Molecular Evolution of the Human Immunoglobulin E Response: High Incidence of Shared Mutations and Clonal Relatedness among ε V_H5 Transcripts from Three Unrelated Patients with Atopic Dermatitis

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Summary

We have analyzed the nucleotide sequences of 19 ε V_H5 transcripts derived from in vivo isotype switched peripheral blood B cells of three patients with atopic dermatitis. Comparison with the patients' own germline V_H5 gene segments revealed that the ε transcripts were derived from both functional members of the human V_H5 gene family and harbored numerous somatic mutations (range 5–36 per V_H5 gene). In two patients, we detected clonally related but diverged transcripts, permitting the construction of a genealogical tree in one patient. We observed a high proportion of shared silent (S) and replacement (R) mutations among ε V_H5 sequences derived from all three individuals, even among transcripts descending from the two different germline V_H5 gene segments. A remarkably high number of these mutations is shared with previously reported V_H5 genes encoding antibodies with defined specificities. The shared S mutations, and likely a fraction of the R mutations, appear to mark preferential sites ("hot spots") of somatic hypermutations in human V_H5 genes. The distribution of R and S mutations over complementarity determining region and framework regions in the majority of V_H regions deviated from that characteristic of antigen-driven immune response. We hypothesize that the V regions of immunoglobulin E-bearing B cells have accumulated "selectively neutral" mutations over extended periods of clonal expansion, resulting in unusual R/S ratios. We propose that the molecular characteristics of the ε V_H regions in atopic dermatitis may be representative of antigens that recurrently or chronically stimulate the immune system.

Atopic dermatitis is an inflammatory skin disorder characterized by a chronically relapsing course with frequent exacerbations and a distinctive clinical morphology. Although the primary cause of atopic dermatitis is unknown, substantial evidence suggests that excessive production of IgE antibodies likely contributes to the pathogenesis of this disorder (1, 2). These antibodies are reactive with a wide variety of environmental allergens. The regulatory effects of allergen-reactive T lymphocytes and their lymphokine products appear to be a major cause of the high serum levels of IgE in patients with atopic dermatitis (3–5).

Although the role of allergen-reactive T cells in controlling IgE class switching is beginning to be elucidated, little is known about the B lymphocytes that are recruited into the IgE response. In particular, questions related to the Ig gene segments encoding the V regions of IgE antibodies and the role of somatic hypermutation in the clonal evolution of IgE responses have not been addressed. Similar studies on the molecular origin of human (auto)antibodies have largely depended on hybridoma technology and EBV transformation to generate monoclonal B cell lines secreting relevant specificities. Attempts to immortalize B lymphocytes switched to IgE in vivo have generally been unsuccessful.

In the present study we have employed a cDNA/PCR approach to analyze the molecular structure of V regions expressed in IgE antibodies of atopic dermatitis patients. This strategy permitted the selective amplification of ε V_H region transcripts from RNA extracted from PBMC. Our data show that members of the V_H3–V_H6 gene families may be utilized in IgE antibodies from patients with atopic dermatitis. The small human V_H5 gene family, which contains only two functional members, was abundantly expressed in ε transcripts. Comparison of the nucleotide sequences of 19 ε V_H5 transcripts with the germline V_H5 genes of these patients revealed the accumulation of high numbers of somatic mutations. A surprisingly high proportion of silent (S)[1] and

[1] *Abbreviations used in this paper:* FR, framework; R, replacement mutation; S, silent mutation.

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replacement (R) mutations was shared among sequences derived from different members of the V_H5 gene family. In two patients, we detected evidence for transcripts derived from clonally related but diverged IgE-bearing B cells which, in one patient, permitted the construction of a genealogical tree. The results of these experiments are discussed in the context of the molecular mechanisms contributing to a clinically relevant IgE-mediated chronic immune response.

Materials and Methods

Patients. All patients analyzed suffered from severe atopic dermatitis according to the diagnostic criteria formulated by Hanifin et al. (6). Patient P1 is a female adult with a total serum IgE level of 18×10^3 kU/liter. Patient P2 is a 12-yr-old boy with a total serum IgE level of 5.3×10^3 kU/liter, and patient P3 is a 9-yr-old boy with an IgE serum level of 9.9×10^3 KU/liter. IgE in the serum of all three patients reacted with more than 15 different environmental allergens as detected in Radio Allergosorbent Test (RAST).

DNA and RNA Isolation. PBMC from atopic dermatitis patients were isolated by Ficoll-Hypaque density gradient centrifugation. Total cellular RNA was prepared from 2×10^6 PBMC using RNAzol™ (Cinna/biotecx, Friendwood, TX) as described previously (7). EBV-transformed polyclonal B cell lines were generated from 5×10^6 PBMC, and genomic DNA was isolated from 10^6 EBV-transformed B cells as detailed elsewhere (8).

First-strand cDNA Synthesis and PCR. Total RNA was used as a template for first-strand cDNA with an oligoprimer specific for the first exon of the ϵ constant region (Cϵ, 5′-TGTCCCGTTGAGGGAG CCTGT-3′) and 10 U of avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia, Uppsala, Sweden), according to standard procedures (7). One tenth of the cDNA reaction mixture was amplified in a PCR employing a previously described set of 5′ primers specific for the human V_H3, V_H4, V_H5, and V_H6 gene families (9), in combination with a second, nested 3′ Cϵ-specific primer containing a SalI restriction site (5′-GGGTCGACAGTCACGGAGGTGGCATT-3′). Genomic DNA was amplified with the 5′ V_H5-specific primer and a 3′ primer 100% homologous to sequences in the 3′ flanking region of both functional V_H5 genes (5′-GGGTCGACGGGCTCGGGGCTGG T-3 (10). All PCR reaction mixtures contained 200 ng of each primer and 2.5 U Taq polymerase (Promega Biotec, Madison, WI). Amplifications were performed in a Bioexcellence thermal cycler (Biores., Woerden, The Netherlands) and consisted of 35 cycles of 1 min denaturation at 94°C, 1.5 min primer annealing at 65°C, and 1.5 min extension at 72°C. After the last cycle, reaction mixtures were incubated for 10 min at 72°C to ensure complete extension of all products.

Cloning and Sequencing of PCR-amplified Material. PCR-amplified material was digested with restriction enzymes EcoRI and SalI (Pharmacia) and separated on a 0.8% low-melting agarose gel (Nusieve; FML Bioproducts, Rockland, ME). A fragment of ~450 bp was excised and ligated into Bluescript (Stratagene Inc., La Jolla, CA) using standard procedures (7). After transformation of competent $D_H5\alpha$ cells, colonies were transferred to nitrocellulose and screened with a ^{32}P-labeled V_H5 probe (11). DNA sequence analysis was according to Sanger et al. (12) using the T7 sequencing kit (Pharmacia).

Results

V_H Gene Family Utilization in ϵ V_H5 Transcripts from Atopic Dermatitis Patients. Total RNA extracted from PBMC of three patients with atopic dermatitis was extended with a Cϵ-specific primer and AMV reverse transcriptase, and first-strand cDNA was amplified by PCR using a nested Cϵ-specific primer in combination with 5′ primers specific for the human V_H3–V_H6 gene families. All four V_H primers yielded amplified PCR products of the expected size. The identity of PCR products was confirmed by hybridizing Southern blots of amplified material with ^{32}P-labeled V_H gene family-specific probes, indicating the V_H gene family specificity of all V_H primers used. The results demonstrated that ϵ transcripts encoded by members of the human V_H3–V_H5 gene families were present in three atopic dermatitis patients, whereas V_H6-encoded ϵ transcripts were found in patient P3 only (data not shown). We were unable to amplify ϵ V_H transcripts from PBMC of four nonatopic, healthy donors.

Molecular Analysis of Germline V_H5 Gene in Atopic Dermatitis Patients. In all three patients, we obtained abundant PCR products with the V_H5 gene family-specific primer. The human V_H5 gene family consists of only two functional members both of which are extremely well-conserved in the human population (10). We focused on this gene family to analyze the molecular structure of ϵ V_H5 gene transcripts. To that end, we first determined the nucleotide sequences of V_H5 genes present in the germline of these patients. Genomic DNA from polyclonal, EBV-transformed B lines was used as starting material in PCR reactions employing primers specific for the leader and 3′ end flanking sequences of both functional V_H5 genes. For each patient, a minimum of 10 bacterial colonies hybridizing to a V_H5 probe were randomly picked and used for nucleotide sequencing. In the genome of each patient, the two functional members of the V_H5 gene family were detected, and no other V_H family members or fragments were detected in this sequence analysis. In patients P1 and P2, both members displayed 100% sequence identity to the published germline genes (represented by the sequences of the V_H5-1R1 and V_H5-2R1 rearrangements [11]). In patient P3, we detected a single nucleotide difference in one of the V_H5-1R1 alleles (see legend to Fig. 1). Based on the large number of germline V_H5 genes sequenced, we estimate the PCR-induced error frequency to be <1/1200 in 35 cycles.

Inter- and Intraclonal Somatic Mutations in ϵ V_H5 Transcripts. To investigate the molecular structure of ϵ V_H5 regions, first-strand cDNA was synthesized from total RNA of PBMC and subsequently amplified in the PCR using a Cϵ and V_H5-specific primer pair. PCR-amplified material was cloned into Bluescript vector and after transformation, multiple V_H5-hybridizing colonies were used for nucleotide sequence analysis. All 19 nucleotide sequences contained open reading frames and were derived from ϵ V_H5 transcripts, confirming the specificity of the Cϵ and V_H5 primers. In Figs. 1 and 2, we have compared the nucleotide and deduced amino acid sequences of the expressed and germline V_H5 genes of patients P1, P2, and P3. Table 1 summarizes the distribution of R and S mutations over framework and CDR regions. In this analysis, we excluded somatic mutations occurring in the junctional region, because the patient's germ-

line diversity (D_H) and joining (J_H) gene segments were not available for comparison.

cDNA clones VHP2-57 and VHP1-58 derive from the germline V_H5-2R1 gene, whereas the other 17 cDNA clones descend from the germline V_H5-1R1 gene. All ϵ V_H5 sequences contain somatic mutations, ranging from 6 to 37 in the V_H portion of individual transcripts. Close inspection of sequences from patient P2 reveals that nucleotide sequences P2-51, P2-52, P2-53, and P2-54 are not identical (between 95.7 and 99.2% sequence homology) but utilize the same D_H and J_H gene segments and are indistinguishable at the V_H/D_H and D_H/J_H junctions (Fig. 1). This strongly suggests that the corresponding B cell clones are the progeny of a common precursor (13). By the same criteria, the collection of eight sequences from patient P1 contains two clonally related IgE transcripts, P1-54 and P1-57, that differ by four nucleotides in the V_H segment (Fig. 1).

Comparison of the nucleotide sequences of all ϵ V_H5 transcripts revealed that numerous mutations are shared among clonally related and unrelated sequences within one individual and among individuals. These shared mutations are present in both CDR and framework (FR) regions and are silent or result in amino acid replacements. Among the most extreme examples are mutations that occur in multiple independent ϵ transcripts derived from all three donors (e.g., compare the C→T substitution at position 173, the G→A at position 230, and the C→T substitution at position 180). It is notable that these mutations involve descendants from both the V_H5-1R1 and V_H5-2R1 germline gene segments.

Based on the four clonally related ϵ V_H5 sequences from patient P2, we constructed a genealogical tree that shows the nature and reconstructs the likely order of somatic mutations that occurred during expansion of this B cell clone (Fig. 3). The tree has been constructed to require the lowest number of parallel mutations. A total of 14 mutations (six R and seven S mutations) is shared by all four sequences. At the first branchpoint, four mutations are shared by clones P2-52 and P2-53 (three R and one S mutation). These clones differ from each other by four R and one S mutation. Similarly, clone P2-54 and P2-51 share four R and two S mutations and differ from each other by one S and one R mutation.

D_H and J_H Gene Utilization. All J_H gene segments except J_H2 were present in the ϵ transcripts. The D_H-gene segments were defined as the nucleotide sequences between the V_H- and J_H-genes, and include putative N insertions. The D_H segments varied extensively in nucleotide sequence and length and displayed partial sequence homology with previously published germline or expressed D_H elements (Fig. 4).

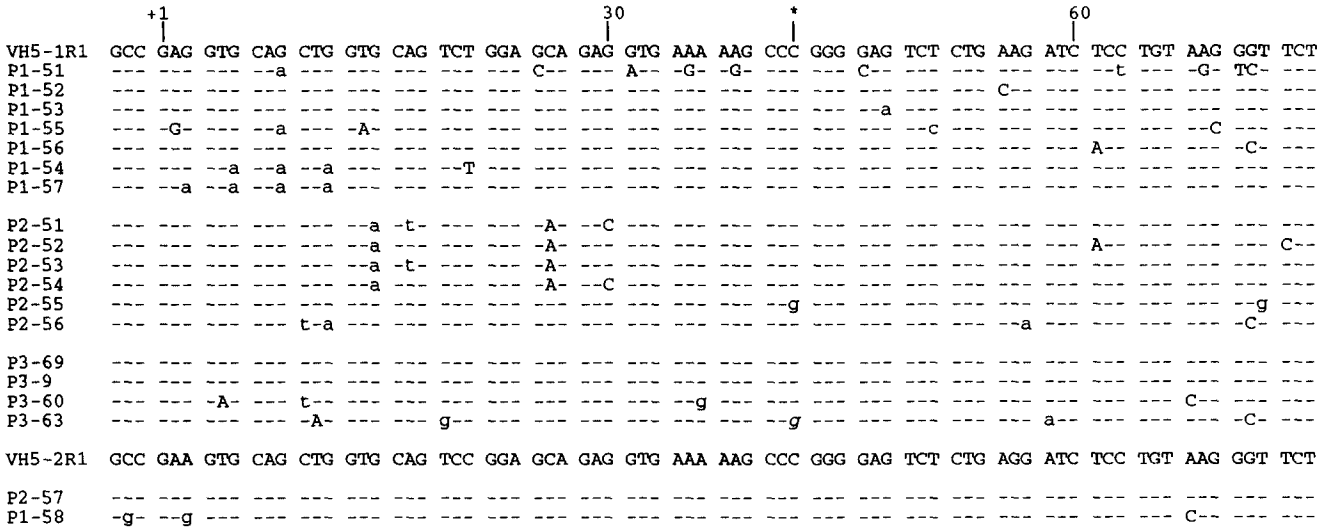
Discussion

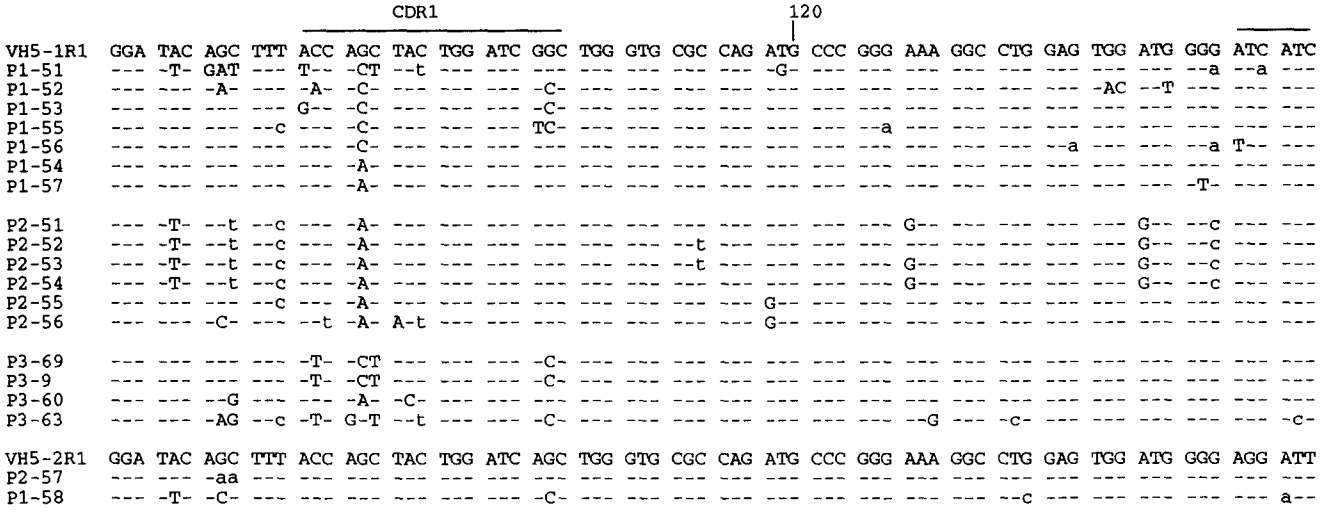
Antibody diversity is generated through a number of mechanisms including somatic assembly and imprecise joining of multiple gene segments, combinatory assortment of different Ig H and L chains, and somatic hypermutation. Although the genetic mechanisms of antibody diversity are fairly well understood, little is known about their contribution to the human antibody response to clinically relevant exogenous antigens. We have analyzed Ig ϵ V_H gene transcripts from peripheral blood B cells of three patients with atopic dermatitis. The sera of these patients contained IgE antibodies reactive with at least 15 different environmental allergens. Our inability to amplify ϵ transcripts from peripheral blood B cells of nonatopic individuals strongly suggests that these ϵ V_H transcripts were relevant to the allergic immune response. The results demonstrate that members of all human V_H gene

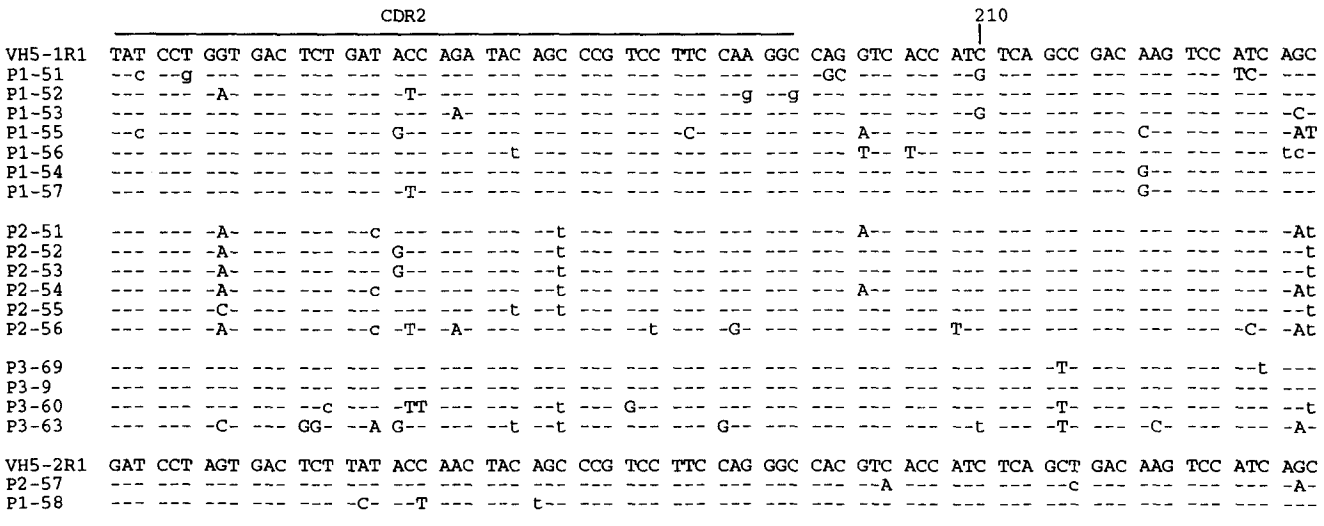
Table 1. *Distribution of Replacement and Silent Mutations in the V_H5 Regions of ϵ Transcripts from three Patients with Atopic Dermatitis*

Sequence	Total no. mutations	Overall R/S	CDR R/S	FR R/S	Sequence	Total no. mutations	Overall R/S	CDR R/S	FR R/S
P1-51	36	2.2	0.6	3.3	P2-51	23	1.1	1	1.1
P1-52	18	2.0	2.0	2.0	P2-52	20	1.2	3	1.0
P1-53	9	8.0	≥3.0	5.0	P2-53	19	0.9	3	0.7
P1-54	7	0.7	≥1.0	≥0.2	P2-54	21	1.0	1	1.1
P1-55	23	2.8	5.0	2.4	P2-55	11	0.8	1	0.8
P1-56	14	1.8	2.0	1.7	P2-56	32	1.6	2	1.5
P1-57	8	1.0	≥2.0	0.5	P2-57	6	1.0	–	1.0
P1-58	17	2.4	1.5	3.0					
					P3-9	6	5.0	≥4.0	1.0
					P3-60	14	1.8	2.5	1.3
					P3-63	36	2.6	2.5	2.7
					P3-69	8	1.7	≥4.0	0.3

For each ϵ transcript, the total number of mutations (columns 2 and 7), the R/S ratio in the overall V_H5 region (columns 3 and 8), the CDR region (columns 4 and 9) and the FR regions (columns 5 and 10) are depicted.


+1
30
*
60
VH5-1R1 GCC GAG GTG CAG CTG GTG CAG TCT GGA GCA GAG GTG AAA AAG CCC GGG GAG TCT CTG AAG ATC TCC TGT AAG GGT TCT
P1-51 --- --- --- --a --- --- --- --- --- C-- --- A-- -G- -G- --- --- C-- --- --- --- --- --t --- -G- TC- ---
P1-52 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- ---
P1-53 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --a --- --- --- --- --- --- --- ---
P1-55 --- -G- --- --a --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --- --- --- --- --C --- ---
P1-56 --- A-- --- --- -C- ---
P1-54 --- --- --a --a --a --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
P1-57 --- --a --a --a --a ---
P2-51 --- --- --- --- --- --- --a -t- --- --- -A- --C --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
P2-52 --- --- --- --- --- --- --a --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --- C--
P2-53 --- --- --- --- --- --- --a -t- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
P2-54 --- --- --- --- --- --- --a --- --- --- -A- --C --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
P2-55 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --g --- --- --- --- --- --- --- --g ---
P2-56 --- --- --- --- t-a --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --a --- --- --- -C- ---
P3-69 ---
P3-9 ---
P3-60 --- --- -A- --- t-- --- --- --- --- --- --- --- --- --g --- --- --- --- --- --- --- --- --- --- C-- --- ---
P3-63 --- --- --- --- -A- --- --- g-- --- --- --- --- --- --- --- --g --- --- --- --- --- a-- --- --- --- -C- ---
VH5-2R1 GCC GAA GTG CAG CTG GTG CAG TCC GGA GCA GAG GTG AAA AAG CCC GGG GAG TCT CTG AGG ATC TCC TGT AAG GGT TCT
P2-57 ---
P1-58 -g- --g --- C-- --- ---


CDR1
120
VH5-1R1 GGA TAC AGC TTT ACC AGC TAC TGG ATC GGC TGG GTG CGC CAG ATG CCC GGG AAA GGC CTG GAG TGG ATG GGG ATC ATC
P1-51 --- -T- GAT --- T-- -CT --t --- --- --- --- --- --- --- --- -G- --- --- --- --- --- --- --- --- --a --a ---
P1-52 --- --- -A- --- -A- -C- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- -AC --T --- --- ---
P1-53 --- --- --- --- G-- -C- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
P1-55 --- --- --- --c --- -C- --- --- --- TC- --- --- --- --- --- --- --- --a --- --- --- --- --- --- --- ---
P1-56 --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --a --- --- --a T-- ---
P1-54 --- --- --- --- --- -A- ---
P1-57 --- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- ---
P2-51 --- -T- --t --c --- -A- --- --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- G-- --c --- ---
P2-52 --- -T- --t --c --- -A- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- G-- --c --- ---
P2-53 --- -T- --t --c --- -A- --- --- --- --- --- --- --t --- --- --- --- G-- --- --- --- --- G-- --c --- ---
P2-54 --- -T- --t --c --- -A- --- --- --- --- --- --- --- --- --- --- --- G-- --- --- --- --- G-- --c --- ---
P2-55 --- --- --- --c --- -A- --- --- --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- ---
P2-56 --- --- -C- --- --t -A- A-t --- --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- ---
P3-69 --- --- --- --- -T- -CT --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
P3-9 --- --- --- --- -T- -CT --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
P3-60 --- --- --G --- --- -A- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
P3-63 --- --- -AG --c -T- G-T --t --- --- -C- --- --- --- --- --- --- --- --G --- -c- --- --- --- --- --- -c-
VH5-2R1 GGA TAC AGC TTT ACC AGC TAC TGG ATC AGC TGG GTG CGC CAG ATG CCC GGG AAA GGC CTG GAG TGG ATG GGG AGG ATT
P2-57 --- --- -aa ---
P1-58 --- -T- -C- --- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --c --- --- --- --- --- a--


CDR2
210
VH5-1R1 TAT CCT GGT GAC TCT GAT ACC AGA TAC AGC CCG TCC TTC CAA GGC CAG GTC ACC ATC TCA GCC GAC AAG TCC ATC AGC
P1-51 --c --g --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -GC --- --- --- --G --- --- --- --- --- --- TC- ---
P1-52 --- --- -A- --- --- --- -T- --- --- --- --- --- --- --- --g --g --- --- --- --- --- --- --- --- --- --- ---
P1-53 --- --- --- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --G --- --- --- --- --- --- -C-
P1-55 --c --- --- --- --- --- --- G-- --- --- --- --- --- --- -C- --- --- --- --- A-- --- --- --- --- C-- --- --- -AT
P1-56 --- --- --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- T-- T-- --- --- --- --- --- --- --- tc-
P1-54 --- G-- --- --- ---
P1-57 --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- G-- --- --- ---
P2-51 --- --- -A- --- --- --c --- --- --- --t --- --- --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- -At
P2-52 --- --- -A- --- --- --- G-- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --t
P2-53 --- --- -A- --- --- --- G-- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --t
P2-54 --- --- -A- --- --- --c --- --- --- --t --- --- --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- -At
P2-55 --- --- -C- --- --- --- --- --- --t --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --t
P2-56 --- --- -A- --- --- --c -T- -A- --- --- --- --t --- -G- --- --- --- --- T-- --- --- --- --- --- --- -C- -At
P3-69 --- -T- --- --- --- --t ---
P3-9 ---
P3-60 --- --- --- --- --c --- -TT --- --- --t --- G-- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --t
P3-63 --- --- -C- --- GG- --A G-- --- --t --t --- --- --- G-- --- --- --- --- --t --- -T- --- -C- --- --- -A-
VH5-2R1 GAT CCT AGT GAC TCT TAT ACC AAC TAC AGC CCG TCC TTC CAG GGC CAC GTC ACC ATC TCA GCT GAC AAG TCC ATC AGC
P2-57 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --c --- --- --- --- -A-
P1-58 --- --- --- --- --- -C- --T --- --- t-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

```
                240                                     270                                               CDR3
                 |                                       |                                        ____________________________
VH5-1R1   ACC GCC TAC CTG CAG TGG AGC AGC CTG AAG GCC TCG GAC ACC GCC ATG TAT TAC TGT GCG AGA
P1-51     G-- --- --t t-a --- --- -C- --- --- --- --- --- --- --- --- --A --- -T- --c --- --- AAA GTC GCA CCA GCC
P1-52     --- --- --t t-- --- --- --- G-a --- --- --- --- --- --- --- --- -T- --t --- --- --- CTG AAG GGG AAA ATA
P1-53     --- --- --- --- --- --- -C- --- --- --- --- C-- --- --- --- --- --- --- --- --- --- CAG GGA CGG GTC CGG
P1-55     --- -T- --- --- --- --- --- --t --- G-- --- --- --- --- --- -CT --- -T- --- --- --- CAA TTA GTT CCA GAT
P1-56     --- --- --- --- --- --- -A- --- --- -C- --- --- --- --- --- --- --- -T- --- --- --- CGT GAT GAC GAC CAG
P1-54     --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- CGC GAC TAC GGT GAC
P1-57     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- CGC GAC TAC GGT GAC

P2-51     --- --- --- --- --- --- --- -A- --- --- --- --t --- -G- --t --- --- --t --- A-- --- CCG AGG GGG TCG GGG
P2-52     -G- --- --- --- --- --- --- --- --- -G- --- --- --- -G- --t --- --- --t --- --- --- CCG AGG GGG TCG GGG
P2-53     -G- --- --- --- --- --- --- --- --- --- --- --- --- -G- --t --- --- --t --- --- --- CCG AGG GGG TCG GGG
P2-54     --- --- --- --- --- --- --- -A- --- --- --- --t --- -G- --t --- --- --t --- --- --- CCG AGG GGG TCG GGG
P2-55     --- --- --- --- --- --- --- --- --- -G- --- --- --- --- --- --C --- --- --- --- --- CAT GGC CTT CAT GGG
P2-56     --t T-- --- --a --T --- -A- --- --- -G- --- --- --- --- -G- --T -Tc -T- --- --- --g CTC GGT GAC AGC AGC

P3-69     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --t --- --a --- CAT GGA ATG GAA TAT
P3-9      --- --- --- t-- --- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- CAT GCC GAT AAT TTT
P3-60     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ACA CAT CGA GGT GGG
P3-63     --- --- -T- --- --C --- --- -C- --- --- --- --- --- --- --- -CA --- -Tt C-- -T- --- CAT CGG AGA AAT GGC

VH5-2R1   ACT GCC TAC CTG CAG TGG AGC AGC CTG AAG GCC TCG GAC ACC GCC ATG TAT TAC TGT GCG AGA
P2-57     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- --- --- CAT CAA ATG TAT AGC
P1-58     --- -TG --- --- --- --- G-- --- --- GC- --- --- --- --- --- --- --- -T- --- --- --- GTC GGG TAT TGT AGT


                                    CDR3
          ____________________________________________________________
JH3                                                                 GCT TTT GAT GTC TGG GGC CAA GGG ACA ATG
P1-51     AAA GTG CAC GGC GAC                                       TAC --- --C TA- --- --- --G --C --C C--
P1-52     GCT ATT ATC TT                                              - --- --C TT- --- --- --G --A --C C--
P1-53     TTG GTC TAC GGA ATC TGG AC                                     -- --C TA- --- --- --G --A --C C--
P1-55     GCT CTC GAG ATG GGC                                           --- --C TA- --- --- --G --A --C C--
P1-56     CTC CTA ATG                                                       --C TA- --- --- --G --A --C C--
P1-54     TAC CAA TCC ACG GGG GGC                                       --C --C CC- --- --- --G --- --C T--
P1-57     TAC CAA TCC ACG GGG GGC                                       --C --C CC- --- --- --G --- --C T--

P2-51     ACT TAT TAT AGT GG                                          - --- --- AC- --- --- --- --- --- ---
P2-52     ACT TAT TAT ACT GG                                          - --- --- A-- --- --- --- --- --- ---
P2-53     ACT TAT TAT ACT GG                                          - --- --- A-- --- --- --- --- --- ---
P2-54     ACT TAT TAT ACT GG                                          - --- --- A-- --- --- --- --- --- ---
P2-55     ACC TCC TTC TCA AGC TG                                      G --C --- T-- --- --- -GG --A --C C--
P2-56     AGA TAC TTC CGT GAT CCC                                       --- --C A-- --- --- --- --- --- ---

P3-69     TAT TAT GGT TCG GGG AGT TCT GAC TAC TAC TAT TAC           TAC A-G --C --- --- --- A-- --- --C -C-
P3-9      GAC                                                       TGG --C C-A A-- --- --- --G --A --C C--
P3-60     ACC AAC TAC                                           GAG TAC --C C-A --- --- --- --G --C --C C-T
P3-63     CAC AGT TCC GCT GGG TGG TAT TAT CAG                       -G- A-G --- --- --- --- --- --- --C -C-

P2-57     AAC TCG                                                           --C TA- --- --- --G --A --C C-A
P1-58     GGT GGT ACC TGC TAT TCC GAA GCT AAA TCG GGG                   --- --C TA- --- --- --G --A --C C--


                                      —Cε—
          GTC ACC GTC TCT TCA    GCC TCC
P1-51     --- --- --- --C --C    --- --- JH4
P1-52     --- --- --- --C ---    --- --- JH4
P1-53     --- --- --- --C ---    --- --- JH4
P1-55     --- --- --- --C --C    --- --- JH4
P1-56     --- --- --- --C ---    --- --- JH4
P1-54     --- --- --- --C --G    --- --- JH5
P1-57     --- --- --- --C --G    --- --- JH5

P2-51     --- --- --- --- ---    --- --- JH3
P2-52     --- --- --- --- ---    --- --- JH3
P2-53     --- --- --- --- ---    --- --- JH3
P2-54     --- --- --- --- ---    --- --- JH3
P2-55     --- --- --- C-C ---    --- --- JH5
P2-56     --- -T- --- --- G--    --- --- JH3

P3-69     --- --- --- --C ---    --- --- JH6
P3-9      --- -T- --- --C ---    --- --- JH4
P3-60     --- --- --- --C ---    --- --- JH1
P3-63     --- --- --- --A C--    --- --- JH4

P2-57     C-- --- --- --C ---    --- --- JH5
P1-58     --- --- --- --C ---    --- --- JH4
```

Figure 1. Nucleotide sequences of ϵ V_H5 transcripts from atopic dermatitis patients P1, P2, and P3. Nucleotide sequences are compared to the rearranged VH5-1R1 and VH5-1R2 genes that represent the two functional V_H5 genes in germline configuration (T. Logtenberg, unpublished data). The nucleotide sequences of the corresponding germline V_H5 gene segments of patients P1, P2, and P3 were 100% identical, except for a single difference at position 42 in one of the alleles of patient P3 (* and *g*). (*Dashes*) Nucleotide identity. The J_H gene segments are aligned with the germline J_H3 gene (35). Upper and lower case letters refer to R and S mutations, respectively.

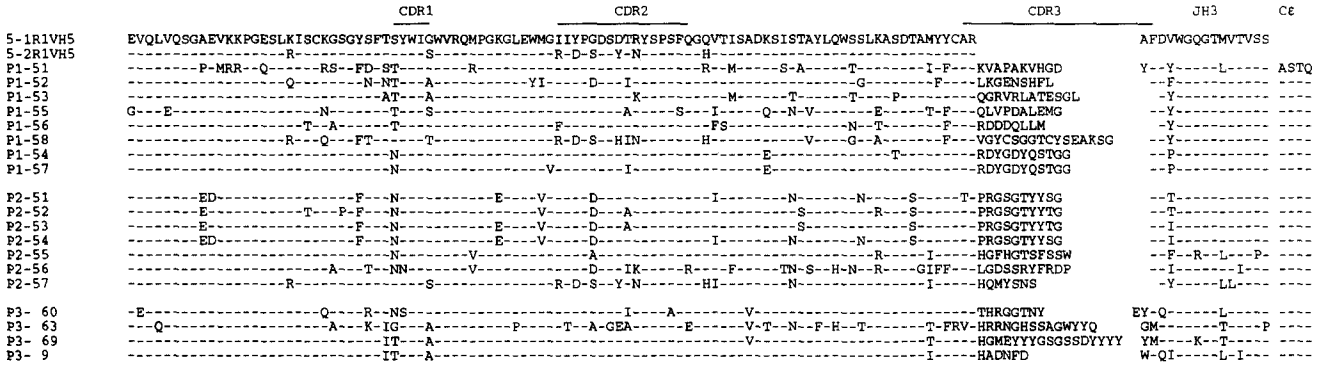


Figure 2. Deduced amino acid sequences of ε V_H5 transcripts. Single-letter amino acid code is used. (*Dashes*) Identity of amino acid residue.

families tested (V_H3–V_H6) may be utilized in the IgE response in atopic dermatitis patients. In PCR experiments, we noted that the small V_H5 gene family, consisting of two functional members, was abundantly expressed in ε transcripts from all three patients, whereas for example V_H6, a family of comparable size, was not detectable in two patients, and generated a scarcely detectable PCR fragment in the third patient.

Nucleotide sequence analysis of the V_H5 genes present in the genome of the atopic dermatitis patients permitted the unequivocal assignment of somatic mutations in the ε V_H5 transcripts. In 19 transcripts analyzed, we detected a total of 328 nucleotide differences (range 5–36 per V_H segment) with the patient's own germline V_H5 genes, including both S and R mutations. Based on a PCR-related error frequency of <1/1,200, virtually all nucleotide differences represent somatic mutations acquired during in vivo growth and differentiation of the corresponding B cells. The distribution and types of mutations in the V regions of a B cell clone have been suggested to reflect the nature of the selective forces causing its expansion (14, 15). In the absence of selection and under the restraint of preserving the Ig molecule's structure, R/S ratios have been estimated to be 2.9 in the CDR and 1.5 in the FR regions (13). R/S ratios in the CDR regions that are substantially higher than 2.9 have been interpreted to reflect a process of positive selection by antigen. The R/S ratios in the CDR and FR regions of the ε V_H5 transcripts do not exhibit a consistent pattern (Table 1). In fact, all combinations of high and low R/S ratios in FR regions with high and low R/S ratios in CDR regions occur (Table 1). Although this appears unusual for an antigen-driven selection process, several notions argue in favor of a role for antigen. Activation of the somatic hypermutation mechanism is associated with antigen stimulation and does not occur in polyclonally stimulated B cells (16, 17). Moreover, the allergen specificity of the IgE antibodies in the sera of these patients suggests an important role for allergen. Finally, the occurrence of clonally related B cells, as observed in two out of three patients, is characteristic for an antigen-driven selec-

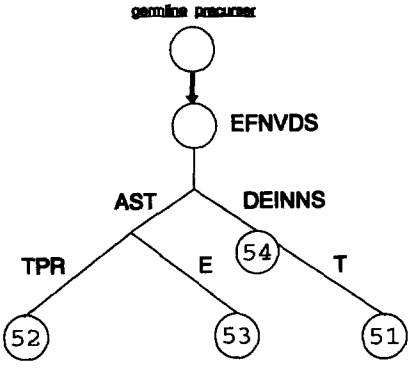


Figure 3. Genealogical tree of clonally related transcripts from patient P2. This tree was constructed to require the fewest independent parallel replacements. Single-letter amino acid code is used to denote R mutations.

Figure 4. Alignment of nucleotide sequences of D_H gene segments utilized in ε transcripts and reported germline D_H elements. The D_H regions of P1-51, P1-53, and P2-55 could not be reliably matched with published germline D_H segments. (*Dashes*) Nucleotide identity.

tion process. Several explanations, not mutually exclusive, for the heterogeneous R/S patterns in ϵ V_H5 transcripts are conceivable. The FR regions, especially FR3, may contribute to antigen binding, as has been argued on the basis of experimental data and theoretical considerations (18–21). In this respect, it is important to realize that antigen determines the shape of the antigen-binding portion of V regions and that current concepts concerning R/S distributions have largely been derived from analysis involving immunization with haptenic groups. The antigen combining site of hapten-binding antibodies may differ profoundly from that of protein-binding antibodies. Examples of nonhaptenic immune responses generating high affinity antibodies with "aberrant" R/S mutations have been reported in the literature (22–24). An alternative interpretation is an extension of a model proposed by Manser (25). It may be envisaged that initially, somatic mutations are efficiently selected by antigen resulting in affinity maturation of the immune response. In case of recurrent or prolonged antigenic stimulation, the extent to which somatic mutations lead to higher binding affinity in an already selected B cell clone becomes limited and finally saturated. At this point, persistent antigenic stimulation may result in the accumulation of "neutral" R and S mutations, i.e., mutations that do not affect the affinity or the structural integrity of the Ig molecule. Such mutations are not selected for or against, and may shift the R/S ratio towards a pattern resembling random mutations. It may be envisaged that such a pattern of mutations of V regions is associated with antigens that chronically and/or recurrently stimulate the immune system, as is the case with allergens and perhaps particular autoantigens (22). In the above scenario, mutations continue to occur in secondary and higher order immune responses as a result of repeated or chronic exposure to the same antigen.

Four out of seven ϵ V_H5 sequences from patient P2 were clonally related as judged by their junctional regions. This set of sequences contained, in addition to many shared somatic mutations, at least three unique mutations. These numbers represent a minimum estimate because somatic mutations in the D_H and J_H segments were not included in our analysis. We constructed a genealogical tree based on the premise that the most shared mutations were acquired early, and the least shared mutations were acquired late in the evolution of the clone. Indeed, the general pattern of mutations appears to reflect the temporal order of somatic mutations acquired during expansion of the clone (17, 26). A small number of shared mutations in the clonally related set of ϵ transcripts likely arose as a result of parallel events (i.e., the A→T at position 17 and the A→G at position 127; Fig. 1).

It has been previously reported that the V regions of murine hybridomas secreting monoclonal antihapten antibodies share somatic mutations, even among hybridomas from different mice and generated in different laboratories (27, 28). In addition, shared mutations have been noted in hybridomas carrying "passenger" transgenes that are not functionally expressed (29, 30). Shared R mutations have been interpreted to reflect the influence of antigen selection conferring proliferative advantage to the B cell clone, whereas shared S mutations may mark positions that are especially prone to somatic mutation, so-called hot spots (28, 31). In the collection of 19 ϵ V_H5 transcripts, we found a remarkably high incidence of shared mutations, even among transcripts derived from different germline members of the V_H5 gene family. The shared mutations were distributed over CDR and FR regions and included both S and R mutations. In the current analysis, it can be excluded that these mutations represent artifacts introduced, for example, as a consequence of cell fusion or in vitro growth of hybridomas. A high number of mutations in the collection of ϵ V_H5 transcripts was also found in two V_H5 expressed genes reported by Andris et al. (32). Thus, out of 17 mutations present in a V_H5 gene encoding an anti-HIV antibody, 12 are also present in the same position in at least one but often multiple ϵ V_H5 transcripts, seven of which constitute identical substitutions (32). Similarly, the majority of mutations present in the V_H5 genes encoding an antiinsulin autoantibody and an antirabies virus antiidiotypic antibody also appear in multiple independent ϵ V_H5 transcripts (33). Thus, the seemingly unprobable event of isolating multiple parallel somatic alterations in V_H transcripts from unrelated individuals and in antibodies with different specificities constitutes, in fact, a frequently occurring phenomenon.

The molecular basis for the high incidence of shared mutations remains to be elucidated. Based on their diversity, multitude, and distribution pattern in the collection of ϵ V_H5 transcripts, it seems unlikely that a somatic gene conversion mechanism, even when considering multiple segmental exchanges, is a major contributor (34). Rather, the accumulation of point mutations, perhaps in combination with intrinsic sequence specificity of the somatic hypermutation mechanism, constitutes the mechanism underlying shared mutations (30). In addition, selection by antigen probably contributes to the occurrence of shared R mutations. Although we have not addressed the antigen specificity of the IgE antibodies encoded by these transcripts, it seems unlikely that they all bind to the same epitope.

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