

Analysis of IgE Antibodies from a Patient with Atopic Dermatitis: Biased V Gene Usage and Evidence for Polyreactive IgE Heavy Chain Complementarity-Determining Region 3¹

Michael R. Edwards,* Wandy Brouwer,* Caroline H. Y. Choi,* John Ruhno,[†] Robyn L. Ward,^{‡§} and Andrew M. Collins^{2*}

To better understand V gene usage, specificity, and clonal origins of IgE Abs in allergic reactions, we have constructed a combinatorial Ab library from the mRNA of an adult patient with atopic dermatitis. Sequence analysis of random clones revealed that 33% of clones used the IGHV6-1 H chain V gene segment, the only member of the V_H6 gene family. IGHV6-1 is rarely used in the expressed adult repertoire; however, it is associated with fetal derived Abs. Features of the V_H6 rearrangements included short complementarity-determining region 3, frequent use of IGHD7-27 D gene, and little nucleotide addition at the D-J junction. There was also a low level of mutation compared with V_H1, V_H3, and V_H4 rearrangements. The library was expressed as phage-Fab fusions, and specific phage selected by panning on the egg allergen ovomucoid. Upon expression as soluble IgE Fabs, 12 clones demonstrated binding to ovomucoid, skim milk, and BSA by ELISA. Nucleotide sequencing demonstrated that the IGHV6-1 V gene segment encoded each of the 12 multiply reactive IgE Fabs. A cyclic peptide was designed from the complementarity-determining region 3 of several of these clones. The cyclic peptide bound both self and nonself Ags, including ovomucoid, human IgG, tetanus toxoid, and human and bovine von Willebrand factor. These results suggest that some IgE Abs may bind more than one Ag, which would have important implications for understanding the multiple sensitivities seen in conditions such as atopic dermatitis. *The Journal of Immunology*, 2002, 168: 6305–6313.

Immunoglobulin E Abs mediate the onset of a wide range of allergic disorders through their recognition of allergens. IgE binds to its high affinity receptor (FcεRI), present upon the surface of mast cells and basophils, the effector cells of allergy (1), and once receptor bound, is most stable on the cell surface. The binding of allergen by specific IgE causes cross-linking of cell-bound IgE, and then aggregation of the FcεRI. This aggregation initiates an enzymatic cascade of events that ultimately leads to cell activation and degranulation (2, 3). Mast cells and basophils contain potent stores of mediators, such as histamine, that are responsible for the immediate symptoms of allergic disorders. These cells may also synthesize IL-4 and 13 that together up-regulate IgE class switching in B cells (4).

Many studies have attempted to characterize the IgE-allergen interaction by focusing on the allergens (5–10), but the fine specificity of the IgE Abs themselves remains largely unknown. As IgE Abs are only present in trace amounts (nanograms per milliliter) in the sera of allergic patients (1), conventional serological ap-

proaches have not been useful in their characterization. B cells producing IgE are also scarce (11, 12), making their isolation and study equally difficult. Therefore, until recently, much of what was known about the structure, specificity, and molecular genetics of IgE Abs had come from the study of IgE myeloma proteins (13–16). A more recent approach to the investigation of the structure and molecular genetics of IgE Abs has been the isolation and analysis of their rearranged Ig V region genes. The V region genes of Abs are formed by the joining of one V, one D, and one J gene segment for the H chain, and one V and one J gene segment for the L chain. In every pre-B cell, this process of recombinational rearrangement generates a functional rearranged H and L chain V gene (17, 18). Diversity is essentially generated by these rearrangements; however, further diversity is added by processing at each end of the recombining elements through N and P nucleotide addition, and exonuclease activity (18). Point mutations made by the process of somatic hypermutation within the germinal center reaction may also introduce diversity (19).

Each rearranged V gene, when expressed, will encode a polypeptide composed of a supporting β-pleated sheet scaffold or framework region and three hypervariable loops termed complementarity-determining regions (CDRs)³ (20). In the majority of Ab-Ag interactions, it is the CDRs that facilitate Ag binding. The CDR3 of the H chain is the most diverse of all the hypervariable loops, and is encoded solely by the V-D-J junction. The human CDR3 can assume a range of shapes and sizes, and can theoretically be 1 of 10¹⁴ different peptides (21).

Schools of *Microbiology and Immunology and †Medicine, University of New South Wales, Kensington, Australia; ‡Royal North Shore Hospital, St. Leonards, Australia; and §Department of Medical Oncology, St. Vincent's Hospital, Darlinghurst, Australia

Received for publication March 27, 2001. Accepted for publication April 19, 2002.

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¹ This study was supported by grants from the National Health and Medical Research Council of Australia, and from Brambles Industries. M.E. was supported by an Australian Postgraduate Award.

² Address correspondence and reprint requests to Dr. Andrew Collins, School of Microbiology and Immunology, University of New South Wales, Sydney, New South Wales, 2052 Australia. E-mail address: a.collins@unsw.edu.au

³ Abbreviations used in this paper: CDR, complementarity-determining region; vWF, von Willebrand factor.

Characterization of the Ig V genes can generate useful information about the specificity and molecular biology of IgE, as well as providing insights into the nature of the B cells that produced them. Our current knowledge of the molecular forces that shape the IgE repertoire comes from a handful of studies on patients with atopic asthma (22–26), atopic dermatitis (27, 28), peanut allergy (29), and allergy to grass pollens (30). Cloning and sequencing the rearranged V genes of IgE sequences from PBMCs (22, 27), a bronchial biopsy (24), and splenocytes (23) of allergic patients have shown a bias to the small V_H5 family. This family is rarely seen in the expressed adult B cell repertoire of normal individuals (31). All of the above studies, however, have sequenced random IgE transcripts, rather than transcripts from allergen-specific IgE. Whether or not specific IgE taking part in an allergic response also demonstrates restricted V gene usage is unknown.

Steinberger et al. (30) have used phage Ab display (32) to isolate specific IgE Ab fragments to the timothy grass pollen allergen Phl p 5. Phage display allows the isolation and characterization of specific Abs by making large V gene libraries in an *Escherichia coli* compatible vector (33, 34). Through manipulation of the *E. coli*-filamentous phage lifecycle, the V gene products are expressed as phage-Ab fusions (35, 36). Specific phage can be isolated by panning against immobilized Ag, and enriched by propagation in *E. coli* (37). The study of IgE by Steinberger represented the first look at allergen-specific IgE Abs in an allergic response. In this study, we have combined rearranged V gene analysis with phage Ab display, to study IgE Abs from an egg-allergic atopic dermatitis patient.

Materials and Methods

Patient sample

After informed consent, 100 ml peripheral blood was collected from a 40-year-old female atopic dermatitis patient. Radioallergosorbent assay demonstrated IgE reactivity to a wide range of allergens, including egg white protein (2+), house dust mite (4+), peanut (2+), and rye grass pollen (4+). Mononuclear cells were harvested from peripheral blood by Ficoll density gradient centrifugation and used as a source of IgE mRNA.

RNA extraction, cDNA synthesis, and PCR amplification

Total RNA was isolated by the method of Chomczynski and Sacchi (38). Synthesis of cDNA was performed as previously described (39). Primers used for PCR amplification involved V region H chain primer sequences reported by Yip et al. (40) and Persson et al. (33). Sequences (5'-3') were V_H1a , CAG GTG CAG CTC GAG CAG TCT GGG; V_H1f , CAG GTG CAG CTC GTG TCT GG; V_H3a , GAG GTG CAG CTC GAG CAG TCT GGG; V_H3f , GAG GTG CAG CTC GAG TCT GGG; V_H4f , CAG GTG CAG CTC GAG TCG GG; V_H4g , CAG GTG CAG CTA CTC GAG TGG GG; V_H6a , GAG GTA CAG CTC GAG CAG TCT GG; V_H6f , CAG GTA CAG CTC GAG TCA GGT CCA; and C ϵ 1 of IgE C ϵ 1, GCT GAA ACT AGT GTT GTC GAC CCA GTC TGT GGA (30). L chain primers used have also been reported by Persson et al. (33) PCR amplification of IgE Fd (H chain variable domain and C ϵ 1) and κ and λ L chains was performed with 5 μ l cDNA template, with 0.6 μ M of each primer, 0.2 mM dNTPs (Promega, Madison, WI), 2.0 mM MgCl₂, 0.4 U *Tth* polymerase (Fischer Biotec, Subiaco, WA), and a buffer supplied by the manufacturer (Fischer Biotec). Cycling was 94°C for 3 min, followed by 94°C for 30 s, 52°C for 1 min, 72°C for 2 min for 40 cycles, and then 72°C for 10 min.

Ab library construction

Ab libraries were constructed essentially as described by Ward et al. (39). Briefly, IgE Fd and L chains of ~660 bp were purified from PCR by agarose gel electrophoresis and then cut with *Xho*I and *Spe*I (IgE Fd) or *Xba*I and *Sac*I (L chains). Cut PCR products (1 μ g) were then ligated with the vector MCO3 (1.5 μ g), which had been digested with similar sets of restriction enzymes. L chain and then Fd H chain were sequentially cloned into the vector using DNA ligase, followed by electroporation into *E. coli* XL-1 (Stratagene, La Jolla, CA) using standard procedures. After 1 h recovery at 37°C, with shaking, an aliquot of the library was titered on 2xYT (yeast extract and trypton medium) plates with 50 μ g/ml carbenicillin, 2%

(w/v) glucose to determine library size. The remaining culture was scaled up to 100 ml with 2xYT 50 μ g/ml carbenicillin and 2% (w/v) glucose and grown for 1 h at 37°C. Insert frequency was determined by the analysis of randomly picked clones for presence of insert by PCR, and by titering colonies produced by mock ligations consisting only of cut vector and no insert, and cut vector with no insert or ligase.

Scaling up of library and selection of specific phage Fabs

After growth for 1 h, the library culture was infected with 1.5×10^{12} VCSM13 helper phage (Stratagene) grown for 2 h at 37°C, then spun at 4000 rpm for 15 min. Cells were resuspended in 2xYT medium with 50 μ g/ml carbenicillin, 70 μ g/ml kanamycin, and 10 μ g/ml tetracycline, selecting for *E. coli* infected with helper phage and maintaining recombinant plasmids. Phage-Fab fusions were produced from the culture by growing overnight at 30°C. Phage were selected on microtiter plates (Polysorb; Nunc, Roskilde, Denmark) coated with purified ovomucoid (a gift from R. Sleight, CSIRO, Sydney, Australia) essentially as previously described (39). One exception was that the amount of Ag decreased over the rounds. In rounds 1 and 2, 100 μ g/ml was used, decreasing to 10 μ g/ml at round 3, 1 μ g/ml at round 4, and 0.1 μ g/ml at round 5. Input and output phage were titered on log phase *E. coli* XL-1 blue (Stratagene) to determine the ratio of input-output phage at each round.

Induction of soluble IgE Fabs, colony screening by ELISA, and inhibition ELISA

Phage isolated from each panning round were used to infect a log phase culture of the nonsuppressor strain *E. coli* HB2151, and single colonies were grown in 96-well plates. The cultures were induced with isopropyl- β -D-thiogalactoside for soluble Fab expression (37). Overnight cultures in 96-well plates were spun at 2750 rpm for 15 min, and 0.1-ml aliquots of culture supernatant were transferred to microtiter plates (polysorp; Nunc) that had been coated in 10 μ g/ml ovomucoid in carbonate buffer and blocked in blocking buffer (2% (w/v) skim milk in PBS, pH 7.4) for 2 h at 37°C. Aliquots were also added to a microtiter plate (polysorp; Nunc) coated in blocking buffer, as described above. A nonspecific Fab (human anti-p53) served as an additional negative control. Supernatants were incubated at room temperature for 1 h before washing three times with PBS, pH 7.4. Purified anti-c-myc Ab (clone 9E10, 0.5 mg/ml) was diluted 1/1000 in diluting buffer (2% (w/v) skim milk in PBS, pH 7.4) and added to each well. Incubation and washing were as above. Goat anti-mouse Ab-HRP conjugate (0.5 mg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) was diluted 1/1000 in diluting buffer, then added and incubated as above. Plates were washed three times with PBS, pH 7.4, HRP substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added, and color was allowed to develop at room temperature for 20 min. The reaction was stopped by addition of 2 M H₂SO₄, and absorbances were read at 450 nm. Clones were considered positive when the OD was 3-fold greater than that seen on blank wells with no Fab sample. To confirm the ELISA results, an inhibition ELISA was performed by a modification of the ELISA in which 50 μ l 2% (w/v) skim milk, 10 μ g/ml ovomucoid, or 2% (w/v) BSA (Sigma-Aldrich, St. Louis, MO) in PBS, pH 7.4, was included in the well at the time of addition of the Fab sample (50 μ l). Inhibition was determined by comparing the absorbances of wells with Fab sample and immobilized Ag, with wells containing Fab sample, immobilized Ag, and Ag in the liquid phase.

PCR amplification of cloned inserts

Plasmids were extracted by an alkaline lysis procedure from overnight cultures grown in 2xYT, 50 μ g/ml carbenicillin, and 2% glucose. Inserts were amplified using PCR with primers pelBFor (CTA CGG CAG CCG CTG GAT TG) and g3pRev (GAT CCT CTT CTG AGA TGA GT), which anneal to the pelB leader sequence and g3p of filamentous phage, respectively, in MCO3. One microliter of plasmid DNA served as template under identical reaction conditions as for cDNA amplification. The cycling conditions used were 94°C for 3 min, then 30 cycles of 94°C for 15 s, 52°C for 50 s, 72°C for 1.5 min, and then one cycle of 72°C for 10 min, on a PerkinElmer (Wellesley, MA) 9600 Gene Amp thermal cycler. PCR products were purified by agarose gel electrophoresis and a gel extraction kit (Qiagen, Chatsworth, CA).

V gene sequencing and V gene sequence alignments

Cloned Fd inserts were sequenced using a Dye Terminator Kit (Applied Biosystems, Foster City, CA), with 90 ng purified PCR product and 7.5 pmol forward (pelBFor) or reverse primer (g3pRev). Sequences were analyzed on an ABI 377 automated sequencer (Applied Biosystems). Cycling conditions were 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min for 25

cycles. Sequences were aligned using the DNAPLOT algorithm available at the V BASE database (41) and are named using the IMGT nomenclature (42). The numbering system for the CDRs and framework regions complies with the system of Chothia et al. (43).

Design of cyclic peptides

The cyclic peptide CP-1 was designed according to the CDR3 sequence of a number of V_H6 -utilizing ovomucoid-binding Fabs. A control peptide CP-N was designed from the CDR3 sequence of a nonbinding V_H3 -utilizing clone (clone 46). Both peptides are illustrated in Fig. 1. The peptides were obtained from Auspep (Parkville, Australia) as a lyophilized powder, and were reconstituted with sterile PBS, pH 7.4, before use.

Peptide-binding assays

Binding of CP-1 and the control peptide CP-N to a range of Ags was assessed by ELISA and dot-blot assay. Microtiter plates (polysorp; Nunc) were coated in 100 μ l 20 μ g/ml ovomucoid, 20 μ g/ml OVA, 10 μ g/ml BSA, 28 μ g/ml tetanus toxoid, 10 μ g/ml recombinant human early pregnancy factor, human *bcl-2* (DAKO, Carpinteria, CA) diluted 1/10, the 4-mer peptide arg-gly-asp-ser (Sigma-Aldrich) diluted 1/10, normal rabbit serum diluted 1/100, Intragram (60 μ g/ml human polyclonal IgG; CSL, Victoria, Australia) diluted 1/100, 8 μ g/ml extracellular domain of human erbB-2, 20 μ g/ml purified bovine von Willebrand factor (vWF), 7 μ g/ml purified human vWF, and 8.5 μ g/ml recombinant human p53. All Ags were diluted in carbonate-coating buffer, and coating occurred overnight at 4°C. After three washes in PBS, pH 7.4, containing 0.05% Tween 20 (PBS/Tween), the wells were blocked in PBS containing 0.1% (w/v) casein hydrolysate for 2 h at 37°C. One hundred microliters of CP-1 or CP-N (diluted to 10 μ g/ml in PBS, pH 7.4, containing 0.1% (w/v) casein hydrolysate) were added per well, and binding was allowed to occur at room temperature for 1 h before washing three times with PBS/Tween. Streptavidin-HRP conjugate (DAKO) was diluted 1/1000 in PBS/casein hydrolysate, added to all wells, and incubated for 1 h at room temperature. After three washes of PBS/Tween, tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories) was added. Color was allowed to develop for 20 min, and the reaction was then stopped by the addition of 1 M H_2SO_4 . Absorbances were read at 450 nm.

Dot-blot assays were conducted on polyvinylidene difluoride membrane (Immun-Blot; Bio-Rad, Hercules, CA). Membranes were blocked overnight at 4°C in 0.1% (w/v) casein hydrolysate and washed four times in PBS/Tween. A total of 10 μ l 200 μ g/ml ovomucoid, 200 μ g/ml OVA, 100 μ g/ml BSA, 280 μ g/ml tetanus toxoid, 100 μ g/ml human recombinant early pregnancy factor, human *bcl-2*, 4-mer peptide arg-gly-asp-ser, normal rabbit serum diluted 1/10, Intragram diluted 1/10, 80 μ g/ml extracellular domain of human erbB-2, 200 μ g/ml bovine vWF, 70 μ g/ml human vWF, and 85 μ g/ml recombinant human p53 diluted in PBS, pH 7.4, were blotted onto the membrane. Membranes were incubated in CP-1 or CP-N at 10 μ g/ml in PBS, pH 7.4, for 1 h at room temperature. A peptide-free control membrane was also included. After washing four times in PBS/Tween,

membranes were incubated in streptavidin-alkaline phosphatase conjugate (Zymed Laboratories, San Francisco, CA) diluted 1/100 in 0.1% (w/v) casein hydrolysate for 1 h at room temperature. Membranes were washed four times in PBS/Tween, and incubated in nitroblue tetrazolium chloride (Research Organics, Cleveland, OH)/5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim, Indianapolis, IN) substrate (0.2 mg/ml each of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate in carbonate buffer). Color was allowed to develop for 10 min, and stopped by immersing membranes in water.

The ability of CP-1 to inhibit binding by serum IgE was assessed using the Pharmacia ImmunoCAP system (Pharmacia and Upjohn, Uppsala, Sweden). Immunocaps specific for ovomucoid were incubated with a 1/4 dilution of serum in PBS, pH 7.4, containing CP-1 at 20 μ g/ml, CP-1 at 10 μ g/ml, CP-1 at 5 μ g/ml, CP-1 at 2.5 μ g/ml, CP-N at 20 μ g/ml, or PBS only for 30 min at room temperature. Immunocaps were washed in an Assay Washer 96 (Pharmacia and Upjohn), and incubated for 2.5 h at room temperature in β -galactosidase/anti-IgE conjugate (Pharmacia and Upjohn). After washing, immunocaps were incubated in 4-methylumbelliferyl- β -D-galactoside substrate for 10 min at room temperature. Fluorochrome was eluted from immunocaps with sodium carbonate buffer, and fluorescence of the eluate was measured at 450 nm.

Statistical analysis

Where appropriate, data were analyzed for statistical significance using either ANOVA with Bonferroni's multiple comparison test, or the unpaired two-tailed *t* test. A confidence interval of 95% was used for all tests.

Results

Ab library construction

DNA encoding the Fd and L chain was amplified from cDNA using PCR. A phage display library containing IgE Fd and L chains was constructed using the vector MCO3. The size of the library was 4.4×10^7 primary clones with an insert frequency of >99%, as determined by titering *E. coli* XL-1 blue after transformation and comparison with mock ligations (data not shown). Presence of H chain and L chain inserts was verified using PCR (data not shown).

Sequence analysis of random clones

The rearranged H chain V genes of 51 primary transformants from the library were sequenced, and the closest matching germline V, D, and J gene segments were aligned. All rearrangements were inframe, functional rearrangements, and all clones were of the IgE isotype (data not shown). Of 51 rearrangements sequenced, 31 unique CDR3 were observed. A total of 19 rearrangements had CDR3 sequences shared by other rearrangements, but differed in the number or position of point mutations across the V gene, and were therefore considered to be independent sequences. Only two pairs of rearrangements (two V_H3 and two V_H6 rearrangements) shared identical CDR3 and mutation pattern, and were considered to be duplications of the same sequences. The two duplications

Table I. Summary of H chain family usage and diversity in 51 IgE H chain rearrangements

Family	<i>n</i>	Number of V_H Rearrangements with ^a		
		Different CDR3	Shared CDR3 ^b	Shared CDR3 and mutation pattern ^c
V_H1	8	4	4	0
V_H3	20	14	6	2
V_H4	6	6	0	0
V_H6	17	4	13	2
Total	51	28	23	4

^a Codons of all V_H rearrangements are numbered according to Chothia et al. (43).

^b Rearrangements that had CDR3s that were identical to one or more other rearrangements.

^c Rearrangements that had identical CDR3s and a pattern and number of mutations that were identical to one other rearrangement.

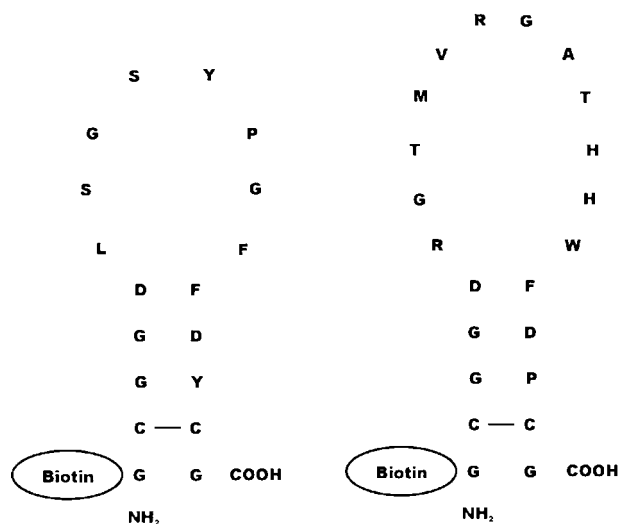


FIGURE 1. Structure of the 17-mer cyclic peptide CP-1 (left) and the 21-mer cyclic peptide CP-2 (right). The N-terminal glycine residues are biotinylated.

were not included in further analysis. There was a higher degree of shared CDR3 sequences in rearrangements of the V_H6 family, as only 4 of 17 had unique CDR3 sequences. Table I shows the number of unique CDRs, and the number of unique individual rearrangements.

V, D, and J gene usage

H chain family usage was 16% (8 of 49) V_H1 , 39% (19 of 49) V_H3 , 12% (6 of 49) V_H4 , and 33% (16 of 49) V_H6 , and in total 11 different V gene segments were used. Tables II and III show the closest matching germline V, D, and J gene segment for each clone. In all, 16 different IGHD genes were identified in the IgE rearrangements. Interestingly, a number of the V_H6 rearrangements also used the IGHD7-27 D gene. There was no IGHD7-27 D gene usage in V_H1 or the V_H4 rearrangements. No germline D gene segment could be aligned for clone 47. All J genes except for IGJH1 were used. V_H1 , V_H3 , and V_H4 rearrangements used a range of IGJH genes including IGJH6*02. In contrast, V_H6 rearrangements did not use the IGJH6*02 segment and were dominated by IGJH4*02. Clone 47 used the IGJH5*01 gene segment.

N nucleotide addition

V_H6 rearrangements demonstrated a low level of N nucleotide addition at the D-J junction (2.06 ± 1.83 ; mean $\pm$ SD) (Fig. 2). Using a one-way ANOVA, this was found to be significantly different from N addition at the D-J junction of V_H1 (9.13 ± 7.88 ; $p < 0.01$), of V_H3 (7.90 ± 4.15 ; $p < 0.01$), and V_H4 rearrangements (8.17 ± 4.49 ; $p < 0.05$). The V_H1 , V_H3 , and V_H4 families

Table III. V, D, and J gene usage of the V_H4 and V_H6 rearrangements^a

Clone	Closest Germline Gene ^b			% Identity with V Gene ^c
	IGHV	IGHD	IGHJ	
16	IGHV4-39	IGHD2-15	IGHJ6*02	96.7
32	IGHV4-31	IGHD3-10	IGHJ4*02	90.9
33	IGHV4-39	IGHD5-12	IGHJ4*02	93.6
38	IGHV4-31	IGHD1-14	IGHJ6*02	96.7
49	IGHV4-31	IGHD5-24	IGHJ4*02	89.6
61	IGHV4-4	IGHD4-17	IGHJ5*02	89.0
1 ^d	IGHV6-1	IGHD7-27	IGHJ4*02	98.9
4	IGHV6-1	IGHD1-26	IGHJ4*02	96.4
12	IGHV6-1	IGHD6-13	IGHJ4*02	99.6
13	IGHV6-1	IGHD6-13	IGHJ4*02	99.3
15	IGHV6-1	IGHD6-13	IGHJ4*02	99.3
18	IGHV6-1	IGHD7-27	IGHJ4*02	98.9
21	IGHV6-1	IGHD6-19	IGHJ4*02	100
23	IGHV6-1	IGHD6-19	IGHJ4*02	99.3
42 ^d	IGHV6-1	IGHD7-27	IGHJ4*02	98.9
44	IGHV6-1	IGHD2-21	IGHJ4*02	98.6
45	IGHV6-1	IGHD7-27	IGHJ4*02	97.8
47	IGHV6-1	UN	IGHJ5*01	95.9
50	IGHV6-1	IGHD6-19	IGHJ4*02	99.3
51	IGHV6-1	IGHD6-13	IGHJ4*02	98.6
54	IGHV6-1	IGHD7-27	IGHJ4*02	99.6
55	IGHV6-1	IGHD7-27	IGHJ4*02	98.6
57	IGHV6-1	IGHD7-27	IGHJ4*02	98.2

^a The percentage identity with each germline V gene is also shown.

^b Comparisons with germline V genes were made using the V BASE database (see Ref. 41), and genes are identified using the IMGT nomenclature (see Ref. 42).

^c Percentage of identity does not include 5' primer sequence.

^d Denotes clones that are identical, having the same CDR3 sequence and pattern of mutation.

Table II. V, D, and J gene usage of the V_H1 and V_H3 rearrangements^a

Clone	Closest Germline Gene ^b			% Identity with V Gene ^c
	IGHV	IGHD	IGHJ	
22	IGHV1-69	IGHD4-11	IGHJ4*02	94.8
28	IGHV1-69	IGHD4-11	IGHJ4*02	95.1
36	IGHV1-69	IGHD7-27	IGHJ4*02	92.5
40	IGHV1-2	IGHD3-22	IGHJ6*02	92.3
41	IGHV1-2	IGHD3-22	IGHJ6*02	90.7
48	IGHV1-2	IGHD3-22	IGHJ6*02	93.3
53	IGHV1-69	IGHD1-26	IGHJ4*02	91.4
63	IGHV1-69	IGHD6-25	IGHJ6*02	97.0
2	IGHV3-30	IGHD5-12	IGHJ3*02	95.6
3 ^d	IGHV3-33	IGHD7-27	IGHJ4*02	94.8
11 ^d	IGHV3-33	IGHD7-27	IGHJ4*02	94.8
14	IGHV3-23	IGHD2-2	IGHJ6*02	94.4
24	IGHV3-30	IGHD2-2	IGHJ4*02	94.4
25	IGHV3-11	IGHD3-10	IGHJ6*02	97.4
26	IGHV3-23	IGHD3-16	IGHJ4*02	97.4
29	IGHV3-33	IGHD6-19	IGHJ4*02	94.8
30	IGHV3-30	IGHD6-13	IGHJ6*02	94.4
31	IGHV3-23	IGHD4-17	IGHJ6*02	95.9
34	IGHV3-23	IGHD6-13	IGHJ4*02	95.4
37	IGHV3-30	IGHD4-17	IGHJ6*02	95.1
39	IGHV3-21	IGHD3-16	IGHJ4*02	96.3
43	IGHV3-23	IGHD1-26	IGHJ4*02	98.1
46	IGHV3-23	IGHD3-10	IGHJ5*02	92.3
52	IGHV3-23	IGHD3-16	IGHJ4*02	94.1
56	IGHV3-23	IGHD5-12	IGHJ2	93.3
59	IGHV3-23	IGHD4-11	IGHJ4*02	89.5
60	IGHV3-33	IGHD4-17	IGHJ5*02	96.7
62	IGHV3-30	IGHD4-17	IGHJ6*02	97.0

^a The percentage identity with each germline V gene segment is also given.

^b Comparisons with germline V genes were made using the V BASE database (see Ref. 41), and genes are identified using the IMGT nomenclature (see Ref. 42).

^c Percentage of identity does not include 5' primer sequence.

^d Denotes clones that are identical, having the same CDR3 sequence and pattern of mutation.

showed no significant difference in N additions at the D-J junction when compared with each other.

CDR3 length

The number of amino acids encoded by the CDR3 was determined for each rearrangement, and the results are presented in Fig. 3. There was a significant difference between V_H6 rearrangements (11.9 ± 0.25 ; mean $\pm$ SD) when compared with V_H1 (17.5 ± 3.74 ; $p < 0.001$), V_H3 (14.2 ± 3.39 ; $p < 0.001$), and V_H4 rearrangements (15.2 ± 4.17 ; $p < 0.001$). Statistical analysis was performed using an unpaired, two-tailed *t* test.

Mutation analysis

All rearrangements except one V_H6 rearrangement showed the presence of point mutations occurring across the V gene. The percentage of identity with germline V genes for each rearrangement is summarized in Tables II and III. The degree of mutation was

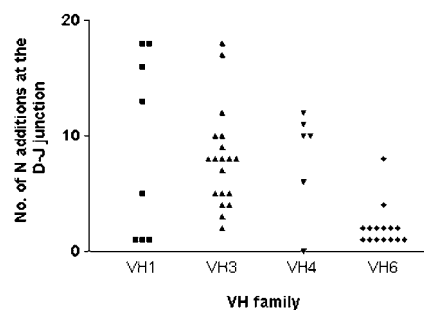


FIGURE 2. The number of N nucleotide additions at the D-J junction of IgE H chain rearrangements. Results are presented for 49 rearrangements and are analyzed according to the V_H gene family identified in each rearrangement.

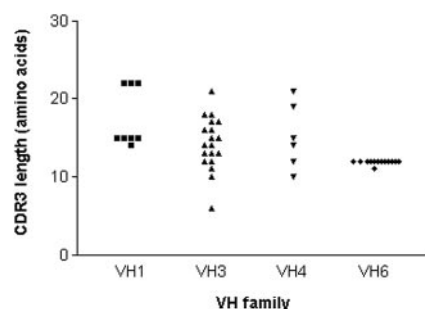


FIGURE 3. CDR3 length in amino acids of IgE H chain rearrangements. Results are presented for 49 rearrangements and are analyzed according to the V_H gene family identified in each rearrangement.

markedly different between V_H6 ($1.36\% \pm 1.23$; mean $\pm$ SD) rearrangements and rearrangements from other families. Fig. 4 represents a scattergraph of the percentage of mutations for each family. The differences, with respect to the V_H6 family, were shown to be highly significant using a one-way ANOVA, for V_H1 ($6.56\% \pm 2.08$; $p < 0.001$), V_H3 ($4.92\% \pm 2.01$; $p < 0.001$), and V_H4 rearrangements ($7.42\% \pm 3.57$; $p < 0.001$).

Selection and specificity of IgE Fabs produced by panning

The IgE Ab library was panned against immobilized ovomucoid over five successive rounds. Ninety-six clones per round were screened for ovomucoid binding in an ELISA. The clones were also tested for binding to the blocking agent (skim milk), and all clones that bound ovomucoid also demonstrated binding to skim milk. Antiovomucoid and skim milk clones were observed in every panning round, peaking in number at round 3. Fig. 5 shows the binding of 12 positive clones from rounds 4 and 5, as well as a negative clone (4-85) representative of the 468 negative clones. The 12 positive clones were then subjected to further analyses. The original assay was repeated (data not shown) and then clones were tested for binding to BSA in an inhibition ELISA. The specificity of the binding was further explored for the 12 clones in a number of inhibition assays with different combinations of immobilized Ag, and Ag in the liquid phase. Binding to immobilized ovomucoid could be inhibited by incubating with BSA and skim milk protein (Fig. 6), and binding to immobilized skim milk protein could be inhibited by incubating with skim milk (data not shown). Binding to immobilized ovomucoid could be inhibited by incubating with ovomucoid in supernatant. This assay was performed on a limited number of clones (clones 5, 8, 37, and 38), and the results are presented in Fig. 7. Further investigations of Fab binding were prevented by our inability to scale up cultures of the V_H6 clones, perhaps as a result of intracellular binding by highly reactive Fabs,

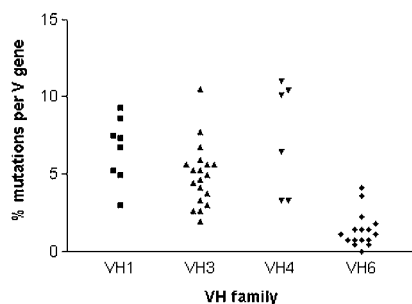


FIGURE 4. The percentage of mutations per V gene segment of IgE H chain rearrangements. Results are presented for 49 rearrangements and are analyzed according to the V_H gene family identified in each rearrangement.

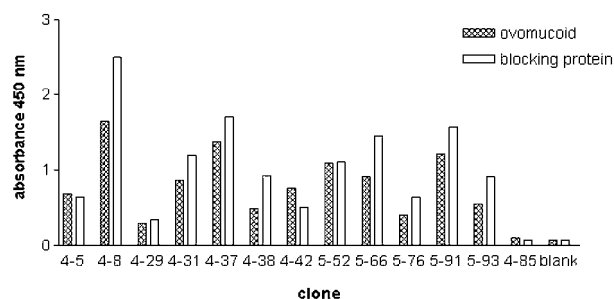


FIGURE 5. Results of ELISA of Fab clones showing ODs from binding to both ovomucoid and blocking protein (skim milk)-coated wells.

leading to cell death. Subsequent investigations of the binding were confined to studies using cyclic peptides designed from the CDR3 regions of a multiply reactive ovomucoid-binding and a control Fab.

Sequences of ovomucoid-binding clones

The H chain V gene amino acid sequences are presented in Fig. 8 for each of the 12 ovomucoid-binding clones. Sequence analysis showed that they all use the V gene segment IGHV6-1, the only member of the V_H6 family. Four clones were identical. The 12 clones isolated by panning had CDR3 regions, and patterns of mutation previously seen in rearrangements identified in the random sequencing experiment (data not shown). Nine of the twelve clones were encoded by the IGHD7-27 D segment, and all clones use the J_H4b J gene (data not shown). All of the polyreactive clones also had a consensus CDR3 sequence, consisting of **DL(P)S(G)G(D)SYP(S)S(G)F(H)FDY**, in which amino acids common to all CDR3s are presented in boldface type. In all clones, the CDR3 was 12 aa long. A variety of L chains was used by the various polyreactive clones (data not shown).

Peptide-binding experiments

To investigate the role of the H chain CDR3 in binding to ovomucoid and other Ags, a cyclic peptide (CP-1) was designed from a CDR3 sequence of several of the ovomucoid-binding IgE Fabs. A control peptide (CP-N) was designed from the CDR3 of a V_H3 -utilizing clone (clone 46). Using an ELISA, the peptide CP-1 gave ODs at least 2-fold greater than control wells (Ag free) when incubated with ovomucoid, tetanus toxoid, human IgG, and human as well as bovine vWF (Figs. 9 and 10). The control peptide CP-N showed no elevated ODs in the ELISA against any Ag specificity.

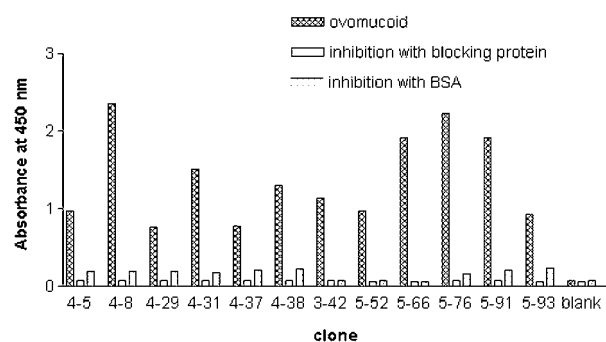


FIGURE 6. The results of inhibition ELISA showing ODs for each of the 12 clones comparing binding to immobilized ovomucoid, with immobilized ovomucoid in the presence of either BSA or skim milk protein in the liquid phase.

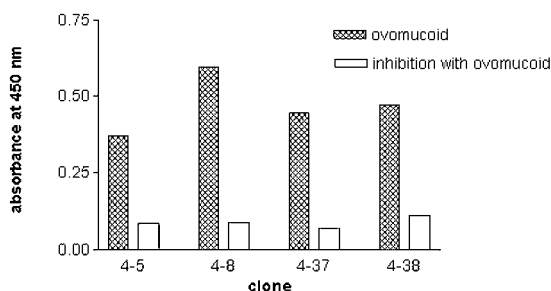


FIGURE 7. Results of inhibition ELISA for four clones showing ODs comparing binding to immobilized ovomucoid, with binding to immobilized ovomucoid in the presence of ovomucoid in the liquid phase.

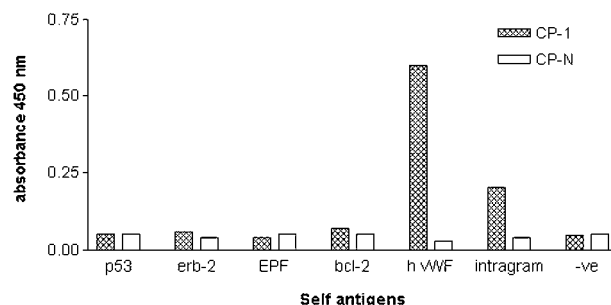


FIGURE 9. ELISA results showing ODs for binding of the cyclic peptides CP-1 and CP-N to self Ags.

Results of the ELISA were confirmed in all cases by dot-blot assay (data not shown).

CP-1 inhibition of binding by serum IgE was assessed using the Pharmacia ImmunoCAP assay. Patient serum was shown to contain 2.94 kU/L anti-ovomucoid IgE, which is in the moderate (class 2) range of specific IgE concentrations. Inhibition of serum IgE ovomucoid binding was observed over a range of peptide concentrations, with 99% inhibition of binding by serum IgE to ovomucoid observed at a peptide concentration of 10 μ g/ml, and 31% inhibition at a concentration of 1.25 μ g/ml (Fig. 11).

Discussion

DNA sequencing and phage Ab display were used to investigate the V gene usage and specificity of IgE Abs from a patient suffering from atopic dermatitis. DNA sequencing of rearranged V genes from 51 random clones revealed an abundant usage of the IGHV6-1 gene segment, the only member of the V_H6 H chain gene family (41). Given the rarity of IGHV6-1 use observed in normal

adult peripheral blood B cells (31), the V_H6 gene usage in the IgE repertoire of this patient is striking, and raises interesting questions regarding the origins of the B cell clones expressing this rarely observed H chain V gene family. It is possible, but unlikely, that the V_H6-containing sequences were produced from a bias in PCR or cloning. The original PCR products produced from template cDNA demonstrated that strong amplifications were generated with CE1- and V_H6-specific primers (data not shown). Previously in the laboratory, PCR amplifications with V_H6-specific primers have generated little or no product for other samples (40), suggesting that strong amplifications are dependent upon the presence and amount of V_H6-encoded cDNA, rather than being produced by a bias in PCR. Cloning bias is another possible explanation for the observed results, and such a possibility has been raised in other studies (31). It is unlikely though that the results presented in this work are due to a cloning bias, as a range of H chain primer combinations was used. All PCR products from each primer combination were pooled before restriction enzyme digest and ligation,

	FR 1	CDR 1	FR 2	CDR 2
IGHV6-1	QQSGPGLVKPSQTLSTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYNDYAVSVKS
4-5	LESGPGLVKPSQTLSTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYDYAVSVKS
4-8	LESGPGLVKPSQTLSTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYNDYAVSVKS
4-29	LEESGPGLVKPSQTLSTCAISGDSVC	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYNDYAVSVKS
4-31	LEESGPGLVKPSQTLSTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYNDYAVSVKS
4-37	LESGPGLVKPSQTLSTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYSDYAVSVKS
4-38	LESGPGLVKPSQTLSTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYNDYALSVKS
5-52	LESGPGLVKPSQTLSTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYNDYAVSVKS
5-66	LESGPGLVKPSQTLSTCAISGDNVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYNDYTVSVKS
5-76	LESGPGLVKPSQTLSTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYSDYAVSVKS
5-91	LESGPGLVKPSQTLSTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYNDYAVSVKS
5-93	LESGPGLVKPSQTLSTCAISGDNVS	SNSAAWN	WIRQSPSRGLEWL	RTYYRSKWYNDYTVSVKS
	FR 3	CDR 3	FR 4	
IGHV6-1	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR			
4-5	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	DPGDSYSSHFDY	WGQGTLLTVSS	
4-8	<u>R</u> MTINPDTSKNH <u>F</u> SLQLNSVTPEDTAVYYCAR	DLSGSYPGFFDY	WGQGTLLTVSS	
4-29	RMTINPDTSKNH <u>F</u> SLQLNSVTPEDTAVYYCAR	DLSGSYPGFFDY	WGQGTLLTVSS	
4-31	<u>R</u> MTINPDTSKNH <u>F</u> SLQLNSVTPEDTAVYYCAR	DLSGSYPGFFDY	WGQGTLLTVSS	
4-37	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	DPGDSYSSHFDY	WGQGTLLTVSS	
4-38	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	DLSGSYPGFFDY	WGQGTLLTVSS	
4-42	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	DLSGSYPGFFDY	WGQGTLLTVSS	
5-52	<u>R</u> MTINPDTSKNH <u>F</u> SLQLNSVTPEDTAVYYCAR	DLSGSYPGFFDY	WGQGTLLTVSS	
5-66	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	DLSGSYPGFFDY	WGQGTLLTVSS	
5-76	RITINPDTSKNQFSLQLNSVTPEDTAVYYC <u>T</u> R	DPGDSYSSHFDY	WGQGTLLTVSS	
5-91	RMTINPDTSKNH <u>F</u> SLQLNSVTPEDTAVYYCAR	DLSGSYPGFFDY	WGQGTLLTVSS	
5-93	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	DLSGSYPGFFDY	WGQGTLLTVSS	

FIGURE 8. Amino acid alignments of the H chain for 12 polyreactive clones with the germline V gene IGHV6-1. CDRs are indicated. Mutations are underlined. Mismatches due to primer sequences were not counted as mutations.

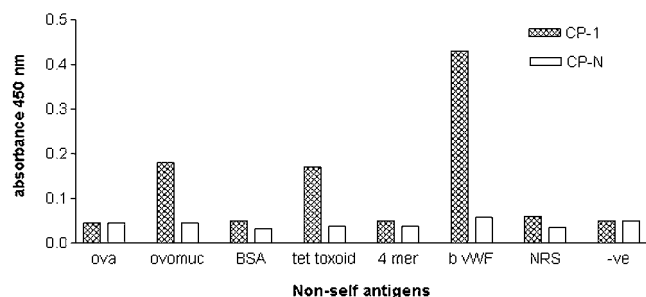


FIGURE 10. ELISA results showing ODs for binding of the cyclic peptides CP-1 and CP-N to nonself Ags.

and were cloned into the vector MCO3 using a common cloning strategy. The preferential use of V_H6 rearrangements in the library is most likely due to the amount of V_H6 -encoded transcripts present in the original starting material.

The use of particular V genes has been noted in a range of diseases, including autoimmunity (44–48) and malignancy (49). IGHV6-1, for example, has recently been associated with idiopathic thrombocytopenic purpura (50). IGHV6-1, the most D-proximal V_H gene segment (51), has also been found to be commonly expressed by fetal B cells (52, 53), and this expression pattern may be associated with its position on chromosome 14 (52).

This is not the first report of the use of the V_H6 gene family in the IgE repertoire. IGHV6-1 has been identified in IgE rearrangements among 9 of 10 patients suffering from a variety of allergic diseases, including atopic dermatitis (54). The other small H chain V gene family (V_H5) has also been observed encoding IgE Abs (22–24, 27). Like the V_H6 gene family, the V_H5 gene family (55) is rarely observed in the adult B cell repertoire (31). In one study, some V gene rearrangements lacked mutations and were in germline configuration (23) similar to results presented in the present study.

A number of D genes were identified in the IgE library. Most notable was the IGHD7-27 gene segment. This segment has been associated with Abs produced by B-1 cells, including fetal B cells (52, 56). The IGHD7-27 D gene has been shown to be present in up to 40% of first and second trimester fetal Ab transcripts; however, it is found in less than 1% of adult Ab transcripts (57). The bias for IGHD7-27 rearrangement among B-1 cells is thought to be due to its position, as it is the most J-proximal D gene (58). Given the low abundance of IGHD7-27-related Ab transcripts in adults, the frequent presence of the IGHD7-27 D gene in the IgE V genes reported in this work is striking. There was a strong correlation between IGHD7-27 D segment and IGHV6-1 gene usage, with 6 of the 16 unique V_H6 clones using both segments. IGHD7-27 was also observed in 1 of 19 V_H3 -utilizing clones. Two studies have previously reported the existence of IGHD7-27 D genes encoding

IgE H chains. One study observed only 1 of 29 rearrangements that used IGHD7-27 (28), while a second study observed 3 of 74 IgE rearrangements that used IGHD7-27 (22).

Further sequence analysis revealed that the V_H6 rearrangements had other interesting features that were significantly different from the rearrangements involving other V gene families. The CDR3 lengths of the V_H6 rearrangements were shorter than the CDR3 of V_H1 , V_H3 , and V_H4 rearrangements. There are several possible factors that may have contributed to the short V_H6 CDR3s: a lack of N addition; the frequent use of the short IGHD7-27 D gene; the lack of the long IGHJ6 J gene segment in any of the V_H6 rearrangements. It is also possible that the CDR3 size observed reflects Ag selection for a particular CDR3 sequence, as all the V_H6 CDR3s were highly related.

In this study, there was also a significantly lower number of point mutations observed in V_H6 rearrangements compared with rearrangements from the other families. One V_H6 rearrangement had no evidence of mutation, suggesting that affinity maturation by mutation and selection of mutants with higher affinities may not always be occurring in the IgE response of this individual.

The library of H and L chain V genes was expressed as phage Ab fusions and phage were selected on the basis of ovomucoid binding. Surprisingly, the ovomucoid-binding Fabs were also able to bind wells coated with skim milk protein and BSA, suggesting that these clones may be capable of binding to a number of dissimilar Ags.

Sequencing the V genes of each of the 12 ovomucoid/BSA-binding IgE Fabs showed that they were encoded by the IGHV6-1 V gene segment. Several of the 12 clones had rearranged V genes and H chain CDR3s that were identical to the V_H6 rearrangements identified in the earlier random sequencing experiments. The IGHD7-27 D gene was found in 9 of 12 clones. All of the isolated IgE Fabs appeared to have a conserved motif within their CDR3 region and shared common amino acids.

The fact that all 12 ovomucoid-binding clones carried different L chains suggested that the L chain CDRs might not be participating in Ag binding. Polyreactivity has, in fact, been reported to sometimes arise solely from Ag interaction with the H chain CDR3. To investigate the contribution of the H chain CDR3s to ovomucoid binding, a cyclic peptide (CP-1) was designed based on the CDR3 sequence encoded by several of the ovomucoid-binding clones. In an ELISA, the peptide was shown to bind to a range of human and nonhuman Ags, including ovomucoid, while a control peptide (CP-N), based on a different CDR3 sequence, did not bind to any of the test Ags. Binding of CP-1 to BSA was not observed, despite the fact that the IgE Fabs bound BSA. This suggests BSA binding by IgE Fabs may require the other H chain CDRs and the L chain.

The results reported in this work are consistent with other studies, in which the polyreactivity of some Abs has been shown to be dependent upon the sequence and structure of the H chain CDR3. This has been shown for murine CD5⁺ B cells (59), mAbs derived from human CD5⁺ lymphocytes (60–62), leukemias of CD5⁺ B cells (63), and polyreactive recombinant Abs isolated from phage display libraries constructed from adult lymphocytes (64). The exact features of the H chain CDR3 that allow polyreactive binding are unknown; however, the short length of the CDR3 may be an important contributing feature.

It is perhaps significant that the IgE Fabs reported in this work were derived from a library constructed from the lymphocytes of a patient with multiple sensitivities. The patient reacted to many different allergens by radioallergosorbent assay, including egg

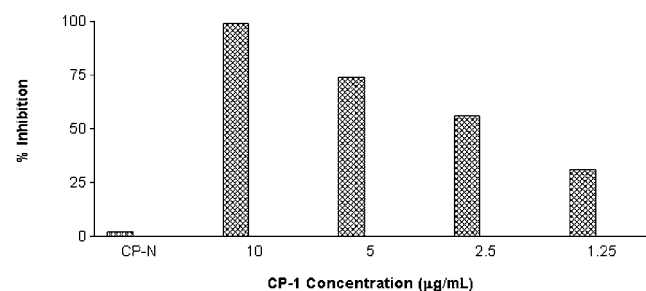


FIGURE 11. Inhibition of serum IgE binding to ovomucoid by the cyclic peptides CP-1 and CP-N, as measured by ImmunoCAP assay.

white proteins, house dust mite, peanut extract, and numerous pollens. It is possible that polyreactive IgE may be, in part, responsible for the multiple reactivities observed. Certainly there is good reason to accept the relevance of H chain CDR3-binding properties to the patient's egg sensitivity, for the cyclic peptide CP-1 was shown to inhibit serum IgE binding to ovomucoid. This suggests that the patient's serum IgE Abs target the same epitopes as those that can be bound by the Fab-derived cyclic peptide.

No monoreactive IgE Fabs were isolated from this study, but their presence in the repertoire of this allergic patient cannot be fully excluded. A possible explanation as to why these Abs were not isolated could be due to competition for Ag during panning. Monoreactive IgE, if present in the library, could possibly have been out-competed by the large number of potentially polyreactive Abs. It is also possible that the conditions used in the panning of this library favored polyreactive rather than monoreactive Abs, and more stringent conditions may be needed to isolate high affinity monoreactive IgE.

The results presented in this study raise interesting issues regarding B cell ontogeny and the genesis of the IgE repertoire. However, it may also have important implications for the sensitization of mast cells and basophils and the etiology of allergy. Mast cell and basophil degranulation is caused by FcεR1 aggregation that occurs when cell-bound IgE binds to allergen. The interaction between surface IgE and allergen is the key to unleashing the mast cell arsenal of proinflammatory mediators. It has been shown in vitro, using the rat basophil leukemia cell line, that degranulation is dependent upon the valency of the Ag (65, 66). If polyreactive IgE Abs exist, FcεR1 aggregation of mast cell- and basophil-bound IgE could more easily occur. A much greater range of both self and nonself Ags could also be responsible for degranulation. Polyreactive IgE may therefore help explain why patients suffering from certain allergies, such as atopic dermatitis, are often reactive to a wide range of allergens. Further analysis of additional patients is now needed to clarify the existence of polyreactive IgE Abs, the extent to which they contribute to IgE production in different allergic disorders, and the source of the B cell clones that produce them.

Acknowledgments

We are grateful to Dave Wanigesekera and Dr. David Coomber for their expertise with phage display techniques and analysis of mutations, and Dr. Michelle Clark for additional advice regarding phage display techniques.

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