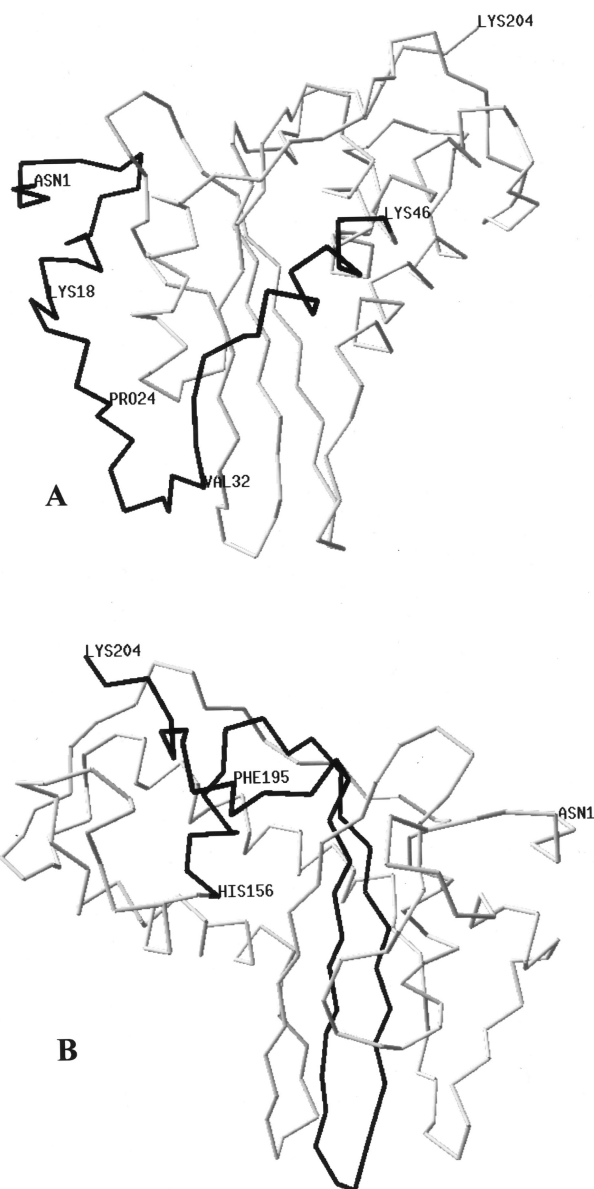


FIGURE 2. Front and back views of C α tracing of the structure of Ves v 5. Regions of Ves v 5 present in hybrids PV₁₋₄₆ and PV₁₅₆₋₂₀₄ are black colored-in patterns in A and B, respectively.

Table I. Hybrid PV₁₋₄₆-encoding cDNA was prepared by ligation of their appropriate *Bsi* HKA I fragments.

PV₁₀₈₋₁₅₅ Ves v 5 and Pol a 5 have a common peptide sequence of KY at positions 106–107 and 109–110, respectively. Their KY sequence is encoded by the nucleotide sequence of AAA TAT. This nucleotide sequence was mutated to an *ApoI* restriction site of AAA TTT encoding the peptide sequence of KF. The mutation of Ves v 5 or Pol a 5 cDNA was made with the primers 9 and 10, or 11 and 12, respectively, shown in Table I. Hybrid PV₁₀₈₋₁₅₅-encoding cDNA was prepared by ligation of the appropriate fragments from *ApoI* digestion of Pol a 5 and PV₁₋₁₅₅-encoding cDNAs.

PV₁₋₁₅₅ and PV₁₅₆₋₂₀₄ Ves v 5 and Pol a 5 cDNAs have a common *EaeI* restriction site encoding amino acid residues 154–156 and 155–157, respectively. The cDNAs encoding these two hybrids were prepared by ligation of the appropriate *EaeI* fragments of their parent cDNAs.

PV₁₋₁₈ and PV₁₉₅₋₂₀₄ Their cDNAs were prepared by PCR with cDNA of Pol a 5 as the template and primers 13 and 6, or primers 4 and 14, respectively, found in Table I.

PV₁₋₂₄, PV₁₋₃₂, and PV₁₇₆₋₁₈₂ Their cDNAs were prepared by the PCR overlap extension method (21) with Ves v 5 and/or Pol a 5 cDNAs as templates and primers 15–20, found in Table I.

Table I. Primers for preparation of Ves v and Pol a 5s and their hybrids^a

Primer Number	Amino Acid Sequence															
1	<u>EcoRI</u> N ¹ N Y C K I K ⁶ CGT GAA TTC AAC AAT TAT TGT AAA ATA AAA															
2	<u>XhoI</u> K R N ¹ N Y C K I K ⁶ CGT CTC GAG AAA AGA AAC AAT TAT TGT AAA ATA AAA															
3	<u>XbaI</u> * K ²⁰⁴ T Q Y L E ¹⁹⁹ CGT TCT AGA TTA CTT TGT TTG ATA AAG TTC															
4	<u>EcoRI</u> V ¹ D Y C K I K ⁶ CGT GAA TTC GTT GAT TAT TGT AAA ATA AAA															
5	<u>XhoI</u> K R V ¹ D Y C K I K ⁶ CGT CTC GAG AAA AGA GTT GAT TAT TGT AAA ATA AAA															
6	<u>XbaI</u> * K ²⁰⁵ K T Y P L ²⁰⁰ CGT TCT AGA TTA TTT TTT TGT ATA AGG TAG															
7	V ⁴⁷ S E H N R F ⁵³ GTA AGC GAG CAC AAT CGG TTT															
8	F ⁵³ R N H E S V ⁴⁷ AAA CCG ATT GTG CTC GCT TAC															
9	V ¹⁰⁴ A K F Q V G ¹¹⁰ GTA GCA AAA TTT CAG GTT GGA															
10	G ¹¹⁰ V Q F K A V ¹⁰⁴ TCC AAC CTG AAA TTT TGC TAC															
11	T ¹⁰⁶ A K F P V G ¹¹² ACC GCA AAA TTT CCA GTT GGA															
12	G ¹¹² V P F K A T ¹⁰⁶ TCC AAC TGG AAA TTT TGC GGT															
13	<u>EcoRI</u> N ¹ N Y C K I K C L K G G CGT GAA TTC AAC AAT TAT TGT AAA ATA AAA TGT TTG AAA GGA GGT															
	V H T A C K ¹⁸ Y ¹⁸ G E ²⁰ GTC CAT ACT GCC TGC AAA TAT GGA GAA															
14	<u>XbaI</u> * K ²⁰⁴ T Q Y L E E N K F ¹⁹⁵ N ¹⁹⁵ G A P ¹⁹² CGT TCT AGA TTA CTT TGT TTG ATA AAG TTC CTC ATT CTT AAA ATT TCC AGC TGG															
15	Y ¹⁹ G S L K P ²⁴ S ²⁵ K N C A ²⁹ TAT GGA AGT CTT AAA CCG AGC AAG AAT TGT GCC															
16	A ²⁹ C N K S ²⁵ P ²⁴ K L S G Y ¹⁹ GGC ACA ATT CTT GCT CGG TTT AAG ACT TCC ATA															
17	L ²² K P N C G N K V V V ³² S ³⁵ V G P ³⁸ CTT AAA CCG AAT TGC GGT AAT AAG GTA GTG GTA TCG GTT GGT CCA															
18	P ³⁸ G V S ³⁵ V ³² V V K N G C N P K L ²² TGG ACC AAC CGA TAC CAC TAC CTT ATT ACC GCA ATT CGG TTT AAG															
19	I ¹⁷⁶ Q E K W H K ¹⁸² H ¹⁸⁴ Y L I ¹⁸⁷ ATT CAA GAG AAA TGG CAC AAA CAT TAC CTC ATA															
20	K ¹⁸² H W K E Q I ¹⁷⁶ V ¹⁷⁶ K L S ¹⁷³ TTT GTG CCA TTT CTC TTG AAT ATA TTT TAG AGA															

^a Amino acid sequence for Ves v 5 is italicized and that for Pol a 5 is not. Restriction enzyme sites are underlined.

Recombinant plasmids encoding Ag 5 or hybrids

Ag 5 or hybrid-encoding cDNAs of the EA or the KR series were digested with restriction enzymes *EcoRI* or *XhoI*, and then inserted into similarly cut pPICZα-A vector (Invitrogen, San Diego, CA). The recombinant plasmids were amplified in TOP10F⁺ bacterial cells. The Ag 5-coding regions of all recombinant plasmids were confirmed by DNA sequencing. They correspond to the sequence data in GenBank with two exceptions for Ves v 5. There are two nucleotide changes at positions 579 and 587: the first change is a silent mutation of G to A substitution, and the second one of T to A substitution results in a codon change, M to K at amino acid residue 196. These two nucleotide changes may represent insect polymorphism rather than random mutations, because the Ag 5 cDNA used in this study was prepared in the same manner as it was done previously.

Molecular biology techniques used in this paper are the same as reported in our previous publication for expression of vespid Ag 5s in yeast (20).

Expression of Ag 5s and hybrids in yeast

Recombinant plasmids (1–2 μg) were linearized by cutting with the restriction enzyme *SacI* and then used to transform competent *Pichia pastoris* KM71 yeast cells (~8 × 10⁹ cells in 40 μl of 1 M sorbitol) by electroporation. Transformed cells were diluted to 2 ml with 1 M sorbitol. They were allowed to recover at 30°C for 1 h without shaking and for 1 more h with shaking at 200 rpm. Next, 50- or 100-μl aliquots were plated out on 100-mm plates with yeast extract, peptone, dextrose, and sorbitol medium containing 1.5 mg/ml Zeocin for selection of multicopy integrants (22). Colonies were picked after a 3- to 4-day incubation and were screened

by small-scale expression to find the best producing colonies. All reagents were obtained from Invitrogen (San Diego, CA).

Yeast cells from selected clones were grown in two 500-ml bottles, each containing 150 ml (pH 6.0) phosphate buffer containing yeast nitrogen base, biotin, glycerol, and histidine at 30°C with orbital shaking at 250 rpm until A_{600 nm} of 10–12. The cells were collected by centrifugation and resuspended in 100 ml similar buffered medium containing methanol in place of glycerol. Incubation was continued at 30°C with shaking at 250 rpm for 4–6 days with a daily addition of 1 ml 50% methanol.

Ag 5s or their hybrids were purified from the culture fluid concentrate following our previously reported procedure, namely ion-exchange chromatography on SE-cellulose (Sigma, St. Louis, MO). About 70% of the main peak was pooled, desalted by reversed phase chromatography on C18 silica, and then lyophilized. Recombinant Ag 5s or hybrids were dissolved in 0.01 M ammonium acetate buffer (pH 4.6) and stored at 4°C. Their concentrations were established from their absorbance at 280 nm, using molar extinctions calculated from their tyrosine and tryptophan contents. The yields of Ag 5s or hybrids ranged from 1 to 7 mg/100 ml of 4-day cultures.

All recombinant Ag 5s or hybrids were characterized by SDS gel electrophoresis, N-terminal sequence analysis, and matrix-assisted laser desorption ionization mass spectrometry. Circular dichroism (CD)⁴ spectra at 0.2 mg/ml of recombinant proteins in 0.01 M acetate buffer (pH 4.6) were

⁴ Abbreviation used in this paper: CD, circular dichroism.

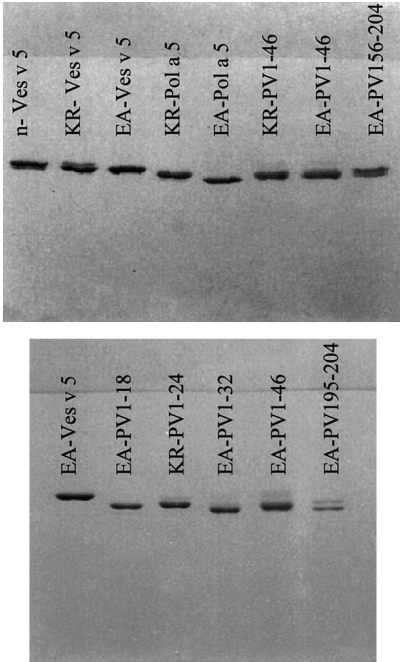


FIGURE 3. SDS gel patterns of Ag5 hybrids. Approximately 2 μ g of each sample was applied.

taken in cells of 1-mm path-length in an AVIV 62DS spectrometer (Aviv Associates, Lakewood, NJ).

Immunological studies

Ag 5- or hybrid-specific sera were collected at week 5 or later from groups of three or four female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) that had received three or more biweekly i.p. injections of 4 μ g immunogen and 1 mg alum in 0.2 ml of PBS. Ag5-specific sera from ASW/sn and P/J mice strains were from a previous study (23). Murine polyclonal Abs specific for natural Ves v 5 were isolated from BALB/c sera by affinity chromatography on Ves v 5-specific immunosorbent, and they were depleted of Pol a 5-cross-reacting Abs by passage through Pol a 5-specific immunosorbent. The immunosorbents were pre-

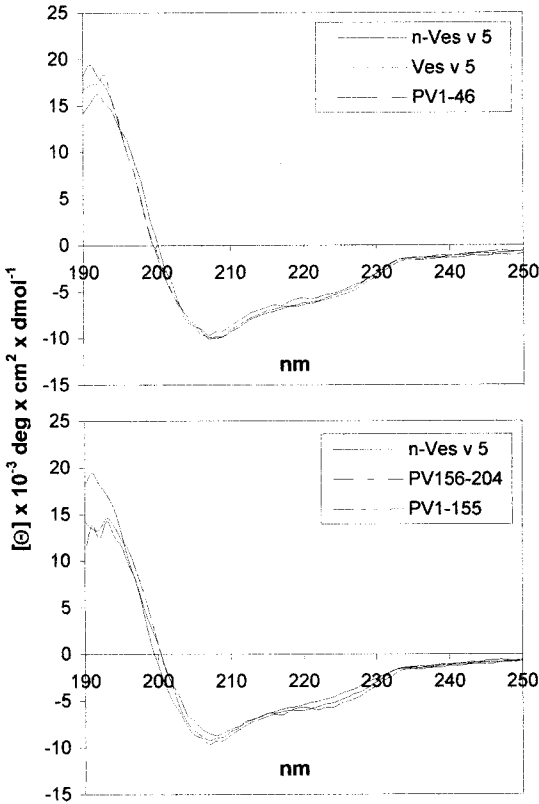


FIGURE 4. CD spectra of natural (n) and recombinant EA-Ves v 5 and recombinant EA-PV hybrids.

pared with cyanogen bromide-activated Sepharose 2B (Pharmacia, Piscataway, NJ). Murine mAbs specific for Ves v 5 were from our earlier work (24). ELISA was made in 96-well plates (Falcon 3911; BD Biosciences, San Jose, CA), and the wells were coated with 4 μ g/ml Ag 5 in 0.05 M Tris-HCl buffer (pH 8). Bound human or mouse IgG was detected with 2 μ g/ml biotinylated goat anti-human IgG (γ -chain specific), or 4 μ g/ml mouse IgG

Table II. Mass spectrometric data of recombinant vespid Ag 5s and hybrids

Protein	Assumed N-terminal Sequence	Abundance ^a (%)	Mass Units	
			Calculated	Found
EA-PV ₁₋₁₈	EAEAEF-PV	100	23,697	23,701
EA-PV ₁₋₃₂	EF-PV	60	22,964	22,930
	EAEF-PV	40	23,151	23,134
EA-PV ₁₋₄₆	EF-PV	10	23,099	23,109
	EAEF-PV	50	23,300	23,327
	EAEAEF-PV	40	23,500	23,515
EA-PV ₁₋₁₅₅	EF-PV	53	23,375	23,334
	EAEF-PV	47	23,575	23,533
EA-PV ₁₅₆₋₂₀₄	EAEF-PV	59	23,776	23,775
	EAEAEF-PV	41	23,932	23,939
	EAEAEF-PV	70	23,700	23,688
EA-PV ₁₉₅₋₂₀₄	REAEAEF-PV	30	23,874	23,844
KR-Ves v 5	Vv	90	23,277	23,274
	EEGVSLEKR-Vv	10	24,305	24,300
KR-Pol a 5	Pa	15	22,935	22,951
	GVSLEKR-Pa	80	23,704	23,698
	EEGVSLEKR-Pa	5	23,962	23,992
KR-PV ₁₋₂₄	PV	85	22,903	22,897
	EEGVSLEKR-PV	15	23,931	23,933
KR-PV ₁₋₄₆	PV	70	22,823	22,834
	KR-PV	30	23,107	23,157

^a Protein abundance was estimated from peak heights of samples in mass spectra. For PV₁₅₆₋₂₀₄ and PV₁₋₁₅₅ samples of EA series, their assumed sequences were confirmed by Edman degradation.

($\gamma 1$ specific), followed with 2 $\mu\text{g/ml}$ avidin-peroxidase conjugate (25). Ab concentration of sera samples was determined by comparison of their ELISA data with that of an immuno-affinity purified sample of Ves v 5-specific Ab as described above.

Proliferation assays were done in triplicates with spleen cells pooled from two to three mice 10 days after five biweekly immunizations. Spleen cells (4×10^5) were cultured with test Ag in 0.2 ml culture medium at 37°C and 5% CO_2 . Tritiated thymidine (1 μCi) was added on day 3, and the thymidine uptake was determined on day 4. The results were expressed as stimulation index values.

Allergenicity was determined by histamine release assay from basophils of yellow jacket-sensitive patients following challenge with Ag 5 or hybrids (26).

Results

Expression of vespid Ag 5s and their hybrids in yeast

The proteins expressed in yeast contain a secretory signal peptide. The signal peptide is linked to the expressed protein via a peptide of KR or KREAEAEF sequence. These two types of proteins are designated as the KR and the EA series, respectively. Upon secretion from the yeast cells, the signal peptide is cleaved from the secreted protein at the KR sequence (*Kex* 2 site) or the two EA sequences (*Ste* 13 site) (22). In our previous work with vespid Ag 5s of the EA series (20), we observed cleavage at the *Kex* 2 site but variable cleavages at the two *Ste* 13 sites. The recombinant proteins had the sequences of EAEAEF-, EAEF-, and EF-protein, where the EF sequence represents the *EcoRI* site for insertion of the cDNA into the vector. Similar findings were made in the present work, as shown by the mass spectrometric data of the six hybrids of the EA series in Table II.

The EAEAEF sequence of recombinant Ves v 5 was found to function as a strong hapten (20). Therefore, we expressed Ves v and Pol a 5s and two hybrids of the KR series. Cleavage at the *Kex* 2 site would have yielded the recombinant proteins with the N-terminal sequence of the natural proteins. The expected cleavage did occur partially together with cleavages at sites 2 or 7–9 residues upstream of the *Kex* 2 site. This is shown by the mass spectrometric data of the proteins of the KR series in Table II. The recombinant proteins of the KR series were of slightly lower yields than those of the EA series.

Two of the hybrids, EA-PV_{108–155} and EA-PV_{176–182}, were secreted in such poor yield that we were not able to isolate them.

Physicochemical characterization of recombinant vespid Ag 5s and their hybrids

The recombinant proteins were characterized by SDS gel electrophoresis (Fig. 3). Several of them showed two closely spaced doublet bands with mobilities similar to that of natural Ves v 5. The doublet bands probably reflect the varying extents of processing at their N-terminal ends, as indicated by the mass spectrometry data in Table II and by N-terminal sequencing of two hybrids, PV_{1–155} and PV_{156–204}.

The recombinant Ag 5s and hybrids showed nearly identical CD spectra as those of the natural Ag 5s. As can be seen in Fig. 4, the spectra of the natural and the EA-Ves v 5 and those of EA-PV_{1–46}, EA-PV_{1–155}, and EA-PV_{156–204} showed the presence of minima at ~ 208 nm with a shoulder at 225 nm. These features are indicative of an ordered structure (27). Proteins with disordered structure have broad minima at 198 nm, as we observed with denatured Ves v 5 (20). Similar CD spectra were observed for Pol a 5 and all other hybrids in Table II; these data are not shown.

The recombinant Ag 5s and hybrids from yeast were as freely soluble in acid or basic buffers as the natural Ag 5s were. This is in contrast to the recombinant vespid Ag 5s from bacteria, which were soluble only in acidic buffer (27).

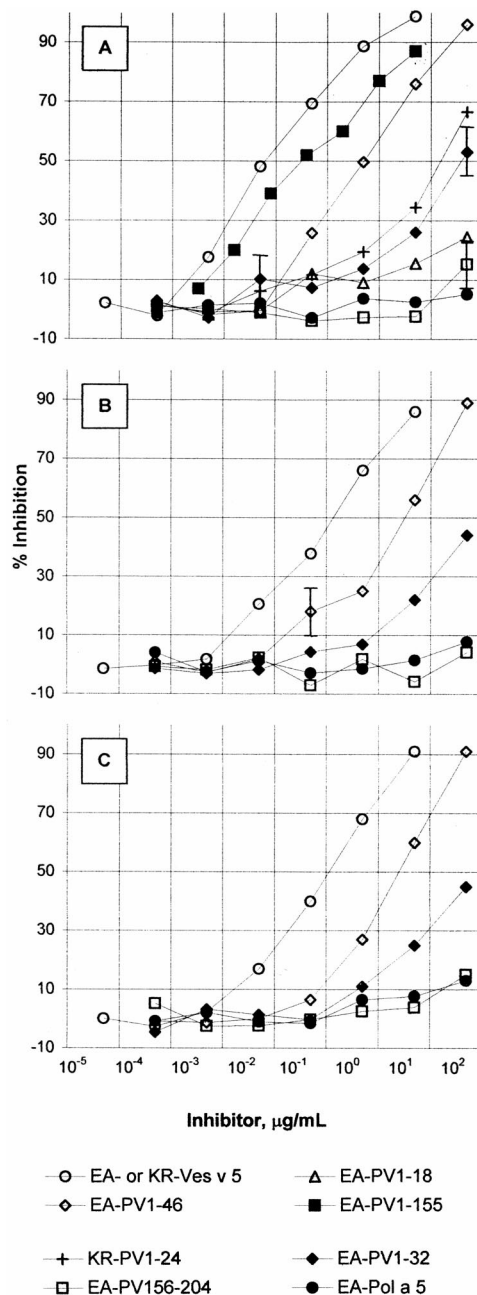


FIGURE 5. Inhibition ELISA with mouse Abs specific for natural Ves v 5. Solid-phase Ag was KR-Ves v 5. A, Ves v 5-specific Abs isolated from BALB/c mice strain and depleted of Pol a 5-cross-reacting Abs; its concentration was 0.1 $\mu\text{g/ml}$. B and C, Antisera from ASW/sn and P/J strains were used directly at reciprocal dilutions of 2×10^5 and 5×10^4 , respectively.

Ves v 5-specific B cell epitopes of hybrids

Ves v 5-specific B cell epitopes of hybrids were detected by the hybrid inhibition of binding of mouse IgG Abs specific for natural Ves v 5 to solid-phase Ves v 5. Both EA- and KR-Ves v 5 were tested as solid-phase Ags with similar results, and the data given below were obtained with solid-phase KR-Ves v 5. Five samples of mouse antisera were tested; three were from mice of BALB/c strains and one each from mice of ASW/sn and P/J strains. Closely similar data were obtained from sera of three groups of BALB/c mice, and the data from sera of one group are shown in Fig. 5A. At the highest concentration of 50 or 500 $\mu\text{g/ml}$ inhibitor tested, the two N-terminal hybrids, EA-PV_{1–46} and EA-PV_{1–155}, showed

maximal inhibition approaching 100%, as EA- or KR-Ves v 5 did. Two other N-terminal hybrids, KR-PV₁₋₂₄ and EA-PV₁₋₃₂, had maximal inhibition of ~60%, and the shortest N-terminal hybrid, EA-PV₁₋₁₈, had maximal inhibition of ~20%. The C-terminal hybrid EA-PV₁₅₆₋₂₀₄ had maximal inhibition of ~15%, and the shorter C-terminal hybrid, EA-PV₁₉₅₋₂₀₄, was not tested. The data from ASW/sn and P/J sera are given in Fig. 5, B and C, and their results are similar to those from BALB/c.

Inhibition ELISA was also made with specific IgGs from sera of six yellow jacket-sensitive patients. The data from three yellow jacket-sensitive patients are shown in Fig. 6, A–C. There are minor individual variations, but the results are similar to those with mouse IgGs. The inhibition data of the N-terminal hybrids in Figs.

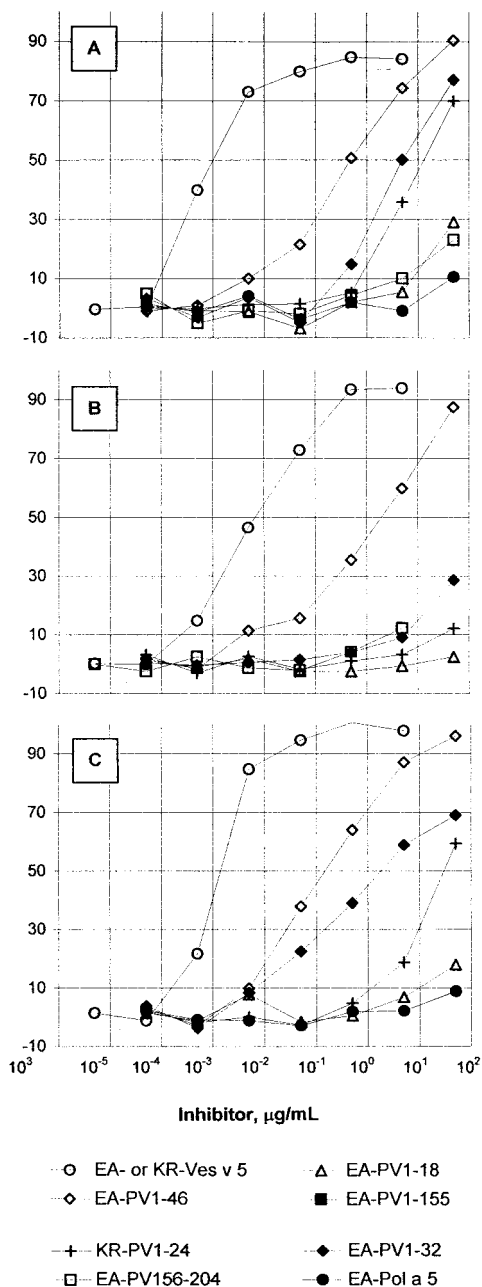


FIGURE 6. Inhibition ELISA with sera from yellow jacket-sensitive patients. Solid-phase Ag was KR-Ves v 5. A, B, and C, Sera from patients 8801, 9079, and DL were used at reciprocal dilutions of 20, 200, and 100, respectively.

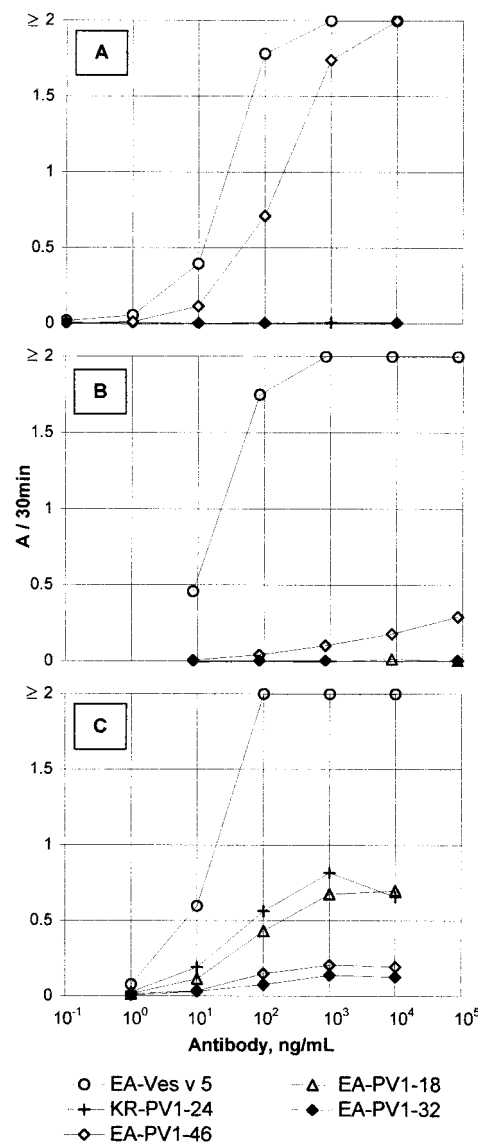


FIGURE 7. Binding of mouse Ves v 5-specific mAbs to solid-phase Ves v 5 or hybrids. A, Ab 870201; B, Ab 870213; and C, Ab 870207.

5 and 6 suggest the immunodominance of the N-terminal region of Ves v 5.

The observed inhibition by the hybrids in Figs. 5 and 6 was not due to cross-reacting epitopes of the Pol a 5 portion of the molecule, because Pol a 5 at 500 $\mu\text{g/ml}$ showed only 4–12% inhibition. The high concentrations of hybrids required for half-maximal inhibition relative to that of Ves v 5 are probably related to their epitope densities, because epitope density is known to strongly influence the affinity constant of a multivalent Ag and a bivalent Ab (28, 29). This may explain why the longest N-terminal hybrid, PV₁₋₁₅₅, showed the lowest concentration for half-maximal inhibition, and it is followed by the next longest hybrids, PV₁₋₄₆, PV₁₋₃₂, PV₁₋₂₄, and PV₁₋₁₈.

Seventeen monoclonal mouse Abs specific for the natural Ves v 5 were tested for their binding of hybrids. ELISA showed one of these mAbs, 870201, bound EA-Ves v 5 and EA-PV₁₋₄₆ with similar affinity and maximal binding (Fig. 7A), and it did not bind any of the other N- or C-terminal hybrids (data not shown). Four other mAbs showed greatly reduced maximal binding to EA-PV₁₋₄₆ but no binding to any of the shorter N-terminal hybrids; the data for one such Ab, 870213, are given in Fig. 7B. Lastly, mAb 870207 in

Table III. Murine Ab responses to vespid Ag 5s and hybrids

Set	Immunogen ^a	Milligram per Milliliter Specific IgG in Sera by ELISA on Solid-Phase ^b		
		EA-Ves v 5	EA-Pol a 5	Hybrid
A	KR-Ves v 5	8.9 (8.5) ^c	0.6	
	KR-Pol a 5	2.8 (1.0)	7.0	
	EA-PV ₁₋₁₅₅	12.0	0.7	
	EA-PV ₁₋₄₆	4.2 (3.5)	1.9	7.6 (5.6)
	EA-PV ₁₋₁₈	1.0 (0.8)	6.9	6.9 (0.7)
	EA-PV ₁₅₆₋₂₀₄	1.6 (0.6)	10.0	2.6 (0.3)
B	EA-PV ₁₉₅₋₂₀₄	1.3 (0.4)	14.0	10.0 (0.3)
	KR-Ves v 5	15.0 (14.0)	0.2	
	KR-PV ₁₋₄₆	0.6 (0.5)	1.0	2.7 (3.0)
	EA-PV ₁₋₃₂	0.9 (0.7)	4.3	8.0 (3.2)
	KR-PV ₁₋₂₄	0.4 (0.3)	4.2	6.5 (0.9)
	EA-PV ₁₋₁₈	0.4 (0.3)	4.5	5.3 (0.7)

^a Sera were collected on wk 7, after three biweekly i.p. injections of immunogen. Set A and B studies were made on separate occasions.

^b Ab concentration was estimated from reciprocal sera concentration required to give an absorbance change of 1.0 in 30 min. Under the conditions used, this change corresponded to a 0.1 µg/ml solution of purified Ves v 5-specific Ab. The estimated Ab concentrations varied by ~40% on repeat measurements.

^c Values in parentheses were obtained after absorption of one-five hundredth diluted sera with 0.2 mg/ml EA-Pol a 5.

Fig. 7C showed greatly reduced binding to EA-PV₁₋₃₂ and EA-PV₁₋₄₆, but it showed moderate binding to EA-PV₁₋₁₈ and EA-PV₁₋₂₄. Data not shown indicated that these mAbs did not bind denatured Ag 5. Therefore, these data suggest the N-terminal hybrids have the native structure of Ves v 5.

Immune responses to hybrids

Mice immunized with hybrids produced Abs specific for the hybrid, Pol a 5 and Ves v 5. The Ab levels of sera samples were measured before and after absorption with Pol a 5 to study their specificity for Ves v 5. These data are summarized in Table III. Mice immunized with natural EA- or KR-Ves v 5 gave nearly the same Ab responses, and only those of the KR-Ves v 5 are given in the Table. EA-PV₁₋₄₆ gave a higher Ab response in set A mice than KR-PV₁₋₄₆ did in set B mice. This difference may be due to the different sets of mice used. EA-PV₁₋₁₈ was used in both sets of experiments, and it gave higher Ab response in set A mice than in set B mice.

Comparison of Ab levels in the N-terminal hybrid-specific sera samples in Table III, before and after Pol a 5 absorption, indicate that 30–80% of their Abs were specific for Ves v 5 when tested on solid-phase Ves v 5, and these values were less when tested on solid-phase hybrid. The data in set A of Table III indicate that, of the three N-terminal hybrids, PV₁₋₁₅₅ was as immunogenic as Ves v 5 was, PV₁₋₄₆ was half as immunogenic, and PV₁₋₁₈ was approximately one-ninth as immunogenic. The data in set B indicate that PV₁₋₄₆ and PV₁₋₃₂ were more immunogenic than PV₁₋₂₄ and PV₁₋₁₈. More importantly, the data from both sets suggest that the longer N-terminal hybrids PV₁₋₄₆ and PV₁₋₃₂ stimulate higher contents of Ves v 5-specific Abs and lower contents of Pol a 5-specific Abs than the two shorter hybrids, PV₁₋₂₄ and PV₁₋₁₈, did.

Proliferation assays were made with spleen cells from mice immunized with vespid Ag 5 or hybrid to study the specificity of T cell responses. The results summarized in Table IV show that the hybrids EA-PV₁₋₄₆, EA-PV₁₋₁₅₅, and EA-PV₁₅₆₋₂₀₄ induced hybrid-specific, as well as vespid Ag 5-specific, T cell responses. The data in Table IV indicate that the best proliferative responses were obtained when the stimulating Ag was the immunogen. This is apparent from comparing the maximal stimulation index values at the highest Ag concentration of 100 µg/ml tested and from com-

Table IV. Vespid Ag 5 or hybrid-stimulated proliferation of murine spleen cells^a

Spleen Cells Specific For	Stimulating Ag		
	EA-Ves v 5	EA-Pol a 5	EA-hybrid
Stimulation index at 100 µg/ml Ag			
KR-Ves v 5	8.2	1.5	
KR-Pol a 5	2.2	6.3	
EA-PV ₁₋₁₅₅	6.1	2.2	5.0
EA-PV ₁₋₄₆	6.0	8.0	13.5
EA-PV ₁₋₁₈	2.3	5.0	6.1
EA-PV ₁₅₆₋₂₀₄	4.1	4.2	6.8
EA-PV ₁₉₅₋₂₀₄	1.7	8.6	4.1
Micrograms per milliliter Ag for stimulation index of 4			
KR-Ves v 5	2.6	>100	
KR-Pol a 5	>100	16	
EA-PV ₁₋₁₅₅	11	>100	0.54
EA-PV ₁₋₄₆	20	2.2	0.26
EA-PV ₁₋₁₈	>100	47	19
EA-PV ₁₅₆₋₂₀₄	60	70	2.3
EA-PV ₁₉₅₋₂₀₄	>100	8	82

^a Background proliferation of spleen cells showed [³H]thymidine uptake of 400–900 cpm.

paring the lowest Ag concentration required for a stimulation index value of 4.

Allergenicity of recombinant vespid Ag 5s and their hybrids in patients

Allergenicity of recombinant vespid Ag 5s and their hybrids in patients was tested by histamine release assay with basophils from ten yellow jacket-sensitive patients. The results in Table V are divided into two groups. The seven patients in group A were ~1000 times more sensitive to Ves v 5 than to Pol a 5, and the three patients in group B were about equally sensitive to both Ag 5s. The complete data from one patient of each group are given in Fig. 8.

Of the three N-terminal hybrids tested, EA-PV₁₋₁₅₅ showed no decrease in allergenicity. EA-PV₁₋₄₆ and EA-PV₁₋₁₈ showed geometric mean reductions of 126- and 583-fold, respectively, in group A patients and 0.7- and 24-fold decreases, respectively, in group B patients. The two C-terminal hybrids, EA-PV₁₅₆₋₂₀₄ and EA-PV₁₉₅₋₂₀₄, had reductions of 1139- and 3207-fold in group A patients, respectively, and 3- and 32-fold in group B patients, respectively.

The different extents of reduction in allergenicity of the N- and C-terminal hybrids reflect both their IgE Ab concentration and their epitope density. The inhibition ELISA data in Fig. 6 suggest a higher concentration of human IgG Abs for the N-terminal region of Ves v 5 than those for the C-terminal region, and this is likely also the case for IgE Abs. Another contributing factor to the greater reduction in allergenicity of the C-terminal hybrid EA-PV₁₅₆₋₂₀₄ as compared with the N-terminal hybrid EA-PV₁₋₄₆ is probably due to its decreased epitope density, because the C-terminal hybrid has fewer surface-accessible residues of Ves v 5 than the N-terminal hybrid does. Similarly, the greater reduction in allergenicity of the shorter N- or C-terminal hybrids, PV₁₋₁₈ or PV₁₉₅₋₂₀₄, as compared with their respective longer ones probably also reflects the influence of epitope density.

The allergenicity of recombinant Ves v 5 from bacteria was compared with that of the natural Ves v 5 and the recombinant Ves v 5 from yeast. In three patients tested, the recombinant

Table V. Summary of histamin release data of hybrids^a

Allergen	Reciprocal Activity Relative to Ves v 5					
	Group A			Group B		
	No. of patients	Mean	Range	No. of patients	Mean	Range
Ves v 5	7	1	1	3	1	1
Pol a 5	7	1154	330–5500	3	0.7	0.2–2
PV _{1–155}	3	1	1–2	2	1	1
PV _{1–46}	5	126	13–3300	2	0.7	0.1–5
PV _{1–18}	3	583	12–5000	2	24	3.0–200
PV _{156–204}	6	1139	1000–3000	3	3	0.4–70
PV _{195–204}	3	3207	50–5000	2	32	20.0–50

^a Proteins of EA series were tested; PV_{1–24} and PV_{1–32} were not tested. Geometric mean values are given.

protein from bacteria was $\sim 10^3$ times less potent than the natural protein or the recombinant protein from yeast. The data, which are not shown, confirm our previous observations that the majority of B cell epitopes are dependent on the conformation of the native allergen.

Discussion

The CD spectral data in Fig. 4 suggest that the hybrids have secondary structures that are closely similar to those of vespid Ag 5s. The inhibition data in Figs. 5 and 6 with Ves v 5-specific human and mouse Abs support that the hybrids have tertiary structures that are closely similar with that of Ves v 5, as data not shown indicate these Abs do not bind the denatured Ves v 5. Additional evidence came from screening with 17 monoclonal mouse IgG1 Abs specific for the natural Ves v 5; six of them bound the N-terminal hybrid PV_{1–46} (Fig. 7). The mAbs have the same specificity as the polyclonal Abs; they do not bind the denatured Ag 5. Therefore, these data together indicate that the hybrids have the native structure and they contain the discontinuous B cell epitopes of Ves v 5.

The inhibition data with polyclonal Abs and the binding data with mAbs in Figs. 5–7 indicate that the dominant B cell epitopes of Ves v 5 are in its N-terminal region. Inspection of the structure of Ves v 5 in Fig. 2 shows that nearly all residues in the N-terminal hybrid PV_{1–46} are surface accessible. This is in contrast to the C-terminal hybrid PV_{156–204}, in which only segments of Ves v 5 are surface accessible. This difference in surface accessibility may explain the immunodominance of the N-terminal region of Ag 5. Others have shown that the entire surface of a protein is potentially antigenic, but the regions with high surface accessibility and surface protrusion are dominant (30, 31).

There is only one known way to map discontinuous epitopes. It is by x-ray crystallography of Ag-Ab complexes (32), and this requires having specific mAbs. The discontinuous epitopes of CD39 were mapped with a series of mouse-human hybrids; mouse and human CD39 molecules have 75% sequence identity, and they share limited antigenic cross-reactivity (33). These findings with CD39 and Ag 5 indicate that hybrids of two homologous proteins represent a useful alternate approach to map their discontinuous B cell epitopes. Advantages of this approach are the use of polyclonal Abs and the detection of the immunodominant regions, but this approach can map only the residues that are contiguous to each other in the peptide chain. Crystallographic studies of protein Ag-Ab complexes have shown that the contact residues of an epitope may contain as many as 17 residues on the surface of an Ag, and these residues may or may not be contiguous to each other in the peptide chain (32).

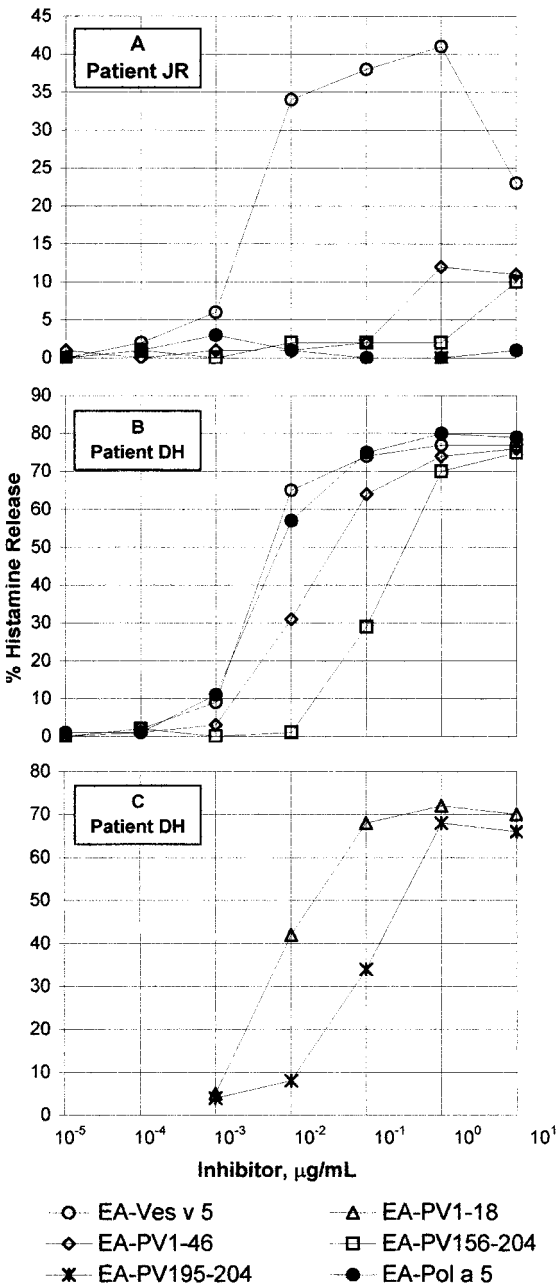


FIGURE 8. Histamine release assay of Ves v 5, Pol a 5 and their hybrids. Data from two patients, JR (group A) and DH (group B), are shown.

Our results with hybrid Ag 5s demonstrate that hybrid allergens can have a 100- to a 1000-fold reduction in allergenicity while retaining the immunogenicity of the natural allergens. The reduction in allergenicity of hybrids is due to a decrease of B cell epitope density, and this is suggested by the decreasing allergenicity of the N- and C-terminal PV hybrids as the Ves v 5 segment is shortened (Table V). As to be expected, the immunogenicity of the hybrids appeared to depend on the length of Ves v 5 segment (Table III). Our data suggest that a PV hybrid with 20–30 residues of Ves v 5 can have maximal reduction in allergenicity while still retaining adequate immunogenicity for Ves v 5.

Each of the hybrids we have studied has only a portion of the B and T cell epitopes of Ves v 5, and a mixture of appropriately chosen hybrids can, in principle, reconstitute the complete epitope library. This is in contrast to the allergens modified by the other procedures described in the introduction to this paper, as most known modified allergens lack part or all of their discontinuous B cell epitopes. It is known from studies in animals or humans that denatured allergens (4, 34) or T cell epitope peptides of allergens (35–38) did not induce allergen-specific Ab responses, although they did stimulate allergen-specific T cell responses. Thus, the hybrids may represent a better way to prepare modified allergens for use as vaccines, because they can induce both cellular and Ab responses. Because the hybrids can induce immune responses to both Ves v 5 and Pol a 5 in mice, studies need to be made to discover whether such treatment with hybrids may lead to wasp sensitivity and/or protection in yellow jacket-sensitive patients. The hybrid approach, if shown to be useful, is applicable to other allergens because many allergens have sequence homology with proteins from diverse sources (39).

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