

The Journal of Immunology

This information is current as
of July 30, 2010

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J. Immunol. published online Jul 21, 2010;
doi:10.4049/jimmunol.0902942

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IgE Transcripts in the Circulation of Allergic Children Reflect a Classical Antigen-Driven B Cell Response and Not a Superantigen-Like Activation

Sebastian Kerzel,¹ Tobias Rogosch,¹ Benjamin Struecker, Rolf F. Maier and Michael Zemlin

Allergic asthma is the most frequent chronic disorder in childhood. Although IgE is a central effector molecule in allergic diseases, the nature of the IgE response is still under debate. The objective of our study was to clarify whether the IgE repertoire in the circulation of allergic children represents a classical Ag-driven and oligoclonal B cell response, a superantigen-like activation of a subset of B cells, or a polyclonal B-1 cell expansion. Using a highly sensitive RT-PCR method, we amplified, cloned, and sequenced IgE H chain transcripts from 13 children with allergic asthma. We gained 1366 functional IgE sequences, which currently represent the most extensive collection of human IgE transcripts. Compared to IgM transcripts from the same children, the somatic mutation rate was significantly enhanced in IgE transcripts (21‰ versus 72‰; $p < 0.001$), which renders a polyclonal B-1 response unlikely. Moreover, IgE sequences displayed significantly enhanced Ag selection and hence were indicative of a classical Ag-driven immune response with affinity maturation ($p < 0.001$). In contrast to several recent studies, the usage pattern of variable gene segment of the H Ig chain in IgE transcripts followed the germline complexity, arguing against a superantigen-like interaction. We conclude that IgE transcripts in the circulation of children with allergic asthma reflect a classical adaptive B-2 cell response. This study provides reference data for a better characterization of the IgE response under immunomodulating therapies, such as anti-IgE therapy or allergen-specific immunotherapy. *The Journal of Immunology*, 2010, 185: 000–000.

Although several studies have addressed the characterization of the IgE repertoire (1), it is subject to ongoing discussion whether the IgE production in allergic disorders is due to an oligoclonally expanded B cell response (2). This is particularly interesting in the context of allergen-specific immunotherapy and the emerging anti-IgE therapy, which has redirected the spotlight in allergy treatment to the B cell (1). In a recent study, Lim et al. (3) showed that the IgE repertoire in patients with atopic dermatitis follows a similar variable gene segment of the H Ig chain (V_H) gene family usage pattern as other Ig isotypes. Davies and O'Hehir (4) found indication that the IgE response could be oligoclonal and Ag-driven. However,

this view is challenged by observations that imply two alternative aspects of immune activation. First, evidence of a heavily biased use of specific V_H gene families in IgE transcripts from allergic patients (5–9) has been interpreted as indicative of a superantigen-driven B cell proliferation. Second, IgE sequences were found to contain a strikingly low level of somatic hypermutation and include significantly shorter CDR-3 (8). These characteristics have also been reported for transcripts from $CD5^+$ B cells (10), which share similarities with murine B-1a cells (11) and arise from polyclonal activation (12). Therefore, it was hypothesized that B-1 cell activation might also play a role during allergic immune responses (8, 13).

Most studies on the human IgE repertoire analyzed a limited number of sequences (<100), often from only one individual patient. Moreover, hitherto almost all studies focused on adult patients. However, the most critical period for developing allergies is childhood. Therefore, we have analyzed the IgE-expressing B cell repertoire of allergic children. The central question was whether the IgE response in children with allergic asthma follows the traditional B-2 pathway. Our aim was to gain a sufficient number of functional IgE sequences to provide a reliable database for statistical analysis of the immunogenetic characteristics. This approach is challenging because IgE-producing plasma cells are extremely rare (14). Therefore, most such studies chose patients with atopic dermatitis with extremely high IgE titers (3, 9). We have recently found indication that the IgE response might differ between allergic sensitization and helminth infection (15), but it is still unknown if the IgE response also differs between different allergic disease entities (e.g., allergic asthma, allergic rhinitis, atopic dermatitis). Systematic analyses of IgE transcripts in patients with moderately elevated IgE levels, especially in children, have not yet been published. Moreover, blood sample volumes of 50 ml or 100 ml, as used in recent studies (3, 4), are not suitable in children.

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Received for publication September 4, 2009. Accepted for publication June 16, 2010.

This work was supported by SFB/TR22 (TP A17) from the Deutsche Forschungsgemeinschaft, by a research grant from University Hospital Giessen and Marburg, and by Behring-Roentgen-Stiftung.

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Abbreviations used in this paper: AA, allergic asthma; AD, atopic dermatitis; AF, *Aspergillus fumigatus*; Al, alder; Alt, *Alternaria alternaria*; Ap, apple; ARC, allergic rhinoconjunctivitis; Be, beech; Bi, birch; Car, carrot; CD, cat dander; CDR-H3, CDR-3 of the H chain; Ce, celery; CH, *Cladosporium herbarum*; CM, cow's milk; CowD, cow dander; D, diversity; DD, dog dander; F, female; FA, food allergy; FR, framework region; GAPDH, GAPDH control showing a 238-bp PCR product; GP, grass pollen; h, human; HDM, house dust mite; HE, hen's egg; HN, hazelnut; Ho, horse; HZ, hazel; IGHD, D gene family; IGHI, J_H gene family; IGHV, V_H gene family; JH, joining gene segment of the H Ig chain; M, male; M_V , number of total mutations in the V region; MW, mugwort; neg, DNA-free negative control; PN, peanut; Rab, rabbit; RAST, radioallergosorbent test; R_{CDR} , ratio of replacement mutations in the CDR; RP, rye pollen; RW, ribwort; V_H , variable gene segment of the H Ig chain; WF, wheat flour.

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In this paper, we describe a highly sensitive PCR that allows for the amplification of IgE transcripts from small blood samples. To the best of our knowledge, we present in the following article the hitherto most extensive collection of human IgE sequences. Demonstrating that IgE transcripts from children with allergic asthma are highly mutated, Ag selected, and clonally restricted, we will provide evidence that the IgE response follows the traditional B-2 adaptive B cell pathway.

Materials and Methods

Patient samples

Children were seen in the outpatient clinic of our Department of Pediatric Pulmonology and Allergology from December 2006 to December 2007. We included 13 children between 3 and 16 y (average age 8.9 y) who, after diagnostic workup, were diagnosed with allergic asthma [based on the criteria of the PRACTALL consensus report (16) and the Global Initiative Against Asthma]. Moreover, we have included peripheral blood samples from four patients with atopic dermatitis between 1 and 4 y (average age 2.3 y). After informed consent, 1.2 ml blood was collected subsequent to a routinely performed blood withdrawal. No additional punctures were performed. The study was approved by the Ethics Committee of the Philipps-University Marburg (Marburg, Germany). Detailed patient characteristics are summarized in Table II.

Preparation of RNA and RT-PCR

Erythrocytes were lysed, and leukocytes were recovered by centrifugation. Total RNA was isolated using the QIAamp RNA Blood Mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA according to standard protocols with SuperScriptIII (Invitrogen, Karlsruhe, Germany).

Seminested IgE PCR

A combination of primers for each V_H gene family (IGHV1–IGHV7) (modified after Ref. 17) was used together with an antisense primer specific for the first exon of the ϵ C region (Ce reverse primer 1) (Table I). In a second PCR, products were reamplified using a combination of the V_H gene family-specific forward primers together with an inner antisense primer specific for the first exon of the ϵ C region (Ce reverse primer 2). Platinum Taq polymerase (Invitrogen) was used with the following program on a thermocycler (Sensquest, Göttingen, Germany): 5 min at 94°C; 30 cycles for 30 s at 94°C, 50 s at 60°C, and 90 s at 72°C; and 10 min at 72°C. GAPDH gene products were amplified using human (h)GAPDH-1 and hGAPDH-2 primers (Table I). PCR products were gel purified, and DNA was extracted with the QIAquick gel extraction kit (Qiagen).

Cloning of PCR products

Ligation and transfection were performed using standard protocols according to the manufacturer's instructions (TOPO-TA cloning kit, Invitrogen).

Sequence analysis

Approximately 120 randomly selected cloned PCR products per patient were sequenced using an ABI capillary sequencer (Applied Biosystems, Darmstadt, Germany). Gene segments were aligned to germline gene segments using the ImMunoGeneTics database with V-QUEST (18). A minimum of six nonmutated nucleotides with at least two nonmutated nucleotides at each end was required to identify a diversity (D) gene (19). The CDR-3 of the H chain (CDR-H3) was defined to include those residues between the conserved cysteine (C104) of framework region (FR)-H3 and the conserved tryptophan (W118) of FR-H4. To analyze Ag selection, we used the algorithms of Lossos et al. (20) and Chang and Casali (21). Phylogenetic trees were compiled using ClustalX (22) and Treedyn (23).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad, La Jolla, CA) and SPSS 17.0 (SPSS, Chicago, IL). Normality distribution was assessed with the Kolmogorov-Smirnov test. Differences between populations were assessed by a two-tailed Student *t* test for normally distributed data or a Mann-Whitney *U* test for nonnormally distributed data. For categorical data, a χ^2 test with post hoc analysis was applied as described by Collis et al. (24). $p \leq 0.05$ was accepted as significant. Means are given with SDs.

Results

Characterization of IgE transcripts from children with asthma

A representative gel demonstrating the amplification of the IgE and IgM H chain mRNA by seminested PCR (for primers see Table I) is shown in Fig. 1. In total, we gained 1366 functional sequences of IgE transcripts from 13 individual children and adolescents with allergic asthma. Of these 1366 sequences, 473 were unique (GenBank accession numbers FJ839966–FJ840439, www.ncbi.nlm.nih.gov/nucleotide/). As a reference, 308 productive $V_HDJ_HC\mu$ sequences (IgM transcripts) were cloned, of which 281 were unique (GenBank accession numbers FJ873815–FJ874095).

V_H family usage pattern in IgE transcripts reflects germline complexity

The frequency distribution of V_H gene family usage of unique IgE transcripts was compared with the repertoire of IgM transcripts. Thirty individual V_H genes from all amplified families (IGHV1, -2, -3, -4, -5, and -7), except for IGHV6, were used by IgM and IgE transcripts. The overall V_H gene family usage in IgE reflected the germline complexity (11) and was similar but not identical to the pattern in IgM (Fig. 2A). IGHV3 was the most dominating V_H family and accounted for 56% (IgM) and 55% (IgE), respectively. However, the usage of the less dominant V_H2 family was diminished in IgE transcripts in comparison with IgM transcripts ($p < 0.001$). A D gene segment could be identified in 471 unique IgE sequences (99.6%) and 272 unique IgM sequences (96.8%). The overall joining gene segment of the H Ig chain (J_H) gene family (IGHJ) and D gene family (IGHD) usage in IgE was similar to the pattern in IgM (Fig. 2B, 2C), although the predominance of theIGHD3 usage was pronounced in IgE ($p < 0.05$).

IgE transcripts have longer N regions and a longer D segment

Both IgM and IgE transcripts displayed a broad range of CDR-H3 length distribution from 18–81 nt (Fig. 3A). However, the focus of distribution was shifted significantly toward longer CDR-H3 regions in IgE sequences. Compared to IgM (42.94 ± 9.79 nt), the average length of CDR-H3 was increased by five bases in IgE transcripts (48.60 ± 12.52 nt) ($p < 0.001$). To assess the relative contribution of V_HDJ_H germline sequence, exonucleolytic nibbling, and N-nucleotide addition to the shifts in CDR-H3 length, we deconstructed CDR-H3 of those transcripts that contained identifiable D genes (Fig. 3B). Whereas the general pattern of CDR-H3 length composition was preserved in IgE transcripts, differences in CDR-H3 length were due to an increased length of the D segment ($p < 0.01$) and an enhanced N-nucleotide addition ($p < 0.01$) in IgE transcripts, shifting the N length distribution toward longer

Table I. Oligonucleotide primers

V_H Sense Primer	
IGHV1/7	5'-CCT CAG TGA AGG TYT CCT GCA AGG C-3'
IGHV2	5'-GTC CTG CGC TGG TGA AAY CCA CAC A-3'
IGHV3	5'-GGG GTC CCT GAG ACT CTC CTG TGC AG-3'
IGHV4	5'-GAC CCT GTC CCT CAC CTG CRC TGT C-3'
IGHV5	5'-AAA AAG CCC GGG GAG TCT CTG ARG A-3'
IGHV6	5'-ACC TGT GCC ATC TCC GGG GAC AGT G-3'
C_H Antisense Primer	
Ce reverse 1	5'-CAG CAA GCT GAT GGT GGC ATA GTG-3'
Ce reverse 2	5'-ATG TTT TTG CAG CAG CGG GTC AAG-3'
GAPDH Primer	
hGAPDH-1	5'-TTG ATT TTG GAG GGA TCT CG-3'
hGAPDH-2	5'-GAG TCA ACG GAT TTG GTC GT-3'

Y, C or T; R, A or G.

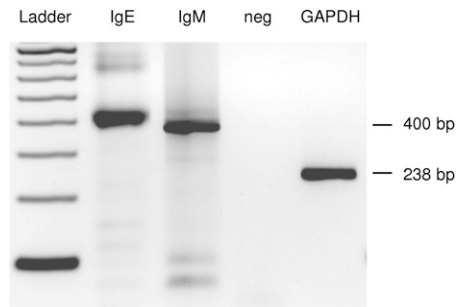


FIGURE 1. Representative gel showing the amplification of IgE and IgM H chain cDNA. IgE transcripts were amplified by seminested RT-PCR, giving PCR products of ~400 bp; as a reference, we amplified IgM transcripts from the same patients. GAPDH, GAPDH control showing a 238-bp PCR product; neg, DNA-free negative control.

N regions (Fig. 4A). Exonuclease nibbling of the germline sequence was similar in IgM and IgE transcripts (data not shown). The pattern of amino acid utilization in the CDR-H3 loop was similar in IgM and IgE, rendering tyrosine, glycine, serine, aspartic acid, and alanine the most frequent amino acids (Fig. 4B), but tyrosine ($p < 0.001$) and serine ($p < 0.001$) were both used

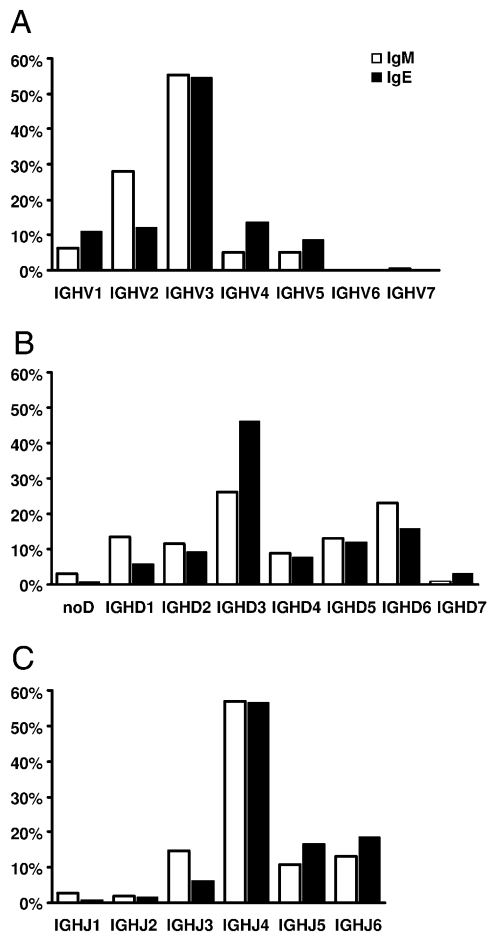
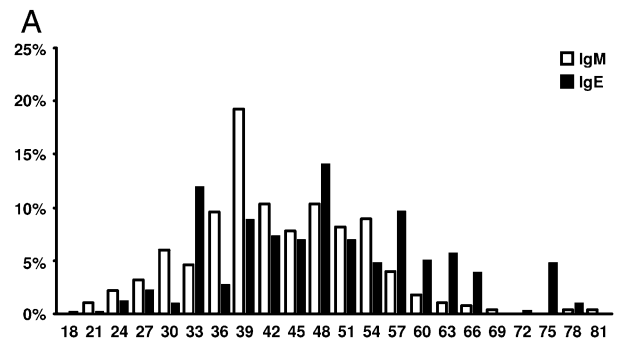


FIGURE 2. IGHV (A), IGHD (B), and IGHJ (C) usage in IgM and IgE transcripts. The overall IGHV usage in IgE reflected the germline complexity and was similar but not identical to the pattern in IgM. No significant VH bias was found. Although IGHV3 was the most dominating VH family in both isotypes, the usage of the V_H2 family was diminished in IgE transcripts. $p < 0.001$. The overall D and J_H gene family usage in IgE was also similar to the pattern in IgM, although the predominance of the IGHD3 gene family usage was pronounced in IgE. $p < 0.05$.



B

	IGHV	N1	IGHD	N2	IGHJ	
IgM	TGT					TGG
	(nucleotides)	6.3	6.6	14.0	5.1	11.3
IgE	TGT					TGG
	(nucleotides)	6.4	8.2	15.7	6.1	12.4

FIGURE 3. A, CDR-H3 length distribution. Both isotypes displayed a broad range of CDR-H3 length distribution. However, the focus was shifted significantly toward longer CDR-H3 regions in IgE transcripts. $p < 0.001$. B, CDR-H3 composition. To analyze the putative contribution of the V_H gene, D gene, and J_H gene, as well as P- and N-nucleotide addition to the total CDR-H3 length, we deconstructed CDR-H3. Whereas the general pattern of CDR-H3 length composition was preserved in IgE transcripts, the differences in CDR-H3 length were due to an enhanced N-nucleotide addition and an increased length of the D segment in IgE transcripts. $p < 0.01$.

significantly more often in IgE than in IgM. The usage of lysine ($p < 0.001$), in contrast, was diminished in IgE transcripts.

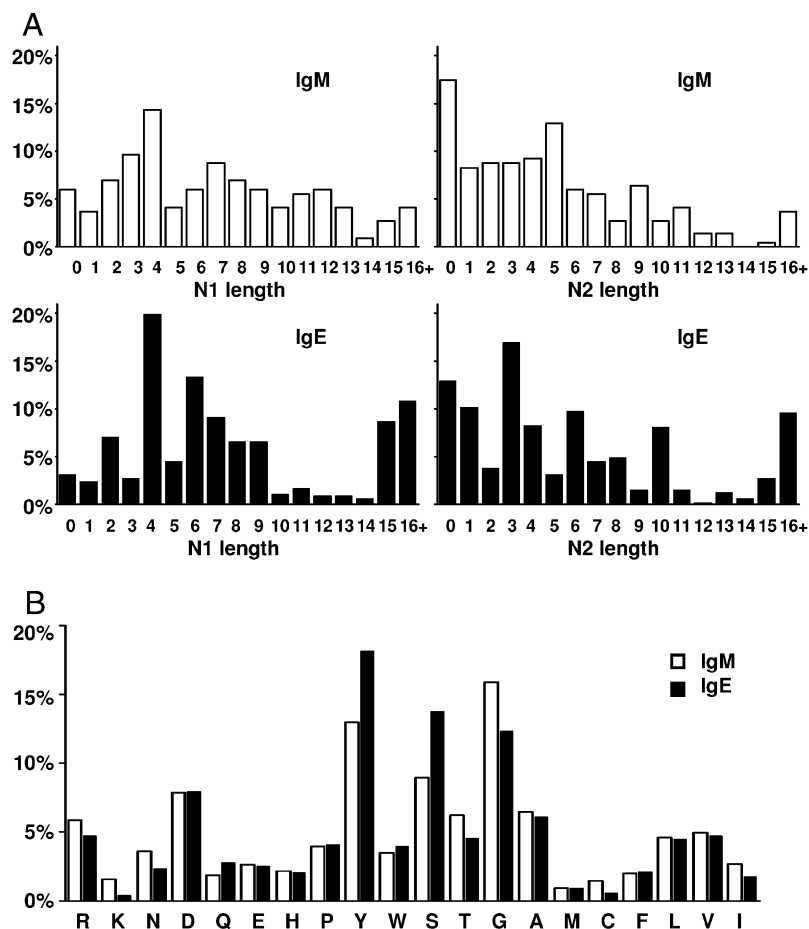
IgE sequences are highly mutated

In IgM transcripts, a considerable amount of sequences (31%) were completely unmutated. In contrast, we found no unmutated sequences at all in IgE transcripts (0%). The average mutational rate in IgE transcripts was 72‰ and thus more than three times as high as in IgM transcripts (20‰; $p < 0.001$) (Fig. 5). Additionally, 59% of IgE sequences contained >50 mutations/1000 nt. In contrast, among IgM transcripts, only 14% of the sequences belonged to this highly mutated group ($p < 0.001$). To exclude significant biasing by *Taq* polymerase error, we calculated the *Taq* error within the C region. *Taq* error rates were 0.99/1000 nt in IgE and 0.97/1000 nt in IgM transcripts. Therefore, the observed differences between the isotypes cannot be explained by *Taq* polymerase error. Detailed analyses revealed no correlation between somatic mutation rate and IgE level, age, or sex.

IgE transcripts display a high degree of Ag selection

To evaluate whether IgE sequences evolved under the influence of Ag selection, we analyzed the distribution of replacement and silent mutations between FRs and CDRs (25). Using the method of Lossos et al. (20), we determined the replacement frequency and the relative length of FR and CDR of each germline V_H gene. The average probability that a random mutation would allocate in CDR was calculated to be 0.23 ± 0.012 , and the sequence-inherent probability that a mutation in the CDR would be a replacement mutation was estimated to be 0.79 ± 0.01 . Therefore, the chance for a random mutation to introduce a replacement mutation into the CDR was 0.18. The binomial distribution method of Chang and Casali (21) was used to calculate the 95% confidence limits for the ratio of replacement mutations in the CDR to the number of total mutations in the V region (M_V) as described by Dahlke et al. (9). This confidence interval is shown as the shaded area in Fig. 6. A data point falling outside these confidence limits represents a sequence that

FIGURE 4. The N length distribution was shifted toward longer N regions in IgE sequences (A). The pattern of amino acid utilization in the CDR-H3 loop was similar in IgM and IgE, rendering tyrosine, glycine, serine, aspartic acid, and alanine the most frequent amino acids (B).



has a high proportion of replacement mutations in the CDR. The probability that such a sequence has accumulated as many replacement mutations in the CDR by mere random mutation is <0.05 . Therefore, an allocation above the upper confidence limit was considered indicative of Ag-driven selection.

According to this definition, 91% of the IgM transcripts showed no signs of Ag selection (Fig. 6A). Notably, more than half of the IgM sequences (58%) had no replacement mutation in the CDR at all and thus allocated in the left lower corner of the plot. Interestingly, the picture for IgE was strikingly different; the IgE sequences displayed a higher degree of significant Ag selection, with 29% of the unique IgE sequences falling above the 95% confidence limit (Fig. 6B), suggesting that these sequences evolved

under the influence of Ag selection. The proportion of Ag-selected sequences in IgE was three times as high as in IgM ($p < 0.001$). Detailed analyses revealed no correlation between Ag-selection and IgE level, age, or sex.

The IgE repertoire is clonally restricted

To determine clonal relationships of the transcripts, we calculated genetic distance dendrograms. Clones were considered clonally related if they: 1) used the same V_H gene; 2) had a highly homologous CDR-H3; and 3) had an identical CDR-H3 length. The 281 unique IgM transcripts pertained to 227 different clonotypes. The IgE repertoire encompassed considerably fewer clonotypes but was composed of multiple related sequences. Among the 473 unique IgE transcripts, only 142 different clonotypes were identified. In Fig. 7A and 7B, we show the phylogenetic trees for IgM and IgE sequences from one representative child (patient six in Table II). From this patient, we gained a total of 36 functional IgM sequences, 33 of which were unique, belonging to 31 different clonotypes. For IgE, the pattern of clonal relatedness displayed a much higher degree of clonal relationship. A total of 114 functional IgE sequences yielded only 38 unique sequences, which pertained to just 15 different clonotypes. The most predominant clonotype alone accounted for 18 of the 38 unique sequences. Notably, this pattern of distribution among total sequences, unique sequences, and different clonotypes was strikingly similar in every single patient. The diversity plots for each patient are provided in Fig. 7C.

IgE response in four patients with atopic dermatitis

To evaluate whether the characteristics of the IgE response differ among distinct atopic entities, we also compared the IgE transcripts from the asthma patients to transcripts from children with atopic

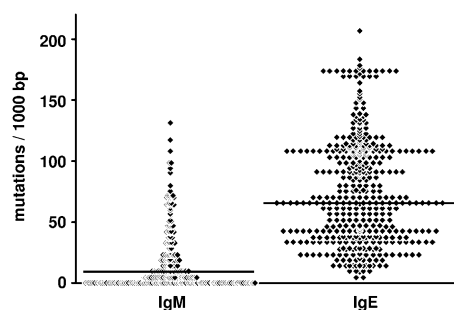
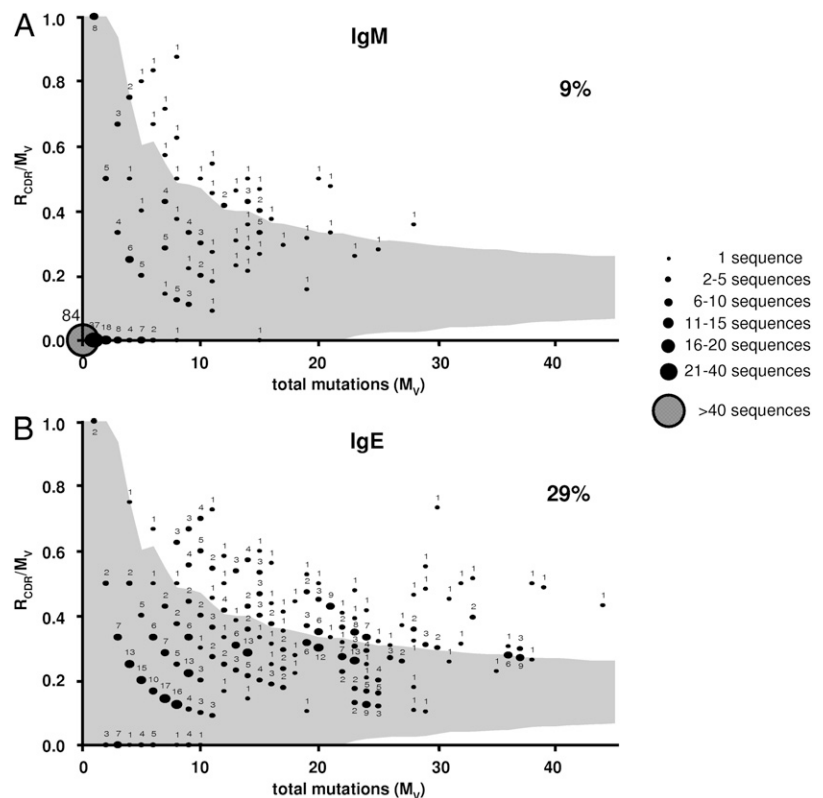


FIGURE 5. Somatic mutation rate of IgM and IgE transcripts (mutations per 1000 nt). Whereas a substantial amount IgM sequences (31%) were completely unmutated, no unmutated sequence at all was found in 1366 functional IgE transcripts (0%). The average mutational rate in IgE transcripts was 72‰ and thus more than three times as high as in IgM transcripts (20‰; $p < 0.001$). Bars represent medians.

FIGURE 6. Inference of Ag selection in IgM (A) and IgE (B) transcripts. Shown is the ratio of replacement mutations in CDR-H1 and CDR-H2 (R_{CDR}) to the total number of mutations in the V region (M_V) plotted against M_V . The shaded area represents the 95% confidence limits for the probability of random mutations. A data point falling outside these confidence limits represents a sequence that has a high proportion of replacement mutations in the CDR. The probability that such a sequence has accumulated as many replacement mutations in the CDR by mere random mutation is <0.05 . An allocation above the upper confidence limit was considered indicative of Ag selection. Data points are accompanied by their observed frequency. Whereas only 9% of the IgM sequences were Ag selected, IgE sequences showed a high degree of significant Ag selection. With 29%, the proportion of Ag-selected sequences in IgE sequences was three times as high as in IgM sequences. $p < 0.001$.



dermatitis. In total, we obtained 129 unique IgE transcripts from four patients with atopic dermatitis (GenBank accession numbers HM116098–HM116226). Initial studies revealed that the IgE

response was less focused in the atopic dermatitis patients: 1) the clonotypic diversity (number of clonotypes divided by number of unique sequences) in IgE transcripts from patients with atopic dermatitis (0.605) was twice as high as in IgE transcripts from patients with allergic asthma (0.302) (Table III), indicating a more polyclonal expansion in atopic eczema; 2) a considerable number of IgE sequences from patients with atopic dermatitis (9%) were completely unmutated (0% in asthma patients). The average somatic mutation rate of IgE sequences from patients with atopic dermatitis was halved compared with transcripts from patients with asthma (35.2 versus 72.1%) (Table III); and 3) The proportion of replacement mutations in the CDR was reduced, and hence fewer transcripts were considered indicative of significant Ag selection (14.0 versus 29.0%) (Table III).

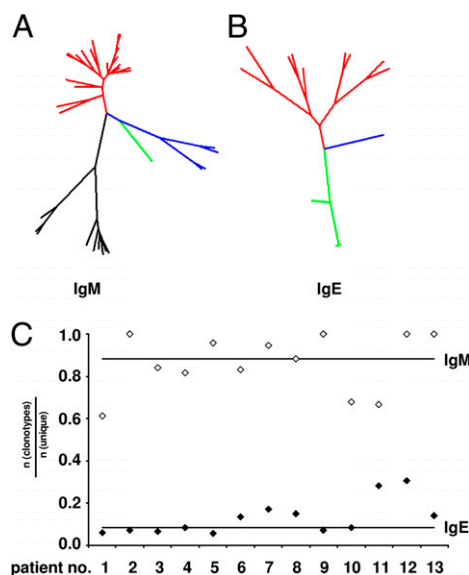


FIGURE 7. The IgE repertoire is clonally restricted. Phylogenetic trees showing the clonal relationship between IgM (A) and IgE (B) transcripts from one representative child (patient six). Although almost every single IgM sequence pertained to a different clonotype, the IgE repertoire was composed of multiple related sequences. In this patient, the 36 functional IgM transcripts belonged to 31 different clonotypes, whereas the 114 functional IgE transcripts displayed a much higher degree of clonal relationship and pertained to just 15 different clonotypes (green, IGHV1; black, IGHV2; red, IGHV3; blue, IGHV5). This pattern of distribution among total sequences, unique sequences, and different clonotypes was strikingly similar in every single patient. The diversity plots for each patient are provided in C. Shown is the ratio between number of clonotypes and number of unique sequences.

Discussion

Although IgE is a central effector molecule of the allergic reaction, it remains controversial whether the IgE response represents a classical Ag-driven immune reaction, a superantigen-like activation, or a polyclonal B-1 cell expansion. In this study, we present evidence that the IgE response in children with allergic asthma represents a classical Ag-driven B cell expansion that follows the traditional B-2 adaptive pathway. The strengths of our study are: 1) that we describe in this paper a highly sensitive PCR method that allows for the amplification of IgE transcripts in small blood samples from children; 2) that we analyzed the IgE response of 13 individual children and adolescents with allergic asthma and compared it to the IgM repertoire from the same patients; and 3) that we gained 1366 functional IgE sequences, which represents the hitherto most extensive collection of human IgE sequences. The main limitation of our study is that the analysis is focused on the systemic IgE repertoire in PBMCs, which might not be identical to the local B cell response in the inflamed tissue.

Several studies found that IgE transcripts from allergic patients preferentially use minor V_H gene families (reviewed in Ref. 1).

Table II. Patient characteristics

Patient Number	Age (y)	Sex	Total IgE (kU/l)	Primary (Secondary) Diagnosis	Skin Prick Test (≥ 0.4 Histamine $\varnothing$)	Unique Sequences
1	12	M	1,054	AA (AD)	HDM, GP, RP, Bi, Rab	39
2	13	M	941	AA	GP, RP, CD, Rab	28
3	5	F	311	AA	HDM, GP, RP, Be, DD	48
4	14	F	375	AA (ARC)	Bi, Al, Hz, Be	43
5	8	M	94	AA	HDM, GP, RP, DD	39
6	5	M	184	AA	GP, RP, MW, Bi, Al, Hz, CD, Rab, CH, Alt, AF	38
7	3	M	393	AA	GP, RP	29
8	5	F	932	AA (AD, FA)	HDM, CM, HN	48
9	13	M	2,257	AA (ARC)	HDM, GP, RP, MW, Bi, Al, Hz, CD, DD	31
10	7	M	671	AA (AD)	HDM, GP, RP, Bi, Be, CD, Ho	29
11	4	M	3,000	AA (AD, ARC, FA)	GP, RP, MW, Bi, Be, DD, HE	33
12	6	M	6,931	AA (AD, ARC)	HDM, GP, CD	37
13	16	M	2,945	AA (AD, ARC)	GP, RP, RW, CD, DD, Ho, Rab, CH	31
14	4	M	2,883	AD	Significant Sensitization in RAST Hz, HN, DD, CD, GP, RP, Ap, Ca, Se	32
15	1	M	750	AD	HE, PN, HN, CM, soy, DD, CD, CowD	35
16	2	M	7,902	AD	Ce, HDM, CD, DD, HE, CM, cod, WF, PN, soy, HN, Car, Ap, GP, Bi, Hz, MW	32
17	1	F	19,100	AD	CM, WF, Hz, HN, GP, RP	30

AA, allergic asthma; AD, atopic dermatitis; AF, *Aspergillus fumigatus*; Al, alder; Alt, *Alternaria alternaria*; Ap, apple; ARC, allergic rhinoconjunctivitis; Be, beech; Bi, birch; Car, carrot; CD, cat dander; Ce, celery; CH, *Cladosporium herbarum*; CM, cow's milk; CowD, cow dander; DD, dog dander; F, female; FA, food allergy; GP, grass pollen; HDM, house dust mite; HE, hen's egg; HN, hazelnut; Ho, horse; Hz, hazel; M, male; MW, mugwort; PN, peanut; Rab, rabbit; RAST, radioallergosorbent test; RP, rye pollen; RW, ribwort; WF, wheat flour.

Particularly the V_H5 gene family was shown by multiple studies to be overrepresented in IgE sequences (5, 26, 27). However, except for V_H2 and V_H7 , each V_H gene family has been found predominant in at least one study. This biased usage of V_H family genes has been interpreted as an indicator for superantigen-driven B cell proliferation. Consequently, the term superallergen was proposed for this alternative way of immune interaction between allergen and IgE (28). Moreover, microbial Ags such as staphylococcal Ag were discussed as the cause of a superantigen-driven B cell expansion in allergic diseases (29). However, one has to take into account that many of these observations rely on the analysis of a limited number of IgE sequences (<100), mostly derived from one or two individual patients. In our current analysis of 1366 functional IgE sequences from 13 allergic children, the V_H gene usage was not relevantly biased but reflected the germline complexity (30), rendering the V_H3 family the most dominating V_H family in IgE transcripts. This is in accordance with a recent study by Lim and coworkers (3), who found that $\sim 80\%$ of the IgE transcripts from adults with atopic dermatitis used the V_H3 family. In a pediatric study with 36 IgE sequences, Bando et al. (31) also reported no bias in the V_H family usage. In our IgE sequences, a detailed analysis of the individual V_H gene usage revealed a pattern that has been described for other Ig isotypes: in the primary human B cell repertoire, V3-23 is the most frequent V_H gene (32). This frequency pattern was preserved in our IgE sequences, 14% of which used the V3-23 gene. In summary, the IgE repertoire of allergic children reflects germline complexity and

follows the V_H usage pattern seen for the primary Ig repertoire, arguing against a superantigen-like activation of the IgE response.

The IgE sequences in our study were highly mutated. This is consistent with the findings of Davies and O'Hehir (4), who found a high rate of somatic mutations in IgE transcripts from three patients with allergic rhinitis. They also reported a subset of IgE clones, sharing 98–100% homology with the germline sequence, which was interpreted suggestive of the involvement of CD5⁺ B cells. These cells share similarities with murine B-1a cells (11) and are functionally characterized by polyclonal activation and production of polyreactive IgM Abs (12). As the emergence of these Abs is independent of immunization with external Ags, they have been termed natural Abs (33). B-1 cells are normally produced during fetal and neonatal development (34) and represent a major B cell subset in peripheral blood throughout childhood (35). The first study to imply a potential role for B-1 cells in the allergic response came from Edwards et al. (8), who analyzed 31 unique IgE sequences from one 40-y-old patient with atopic dermatitis. In uncommonly overrepresented sequences containing the otherwise scarce IGHV6-1 gene segment, they found a low level of somatic hypermutation in the V_H region and very short CDR-H3 regions due to reduced N nucleotide addition, which both have also been reported for transcripts from B-1 cells (10, 11). In our present study on pediatric patients, however, the IgE transcripts from children with allergic asthma had longer CDR-H3 regions due to enhanced N-nucleotide addition. Moreover, only a very small part of the IgE sequences showed sequence homology to germline $>98\%$. Notably, out of 1366 functional IgE sequences, we did not find one single unmutated sequence. We therefore conclude that the IgE transcripts in our study are not suggestive of a polyclonal B-1 response.

This conclusion is further supported by the observation that IgE transcripts from allergic children display evidence of enhanced Ag selection. We found a significantly enhanced clustering of replacement mutations into the CDR in IgE transcripts. For all V_H families, the IgE transcripts displayed a higher level of somatic mutations with more signs of significant Ag selection than the

Table III. Clonotypic diversity, somatic mutation rate, and Ag selection of the IgE-producing peripheral B cells from patients with allergic asthma versus atopic dermatitis

	AA	AD	p Value
Clonotypic diversity	0.302	0.605	<0.0001
Somatic mutation rate (%)	72	35	<0.0001
Percent Ag selected	29.0	14.0	<0.001

AA, allergic asthma; AD, atopic dermatitis.

corresponding IgM transcripts. This again strongly argues against a superantigen-like selection. How can these observations be reconciled with Dahlke et al. (9), who found that IgE sequences from both nonallergic and allergic individuals display few somatic mutations and show almost no signs of Ag selection? They concluded that the reduced selection for replacement mutations in the CDR of IgE transcripts suggests a generally lower affinity of the IgE response and implies a distinct evolution of IgE Abs, which differs fundamentally from other Ig isotypes. However, we see two pivotal differences between the study of Dahlke et al. (9) and our study: first, the number of included patients and analyzed sequences might explain part of the observed differences. In the study by Dahlke et al. (9), 76 IgE sequences were gained from one allergic individual (age not stated), whereas we analyzed 1366 sequences from 13 allergic children. Second, the focuses were on different allergic entities: whereas we focused on children with allergic asthma, the one patient in the study by Dahlke et al. (9) suffered from atopic dermatitis.

To address the question whether the characteristics of the IgE response depend on the atopic entity, we examined IgE transcripts from four children with atopic dermatitis and high IgE levels. The IgE repertoire in peripheral blood from patients with atopic dermatitis showed significantly more clonal diversity, a lower somatic mutation rate, and less signs of Ag selection than in patients with asthma. These initial results indicate that the IgE response is less focused in atopic dermatitis compared with allergic asthma. Although further experiments with more patients with atopic dermatitis are necessary to confirm these findings, our initial results suggest different immunomechanisms of allergic sensitization in allergic asthma and atopic dermatitis.

Our observation that the IgE sequences from allergic children display signs of enhanced Ag selection is further sustained by the finding that the level of clonal relatedness was remarkably high in IgE transcripts. Whereas almost every unique IgM sequence pertained to a different clonotype, the 473 unique IgE sequences belonged to only 142 clonotypes. This is in harmony with Davies and O'Hehir (4), who found that the IgE repertoire from three patients with hay fever displays a high degree of clonal relatedness. This and our observations indicate that the IgE response is much more focused than the primary repertoire and suggest an oligoclonal expansion of B cells in the sense of an adaptive immune response, as seen for other secondary repertoires. We conclude that the IgE response in children with allergic asthma is indicative of a classic Ag-driven and oligoclonal B cell response.

In summary, we found: 1) that the V_H gene utilization in IgE transcripts argues against a superantigen-like activation of the IgE response; 2) the high level of somatic hypermutation throughout the V region and the significantly longer CDR-H3 regions of IgE sequences render a relevant participation of a polyclonal B-1 response unlikely; and 3) IgE transcripts display signs of significant Ag selection and are therefore indicative of a classical Ag-driven immune response with affinity maturation. We conclude that the IgE response in PBMCs from children with allergic asthma follows the traditional adaptive B-2 cell pathway. Although our study presently has no direct clinical implication, it provides the methods and the reference data required for a better characterization of the immunomechanisms in therapeutic approaches that aim to modify the IgE response, such as anti-IgE therapy or allergen-specific immunotherapy.

Acknowledgments

We thank Regina Stoehr and Sabine Jennemann for excellent technical assistance, Dr. Andreas Borta for advice with the statistical analysis, and the team of the Kindertagesklinik Marburg for committed support.

Disclosures

The authors have no financial conflicts of interest.

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