

ORIGINAL ARTICLE

Diversity of immunoglobulin E-encoding transcripts in sinus mucosa of subjects diagnosed with non-allergic fungal eosinophilic sinusitis

M. Levin¹, L. W. Tan², L. Baker², P.-J. Wormald², L. Greiff³ and M. Ohlin¹

¹Department of Immunotechnology, Lund University, Lund, Sweden, ²Department of Surgery-Otorhinolaryngology, Head and Neck Surgery, University of Adelaide, Adelaide, Australia and ³Department of Otorhinolaryngology, Head and Neck Surgery, Skåne University Hospital/Lund University, Lund, Sweden

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Summary

Background The role of allergy in the aetiopathogenesis of chronic rhinosinusitis (CRS) remains controversial. For example, in some cases with sinus fungal infections allergy can be demonstrated by standard tests. In other cases, such signs can be absent despite elevated levels of IgE-positive cells in sinus tissue and the presence of IgE and eosinophils in the sinus mucous.

Objective To define the nature of molecular diversity in antibodies of the IgE isotype at the site of local inflammation in subjects diagnosed with non-allergic fungal eosinophilic sinusitis (NAFES).

Methods The local occurrence and sequence characteristics of IgE-encoding transcripts in NAFES patients were investigated and compared with sequences found in subjects diagnosed with CRS featuring systemic allergy. These sequences have also been compared with other reported IgE-encoding transcriptomes.

Results IGHV genes derived from major subgroups 1, 3, 4 and 5 and a diverse set of IGHD and IGHJ genes were shown to create the IgE repertoire in patients diagnosed with NAFES and CRS. The average lengths of the third hypervariable loop in these populations were 15.8 and 14.6 residues. The sequences showed evidence of extensive somatic hypermutation (mutation frequency: NAFES, $6.4 \pm 3.2\%$; CRS, $7.0 \pm 4.4\%$) with substitutions targeted to complementarity-determining regions. These sequence collections thus show extensive similarities to those found in other polyclonal Ig repertoires including those encoding IgE.

Conclusion and Clinical Relevance We conclude that sinus IgE-encoding transcripts in subjects diagnosed with NAFES show evidence of conventional IgE responses and we suggest that allergens with characteristics of classical antigens should be investigated for a role in the local response occurring in NAFES. This investigation illustrates that assessment of local immunity might be an important diagnostic tool in conditions like NAFES with no systemic signs of allergy to identify or rule out an allergic component of the patient's disease.

Keywords chronic rhinosinusitis, IgE repertoire, local immunity, molecular diversity, mucosa, non-allergic fungal eosinophilic sinusitis, transcriptome analysis

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Correspondence:

Dr Mats Ohlin, Department of Immunotechnology, Lund University, BMC D13, S-22184 Lund, Sweden.
E-mail: mats.ohlin@immun.lth.se

Introduction

In type I allergic responses, conditions affecting up to one-third of the world-wide population [1], IgE antibodies play a key role as allergen recognizer. Cross-linking by allergen of antigen-specific IgE bound to high-affinity IgE receptors (FcεRI) on mast cells and basophils triggers a cascade of events, eventually leading to activation and degranulation of these cells. The release of biologically

active compounds such as histamines, proteases, cytokines and chemokines, at both local and systemic sites, gives rise to the symptoms of an allergic response [2, 3]

Patients suffering from chronic rhinosinusitis (CRS), a set of diseases characterized by a prolonged inflammation of the sinonasal mucosa, is a considerable clinical problem [4]. CRS can be divided into subgroups depending on the involvement or not of well-defined allergic and infectious conditions. The aetiology of CRS is, however,

often obscure, resulting in difficulties in the diagnosis and treatment of this condition.

While allergic reactions are responsible for a fraction of CRS conditions, many subjects diagnosed with CRS lack signs of systemic allergy [5–7]. It can, however, not be excluded that local production of IgE in the absence of circulating IgE may contribute in these cases. Indeed, substantial local production of IgE has been observed in subjects with conventional allergic rhinitis (AR) [8]. Local presence of allergen-specific IgE with minimal correlation to systemic levels of IgE has also been noted e.g. in atopic subjects in response to local challenge with conventional allergen [9] and in nasal polyp tissue [10]. Evidence of localized allergen-specific IgE has intriguingly also been found in studies of patients with non-allergic, idiopathic rhinitis [11, 12]. However, little is known about the molecular nature of localized IgE repertoires and their development in systemically non-allergic subjects. Are these IgE the product of a classical immune response involving T cell-dependent antigens or are they a result of polyclonal activation of B lymphocytes by other means? Such an involvement of alternative means of IgE induction has even been proposed to occur in conventional AR [13] and such mechanisms cannot be excluded in systemically non-allergic subjects, in particular in cases where infection with bacteria and fungi may play a role in disease development.

Fungal rhinosinusitis is characterized by an eosinophilic mucosal infiltration and the production of eosinophilic mucous. Eosinophilic mucous CRS (EMCRS) can be divided into the following subgroups: (1) Allergic fungal sinusitis (AFS): fungal allergy with fungus; (2) Allergic fungal sinusitis-like (AFS-like): fungal allergy but no fungus; (3) Non-allergic fungal eosinophilic sinusitis (NAFES): no allergy but with fungus; and (4) Non-allergic non-fungal chronic sinusitis (NANFS): no allergy and no fungus. Allergic fungal sinusitis has been thought to be mediated by a local IgE response to fungus in the sinus mucosa. However, it is diagnosed by a positive IgE response seen in the blood. The other major group making up fungal sinusitis is NAFES. The mucosa of these systemically non-allergic patients feature an inflammatory response characterized by elevated numbers of IgE-positive cells, the presence of IgE and eosinophilic granulocytes, suggesting a potential involvement of a typical IgE-mediated allergic response [14, 15]. This study attempts to describe the local IgE-producing capability by characterizing sequences of transcripts encoding the variable heavy (H) chain domain of IgE as found in sinus mucosa of individuals diagnosed with NAFES and CRS. This is carried out in order to define whether or not IgE responses in subjects not diagnosed with allergy, but who nevertheless have local IgE production, are similar in nature to T cell dependent, somatically mutated antibody responses.

Methods

Subjects, classification, clinical parameters and sample collection

Sample (Table 1) collection was conducted in the hospitals of a tertiary rhinological practice in Adelaide, South Australia, with the approval of the hospital-based Ethics of Human Research Committee. Patient categories were classified according to the following criteria: CRS: No eosinophilic mucus, defined as the presence of Charcot–Leyden crystals detected by microscopy, was present at the time of surgery and the patient was negative for culture and staining of fungal hyphae ($n = 6$). NAFES: Patients with eosinophilic mucus, from whom fungus was identified either on microscopy or culture and who showed an absence of systemic allergy to fungus, as determined by a negative IgE test against Mould Mix ($n = 5$).

Sinus tissue was harvested from patients undergoing endoscopic sinus surgery. The tissue was immediately placed in Dulbecco's modified Eagle's medium (Sigma, Sydney, Australia) on ice and transported to the laboratory. For long-term preservation, the tissue was immersed in RNAlater (Ambion, Austin, TX, USA) at 4 °C overnight and then stored at –70 °C.

Reversed transcription, polymerase chain reaction, gene cloning and gene sequencing

Up to 100 mg wet weight of tissue was homogenized in 1 mL of QIAzol reagent (Qiagen, Hilden, Germany) using the TissueRuptor (Qiagen). RNA was extracted using the RNeasy Lipid Tissue mini kit according to the manufacturer's instructions (Qiagen). RNase Inhibitor (Qiagen) was added to prevent degradation and the RNA was stored at –70 °C. Complementary DNA was reverse transcribed from 400 µg of RNA template using a Quantitect Reverse Transcription Kit (Qiagen) on the BioRad (Hercules, CA, USA) My Cycler using the blend of oligo-dT and random primers included in the kit. A no template control was generated for each patient by including a reaction that did not contain the reversed transcriptase. cDNA was stored at –20 °C. PCR amplification of GAPDH-encoding cDNA (primers: 5'-TGGTATCGTGGAAGGACTC-3' and 5'-AGTAGAGGCAGGGATGATG-3') was used to confirm that successful cDNA synthesis had been achieved using all mRNA preparations (data not shown).

Primers [16] specific for the variable domains of Ig-encoding genes were synthesized by Eurofins MWG Operon (Ebersberg, Germany) (Table 2). Nested PCR was performed using PfuUltra High-Fidelity DNA Polymerase (Stratagene; Agilent Technologies, Santa Clara, CA, USA) with a set of seven primers annealing to the 5'-end of the seven IGHV subgroups and one of three primers annealing

Table 1. Patient samples used in this study and summary of PCR amplification of IgE-encoding transcripts

	Systemic allergy tests*							
Patient	Positive mould response	Other positive responses	Positive fungal status [†]	Eosinophilic mucin status	IgE [‡]	PCR product	Sequences analysed	No. of clonotypes
CRS								
1	Nil	House dust mite, grass	Nil	Nil	107 kU/L	Yes	18	4
2	Nil	House dust mite	Nil	Nil	854 kU/L	Yes	17	8
3	Nil	Nil	Nil	Nil	25 kU/L	No	–	–
4	Nil	Grass	Nil	Nil	1 kU/L	No	–	–
5	Nil	Grass	Nil	Nil	248 kU/L	Yes	16	12
6	Nil	Cat, weeds	Nil	Nil	ND	Yes	29	3
NAFES								
7	Nil	Nil	Positive	Positive	10 kU/L	Yes	32	9
8	Nil	Nil	Positive	Positive	1188 IU/mL	Yes	30	6
9	Nil	Nil	Positive	Positive	21 IU/mL	No	–	–
10	Nil	Nil	Positive	Positive	15 IU/mL	Yes	25	14
11	Nil	Nil	Positive	Positive	ND	Yes	28	7

*Detailed analytical results provided in supporting information Table S1.

†Positive on culture and/or fungal hyphae staining.

‡Different test methodologies reporting results with different units were used by different diagnostic laboratories.

ND, not determined; CRS, chronic rhinosinusitis; NAFES, non-allergic fungal eosinophilic sinusitis.

Table 2. Sequences of primers used for amplification of genes encoding variable domains of IgE

Primer specificity	Sequence
IGHV1	CAGGTGCAGCTGGTGCAGTCTGG
IGHV2	CAGRTCACCTGAAGGAGTCTGG
IGHV3	GAGGTGCAGCTGGTGGAGTCTGG
IGHV4	CAGGTGCAGCTGCAGGAGTCGGG
IGHV5	GAGGTGCAGCTGGTGCAGTCTGG
IGHV6	CAGGTACAGCTGCAGCAGTCAGG
IGHV7	CAGGTGCAGCTGGTGCATCTGG
IGHE ver. 1	ATAGTGACCAGAGAGCGTGAGGGT
IGHE ver. 2	GGAGGTGGCATTGGAGGGAATGTT
IGHE ver. 3	AAGGGGAAGACGGATGGGCTCTG

within the part of the IGHE gene encoding the first constant domain of IgE. Two or three rounds (the number of cycles were 40, 20–25 and 10–15, respectively, for the first, second and third round of amplification) of PCR were carried out using the same set of 5'-primers and a 3'-primer annealing inside the 3'-primer used previously, but still within the IGHE gene. Negative controls without added template or with the negative reverse transcription template, described above, were used to rule out contamination of the PCR reaction. Negative controls were allowed to proceed through the nested PCR reaction to ensure that very rare contaminants did not result in an amplified product. PCR products were cloned directly into pCR.Blunt II-TOPO vector and transformed into One Shot Electrocomp Top10 *Escherichia coli* according to protocols of the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Paisley, UK). Colonies were picked at random and plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen).

To examine the frequency of mutations introduced as an artefact of the PCR process, a cloned heavy chain variable-domain encoding gene was exposed to a total of 80 cycles over three rounds of PCR, resembling the conditions used in the amplification of genes encoding variable domains of IgE. The PCR product was cloned and transformed as above. Random colonies were picked and plasmids purified using QIAprep Spin Miniprep Kit (Qiagen).

To enable future construction of antibody libraries encoding single-chain fragment variables (scFv), amplified transcripts encoding the variable H chain domain of IgE, obtained as described above, were also modified for cloning in phagemid vectors in a PCR with seven primers annealing to the IGHV subgroups and one annealing to all IGHJ genes [16]. V-KAPPA and V-LAMBDA-encoding genes were amplified in a similar manner using primers annealing to the IGKV and IGLV subgroups and distinct IGKJ and IGLJ genes, separately [16]. Heavy- and light-chain variable domain-encoding genes were assembled in an eight-cycle overlap extension PCR to produce genes encoding a fusion protein with a (glycine₄-serine)₃-linker between the two variable domains. PCR products were cloned into a modified version [17] of the pFab5c.His phagemid vector [18] as described [16]. Ligated DNA was transformed into electro-competent Top10F' *E. coli* (Invitrogen). Colonies were picked at random and plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen). Sequencing of genes cloned in the pCR.Blunt II-TOPO and pFab5c.His vectors was performed by Eurofins MWG Operon using vector-specific primers. Sequences (deposited in GenBank: accession numbers HQ703971–HQ704052 and HQ704054–HQ704068) encoding the H chain variable domain of IgE were pooled from data obtained from the two sets of sequences described above.

Analysis of immunoglobulin gene usage, gene rearrangements and statistical analysis

Sequences encoding the H chain variable domain in sequence collections of sinus NAFES and CRS mucosal tissue IgE (this study) were compared with other IgE transcriptomes. One of these transcriptomes included peripheral blood mononuclear cell (PBMC) IgE-A studied previously by us [16]. Sequence collections PBMC IgE-K [19] and PBMC IgE-W [20] were downloaded from GenBank. In cases where there were several clonally related members [i.e. sequences originating from a common IGHV gene, a common IGHJ gene and with a sequence within the third complementarity-determining region (CDR) of the H chain (CDRH3) of identical length and ≥ 80 nucleotide identity], one of the several members was selected at random for analysis of gene usage, CDRH3 length distribution and mutational status of each population to avoid bias as a consequence of high prevalence of certain related sequences in the transcriptomes. By these standards, these transcriptomes contained 72 (PBMC IgE-A), 105 (PBMC IgE-K) and 116 (PBMC IgE-W) different clonotypes. IGHV, IGHD and IGHJ gene usage and mutational status were determined using the IMGT/V-QUEST web tool (program version: 3.2.15; reference directory release: 201030-3) [21]. PCR-primer encoded sequences were removed before analysis. In sequences where the automated IMGT/V-QUEST tool suspected insertions or deletions, the analysis was repeated using the 'Search for insertions and deletions in V-REGION'-advanced setting. Data for PBMC IgE-C and AR IgE [13] and from studies of Igs made by Yamada et al. [22] and Boyd et al. [23] were obtained directly from data presented in those publications. Similarly, CDRH3 amino acid composition data of PBMC IgE-K and a corresponding IgM repertoire (PBMC IgM-K) were obtained from the literature [19]. Statistical comparisons of IGHV subgroup, IGHD subgroup and IGHJ gene usage, CDRH3 length and IGHV gene mutational status were performed using the non-directional Mann-Whitney *U*-test on the VassarStats web site (<http://faculty.vassar.edu/lowry/VassarStats.html>). Significant differences were found at the level of $P < 0.05$ without and with correction for multiple statistical tests using the Benjamini-Hochberg [24] false discover method (<http://sdmproject.com/utilities/?show=FDR>). CDR are defined as described in the IMGT nomenclature [25].

Results

Amplification of immunoglobulin E-encoding transcripts found in sinus tissue

PCR products of IgE transcripts were obtained from four out of five patients diagnosed with NAFES and from four out of six patients diagnosed with CRS. The negative

controls included in the PCR procedure did not give rise to such products. In the case of CRS, a PCR product was only obtained from cDNA derived from subjects with signs of systemic allergy (Table 1). Sequence analysis of cloned PCR products (115 and 80 sequences derived from mucosa of NAFES and CRS, respectively) was carried out. From these, 36 and 27 clonally independent sequences were identified from material obtained from NAFES and CRS patients, respectively. Consequently, even when sequencing a limited number of PCR products, several clonally related products were identified, indicating that the sizes of the IgE-encoding repertoires were small. This agrees well with findings of limited sizes of IgE repertoires encoded by B lymphocytes found in peripheral blood [16, 19, 26, 27].

Diversity of genetic origin of immunoglobulin E-encoding transcripts in sinus mucosa

Despite the limited size of the clonotypes, they were diverse with respect to their origin in germline genes. The repertoires found in patients diagnosed with NAFES as well as CRS were dominated by antibodies encoded by genes belonging to subgroup IGHV3 with IGHV1 representing the second most common subgroup (Fig. 1a). These IGHV subgroups are also commonly used to encode antibody repertoires in other lymphocyte populations such as peripheral blood B lymphocytes producing IgG and IgE [16, 19, 28]. The distribution of IGHV subgroups in the IgE transcriptome of NAFES and CRS patients did not differ significantly (Table 3). Furthermore, these IGHV gene distributions did not differ significantly from that found in IgE-encoding transcripts in peripheral blood B lymphocytes of allergic individuals [16] that had been amplified using a very similar primer set (Table 2). No extensive overrepresentation, as observed in other studies [16, 29], of genes derived from IGHV5 was seen (Fig. 1a). Not only were diverse IGHV subgroups used to encode IgE in sinus tissue of subjects diagnosed with NAFES or CRS but also within these gene subgroups a diverse set of members were used (Fig. 2). We were able to identify IgE-encoding transcripts derived from five and six members of the IGHV1 subgroup and from 13 and seven members of the IGHV3 subgroup in patients diagnosed with NAFES and CRS, respectively. Only the IGHV4 subgroup was substantially underrepresented in comparison with its use in rearrangements in normal B lymphocytes, as determined by others [23, 28]. The distribution of IGHJ gene usage (Fig. 1b) did not differ between IgE transcriptomes in sinus tissue in subjects diagnosed with NAFES and CRS nor did their distribution differ from that observed in the transcriptome of IgE transcripts from peripheral blood B lymphocytes [16, 19] or other isotypes [22] (Table 3). The IGHD gene usage was similarly diverse, using all IGHD subgroups except the otherwise rarely used IGHD7 subgroup (Fig. 1c) and 16 and

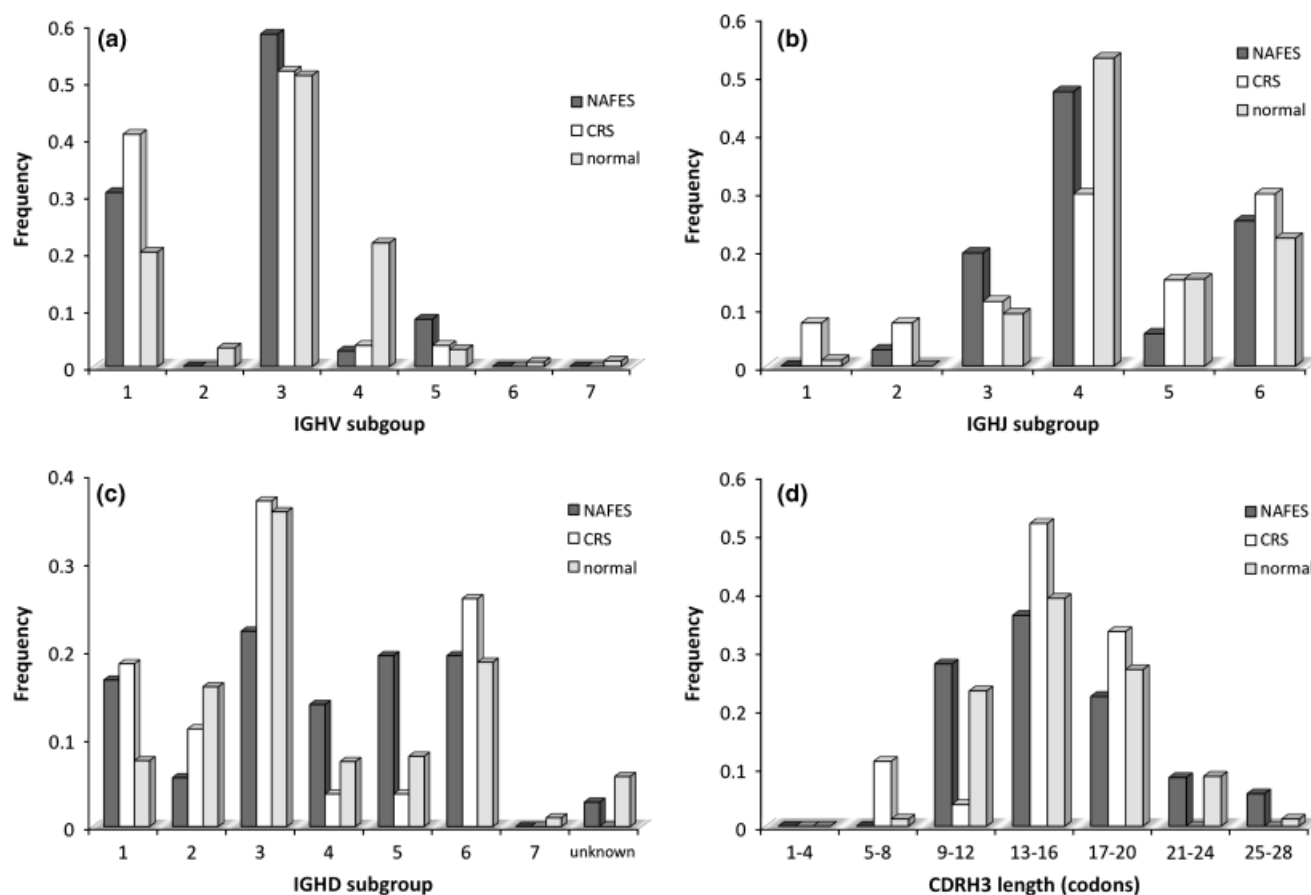


Fig. 1. Diversity of IgE-encoding transcripts in sinus mucosa. Origin in different IGHV (a), IGHJ (b) and IGHD (c) subgroups among different transcripts derived from mucosal tissue of subjects diagnosed with non-allergic fungal eosinophilic sinusitis (NAFES) (dark grey bars) and chronic rhinosinusitis (CRS) (white bars). The distribution of lengths of the critical CDRH3 loop in the populations of IgE-encoded transcripts is shown in (d). The results are compared with other Ig repertoires as assessed by Boyd et al. [23] in the case of IGHV and IGHD subgroup usage (a, c) and Yamada et al. [22] in the case of IGHJ subgroup usage and CDRH3 length (b, d). Altogether, NAFES and CRS both feature mucosal IgE-encoding transcripts with a character similar to those found in other Ig repertoires.

14 out of 27 IGHD genes were used in the NAFES and CRS IgE-encoding gene sets, respectively. Altogether, the gene repertoire encoding IgE H chains in sinus tissue, both in subject suffering from CRS and NAFES, is diverse with respect to its genetic origin.

The product of the gene rearrangement, the CDRH3, believed to be of substantial importance for antibody specificity [30], did not differ in length between IgE repertoires encoded by B lymphocytes in the sinus tissue of patients suffering from NAFES and CRS (Fig. 1d, Table 3). The average lengths of CDRH3 in these populations were 15.8 and 14.6 residues, in agreement with the length of CDRH3 found in IgE produced by peripheral blood B lymphocytes (14.8–16.1 residues) as exemplified by the PBMC IgE-A, PBMC IgE-C and PBMC IgE-W transcriptomes and in other Ig repertoires (15.1–15.3 residues) [22, 31] (Table 3). The amino acid composition of these polyclonal repertoires was also reasonably in agreement with each other and with other reported repertoires (supporting information Fig. S1).

Mutational status of immunoglobulin E-encoding transcripts in sinus mucosa

In order to assess the mutational status of IgE-encoding transcripts in sinus tissue, we identified the frequency of mutations among the different clonotypes. All clonotypes carried mutations in relation to the closest IGHV germline gene (1.9–23%), but we observed no difference in mutational status of sequences derived from the tissue of NAFES (mutational frequency: $6.4 \pm 3.2\%$) and CRS (mutational frequency: $7.0 \pm 4.4\%$) (Fig. 3, Table 3). Control experiments indicated that the PCR process induced 0.22% mutations in an amplified product, confirming that the vast majority of all mutations were not experimental artefacts but rather occurred as a result of a somatic hypermutation process occurring *in vivo*. Mutations were targeted to CDR as they, in comparison with framework regions (FR), carried 2.7 and 3.1 times higher frequencies of base substitutions in transcripts derived from subjects diagnosed with CRS and NAFES, respectively. As expected, non-silent mutations

Table 3. Statistical comparisons of IGHV subgroup, IGHD subgroup, and IGHJ gene usage, CDRH3 length and mutation frequency of IgH chain-encoding transcriptomes

Parameter	Compared sets of sequences		Significance of difference*	
			Without correction	With Benjamini–Hochberg correction
IGHV subgroup	Mucosal NAFES IgE	Mucosal CRS IgE	NS	NS
	Mucosal NAFES IgE	PBMC IgE-A	NS	NS
	Mucosal CRS IgE	PBMC IgE-A	NS	NS
	Mucosal NAFES IgE	PBMC IgE-K	$P = 0.032$	NS
	Mucosal CRS IgE	PBMC IgE-K	$P = 0.004$	$P = 0.025$
	Mucosal NAFES IgE	PBMC IgE-W	NS	NS
	Mucosal CRS IgE	PBMC IgE-W	NS	NS
	Mucosal NAFES IgE	PBMC IgE-C	$P = 0.0124$	NS
	Mucosal CRS IgE	PBMC IgE-C	$P = 0.0023$	$P = 0.016$
	Mucosal NAFES IgE	Nasal AR IgE	$P = 0.0001$	$P = 0.001$
	Mucosal CRS IgE	Nasal AR IgE	$P < 0.0001$	$P < 0.001$
	Nasal AR IgE	PBMC IgE-A	$P < 0.0001$	$P < 0.001$
	Nasal AR IgE	PBMC IgE-K	$P = 0.0008$	$P = 0.0069$
	Nasal AR IgE	PBMC IgE-W	$P < 0.0001$	$P < 0.001$
	Nasal AR IgE	PBMC IgE-C	NS	NS
	PBMC IgE-A	PBMC IgE-K	$P = 0.017$	NS
	PBMC IgE-A	PBMC IgE-W	NS	NS
	PBMC IgE-A	PBMC IgE-C	$P = 0.008$	$P = 0.043$
	PBMC IgE-K	PBMC IgE-W	$P = 0.026$	NS
	PBMC IgE-K	PBMC IgE-C	NS	NS
	PBMC IgE-W	PBMC IgE-C	$P = 0.014$	NS
IGHJ gene	Mucosal NAFES IgE	Mucosal CRS IgE	NS	NS
	Mucosal NAFES IgE	PBMC IgE-A	NS	NS
	Mucosal CRS IgE	PBMC IgE-A	NS	NS
	Mucosal NAFES IgE	PBMC IgE-K	NS	NS
	Mucosal CRS IgE	PBMC IgE-K	NS	NS
	Mucosal NAFES IgE	PBMC Ig [†]	NS	NS
IGHD subgroup	Mucosal CRS IgE	PBMC Ig [†]	NS	NS
	Mucosal NAFES IgE	Mucosal CRS IgE	NS	NS
	Mucosal NAFES IgE	PBMC IgE-A	$P = 0.02$	NS
	Mucosal CRS IgE	PBMC IgE-A	NS	NS
	Mucosal NAFES IgE	PBMC IgE-K	NS	NS
CDRH3 length	Mucosal CRS IgE	PBMC IgE-K	NS	NS
	Mucosal NAFES IgE	Mucosal CRS IgE	NS	NS
	Mucosal NAFES IgE	PBMC IgE-A	NS	NS
	Mucosal CRS IgE	PBMC IgE-A	NS	NS
	Mucosal NAFES IgE	PBMC IgE-K	NS	NS
	Mucosal CRS IgE	PBMC IgE-K	NS	NS
	Mucosal NAFES IgE	PBMC IgE-W	NS	NS
	Mucosal CRS IgE	PBMC IgE-W	NS	NS
Mutation frequency	Mucosal NAFES IgE	PBMC Ig [†]	NS	NS
	Mucosal CRS IgE	PBMC Ig [†]	NS	NS
Mutation frequency	Mucosal NAFES IgE	Mucosal CRS IgE	NS	NS

*Mann–Whitney *U*-test[†]Yamada *et al.* [22].

NS, not significant; NAFES, non-allergic fungal eosinophilic sinusitis; AR, allergic rhinitis; PBMC, peripheral blood mononuclear cells; CRS, chronic rhinosinusitis.

were targeted to CDR (Fig. 4). These levels are in close agreement with the level of mutation (7.2%; range: 0.5–21%) found in IgE-encoding transcripts produced by peripheral blood B lymphocytes [19]. The high frequency of mutation indicates that IgE-encoding transcripts found in the sinus mucosa in patients diagnosed not only with CRS

but also with NAFES are produced by B lymphocytes that have undergone an affinity maturation process and not just by B lymphocytes that have been clonally expanded in the absence of a germinal centre reaction.

In some cases, clonally related sequences, not only as determined by an origin in identical IGHV and IGHJ genes

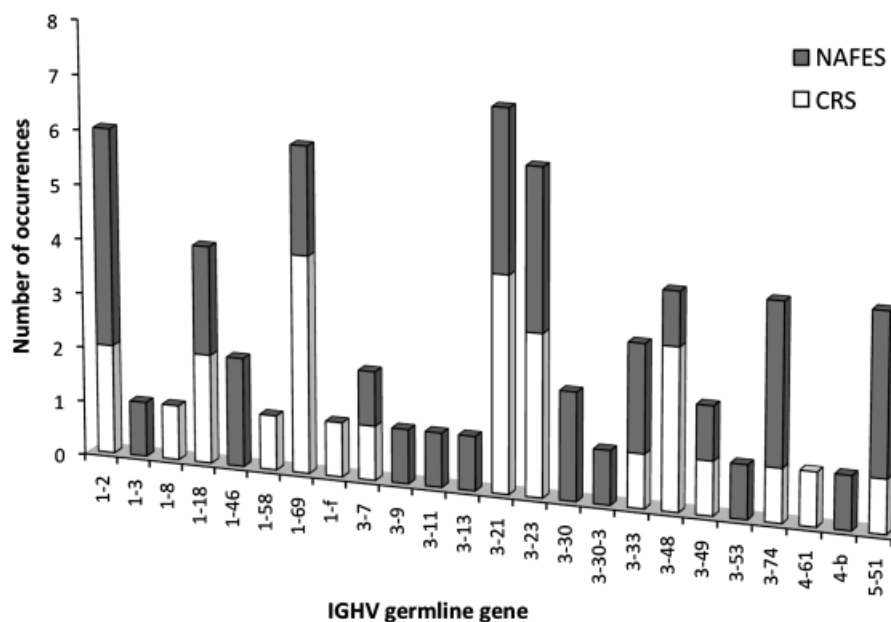


Fig. 2. IGHV germline gene origin among IgE-encoding sequences derived from mucosal tissue of patients diagnosed with non-allergic fungal eosinophilic sinusitis (NAFES) (grey bars) and chronic rhinosinusitis (CRS) (white bars). Both conditions are characterized by diverse IGHV gene usage.

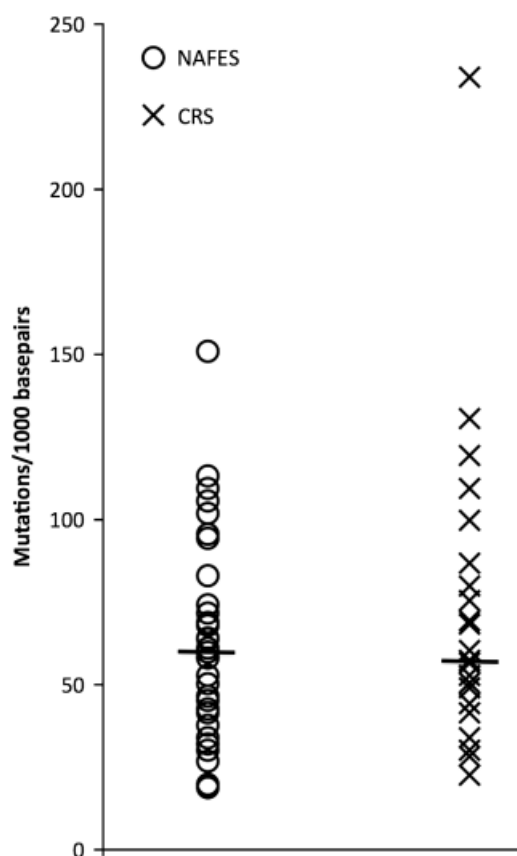


Fig. 3. Mutational status of the different clonotypes derived from subjects diagnosed with non-allergic fungal eosinophilic sinusitis (NAFES) (O) and chronic rhinosinusitis (CRS) (X), indicating a high and similar level of mutation in such IgE repertoires. The median level of mutation for each population is illustrated with a horizontal line.

but also with extensive similarity in CDRH3 sequence, with different mutational status were identified in material derived from individuals diagnosed with NAFES and CRS (Fig. 5 and supporting information Fig. S2). These findings demonstrate that cells that have diversified from each other through a mutational process are present simultaneously in sinus tissue of such subjects.

Discussion

CRS represents a heterogeneous set of diseases that in many cases have a poorly defined aetiology. IgE-mediated allergy contributes to some of these conditions but many subjects show no evidence of systemic allergic disease. We here describe that IgE-encoding transcripts derived from the sinus mucosa of subjects diagnosed with one such supposedly non-allergic condition, NAFES, show features similar to those of IgE repertoires encoded in sinus mucosa of patients diagnosed with allergic CRS as well as in peripheral blood B lymphocytes of allergic subjects. These similarities include a very diverse gene usage, length of the critical CDRH3 sequence and the mutational status of these IgE-encoding genes. The results implicate that NAFES is accompanied by the local production of antigen-specific IgE and that the condition involves a response similar to a conventional IgE response.

There are some differences in particular between the distribution of IGHV subgroups between the different IgE populations assessed in this study (Table 3). The major differences, that even withstand a Bonferroni-Hochberg

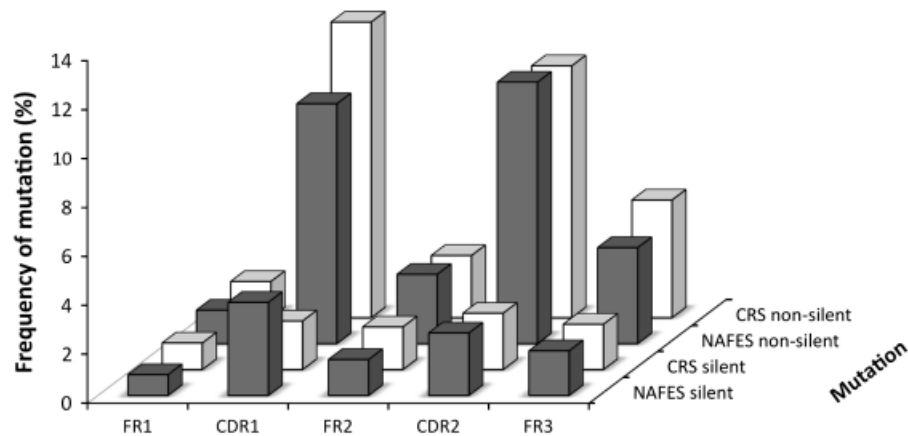


Fig. 4. Frequency of base substitutions in framework regions (FR) and complementarity determining region (CDR) of transcripts encoding IgE in mucosal tissue derived from subjects diagnosed with non-allergic fungal eosinophilic sinusitis (NAFES) (dark grey bars) and chronic rhinosinusitis (CRS) (white bars).

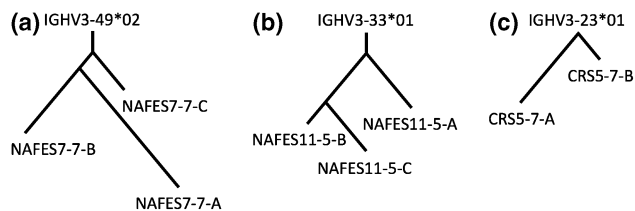


Fig. 5. Evidence for the presence of multiple, clonally related sequences in the sinus mucosa. Proposed evolution paths of different members of three clonotypes derived from patients 7 (a) and 11 (b), both diagnosed with non-allergic fungal eosinophilic sinusitis (NAFES), and patient 5 (c) diagnosed with chronic rhinosinusitis (CRS). The length of the lines is proportional to the number of mutations from the end of the 5'-primer to codon 106 (the second codon in CDRH3) in relation to the closest IGHV germline gene sequence. A detailed sequence comparison is given in supporting information Fig. S2.

correction that deal with false discovery when applying multiple statistical comparisons, are those between the nasal AR IgE transcriptome [13] and a variety of other IgE repertoires, including those found in the sinus mucosa of subjects diagnosed with NAFES and CRS (Table 3). Indeed, skewed repertoires have been taken as evidence for modes of B lymphocyte stimulation other than those encountered in regular T cell-dependent antibody responses involving conventional allergens. Microbial superantigens have been proposed as one modality that may cause such abnormal responses by polyclonal activation of B lymphocytes encoding antibodies with an origin in specific IGHV gene subgroups, irrespective of their antigen specificity. Such indications have in particular been implied by preferential expansion of an IgE population with an origin in not only IGHV5 [13, 29] but also IGHV6 [32]. However, a potential problem of technical nature in studies of IgE repertoires is that complex primer mixtures have to be used for extensive amplification or rare cDNA sequences, mixtures with less than perfect fit to the many different gene members of the different families. Different studies

use different primer sets, and differences in amplification efficiency of the different primer sets with respect to different IGHV subgroups have in many cases not been formally ruled out. Such differences may in particular affect the results of IGHV gene and subgroup utilization. Importantly, IgE repertoires found in mucosal tissue of subjects with NAFES can neither, as outlined above, be differentiated from those found in mucosal tissue of subjects with CRS nor from at least two independently investigated IgE repertoires of peripheral blood B lymphocytes. We conclude that there currently is no evidence for extensively skewed IgE repertoires in subjects diagnosed with NAFES. Our studies thus lend little support for an involvement of a common superantigen activity in NAFES. One cannot formally exclude the possibility of the existence of many different superantigens with an origin in the different pathogens that colonize the sinus mucosa, which together target and expand all major IGHV subgroups. However, the preferential localization of mutations to CDR would, if anything, speak against an extensive involvement of mainly FR-binding superantigens as a driver for polyclonal B lymphocyte activation and Ig gene hypermutation resulting in mucosal IgE production. The high level of mutation also argues against an involvement of B-1-like B cells as a source of IgE in the sinus mucosa of subjects diagnosed with NAFES, a population of lymphocytes that have been implicated previously in largely unmutated IgE responses [32]. These findings suggest that mucosal IgE repertoires involved in NAFES also in this respect are similar to conventional, hypermutated IgE repertoires found in peripheral blood lymphocytes of allergic subjects [16, 19].

The fact that we do find a substantial amount of clonally related sequences, even when sequencing a limited number of PCR products, indicates that only few B lymphocytes are recruited to participate in such mucosal IgE responses. Such restricted repertoire sizes suggest a

repertoire that is targeted against a limited number of epitopes. This is in agreement with studies of the limited sizes of IgE-encoding repertoires found in peripheral blood of allergic individuals [16, 19, 26, 27] and again argues against an extensive polyclonal activation by superantigens. The fact that clonally related, but different, sequences are found implies either that clones with a common clonal origin evolved in secondary lymphoid organs migrate to adjacent sites in the mucosa, or that these clones actually evolve their antibody-encoding genes within the mucosa itself. Indeed, evidence of local hypermutation and Ig class switch has been identified in nasal mucosa of subjects with AR [33]. Further studies that measure activation-induced cytidine deaminase activity as an indicator of ongoing hypermutation in the sinus mucosa of subjects with evidence of NAFES are therefore warranted.

Although the number of sequenced IgE-encoding transcripts is relatively small, some IGHV genes that are not among the most commonly used IGHV genes, were repeatedly encountered in some instances (Fig. 2). For example, three independent clones originating from IGHV1-2, which normally makes up only 0.3–3.9% of a single individual's repertoire [23], were identified in donor 10's IgE-encoding transcriptome. At this point, we can only speculate on whether or not this is a coincidence or whether it relates to a selection process *in vivo*, possibly caused by a superantigen or, in our view more likely, by recruitment of a restricted IGHV gene repertoire by an immunodominant allergen of e.g. fungal or bacterial

origin. Such restricted repertoires targeting immunodominant epitopes have been identified in the past in allergic responses [34] as well as in a number of other antibody repertoires (as exemplified in Persson *et al.* [34]). A future assessment of the specificity of such clonotypes, e.g. by combinatorial antibody library technology, as demonstrated in the case of other IgE repertoires [27], will be required to resolve this finding properly.

In conclusion, IgE repertoires encoded in the sinus mucosa of patients suffering from NAFES do not appear different from other polyclonal Ig repertoires raised upon encounter with antigen indicating that they have developed as a consequence of a conventional response against antigen exposure. Future detailed investigations of the specificity of these antibodies in the quest to identify allergic components potentially involved the development NAFES in different subjects should allow us to assess this disease by the use of new diagnostic tools measuring local, mucosal IgE. Such information might aid clinical decision-making, improve management of the disease and allow targeting of the causes of disease for instance by use of specific immunotherapy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Amino acid composition of CDRH3 of IgE as assessed by analysis of transcripts in mucosal tissue of subjects diagnosed with NAFES (green bars) and (blue bars) in comparison with the amino acid composition of CDRH3 in transcripts found in peripheral blood mononuclear that encode IgE (yellow bars (PBMC-IgE-W[1]) and red bars (PBMC IgE-K [2])) or IgM (white bars (PBMC IgM-K [2])). Amino acids are defined by the standard one-letter code. No extensive misrepresentation of residues is seen in IgE-encoding transcriptomes in NAFES and CRS.

Figure S2. Different levels of mutations in different PCR-products with an origin in three different immunoglobulin gene rearrangements (clonotypes) identified from patients 7 (A) and 11 (B), both diagnosed with NAFES, and patient 5, diagnosed with CRS (C). CDR, as

defined by the IMGT nomenclature [3], are highlighted with black lines. Note the presence of substitutions shared by all members of the clonotypes derived from subjects 7 (A) and 11 (B) in comparison to their germline gene origin (highlighted by stars), as well as substitutions differentiating the different sequences in all three clonotypes, each with a likely origin in a common B lymphocyte rearrangement. The grey line indicates the part of the amplified sequences that encodes the constant domain and thus defines that these clonotypes have been amplified from the IgE-encoding transcriptome.

Table S1. Compilation of allergy diagnostic tests performed on subjects included in this study.

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Supporting information for

Diversity of IgE-encoded transcripts in sinus mucosa of subjects diagnosed with non-allergic fungal eosinophilic sinusitis

Mattias Levin¹, Lor Wai Tan², Leonie Baker², Peter-John Wormald², Lennart Greiff³ and
Mats Ohlin^{1,*}

Dept. of Immunotechnology¹, Lund University, Lund, Sweden; Department of Surgery-
Otorhinolaryngology, Head and Neck Surgery², University of Adelaide, Adelaide,
Australia; Dept. of Otorhinolaryngology, Head and Neck Surgery³, Skåne University
Hospital/Lund University, Lund, Sweden

Supplementary Table 1. Compilation of allergy diagnostic tests performed on subjects included in this study.

	Patient	Systemic allergies	IgE ^a	Allergen-specific IgE in blood ^a
CRS ^c	1	allergic symptoms SPT ^b : mould: - house dust/mite: + grass: + animal dander: -	107 kU/L	mould mix: <0.35 kUA/L mite/house dust mix: 7.0 kUA/L grass mix: 23.8 kUA/L animal mix: <0.35 kUA/L
	2	allergic symptoms SPT: mould: - house dust/mite: +	854 kU/L	mould mix: 0.66 kUA/L mite/house dust mix: 6.2 kUA/L grass pollen mix: 0.71 kUA/L tree pollen mix: 0.63 kUA/L weed mix: 0.7 kUA/L
	3	no allergic symptoms	25 kU/L	mould mix: <0.10 KU/L mite/house dust mix: <0.10 KU/L grass mix: <0.10 KU/L weed mix: <0.10 KU/L
	4	allergic symptoms SPT: mould: - house/dust mite: - grass: + animal: -	1 kU/L	mould mix: <0.35 kUA/L mite/house dust mix: <0.35 kUA/L grass mix: 2.4 kUA/L animal mix: <0.35 kUA/L
	5	allergic symptoms SPT: mould: - grass: + others: -	248 kU/L	Alternaria (m6): <0.35 kUA/L house dust mite (d1): <0.35 kUA/L couch grass (g2): 1.5 kUA/L rye grass (g5): 3.9 kUA/L cat dander (e1): <0.35 kUA/L
	6	allergic symptoms SPT: mould: - cat: + weeds: +	ND ^e	ND
NAFES ^d	7	no allergic symptoms	10 kU/L	mould mix: <0.35 kUA/L mite/house dust mix: <0.35 kUA/L grass mix: 0.44 kUA/L animal dander mix: <0.35 kUA/L
	8	no allergic symptoms	1188 IU/mL	mould mix: negative mite/house dust mix: negative pollen mix: negative animal dander mix: positive
	9	no allergic symptoms	21 IU/mL	mould mix: <0.35 kUA/L mite/house dust mix: <0.35 kUA/L pollen mix: <0.35 kUA/L weed mix: <0.35 kUA/L
	10	no allergic symptoms	15 IU/mL	mould mix: negative mite/house dust mix: negative pollen mix: negative weed mix: positive
	11	no allergic symptoms	ND	ND

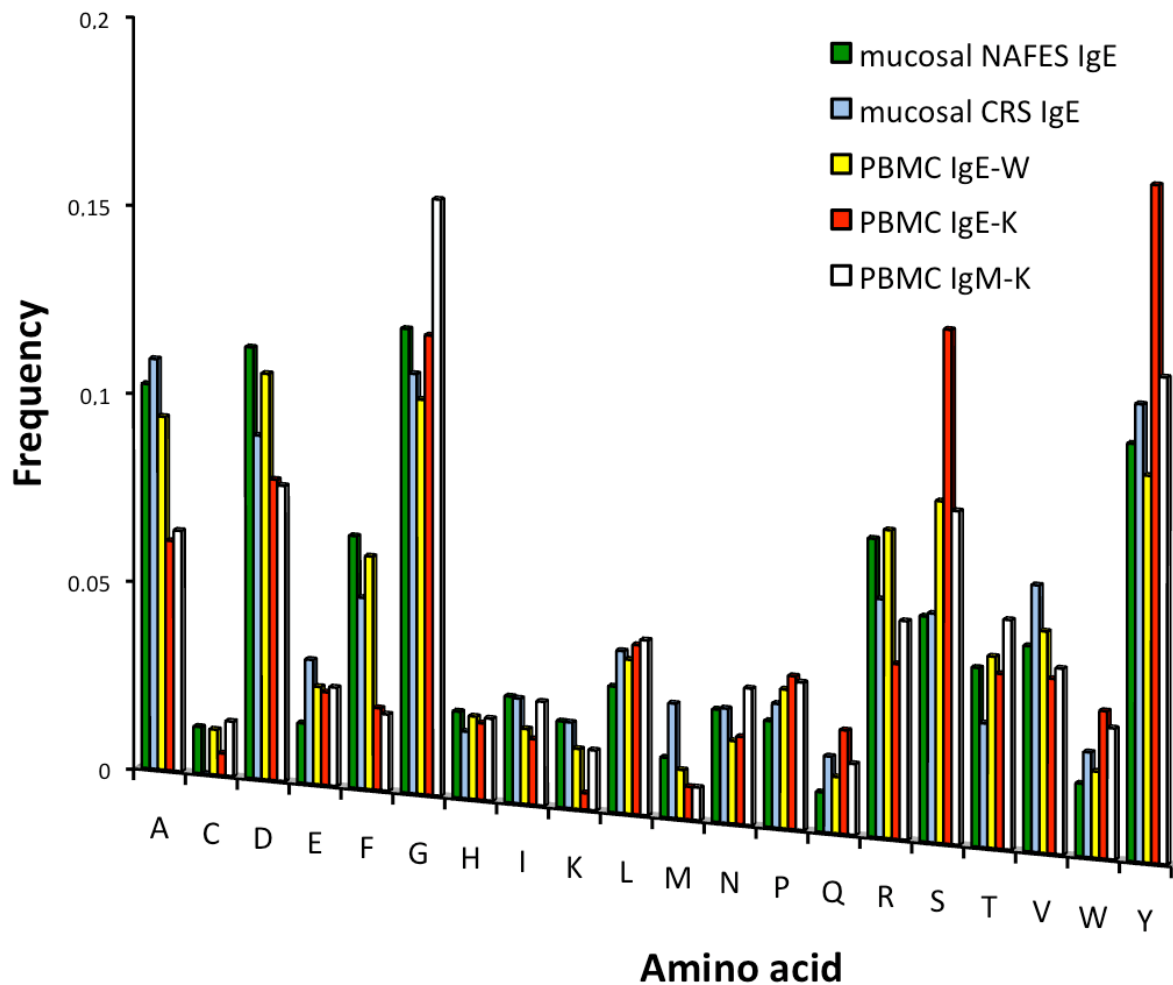
^a different test methodologies reporting results with different units were employed by different diagnostic laboratories

^b skin prick test

^c subjects negative for eosinophilic mucus and for culture and staining of fungal hyphae

^d subjects positive for eosinophilic mucus and for either culture or staining of fungal hyphae or for both

^e not determined at time of surgery



Supplementary Figure 1. Amino acid composition of CDRH3 of IgE as assessed by analysis of transcripts in mucosal tissue of subjects diagnosed with NAFES (green bars) and CRS (blue bars) in comparison with the amino acid composition of CDRH3 in transcripts found in peripheral blood mononuclear that encode IgE (yellow bars (PBMC-IgE-W [1]) and red bars (PBMC IgE-K [2])) or IgM (white bars (PBMC IgM-K [2])). Amino acids are defined by the standard one-letter code. No extensive misrepresentation of residues is seen in IgE-encoding transcriptomes in NAFES and CRS.

A IGHV3-49*02 GAGGTCAGCTGGTGGAGTCTGGGGAGGCTTGGTACAGCCAGGGCGGTCCCTGAGACTCTCCTGTACAGCTTCTGGATT
NAFES7-7-A GGGAGGCTTACTACAGCCAGGGCGATCCGTGAGACTCTCCTGTGCAACTTCTGGATT
NAFES7-7-B GGGAGGCTTGGTACAGCCAGGGCGGTCTCTGACACTCTCCTGTTCAGTTCTGGATT
IGHJ6*02 GGGAGGCTTGGTACAACAGGGCGGTCCCTGAGACTCTCCTGTACAACTTCTGGATT

IGHV3-49*02 CACCTTTGGTGATTATGCTATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTAGGTTTCATTAGAAGCA
NAFES7-7-A CACCTTTGGTGATTACGCGATGAGTTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTGGGTTTCATTAGAAGCA
NAFES7-7-B AACCTTTGGTGATTATGCTATGAGTTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTGGGTCTCATAGAAGCA
NAFES7-7-C CACCTTTGGTGATTATGCTATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTAGGTTTCATTAGAAGCA
IGHJ6*02

IGHV3-49*02 AAGCTTATGTTGGGACAACAGAAATACGCCGCGTCTGTGAAAGGCAGATTCACTATCTCAAGAGATGATTCAAAAAGCATC
NAFES7-7-A AGACTTATCGTGGGATTAATAGACTACGCCGCGTCTGTGAAAGGCAGATTCAACATTTCAAGAGATGATTCAAAAAGCATC
NAFES7-7-B AACCTTATCGTGGGACAACAGACTACGCCGCGTCTGTGAAAGGCAGATTCAACATCTCAAGAGATGATTCAAAAAGCATC
NAFES7-7-C AAACCTTATCGTGGGACAACAGAAATATGCGCGCTCTGTGAAAGGCAGATTCACTATCTCAAGAGATGATTCAAAAAGCATC
IGHJ6*02 * *

IGHV3-49*02 GCCTATCTGCAAAATGAACAGCCTGAAAAACGAGGACACAGCCGTGTATTACTGTACTAGAGA
NAFES7-7-A GCCTATCTGGAATGAACAGCTGTGAAAGTCGAGGACACAGCCCTGTATTATTGTTGTAGAGTTAGAGGAAGCGGCTACGG
NAFES7-7-B GCCTATCTGCAAAATGAACAGCCTGAAAAACGAGGACACAGCCGTGTATTACTGTGGTAGAACCTTAGGGAGCGGCAACGC
NAFES7-7-C GCCTATCTGCAAGTGAACAGCCTGCAAAACGAGGACACAGCCGTGTATTACTGTGTAGAGTTTTAGGGAGCGGCAACGC
IGHJ6*02 *

IGHV3-49*02 CTCAAATACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGCCTCCACA
NAFES7-7-A CATATCTACTACTACTACGGTATGGACGTCTGGGGCCTAGGGACCACGGTCACCGTCTCCTCAGCCTCCACA
NAFES7-7-B GGCATACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCA
IGHJ6*02 TTACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAG

B IGHV3-33*01 CAGGTGCAGCTGGTGGAGTCTGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCGTCTGGATT
NAFES11-5-A GGGAGGCGTGGTCCAGCCTGGGAAGTCCCTGAGACTCTCCTGTGCAGCGTCTGGATT
NAFES11-5-B GGGAGGCGGACCCAGCCTGGGAGGTCACTGAGACTCTCCTGCAAGCGTCTGGATT
IGHD5-24*01 GGGAGGCGGCGCCAGCCTGGGAGGTCCCTGAGACTCTCCTGCGAAGCGTCTGGCTT
IGHJ3*02

IGHV3-33*01 CACCTTCAGTACCTATGGCATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATGGTATG
NAFES11-5-A CACCTTCAGTGTCTTTGGCATGCACTGGGTCCGCCAGACTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATGGTATG
NAFES11-5-B CACCTTCAGTAGCTATGGCATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATGGTATG
NAFES11-5-C CACCTTCAGTACCTATGGCATGCACTGGGTCCGTCAAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGCTATATGGTATG
IGHD5-24*01
IGHJ3*02

IGHV3-33*01 ATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGGCCGATTACCATCTCCAGAGACAATTCCAAGAACAAGCTGTAT
NAFES11-5-A ATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGGTTCGATTACCATCTCCAGAGACAATTCCAAGAACAATACTAT
NAFES11-5-B ATGGAAGTATAGACTCCGTGAAGGGCCGATTACCATCTCCAGAGACAATTCCAAGAACAATACTAT
NAFES11-5-C ATGGAAGTATAGACTCCGTGAAGGGCCGATTACCATCTCCAGAGACAATTCCAAGAACAATACTAT
IGHD5-24*01 **
IGHJ3*02

IGHV3-33*01 CTGCAAAATGAACAGCCTGAGAGCCGACGACACGGCTGTGTATTACTGTGCGAGAGA
NAFES11-5-A CTGCAAAATGAACAGCCTGAGAGCCGACGACACGGCTGTATATTACTGTGCGAGAGCTGTCCCCAAATACACTCCGCAAGA
NAFES11-5-B CTGCAAAATGAACAGCCTGAGAGTCCGACGACACGGCTGTGTATTACTGTGCGAGAGCTGTCCCCAAATACACTCCGCAAGA
NAFES11-5-C CTGCAAAATGAACAGCCTGAGAGCCGACGACGAGCTGTATATTACTGTGCGAGAGCTGTCCCCAAATACACTCCGCAAGA
IGHD5-24*01 * GTAGAGATGGCTACAAATTAC
IGHJ3*02 GA

IGHV3-33*01 TGTTTTTGATATCTGGGGCCAAGGGACAATGGTACCCGTCTCTTCAGCCTCCACA
NAFES11-5-A TGTTTTTGATATCTGGGGCCAAGGGACAATGGTACCCGTCTCTTCAGCCTCCACA
NAFES11-5-B TGTTTTTGATATCTGGGGCCAAGGGACAATGGTACCCGTCTCTTCAGCCTCCACA
IGHD5-24*01
IGHJ3*02 TGTCTTTGATATCTGGGGCCAAGGGACAATGGTACCCGTCTCTTCAG

C IGHV3-23*01 GAGGTGCAGCTGTTGGAGTCTGGGGAGGCTTGGTACAGCCTGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATT
CRS5-7-A GGGAGGCGTGGTCCAGCCTGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATT
CRS5-7-B GGGAGGCTTGGTACAGCCTGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATT
IGHD2-2*01
IGHJ5*02

IGHV3-23*01 CACCTTTAGCACTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTA
CRS5-7-A CACCTTTAGTGCCTATCTCTATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTA
CRS5-7-B CACCTTTAGCAAAATATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTA
IGHD2-2*01
IGHJ5*02

IGHV3-23*01 GTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTACCATCTCCAGAGACAATTCCAAGAACACGCTGTAT
CRS5-7-A GTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTACCATCTCCAGAGACAATTCCAAGAACACGCTGTAT
CRS5-7-B GTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTACCGTCTCCAGAGACAATTCCAAGAACACGCTGTAT
IGHD2-2*01
IGHJ5*02

IGHV3-23*01 CTGCAAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAGA
CRS5-7-A CTGCAAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAGCGTGGGACCATAAACAGCTGCCCT
CRS5-7-B CTGCAAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAGCGTGGGACCATAAACAGCTGCCCT
IGHD2-2*01 AGGATATTGTAGTAGTACCAGCTGCTAT
IGHJ5*02

IGHV3-23*01 CTACAACCTGGTTCGACCCCTGGGGCCAGGGAACCTGGTCACCGTCTCCTCAGCCTCCACA
CRS5-7-A CTACAACCTGGTTCGACCCCTGGGGCCAGGGAACCTGGTCACCGTCTCCTCAGCCTCCACA
CRS5-7-B GGC
IGHD2-2*01 ACAACCTGGTTCGACCCCTGGGGCCAGGGAACCTGGTCACCGTCTCCTCAG
IGHJ5*02

Supplementary Figure 2. Different levels of mutations in different PCR-products with an origin in three different immunoglobulin gene rearrangements (clonotypes) identified from patients 7 (A) and 11 (B), both diagnosed with NAFES, and patient 5, diagnosed with CRS (C). CDR, as defined by the IMGT nomenclature [3], are highlighted with black lines. Note the presence of substitutions shared by all members of the clonotypes derived from subjects 7 (A) and 11 (B) in comparison to their germline gene origin (highlighted by stars), as well as substitutions differentiating the different sequences in all three clonotypes, each with a likely origin in a common B lymphocyte rearrangement. The grey line indicates the part of the amplified sequences that encodes the constant domain and thus defines that these clonotypes have been amplified from the IgE-encoding transcriptome.

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