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Local Somatic Hypermutation and Class Switch Recombination in the Nasal Mucosa of Allergic Rhinitis Patients

Heather A. Coker,1,* Stephen R. Durham, † and Hannah J. Gould 2 *

Immunoglobulin E is produced by nasal B cells in response to allergen. We have analyzed IgE V H region sequences expressed in the nasal mucosa of patients suffering from allergic rhinitis. V H region sequences were amplified by RT-PCR from IgE+ B cells from nasal biopsies. In two of six patients, sequence analysis clearly demonstrated the presence of closely related IgE+ B cell clones: cells displaying identical signature regions across CDR3/FWR4, indicating a common clonal ancestry, but a mixture of shared and diverse somatic mutations across the V H region. Furthermore, in one of the two patients exhibiting related IgE+ B cell clones, five IgA+ B cell clones, related to the IgE+ B cell family, were also isolated from the patient’s nasal mucosa. This evidence, combined with the local expression of mRNA transcripts encoding activation-induced cytidine deaminase, suggests that local somatic hypermutation, clonal expansion, and class switch recombination occur within the nasal mucosa of allergic rhinitics. The presence of related B cells in the nasal mucosa does not appear to result from the random migration of IgE+ cells from the systemic pool, as analysis of a nonatopic subject with highly elevated serum IgE did not exhibit any detectable V H-Ce transcripts in the nasal mucosa. We have provided evidence that suggests for the first time that the nasal mucosa of allergic rhinitics is an active site for local somatic hypermutation, clonal expansion, and class switch recombination, making it of major significance for the targeting of future therapies. The Journal of Immunology, 2003, 171: 5602–5610.

Allergic reactions occur mainly in the mucosal tissues of the respiratory tract, gut, and skin. Susceptibility at these sites is due to the presence of mast cells bearing the high affinity IgE receptor, FcεRI, sensitized by Abs of the IgE class. Cross-linking of the receptors by allergen binding to IgE Abs triggers immediate hypersensitivity. Mast cells in the nasal mucosa of patients with allergic rhinitis are sensitized by IgE Abs that are produced locally by resident plasma cells (1). In an allergic individual, local IgE production persists for long periods in the absence of allergen, enabling an immediate response upon re-exposure to allergen. However, little is known about the history of the IgE-producing B cells, in particular when and where the precursor cells underwent class switch recombination (CSR)3 to IgE (involving rearrangement of the C region genes encoding the various Ab classes, e.g., Cμ to Cε) and affinity maturation by somatic hypermutation (SHM).

Both SHM and CSR are stimulated by Ag in the germinal centers of lymphoid tissue (2), but it is becoming increasingly apparent that these processes may also occur locally at sites of chronic or recurrent Ag stimulation, as has been clearly demonstrated for rheumatoid arthritis (3, 4). In the local mucosal environment, signals to initiate SHM and CSR are available (5, 6), as in vitro studies have demonstrated that, in the presence of Ag, T cells have the ability to induce SHM and CSR by the production of cytokines (such as IL-4) and also their interaction via CD40:CD40-ligand and CD80:CD28 with B cells (7). Evidence suggesting local CSR has been presented for IgA in the murine gut mucosa (8), for IgE in the human nasal mucosa of allergic rhinitics (9–11), and in the human lung mucosa in allergic asthma (12, 13). Evidence for local SHM has been presented for IgE in the lung mucosa of an allergic asthmatic patient (13). We have examined the evidence for local SHM and CSR in six patients with allergic rhinitis.

SHM results from the stepwise accumulation of predominantly single nucleotide substitutions into the V region DNA. This stepwise accumulation of mutations enables the genealogy of a B cell to be traced, relying on the unique complementarity-determining region 3/framework region 4 (CDR3/FWR4) clonal signature generated by the VDJ recombination of the progenitor cell. It is estimated that mutations are introduced at a rate of 10–4–10–3 per base pair per generation (14). A single mutation may bring about as much as a 10-fold increase in Ab affinity (14, 15). Mutations are introduced at a high level across the CDRs, but additionally the RGYW motif (in which R = A or G, Y = C or T, W = A or T, and G is mutated) or the reverse complement WRCY is a frequent target of mutation, particularly at the serine codons AGC and AGT (16). The molecular mechanism of SHM has not been fully elucidated, although errors are thought to be introduced by an activation-induced cytidine deaminase (AID) and error-prone polymerase-dependent process (17–19). Several DNA polymerases have been suggested as candidates, including DNA polymerase η, κ, τ, and ζ (20–23). AID has also been shown to be required for CSR (17, 18).

We present evidence generated by RT-PCR and DNA sequencing of V H-Ce sequences from the nasal mucosa of allergic rhinitis patients. Our work is the first to demonstrate in two allergic rhinitis
patients the clear presence of related IgE+ B cell clones in the nasal mucosa, B cells that exhibit shared ancestry (judged by identical CDR3/FWR4 motifs), and both shared and diverse somatic mutations. In addition, detailed investigation enabled the detection of sequences from IgA- B cell clones from the nasal biopsy of one patient. These sequences exhibited shared ancestry and both shared and diverse somatic mutations with the related IgE+ B cell clones isolated from the same nasal biopsy sample. IgE V H-C θ region amplification from the nasal mucosa of a healthy nonatopic subject with high elevated serum IgE was negative, implying that the families of related clones seen in allergic patients were unlikely to have resulted from the random migration of IgE+ B cells from a systemic pool.

Furthermore, RT-PCR analysis demonstrated the presence of mRNA-encoding AID in the nasal mucosa in five of seven allergic rhinitis patients, the first reported instance of local AID expression in humans. We propose that local somatic hypermutation, clonal expansion, and class switch recombination take place in the nasal mucosa of allergic rhinitis patients. We suggest that this local activity is fundamental to the pathogenesis of allergic disease.

Materials and Methods

Samples from allergic rhinitis patients

Male and female donors between the ages of 18 and 55 were recruited from the Royal Brompton Hospital Allergy Clinic (London, U.K.) or by advertisement in the local press. None had received immunotherapy, and any medication was discontinued at least 2 wk before nasal biopsy. Biopsies were performed at the Royal Brompton Hospital with the approval of the local Ethics Committee and the patients’ written informed consent. The allergic status of the donors was assessed by medical history, skin-prick tests, and serum allergen-specific IgE (radioallergosorbent test [RAST]). Six patients who exhibited a total IgE of over 200 IU/ml were selected for the study of V<sub>H</sub>-C<sub>θ</sub> transcripts. Biopsies were taken according to a previous detailed protocol (24) in which a 2.5-mm<sup>2</sup> biopsy was randomly taken from the undersurface of the inferior turbinate, behind the anterior insertion into the lateral wall of the nose and 8–10 cm distal to the nearest lymphoid tissue, Waldeyer’s ring in the pharynx. The random nature of the biopsies prevented the sampling of any defined cell populations from within the inferior turbinate. Biopsies were placed in 1.5 ml HBSS (Invitrogen, Paisley, U.K.) and washed to remove blood before being transferred to a cryo-tube and snap frozen in liquid N<sub>2</sub> before being stored at −70°C. A total of 25 ml blood was taken from each patient, and PBMC was isolated by Ficol-Paque density-gradient centrifugation. The PBMC pellet was snap frozen and stored at −70°C. The samples were treated to extract RNA and manufacture cDNA as for the tissue samples, with the exception that 1 ml RNAWIZ buffer was added to the PBMC pellet (4 × 10<sup>6</sup> cells) and the cells were resuspended by pipetting before dividing into four 250-μl aliquots, one of which was used for future molecular analysis.

Samples from the nonatopic subject

A healthy 55-year-old male (G.J.) was recruited with no previous medical history of any allergic symptoms and a negative skin-prick test and RAST for all common allergens (a slightly raised RAST of 0.71 IU/ml (normal 0.35 IU/ml) was detected for Aspergillus fumigatus). No other abnormal medical conditions were reported, although the subject’s total serum IgE was 1834 IU/ml. A nasal biopsy and PBMC sample were taken according to the protocol above.

Amplification of GAPDH from the samples used the following PCR method: 5 μl of cDNA was added to a 50 μl PCR, which included both a GAPDH forward and reverse primer each at 1 μM (GAPDH forward, 5′-ATTTGTTGCTATTGGCCGCTTGGTC-3′; GAPDH reverse, 5′-TCACTACTTTGCTATTGGCCGCTTGGTC-3′), dNTPs at 0.25 mM, and included 1.25 U PFU DNA polymerase (Promega, Madison, WI). The reaction was initially denatured at 95°C for 2 min, then subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 2 min before a final extension at 72°C for 10 min.

RNA extraction

Biopsies were transferred to an RNase-free tube and homogenized in 250 μl RNAWIZ buffer (Ambion, Austin, TX) with 300 vigorous pulses using an Eppendorf homogenizer (Anachem, Luton, U.K.). Total RNA was extracted from each sample, according to the manufacturer’s protocol, and resuspended in 20 μl RNase-free water. The concentration of RNA was determined from the absorbance at 260 nm.

RT-PCR of V<sub>H</sub>-Ce transcripts

RNA (5 μg) was included in a 40 μl cDNA reaction with Moloney murine leukemia virus reverse transcriptase (Inovotrypt) using an oligo(dT) primer (Inovotripe). A total of 5 μl of cDNA was added to a 50 μl PCR, which included each V<sub>H</sub> leader region class-specific primer (V<sub>H</sub>1L-V<sub>H</sub>6L) at 0.5 μM and the Cε-specific primer (Cε) at 0.5 μM, dNTPs at 0.25 mM, and included 1.25 U PFU DNA polymerase (Promega). The reaction was initially denatured at 95°C for 2 min, then subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 2 min before a final extension at 72°C for 10 min. A total of 5 μl of the first PCR was then transferred into a second nested PCR, which differed only in that it included V<sub>H</sub> class-specific primers homologous to FRW1 (V<sub>H</sub>1F-V<sub>H</sub>6F) and an internal Cε primer (Cε2). This reaction was incubated with an initial denaturation at 95°C for 2 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min, extension at 72°C for 2 min, then a final extension at 72°C for 10 min. The PCR for amplification of the separate V<sub>H</sub> classes of the V<sub>H</sub>-Ce transcripts in the PBMC of the nonatopic subject GJ29 used the same conditions as above, but using the appropriate V<sub>H</sub> primer in each reaction.

The primers used were as follows: V<sub>H</sub>1L, 5′-CACTGAGCTGAGCCCTGGGATCCTGGTC-3′; V<sub>H</sub>2L, 5′-CACTGAGCTGACATCTTTGCTACAC3′-3′; V<sub>H</sub>3L, 5′-CTGAGGTGAGCTGACCTGGGATCCTGGTC-3′; V<sub>H</sub>4L, 5′-CTGAGGTGACGCTGGGATCCTGGTC-3′; V<sub>H</sub>5L, 5′-CTGAGGTGACGCTGGGATCCTGGTC-3′; V<sub>H</sub>6L, 5′-CTGAGGTGACGCTGGGATCCTGGTC-3′. In the second nested PCR, the first Cε primer was V<sub>H</sub>1F, 5′-CTGAGGTGACGCTGGGATCCTGGTC-3′; V<sub>H</sub>2F, 5′-CTGAGGTGACGCTGGGATCCTGGTC-3′; V<sub>H</sub>3F, 5′-CTGAGGTGACGCTGGGATCCTGGTC-3′; V<sub>H</sub>4F, 5′-CTGAGGTGACGCTGGGATCCTGGTC-3′; V<sub>H</sub>5F, 5′-CTGAGGTGACGCTGGGATCCTGGTC-3′; V<sub>H</sub>6F, 5′-CTGAGGTGACGCTGGGATCCTGGTC-3′.

RT-PCR of V<sub>H</sub>-Ce μ, V<sub>H</sub>-Ce δ, and V<sub>H</sub>-Ce γ transcripts

PCR amplification of sequences from IgM<sup>+</sup>, IgG<sup>+</sup>, or IgA<sup>+</sup> B cells was conducted as separate reactions based on a multiple step PCR published previously (28, 29).

PCR 1

The initial nested PCR was conducted as for the amplification of V<sub>H</sub>-Ce, except that only the V<sub>H</sub>4 primers were used. The PCR for amplification of the separate V<sub>H</sub> classes of the V<sub>H</sub>-Ce transcripts was conducted as separate reactions based on a multiple step PCR published previously (28, 29).

PCR 2

Reaction 2 of the nested PCR for amplification of sequences from IgM<sup>+</sup>, IgG<sup>+</sup>, or IgA<sup>+</sup> cells used an inner V<sub>H</sub>5 primer, V<sub>H</sub>5Fm, with each of the inner Cε<sub>1</sub> primers (Cε1, Cε2, or Cε3) to further amplify the V<sub>H</sub>5-Cε<sub>1</sub> sequences. All other conditions remained the same as those specified above for IgE and the reaction denatured at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 15 min.

PCR 3

The PCR products from each second PCR were subjected to electrophoresis on a 1% agarose gel, and the DNA was excised. A total of 5 μl of the gel-extracted PCR product was carried forward into a third, semi-nested PCR for each isotype.

PCR 4

Reaction 3 was seminested, using the gel-extracted PCR 2 products with the inner end of each set of Cε<sub>1</sub> primers and a CDR3/FWR4 primer specific for each clonal family (e.g., B1605 specific for the SO16 V<sub>H</sub>5 family) to amplify from CDR3 to the 5′ of Cε. The conditions for PCR 3 were as detailed above, except the initial denaturation 95°C was for 2 min, then 20 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 15 min. PCR products were cloned and sequenced as below before proceeding to the following stage.
PCR 4

To obtain the V<sub>H</sub> region sequences from specific IgA clones, 5 μl of the gel-extracted PCR 2 products was used in the seminested reaction 4 with conditions as for the previous reactions, except for the use of the VHS5Fm primer and SO16A4 (specific for the D-J<sub>D</sub>-J<sub>H</sub> junction) with initial denaturation at 95°C for 2 min, then 20 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 15 min.

PCR 5

An additional experiment was conducted to reamplify the complete sequence of the V<sub>H</sub>5-IgA clone 1 from CDR1 in the V<sub>H</sub>-<sub>J</sub>H region to C<sub>H</sub> (previously only analyzed by PCR 3 and 4 generating different fragments of the full sequence). The seminested PCR 5 included: 5 μl of the gel-extracted PCR 2 products with conditions as above, except that the specific CDR1 primer (B16VSC1) was used in conjunction with the inner C<sub>H</sub> primer. The reaction was then incubated for an initial denaturation at 95°C for 2 min, then 20 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 15 min.

The primers used were as follows: C<sub>μ</sub>1, 5'-GCTCTCGATCCGACGGGAAT-3'; C<sub>μ</sub>2, 5'-CGAGGGGGAAGGGGTGTTTA-3'; C<sub>α</sub>1, 5'-GGGAC CACGTTCCCACTC-3'; C<sub>α</sub>2, 5'-CTCACGCCGAAGGACGCTC-3'; C<sub>γ</sub>1, 5'-CCGTTCCGGGAAGTTGTCC-3'; C<sub>γ</sub>2, 5'-CAGGGGAAAGAGCGAT-3' (C<sub>α</sub>2 and C<sub>α</sub>2 based on that published previously (30, 31)); V<sub>δ</sub>SFM, 5'-TGGACGTGCTGACGTCG-3'; B16V5, 5'-AGACATAAGAGTG-GCT-G-3'; SO16A4, 5'-TGGCCCCGATGTAGC-3'; b16VSC1, 5'-TATAGGTGTCACCTAGC-3'.

RT-PCR of AID transcripts

To amplify AID mRNA transcripts by RT-PCR, cDNA was manufactured from nasal biopsy samples taken from seven allergic rhinitis patients, as above. A total of 5 μl cDNA was added to each 25 μl PCR mix, which included the forward primer AID1P at 0.2 μM and the reverse primer AID2P at 0.2 μM, dNTPs at 0.2 μM, MgCl<sub>2</sub> at 1 mM, and included 1.25 U Platinum TaqDNA polymerase (Invitrogen, San Diego, CA). The reaction was initially denatured at 94°C for 5 min, then subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min before a final extension at 72°C for 15 min. A total of 5 μl of the first PCR was then transferred into a second PCR, which differed only in that it included an inner forward primer AID3P at 0.2 μM and an inner reverse primer AID4P at 0.2 μM. This reaction was incubated with an initial denaturation at 94°C for 5 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min, then a final extension at 72°C for 5 min.

Details of the primers are as follows: AID1P, 5'-GAGGCAAGAGA CACTCTGG-3'; AID2P, 5'-GTGACATTCTGGAAGTTGCG-3' (based on AID primers published previously (17)); AID3P, 5'-TAGACCTCGGC CGCTGCTACC-3'; AID4P, 5'-CAAAAGGATGCCTGCGGACGGAGCAGT-3' (based on AID primers published previously (32)).

Cloning and sequencing of PCR products

All PCR mixes were subjected to electrophoresis on 1% agarose gels, except for the C<sub>μ</sub>, C<sub>α</sub>, and C<sub>γ</sub> PCR products from reaction 3 and the AID PCR products that were subjected to electrophoresis on 2 or 1.5% agarose gels, respectively. Bands of the appropriate size were gel purified (Qiagen, Valencia, CA) and cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). EcoRI digests confirmed the presence of the cloned insert, and subsequent minipreps were sequenced using M13F or M13R primers (Molecular Biology Unit, King’s College London, and ABC Sequencing Service, Imperial College).

Identification of sequences

Assignment of V<sub>H</sub>, D, and J<sub>H</sub> genes and their somatic mutations was conducted according to their homology with the germline sequences detailed on the VBase database (www.mrc-cpe.cam.ac.uk). The identity of a D gene given a score of less than 50 by VBase was regarded as undefined (in which +5 is awarded for a nucleotide match and −4 for a mismatch). Where the alignment of the V<sub>H</sub> and D gene or D and J<sub>H</sub> was seen to overlap, precedence was given to V<sub>H</sub> and J<sub>H</sub> so that a truncated D gene sequence was determined (in which D genes are scored above J<sub>H</sub> genes, which are scored above V<sub>H</sub> genes).

Results

V<sub>H</sub> sequence analysis and IgE+ B cell clonality

We have analyzed V<sub>H</sub>-Ce sequences from the nasal biopsy samples of six patients (five multiallergics and one grass pollen monovalent), characterized by skin-prick test, RAST, and a medical history of allergic rhinitis for at least 2 years (Table 1). The selected patients exhibited serum IgE levels >200 IU/ml to ensure success in RT-PCR amplification of V<sub>H</sub> regions (27, 33). Fifteen

Table 1. Patients’ clinical and experimental data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Status at Biopsy&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total Serum IgE (IU/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Specific IgE (IU/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Allergies&lt;sup&gt;c&lt;/sup&gt;</th>
<th>No. of B Cell Families (No. of Members Isolated From Each Clonal Family) in Nasal Biopsy</th>
<th>No. of V Region Sequences Analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB7</td>
<td>O/P</td>
<td>236</td>
<td>G = 9.25</td>
<td>HDM, G, C, D, CL</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>CM10</td>
<td>O/P</td>
<td>382</td>
<td>HDM = 1.09</td>
<td>HDM, G, M, T, B, C, D, H, CL</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>HD14</td>
<td>I/P</td>
<td>787</td>
<td>HDM = 2.68</td>
<td>HDM, G</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>SO16</td>
<td>I/P</td>
<td>514</td>
<td>H = 101</td>
<td>G, T, B, D</td>
<td>2 (2, 3)</td>
<td>15</td>
</tr>
<tr>
<td>HD17</td>
<td>I</td>
<td>2745</td>
<td>G = 101</td>
<td>G</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>AP19</td>
<td>I/P</td>
<td>414</td>
<td>HDM = 1.23</td>
<td>HDM, G, C, D, H, CL</td>
<td>1 (3)</td>
<td>15</td>
</tr>
<tr>
<td>GJ29</td>
<td>Nonatopic</td>
<td>1834</td>
<td>AP = 0.71</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> In season (I), out of season (O), perennial (P).

<sup>b</sup> Determined by RAST for the allergens; house dust mite (HDM), grass (G), cat (C), dog (D), tree (T), A. fumigatus (AF).

<sup>c</sup> House dust mite (HDM), grass pollen (G), mugwort (M), three trees (T), silver birch (B), cat (C), dog (D), horse (H), A. fumigatus (AF), C. herbarum (CL), A. alternata (A), determined by skin-prick test.

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sequences were analyzed from the nasal biopsy samples of each patient.

All samples were PCR amplified using PFU DNA polymerase, which exhibits a proofreading ability resulting in a very low experimental error rate compared with other DNA polymerases. The experimental error rate generated in our system of nested RT-PCR amplification was determined to be ~1 in 4720 bp, the equivalent of 1 mutation introduced experimentally in approximately every 17 VH region sequences amplified in this manner. Experimental error rate was determined by analysis of the number of mutations introduced into the Cε DNA amplified by the VH-Cε PCR (data not shown).

Initial analysis of VH/Cε sequences generated by RT-PCR demonstrated that we were able to detect all VH gene families with the exception of VH2, one of the most infrequently expressed VH gene families (34). VH, D, and J genes were assigned using the VBase.

mucosa of allergic rhinitis patients

Table II. VH region sequences isolated from IgE+ B cells in the nasal mucosa of allergic rhinitis patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sequence</th>
<th>Germline VH Gene with Greatest Homology</th>
<th>Percentage of Mutation from Germline</th>
<th>No. of Times Isolated</th>
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<tbody>
<tr>
<td>JB7</td>
<td>B7C14</td>
<td>1–46</td>
<td>22.7</td>
<td>1</td>
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<td>B7C9</td>
<td>3–23</td>
<td>5.5</td>
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<td></td>
<td>B7C17</td>
<td>3–74</td>
<td>4.8</td>
<td>8</td>
</tr>
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<td></td>
<td>B7C22</td>
<td>4–04</td>
<td>7.3</td>
<td>5</td>
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<tr>
<td>CM10</td>
<td>B1OC7</td>
<td>3–30/3–30.5</td>
<td>2.9</td>
<td>1</td>
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<tr>
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<td>B1OC13</td>
<td>3–30/3–30.5</td>
<td>4.4</td>
<td>1</td>
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<td></td>
<td>B1OC1</td>
<td>3–66</td>
<td>10.9</td>
<td>1</td>
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<td></td>
<td>B1OC2</td>
<td>3–66</td>
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<td>B14C9</td>
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<td>B14C4</td>
<td>3–30</td>
<td>11.0</td>
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<td>B14C2</td>
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<td>7.0</td>
<td>8</td>
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<td></td>
<td>B14C11</td>
<td>4–59</td>
<td>6.4</td>
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<td>B14C19</td>
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<td>B14C12</td>
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<td>B16C3*</td>
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<td>B16C10</td>
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<td>B16C6</td>
<td>3–65</td>
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* VH gene locus detailed such that 5–51 denotes a VH5 gene.*

Identification of related IgE and IgA B cell clones in the nasal mucosa

Further investigation of patients SO16 and AP19 was undertaken to determine whether sequences from cells related to the families of IgE+ B cell clones, but which expressed isotypes other than IgE, could be detected in the nasal mucosa. A three-stage PCR approach based on that documented previously (28, 29) was used to achieve this, using a nested reaction (PCR 1 and 2) to amplify VH5 or VH5/Cε sequences from either IgM+, IgG+, or IgA+ B cells in each cDNA sample. The third PCR in each case used a seminested approach utilizing a forward primer specific for the clonal signature region (VH-D junction) of interest, combined with the inner set of primers for IgM, IgG, or IgA.

No sequences suggesting the presence of B cell clones of any other isotype related to the VH3-Cε family from SO16, or the VH5-Cε family from AP19, were detected. In contrast, successful amplification of a signature region from an IgA+ B cell related to the VH5 family of IgE+ B cell clones was isolated from the nasal mucosa of patient SO16 (Fig. 3A) (nucleotide sequence submitted to EMBL, accession number AJ491916), indicating the presence of at least one IgA+ B cell sharing a common ancestry with the previously isolated family of IgE+ B cells.
Another, separate, seminested PCR (PCR 4) was used to amplify the full \( V_H \) region sequence from the related IgA\(^+\) B cell(s) from which the signature region had been amplified in PCR 3. Sequences were detected from five IgA\(^+\) B cell clones that were related to each other and to the IgE\(^+\) family of B cell clones and that exhibited a complex pattern of shared and unique somatic mutations throughout the full length of the \( V_H \) region sequence (nucleotide sequences submitted to EMBL, accession numbers AJ536081–5). These sequences were aligned, and homology with the sequences from the related IgE\(^+\) B cell clones, also isolated.

**FIGURE 1.** Alignment of \( V_H \)-D-J sequences amplified by RT-PCR from the nasal mucosa of allergic rhinitis patients SO16 and AP19 indicated the presence of clonally related IgE\(^+\) B cells. Sequences are detailed such that, e.g., sequences isolated from AP19 have closest homology with the germline sequence of a \( V_H \) gene from the \( V_H \) family, locus 5-51 (\( V_H \)5-51), the D gene D1-26, and the \( J_H \) gene \( J_H \)3b. (Although the D1-26 germline sequence in reading frame 2 used in AP19 encodes a stop codon, denoted with an asterisk, the nontemplated insertion between the \( V_H \) and D gene is such that rearranged sequences demonstrate an open reading frame.) CDR regions are indicated and PCR primer regions are underlined. Mutations in the primer regions were not included.
from the nasal mucosa of patient SO16, could clearly be seen (Fig. 3 B).

To eliminate the possibility that the IgA clones were PCR artifacts (unlikely as both the shared and diverse mutations were dispersed throughout the VH region), we successfully reamplified in PCR 5 the complete VH-D-JH sequence of CDR1 from the IgA clone C1 that had previously been partially amplified by PCR 4 (nucleotide sequence submitted to EMBL, accession number AJ536086) (data not shown). Both the signature region and the somatic mutations were identical with that amplified previously, confirming that the sequences presented in this study were unlikely to be PCR artifacts.

VH-Ce sequences were not isolated from the nasal mucosa of a nonatopic subject with highly elevated serum IgE To investigate whether random migration of IgE+ B cells from the circulation to the nasal mucosa occurs in subjects exhibiting high systemic IgE levels (a possible explanation for the presence of related B cell clones seen in the nasal mucosa of allergic rhinitis patients), a nasal biopsy was taken from nonatopic healthy subject GJ29, who exhibited extremely elevated serum IgE (total serum IgE 1834 IU/ml). No VH-Ce sequences could be isolated from the nasal biopsy sample (Fig. 4A), although the nasal biopsy exhibited a strong GAPDH signal (Fig. 4C) and VH-Cµ, VH-Cα, and VH-Cγ PCR products, corresponding to IgM, IgA, and IgG+ B cells, respectively, could be amplified from the sample (data not shown). In addition, a strong IgE signal was obtained from PCR amplification of IgE VH regions from the subject’s PBMC (Fig. 4A). When VH-Ce transcripts from the subject’s PBMC were amplified using primers to amplify the different VH class in separate reactions, the subject’s IgE was shown to be comprised of B cells expressing VH1, VH3, VH4, VH5, and VH6 (Fig. 4B), thereby demonstrating that the repertoire of IgE+ B cells in the PBMC was diverse and that a mono/oligoclonal lymphoproliferative disorder did not account for the subject’s raised total serum IgE.

Local AID mRNA transcripts in the nasal mucosa of allergic rhinitis patients

To investigate the presence of local AID mRNA transcripts in allergic rhinitis, a nested PCR technique was applied to nasal biopsy samples from seven patients. Two of these samples (CM10 and AP19) had also been used in the VH-Ce transcript analysis (restricted sample size unfortunately prevented the analysis of the other five nasal biopsies on which VH-Ce analysis had been conducted). All of the patients in which AID analysis was studied were either biopsied within the grass pollen season or suffered from perennial allergic rhinitis. When the PCR products were subjected to agarose gel electrophoresis, AID mRNA was shown to be present in the nasal mucosa of five of the seven patients (Fig. 5). The PCR products were confirmed by Southern blot analysis using a probe that spanned exons 3 and 4 (data not shown).
Distantly related to a family of IgE B cell clones observed in the nasal mucosa of allergic rhinitis patient SO16. The signature region sequence (which was joined to the 5' end of the IgA C region) generated by PCR 3 and also the corresponding V_{H}-D-J_{H} sequences from the related IgA clones (amplified by PCR 4) are both detailed, as the join to IgA was only implicit in the signature region sequence. A, An identical signature region sequence was amplified by PCR 3 from both IgE and IgA B cell clones. Sequences had greatest homology with the germline V_{H} 5 gene V5-51, D4-17, and J_{H} 4b (positions indicated). Primers used in PCR 3 are marked in bold, and regions of homology are indicated by vertical lines. B, The V_{H} region sequences of the related IgA clones amplified by PCR 4 were aligned with the related IgE clones and exhibited both shared and unique V_{H} region mutations. The sequences had highest homology with the germline V_{H} 5 gene V5-51 and D4-17 (indicated). The J_{H} gene J_{H} 4b was only partially amplified and was completely encompassed by the primer region. Primer regions are underlined. Mutations in the primer regions were not included.

**Discussion**

Allergic rhinitis is increasingly prevalent in the Western world. Studies at the molecular level have enabled the investigation of IgE-mediated immediate hypersensitivity at specific locations. Previous work has demonstrated the occurrence of local IgE protein production in the nasal mucosa of patients suffering from allergic rhinitis (1), but no PCR-based investigation has been undertaken until now to determine whether DNA class switch recombination to generate B cells committed to IgE synthesis and concurrent somatic hypermutation also occur locally in the nasal mucosa of allergic rhinitis patients (as has been shown for the asthmatic lung mucosa (13)). These processes are potential therapeutic targets.

To investigate whether local CSR and SHM occur locally in the nasal mucosa of allergic rhinitis patients, we have amplified IgE^{+} V_{H} region sequences by RT-PCR from the nasal mucosa of six allergic rhinitis patients. These sequences have provided clear evidence of families of closely related IgE B cell clones in the nasal mucosa of two of the six patients. This was in stark contrast both to the one instance in which two very distantly related IgE^{+} B cell clones were isolated from the PBMC of one patient and the one instance in which an IgE^{+} clone from the PBMC was found to be distantly related to a family of IgE^{+} clones from the nasal mucosa (possibly resulting from the differentiation of related cells from lymphoid tissue before SHM and clonal expansion in the nose, or from the nasal mucosa into the circulation).

Each family of related B cell clones originates from a common precursor B cell that proliferated and was subjected to differing extents of SHM. We propose that the occurrence of local SHM and subsequent clonal expansion are a likely explanation for the presence of these families of closely related IgE^{+} B cell clones in the mucosa, rather than the migration of all members of each family of related B cell clones from lymphoid tissue to the same location in the nasal mucosa.

Additional evidence supporting the theory of local events in the nasal mucosa was provided by the further investigation of patient SO16. Sequences were identified from five different IgA^{+} B cells that exhibited identical CDR3/FWR4 clonal signature regions to those from one of the IgE^{+} families of related B cell clones also isolated from the same nasal biopsy sample. These IgA sequences exhibited a range of mutations shared with and also different from the IgE sequences. Again, it is unlikely that these IgA^{+} cells were derived from CSR in lymphoid tissue and that they then homed to the same 2.5 mm^{3} of nasal mucosa as the related IgE^{+} B cells. Detection of related IgE^{+} and IgA^{+} B cell clones instead implies that it is more likely that the different Ab isotypes were generated by local CSR, as has been previously suggested, in the lung mucosa of allergic asthmatics (13).

Further studies were conducted to investigate the origin of the related B cell clones observed in the nasal mucosa of the allergic rhinitis patients. Analysis of a healthy nonatopic subject with highly elevated IgE indicated that whereas (as expected) a strong RT-PCR product resulted from the amplification of IgE V_{H} regions from the subject’s PBMC, no IgE V_{H} region sequences could be isolated from the subject’s nasal mucosal sample even though a positive GAPDH signal could be amplified and V_{H} region sequences from other isotypes were detected. This suggests that a
The presence of AID in the nasal mucosa might therefore be expected if the local microenvironment supported CSR and SHM. We detected AID mRNA transcripts in the nasal mucosa of allergic rhinitis patients located in clusters, as observed by immunohistochemical staining of CD19⁺ B cells from nasal biopsy sections. Our preliminary analysis of adjacent halves of a biopsy has suggested that clonal families are highly localized within the mucosa (Coker et al., unpublished observations). Our inability to take more than a single biopsy at any one time from a patient for ethical reasons implies that such clusters may be excluded by the nature of the random sampling of ~0.1% of the inferior turbinate; each turbinate is on average 10 g, and each nasal biopsy, on average, 10 mg (L. Smurthwaite, unpublished observations).

Although this is the first study to investigate the local environment of the nasal mucosa in this number of patients, a previous study being restricted to just two lung biopsies from a single asthmatic patient (13), we suggest that it is highly significant that in many respects a consistent pattern of results is emerging in comparison with that observed in the allergic asthmatic lung (13). Our results additionally describe analysis of the V₁ region repertoire of the nasal mucosa of a healthy nonatopic patient with high systemic levels of IgE, but no detectable local mRNA-encoding IgE, suggesting that IgE⁺ B cells do not randomly migrate to the nasal mucosa from the circulation. We therefore conclude that the cells in the nasal mucosa of allergic rhinitis patients are stimulated with allergen, undergo class switch recombination and somatic hypermutation, and expand locally in the presence of AID. Our data strengthen the argument that the mucosa acts as a microenvironment in which cells from the immune system direct, process, and remove Ag in a localized manner, and we suggest that this is fundamental to the pathogenesis of allergic disease.

**Acknowledgments**

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References


