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Analysis of immunoglobulin E V_H transcripts in a bronchial biopsy of an asthmatic patient confirms bias towards V_H5, and indicates local clonal expansion, somatic mutation and isotype switch events

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SUMMARY

Immunoglobulin E (IgE)-dependent mechanisms play a pivotal role in mediating allergic disease. Previously, V_H-Cε transcripts from blood or spleen of atopic asthmatics have been analysed for V_H gene usage and patterns of somatic mutation. An over-representation of the minor V_H5 family has been observed, consistent with a superantigen drive. As local mucosal events in IgE production may be more significant in the disease process, we have analysed V_H-Cε transcripts from a bronchial biopsy of a patient with severe asthma. V_H5 predominance was confirmed with 10 of 30 unique clones derived from this family. Repeated sequences, some with intraclonal variation, revealed clonal expansion and continuing mutational activity at the site. Unexpectedly, three unmutated V_H-Cε sequences were found, indicating that isotype switching to IgE can occur without mutation. Detection of a sister clone with extensive mutations was again consistent with local mutational activity. Evidence for local isotype switching was obtained by identification of clonally related immunoglobulin M (IgM), immunoglobulin G (IgG) and immunoglobulin E (IgE) sequences. However, in contrast to findings in blood, no IgG4 transcripts clonally related to IgE were detected, suggesting that the balance between synthesis of IgG4 and IgE may differ between systemic and local sites. These data confirm a V_H5 bias in IgE, and support the concept that IgE-synthesizing B cells arise via local differentiation.

INTRODUCTION

Immunoglobulin E (IgE) antibodies are known mediators of allergic disease, including allergic asthma.[1,2] Allergen can cross-link IgE that is bound to its high-affinity receptor (FcεRI) on the surface of mast cells or basophils, resulting in the release of mediators that lead to the symptoms of Type I hypersensitivity.[3] The presence of high- and low-affinity receptors has been reported on many cell types in the bronchial mucosa of asthmatics, with an increased number of FcεRI-expressing cells being found in asthmatics.[4] IgE has the potential to mediate inflammation in the airways by enhancing the release of proinflammatory mediators from activated cells.[5-7] IgE-mediated antigen presentation is another potential way by which IgE is involved in the inflammatory processes of asthma and atopy.[8,9] The central role of IgE in both the early and late responses has been confirmed by studies with non-anaphylactogenic anti-IgE monoclonal antibody (mAb) that binds to free IgE and to IgE on B cells. Treatment of mild asthmatics with this mAb inhibited the late reaction by 60% and also suppressed the early response.[10]

Allergen-specific IgE has been detected in nasal and respiratory secretions,[11,12] with a recent study finding IgE specific for house dust mite (HDM) in the sputum of HDM-sensitive asthmatics, but not in healthy control subjects.[13] However, the origin of IgE-secreting cells is unknown, although IgE-positive B cells have been identified in local tissue.[14,15] It is unclear whether such cells have been recruited from lymphoid tissue or are induced to undergo isotype switching within the mucosal site: recent data supports the latter possibility.[14-16] As locally synthesized IgE may be important in responses to exogenous antigen, the origin and nature of IgE-expressing B cells at local sites of disease is of interest.

Immunogenetic analysis allows us to identify B-cell clones that have undergone isotype switching to IgE. It is then possible to analyse the nature and mutational patterns of V_H genes used. During genetic recombination, one V_H gene from a

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Abbreviations: CDR, complementary-determining region; FWR, framework region; SAg, superantigen.

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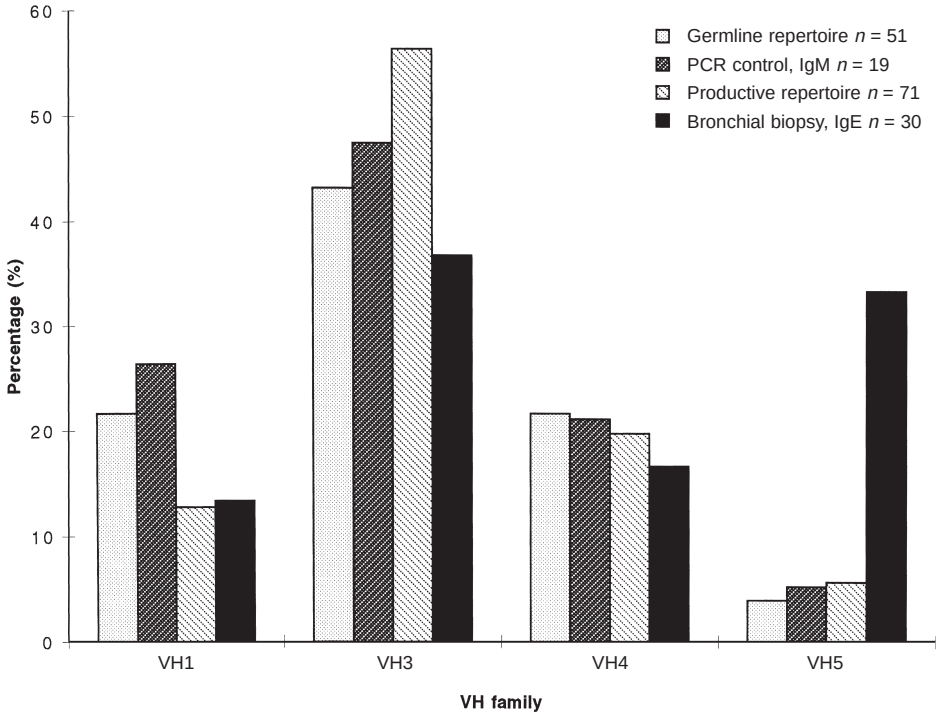

Figure 1. Graph showing a significant over-representation of the V_H5 family by immunoglobulin E (IgE) in the bronchial biopsy when compared with the germline repertoire,[38] a polymerasec chain reaction (PCR) control for immunoglobulin M (IgM)[18] and the productive repertoire from normal B cells.[26]

germline repertoire of 51, in combination with D and J_H genes, is joined to a C-region gene (initially immunoglobulin M [IgM]) to give rise to functional genes that can encode the H chain of antibody. A preferential usage of the minor V_H5 family by IgE was previously observed in the peripheral blood and spleen of atopic asthmatics[17,18] and also in peripheral blood from patients with atopic dermatitis.[19] Bias in V_H gene usage can indicate an influence of superantigen (SAg), which binds V_H via the conserved framework region (FWR) outside the conventional binding sites in the complementarity-determining region (CDR).[20] One suggestion is that allergens, and perhaps parasitic antigens, are acting in this manner.[17] In order to focus on events at the site of disease, we studied a bronchial biopsy from a severe asthmatic. We report clear predominance of V_H5 usage. Analysis of B-cell clones also indicated that somatic mutation and isotype switching are occurring in the local environment.

MATERIALS AND METHODS

Background of the patient

The patient was a 32-year-old male who had suffered with asthma from birth. He has a severe form of perennial asthma and is highly allergic to HDM, grass pollen, cat, dog and feather, with a very high serum IgE level of 5000 IU/ml. At the time of the study his forced expiratory volume in 1 second (FEV_1) was 1·72, which is 49% of predicted. He has daily symptoms with morning chest tightness and significant effect on his physical activity. In addition, at the time of biopsy he had nocturnal symptoms every night, an indicator of poorly controlled asthma. His treatment consisted of budesonide aerosol (2 mg twice daily) delivered by a nebuliser, 10 mg daily of oral prednisolone and regular twice-daily nebulised salbutamol (2·5 mg).

Bronchoscopy procedure

The patient agreed to participate in the study, which was approved by the Joint Southampton University and Hospital Ethics Committee. Following premedication with 2·5 mg of salbutamol and 0·5 mg of ipratropium bromide, delivered by nebuliser, and intravenous atropine (0·6 mg) the patient underwent bronchoscopy according to the guidelines issued by the National Institutes of Health,[21] as previously reported,[22] using lignocaine for topical anaesthesia. A fibreoptic bronchoscope was introduced and two biopsies of subcarinae were taken using alligator forceps. The biopsies were snap-frozen and stored in liquid nitrogen until analysis.

Preparation of cDNA and amplification of V_H genes

The biopsies were homogenized with 200 µl of RNAzol and the RNA was then extracted as previously described.[23] cDNA was then prepared with oligo dT primer and a first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). Superscript II was used as the reverse transcriptase enzyme (Gibco BRL, Life Technologies Inc., Paisley, Strathclyde, UK). Four separate nested polymerase chain reactions (PCRs) were carried out for amplification of V_H 1-7 families in combination with Cε. The products were cloned as previously described[17] and sequenced using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA), with M13 forward and reverse primers. The V_H primers had previously been checked for any inherent bias for the V_H5 family by a nested PCR using IgM primers in place

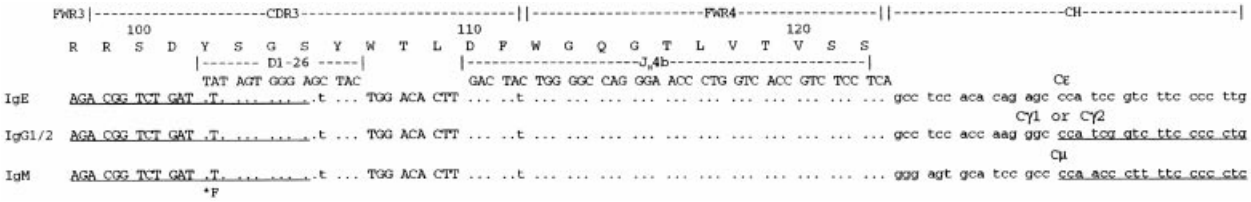

Figure 2. Immunoglobulin M (IgM)-, immunoglobulin G (IgG)- and immunoglobulin E (IgE)-related clones with a common CDR3/FWR4 sequence. The nucleotide sequences of CDR3/FWR4/C_H transcripts are aligned to D-segment and J_H germline genes. Primer sequences are underlined, the Cε primer is 3′ to the sequence shown.

of IgE primers. No asymmetry in V_H family usage was obtained (Fig. 1; reference 18).

Investigation of V_H-Cε related clones

Identification of CDR3-FWR4-CH sequences. For two V_H5-Cε sequences (clones 1 and 2), clonally related transcripts were sought by the use of CDR3-specific oligonucleotides. A nested PCR, specific for V_H5 in combination with IgM, immunoglobulin G (IgG)[23] and IgG4,[24] was carried out as previously described.[25] followed by amplification with 5′ CDR3-specific primers for IgE clones 1 (5′-AGACGGGCTGACTA-TAGGGGGA-3′) or 2 (5′-AGACGGTCTGATTTTAG-TGGGA-3′), with the inner primers for IgM, IgG or IgG4. A control PCR was also carried out, of the CDR3-specific primer with the inner IgE primer. Products obtained were then purified, cloned and sequenced. The nucleotide sequences of the CDR3-C_H transcripts obtained were compared with that of the IgE clones to check for clonal relatedness (Fig. 2).

RESULTS

V_H family utilization in IgE

In the bronchial biopsy, we analysed V_H gene usage in IgE by using a nested PCR. Sequences from 30 distinct B cells were obtained, indicated by the unique CDR3 regions of the V_H-Cε transcripts. To determine V_H family usage, sequences were aligned to the closest germline V gene (Fig. 1). The V_H5 family was significantly over-represented ($P<0{\cdot}001$ by χ^2 analysis) with 10 of 30 (33%) clones derived from the two members V_{5-51} and V_{HVMW} (a polymorphic variant of V_H32). To assess for reproducibility, the PCRs were carried out as four separate reactions and the V_H5 family was evident in all PCRs. To control for primer bias, we compared this pattern with that obtained for IgM using the same V_H primers (Fig. 1), where only one of 19 V_H5-encoded sequences were obtained.[18] Figure 1 also compares these results with the V_H repertoire of productive rearrangements from individual normal B cells, where V_H5 was used in four of 71 B cells (5·6%; reference 26).

Analysis of V_H5-Cε gene sequences

Sequences obtained are shown in Fig. 3, with unique CDR3s indicating clonal relationships (GenBank accession nos: AF110479-AF110491). Groups 1 and 2 appear to be each derived from a single B cell that has undergone further diversification. The similarity between the CDR3s of the two groups is intriguing, and it raises the possibility that they are all derived from the same original B cell. This is underlined by the sharing of several replacement mutations between 1b and Group 2 clones. However, there are too many differences between the CDR3 sequence to confirm a common clonal

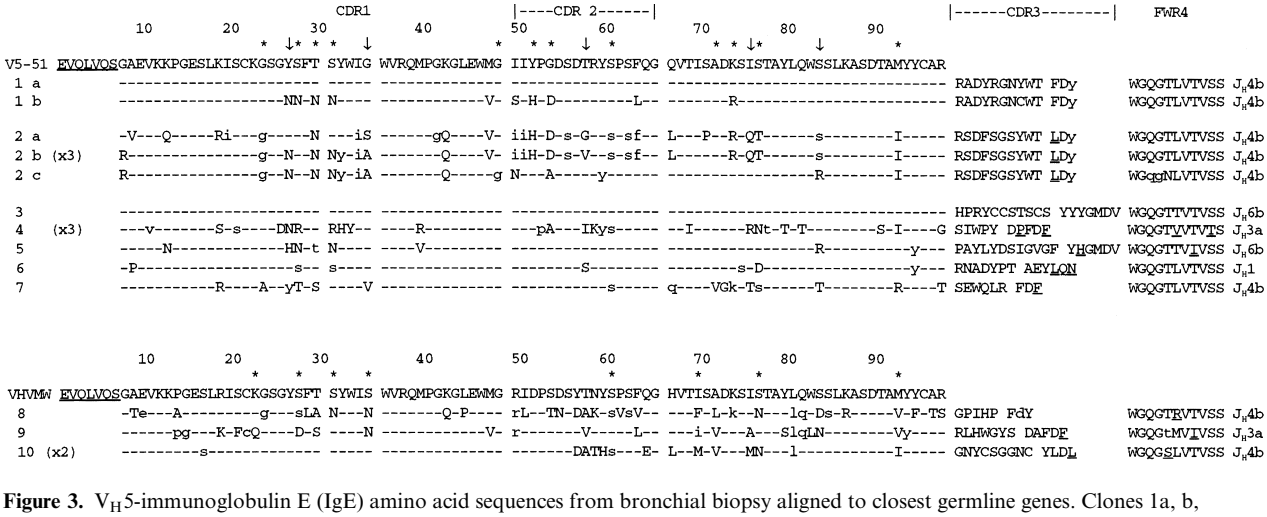

Figure 3. V_H5-immunoglobulin E (IgE) amino acid sequences from bronchial biopsy aligned to closest germline genes. Clones 1a, b, and 2a, b, c are sets of related clones. Identical repeated sequences were found for clones 2b, 4 and 10, with number of repeats shown in brackets. Upper case: replacement mutation; lower case: silent substitution. *Indicates sites that have previously been reported as hot-spots of mutation.[23] ↓ Indicates frequently mutated sites not previously reported.

origin, and the possibility remains that they represent two parallel, independent clones, possibly converging on one epitope.

As mRNA from the low numbers of IgE^+ B cells was limited, we considered the possibility of PCR artefacts arising from cross-over events. In view of the fact that separate PCR reactions were carried out, and that the sequences obtained did not show patterns consistent with cross-over, we thus eliminated this possibility.

The finding of repeated identical sequences both within Group 2 and in other single sequences (4 and 10) is consistent with clonal expansion. However, an alternative explanation may be that plasma cells with increased levels of mRNA were present. Identical repeats occurred not only in V_H5-derived sequences, but were seen in IgE encoded by V_H1 (one instance), V_H3 (two instances) and V_H4 (one instance) (data not shown). It is unlikely, because of the high serum level of IgE in this patient and the large number of different clones obtained, that the repeated sequences indicate the presence of a restricted set of B cells in the mucosa.

There was a generally high level of somatic mutation, both in the V_H5-Cε sequences (mean = 8·2%) and in the other V_H families (6·5%, data not shown). Analysis of the V_H5-derived sequences showed that mutations were dispersed throughout the sequences (Fig. 3), with no significant clustering of replacement mutations in the CDR, characteristic of antigen selection.[27] However, hot-spots of mutational activity were evident, as described previously for V_H5,[23] and are indicated by an asterisk in Fig. 3. The incidence was similar to that previously seen in IgE, with particularly prominent sites at Ser31 and Ser77, known to be susceptible codons. Other sites were also frequently mutated, which had not been observed previously. These are shown by the arrows in Fig. 3, for example Tyr27. However, none of these sites have identical repeated substitutions, which would be indicative of antigen selection,[28] and the sample size is fairly small, preventing any significant conclusions to be made about additional hot-spot incidence.

Clones 1a and 3 had no apparent somatic mutations, a feature that appears to be unique to local tissue, and has not been found in 124 V_H-Cε sequences from blood or spleen.[17,18,23-25] Interestingly, 1b shares the CDR3 of 1a, indicative of the same B cell of origin, but has accumulated 11 replacement mutations (Fig. 3), probably as a result of local somatic mutation events. Clones in Group 2 share the CDR3 sequence, characteristic of a common B-cell origin, but there is a considerable level of intraclonal variation, with both shared and distinct mutations evident (Fig. 3). This is again consistent with local somatic mutation.

Presence of clonally related Cμ and Cγ transcripts

We focused on two V_H5-Cε clones, 1 and 2, to look for transcripts of alternative isotypes related to the IgE clones already obtained. By using a CDR3-specific 5′ primer, together with 3′ primers based in Cμ or Cγ, we obtained IgM- and IgG-related transcripts with a common CDR3 for clone 2 (Fig. 2). In the case of clone 1, the PCRs were negative and no alternative transcripts were detected. Control PCRs for CDR3-Cε transcripts on material from the nested V_H5-C_H PCR products were negative, eliminating the possibility that the IgM and IgG transcripts were actually PCR contaminants derived from the IgE clone by a PCR cross-over event.

The clonal relationship of the alternative isotypes obtained from clone 2 is evident from the identical CDR3-FWR4 sequences (see Fig. 2), when compared with the CDR3/FWR4 of the IgE clone from the end of the 5′-CDR3 primer onwards. Four features underline their shared origin: the common N-additions at positions 107, 108 and 109; common D-segment alignments of the same length and reading frame; a common J_H gene usage; and the common silent nucleotide substitutions in amino acids 105 (Ser, AGC→AGT) and 111 (Phe, TAC→TAT), Fig. 2. The related clones were derived from either clone 2a or 2b, not 2c, as seen by comparison of the FWR4 sequence of the clones (Fig. 3).

The IgG subclass of the transcripts obtained was either IgG1 or IgG2, the consensus IgG primer used being too far upstream to distinguish between these two. No evidence for IgG3 or IgG4 sequences was obtained. In addition, an IgG4-specific PCR was carried out, but CDR3-Cγ4 transcripts were not obtained. The upstream sequences were sought (V_H5-CDR3), as previously described,[25] but we were unable to obtain these. The primers amplified the appropriate V_H5-Cε sequence when tested. However, with the IgM and IgG PCRs there was cross-priming of the CDR3-specific primers with V_H-C_H transcripts that had similar, but not identical, CDR3s. These may have been present in greater frequency than the clones we were seeking.

DISCUSSION

The first striking result from this analysis of V_H-Cε transcripts from a bronchial mucosal biopsy of an atopic asthmatic is the predominance of the small V_H5 family. This is confirmatory of our previous observations of a bias towards V_H5 usage in IgE from the peripheral blood and spleen of HDM-allergic asthmatics, although the bias found here is more evident than that observed in the peripheral blood.[18] This finding supports the concept that there may be a common allergen acting as a B-cell SAg. As SAgs are usually derived from pathogens, it raises the possibility that a pathogen-derived SAg may preferentially recruit V_H5-expressing B cells that are initially expressing IgM. These are subsequently induced to switch to IgE expression in the appropriate cytokine environment.

The pattern of somatic mutation observed in these V_H5-Cε transcripts is similar to that observed previously in clones from the blood and spleen.[17,18] The same hot-spots are observed in these sequences as in V_H5 clones reported previously,[23] with a few additional sites that are commonly mutated. Two of these new sites are in the CDR, and the others are adjacent to, or within, the susceptible serine motif AGC/T, so their presence is not surprising.[28]

An unexpected finding is the absence of somatic mutation in three of the IgE clones, two of which are from the V_H5 family. It appears that isotype switching to IgE in B cells can be reached locally with either a complete absence of somatic mutation or with a high level. Unmutated IgE sequences presumably arise from the isotype switching of germline-encoded IgM antibody to IgE before somatic mutation and affinity maturation. In one of the sets of expanded clones, the unmutated clone has a mutated sister clone. Therefore, it would

appear that somatic mutation is occurring locally, as it is unlikely that the unmutated clone has travelled to a lymph node to undergo somatic mutation and then migrated back to the exact same local sites. Further support for local somatic mutation is seen in the second set of related clones that have distinct, as well as shared, mutations. This is indicative of clonal expansion at a local site with ongoing somatic mutation. This pattern has also been detected in B cells from the peripheral blood, where there are generally far fewer IgE-expressing B cells than obtained in this biopsy.[18,29]

Local isotype switching also appears to be occurring, with IgM-, IgG- and IgE-related clones being found together at this local site. This is demonstrated by the presence of identical nucleotide sequences of CDR3/FWR4 transcripts with IgM, IgG1/2 or IgE C regions. This contrasts with our previous results from the blood where IgG4- and IgE-related clones were detected together, with no evidence of other IgG subclasses.[25] Co-expression of IgG4 and IgE may be expected, as interleukin (IL)-4 and IL-13 induce switching to both isotypes.[30,31] The different pattern at the local site can be explained by the influence of other cytokines on isotype transcript levels. It is known that higher IL-4 : interferon-γ (IFN-γ) levels enhance IgE production, while increased IFN-γ expression favours IgG4 production,[32,33] and this may be reflected at the mRNA level. Therefore, the absence of IgG4 at a disease site may be a result of local cytokine expression, with high IL-4 : IFN-γ ratios inducing greater IgE production. Consistent with this possibility, a T helper 2 (Th2)-dominant cytokine pattern has been reported in the bronchial mucosa of asthmatics with elevated IL-4 and IL-13 levels.[34,35] In contrast, the IgE-expressing B cells in the systemic circulation may have arisen in sites, possibly the spleen, where the IL-4 : IFN-γ ratio is lower, favouring production of IgE and IgG4.

Recent studies provide evidence to suggest that class switching to IgE occurs at local sites of disease. Increases in Iε germline transcripts, in addition to Cε transcripts and IL-4, have been detected in nasal mucosal biopsies of hayfever patients following allergen challenge, while the number of B cells remained constant.[14] Similar results have been obtained in sinus mucosal biopsies from atopic patients with chronic sinusitis.[15] A different group has used PCR detection of Sμ/Sε switch circle DNA as evidence for *in vivo* IgE isotype switching in ragweed-sensitive individuals following nasal challenge with ragweed and diesel exhaust particles.[16] The accumulated evidence for local somatic mutation and isotype switching events is surprising, given that there is little evidence for germinal centre (GC) structures at the mucosal site.[14] Although dependence on the GC is not absolute,[36] the high degree of mutational activity will probably require an environment where CD40 ligand (CD40L), cytokines and antigen are present.[37] At least in patients with asthma, the cellular milieu at the mucosal surface appears to be capable of providing this.

In summary, it appears that at least some IgE-expressing B cells arise within a local site of disease. Our hypothesis from the immunogenetic analysis is that IgM-expressing B cells, particularly those expressing the V_H5 family, are stimulated by allergen and undergo local differentiation, accumulation of mutations and isotype switching to IgE. The relationship with IgE-expressing B cells in blood and spleen is not yet apparent, but local cytokine influences clearly direct B cells in this site where disease is manifest.

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