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IgG4 and IgE Transcripts in Childhood Allergic Asthma Reflect Divergent Antigen-Driven Selection

Tobias Rogosch,* Sebastian Kerzel,[†] Friederike Dey,* Johanna-Josephina Wagner,* Zhixin Zhang,[‡] Rolf F. Maier,* and Michael Zemlin*

The physiologic function of the “odd” Ab IgG4 remains enigmatic. IgG4 mediates immunotolerance, as, for example, during specific immunotherapy of allergies, but it mediates tissue damage in autoimmune pemphigus vulgaris and “IgG4-related disease.” Approximately half of the circulating IgG4 molecules are bispecific owing to their unique ability to exchange half-molecules. Better understanding of the interrelation between IgG4 and IgE repertoires may yield insight into the pathogenesis of allergies and into potential novel therapies that modulate IgG4 responses. We aimed to compare the selective forces that forge the IgG4 and IgE repertoires in allergic asthma. Using an IgG4-specific RT-PCR, we amplified, cloned, and sequenced IgG4 H chain transcripts of PBMCs from 10 children with allergic asthma. We obtained 558 functional IgG4 sequences, of which 286 were unique. Compared with previously published unique IgE transcripts from the same blood samples, the somatic mutation rate was significantly enhanced in IgG4 transcripts (62 versus 83%; $p < 0.001$), whereas fewer IgG4 sequences displayed statistical evidence of Ag-driven selection ($p < 0.001$). On average, the hypervariable CDRH3 region was four nucleotides shorter in IgG4 than in IgE transcripts ($p < 0.001$). IgG4 transcripts in the circulation of children with allergic asthma reflect some characteristics of classical Ag-driven B2 immune responses but display less indication of Ag selection than do IgE transcripts. Although allergen-specific IgG4 can block IgE-mediated allergen presentation and degranulation of mast cells, key factors that influence the Ag-binding properties of the Ab differ between the overall repertoires of circulating IgG4- and IgE-expressing cells. *The Journal of Immunology*, 2014, 193: 000–000.

The least abundant of the IgG subclasses in healthy individuals, IgG4 has been termed the “odd” Ab owing to its unique ability to exchange half-molecules on the protein level (Ref. 1; see Refs. 2–4 for review). Because of this “Fab arm exchange,” approximately half of the circulating IgG4 Abs are bispecific (5). It has been suggested that the formation of the IgG4 repertoire underlies different mechanisms of selection than the other Ig isotypes (6).

The IgG4 subclass accounts for ~4% of the total serum IgG in healthy subjects (2) but was seen at substantially higher concentrations in inhabitants of areas with endemic parasitism (7). The role of IgG4 in physiologic immune responses and in allergic diseases is under investigation. Some studies have found a negative correlation between IgG4 titers and symptom score (8–13) and a positive correlation between IgG4 titers and IL-10-secreting APCs (14) following specific immunotherapy. Nouri-Aria et al. (15) found that during specific immunotherapy, allergen-specific

IgG4 serum levels increase 10- to 100-fold, and they conclude that grass pollen immunotherapy may induce allergen-specific, IL-10-dependent “protective” IgG4 responses. IgG4 can act as a “blocking Ab” at the mast cell level and/or at the level of APCs (12, 16, 17). In harmony with this finding, blockage of the IgE–allergen interaction in sera from patients after hyposensitization is completely abolished if IgG4 is removed (15). IgG4 binds poorly to Fcγ receptors (18) and is unable to activate the complement system and may even inhibit complement activation by other isotypes (19). Intriguingly, both atopic and nonatopic individuals develop allergen-specific IgG4 after long-term exposure to allergens (20). For instance, in nonallergic beekeepers specific IgG4, but no specific IgE, Abs to bee venom are found (20).

Isotype switching of B cells to both IgE and IgG4 is induced by Th2-type cytokines IL-4 or IL-13 and by CD40:CD40-ligand interaction between B and T cells (21–23). IL-10, IL-12, and IL-21 shift the balance between IgG4 and IgE toward IgG4 production (24–26). Recently, it has been shown that IgG4-producing cells essentially develop from IL-10-secreting, CD25⁺, CD71⁺, CD73⁺ B regulatory cells (27). Thus, both IgG4 production and T cell tolerance against allergens during allergen-specific immunotherapy seem to develop in a network of IL-10-producing regulatory T and B cells (28–30). IgG4-producing cells can undergo secondary class switch to IgE (31), as was shown also for IgG1 in mice (32); thus, a fraction of the IgE repertoire may reflect a subset of the IgG4 repertoire in humans. Although IgG4 seems to mediate tolerance during successful treatment in allergic patients, its function during allergen–Ab complex formation in food allergies is unknown (33). Moreover, IgG4 can also mediate autoimmunity (e.g., pemphigus vulgaris) and might play a pathogenic role in newly identified “IgG4-related disease” (34).

We have shown that, in contrast to earlier assumptions, the IgE transcripts in PBMCs of allergic children reflect a classical Ag-driven B2 cell response and not a B1 cell response or a superantigen-like

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Abbreviation used in this article: FR, framework region.

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activation (35). Even in the absence of Ag, B1 cells spontaneously secrete “natural” Abs that are more “germline-like” because of minimal N-region addition and low somatic mutation frequency compared with B2 cell–derived Igs (36). In contrast, B2 cell–derived Abs show longer CDR3 regions, an enhanced number of mutations, and a higher degree of Ag selection. In a recent review, Wu and Zarrin (37) pointed out that there have been very few comparisons of the IgG4 and IgE repertoires. So far, the available collections of IgG4 transcripts originate from patients with IgG4-related sclerosing sialadenitis (38), from people living in areas of endemic parasitism (7), and from healthy, urban residents (39). Although the transcripts from patients with IgG4-related sclerosing sialadenitis contained a high proportion of unmutated or low (<20%) mutated variable gene sequences, arguing against a classical Ag-driven B2 cell response of IgG4-expressing cells, the IgG4 sequences from subjects living in areas of endemic parasitism and from healthy, urban residents show high frequencies of somatic mutation.

In this study, we sought to characterize the IgG4-expressing peripheral B cell repertoire of children with allergic asthma in comparison with previously obtained IgE transcripts from the same blood samples. The central aim was to clarify whether the IgG4 response in children with allergic asthma reflects a B2 cell immune response similar to the IgE response.

Materials and Methods

Patient samples

Ten samples of the 13 children described in Ref. 35, who were diagnosed with allergic asthma according to standardized criteria of the PRACTALL consensus report (40) and the GINA (Global Initiative against Asthma), were accessible for IgG4 analysis. After informed consent, 1.2 ml blood was collected subsequent to a routinely performed blood withdrawal. No additional punctures were performed. This study was approved by the Ethics Committee of the Philipps-University Marburg.

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 SuperScript III (Invitrogen, Karlsruhe, Germany). A combination of framework region (FR) 1–based forward primers for each VH gene family was used (35), modified from Ref. 41: VH1/7: 5'-CCT CAG TGA AGG TYT CCT GCA AGG C-3'; VH2: 5'-GTC CTG CGC TGG TGA AAY CCA CAC A-3' (note: at the eighth position from the 3' end, a C instead of Y [= C/T] and at the second position from the 3' end an S [= C/G] instead of C would match all known human germline VH2 gene segments); VH3: 5'-GGG GTC CCT GAG ACT CTC CTG TGC AG-3'; VH4: 5'-GAC CCT GTC CCT CAC CTG CRC TGT C-3'; VH5: 5'-AAA AAG CCC GGG GAG TCT CTG ARG A-3'; VH6: 5'-ACC TGT GCC ATC TCC GGG GAC AGT G-3'). The reverse primer is specific for the hinge region of the C γ 4 C region (5'-GCA TGA TGG GCA TGG GGG ACC ATA TTT GGA-3'). PCR amplifications were carried out in a total volume of 25 μ l containing 1 μ l cDNA eluate, 1.5 mM MgCl₂, and 0.2 μ M of each forward and reverse primer using 1 U Platinum Taq polymerase (Invitrogen). The following program was performed on a thermocycler (Sensquest, Göttingen, Germany): 5 min at 94°C; 40 cycles with 20 s at 94°C, 30 s at 60°C, and 90 s at 72°C; 10 min at 72°C. As a control for RNA quality, GAPDH transcripts were amplified from each sample, using human GAPDH-1 and human GAPDH-2 primers (35). PCR products were gel purified, and DNA was extracted with 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

After the transformed cells had grown on agar plates, clones from each subject were randomly selected. Plasmid DNA was extracted, linearized, and sequenced using an ABI capillary sequencer. Gene segments were

aligned to germline gene segments, using the ImMunoGeneTics (IMGT) database with HIGHV-QUEST (42). A minimum of six nonmutated nucleotides with at least two nonmutated nucleotides at each end were required to identify a diversity (D) gene (43). The CDRH3 was defined to include those residues between the conserved cysteine (C104) of FR-H3 and the conserved tryptophan (W118) of FR-H4. Somatic mutation rates and Ag selection were studied in the VH genes, using the algorithms of Dahlke et al. (44), Lossos et al. (45), and Chang and Casali (46), as described previously (47). Sequence analyses were performed using IMGT/V-QUEST (42) and the Ig analysis tool (IgAT) (48). Phylogenetic trees were compiled using ClustalX (49) and Treedyn (50).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (La Jolla, CA), SPSS 17.0 (Chicago, IL), and PAST 2.17 (51). 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, respectively. For categorical data, a χ^2 test with post hoc analysis was applied as described by Collis et al. (52). Diversity was assessed by the Shannon index. A *p* $\leq$ 0.05 was considered significant. Means are given with SE. The *p* values have been corrected for multiple hypothesis testing, using the method of Benjamini–Hochberg to detect the false discovery rate (53).

Results

Characterization of IgG4 transcripts

To assess the IgG4 response in asthmatic children, we analyzed a total of 558 functional IgG4 transcripts. Of these transcripts, 286 were unique (GenBank accession nos. JN000406–JN000699, www.ncbi.nlm.nih.gov/genbank). As a reference, we used 341 previously published unique V_HDJ_HC _{ϵ} sequences (IgE transcripts) from the same blood samples (GenBank accession nos. FJ840062–FJ840439) (35).

VH, D, and JH gene usage

A total of 25 individual VH genes from the families IGHV1, 2, 3, and 5 were used by IgG4 transcripts, with IGHV2–5 being the most frequent VH gene (24.5%) (Fig. 1A). An identifiable D gene segment could be found in 284 unique IgG4 sequences (99.3%) and in 340 unique IgE sequences (99.7%).

The general D and JH gene family usage in IgG4 was similar to the pattern in IgE (Fig. 1B, 1C), except for the IGHD3 gene families, which were used less often (*p* < 0.001), and the IGHD4 families, which were used more often in IgG4 than in IgE (*p* < 0.01).

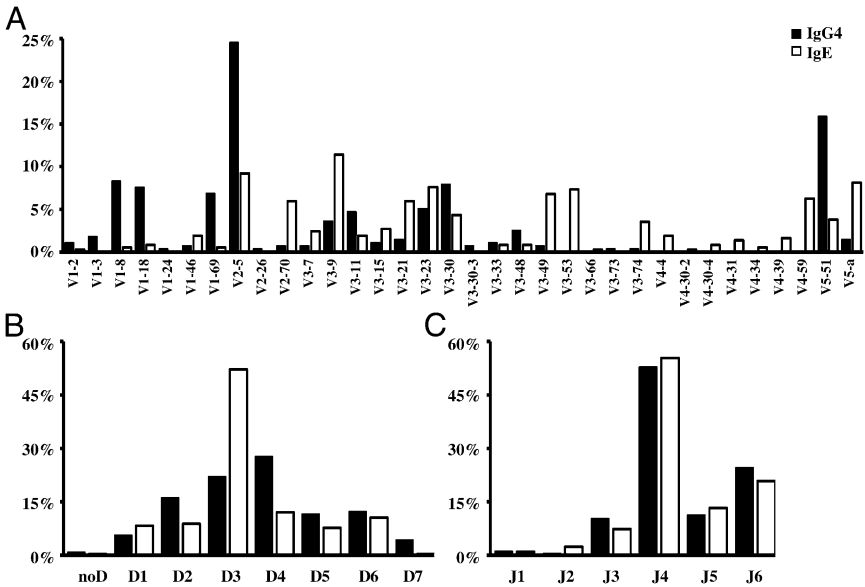
IgG4 transcripts contain shorter CDR-H3 regions but more N nucleotides

Both IgG4 and IgE transcripts displayed a broad range of CDR-H3 length distribution from 18 to 84 nucleotides (Fig. 2A). However, compared with IgE (49.47 $\pm$ 0.69 nt), the average length of CDR-H3 was decreased by four nucleotides in IgG4 transcripts (45.30 $\pm$ 0.71 nt; *p* < 0.001). To assess the relative contribution of VDJ 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. 2B). Whereas IgG4 transcripts contained more N nucleotides (*p* < 0.001, Supplemental Fig. 1), this effect was overcompensated by a decreased length of V and D segments (*p* < 0.001) in IgG4 transcripts. The pattern of amino acid utilization in the CDR-H3 loop showed differences between IgE and IgG4 (Fig. 3): Valine, threonine, arginine, and proline (*p* < 0.001) were used significantly more often in IgG4 than in IgE, and tyrosine, serine, and glutamine less often [*p* < 0.001; χ^2 test with post hoc analysis, as described by Collis et al. (52)].

IgG4 sequences are highly mutated

Analyzing the VH region from CDR1 to FR3, we found no unmutated sequence at all in IgG4 and IgE transcripts (0%). The

FIGURE 1. Gene segment use. **(A)** VH gene, **(B)** JH gene family, and **(C)** D gene family usage of IgG4 and IgE transcripts. IgE transcripts from Ref. 35.



average mutational rate in IgG4 transcripts was 83.1%, thus being significantly higher than in IgE transcripts (62.1%; $p < 0.001$) (Fig. 4). In addition, 92% of IgG4 sequences contained >50 mutations/1000 nucleotides. Among IgE transcripts, in contrast, only 52% 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 1.60/1000 nucleotides in IgG4 and 0.61/1000 nucleotides in IgE transcripts. Therefore, the observed differences between the isotypes cannot be explained by Taq polymerase error.

The IgG4 sequences display signs of Ag selection

To evaluate whether IgG4 sequences evolved under the influence of Ag selection, we analyzed the distribution of replacement and silent mutations between FR and CDR, as described previously (35, 44).

According to this definition ($\alpha=0.05$), 14% of the IgG4 transcripts showed signs of Ag selection (Fig. 5A). Notably, the picture for IgE was different: the IgE sequences displayed a higher degree of significant Ag selection, with 32% of the unique IgE sequences falling above the 95% confidence limit ($p < 0.001$) (Fig. 5B).

Of interest, IgE transcripts with less than the average mutational rate showed fewer signs of Ag selection (20.2%, $n = 188$) compared with the higher mutated group (49.7%, $n = 153$), whereas no difference was noted in Ag selection in the low and high mutated IgG4 transcripts (data not shown).

Phylogenetic trees

To determine clonal relationships of the transcripts, we calculated genetic distance dendrograms. Clones were considered clonally related if they 1) used the same VH gene, 2) had a highly homologous CDR-H3 ($<10\%$ difference in nucleotide sequence), and 3) had an identical CDR-H3 length. Among the 286 unique IgG4 transcripts, 119 different clonotypes were identified (Shannon index: 4.387; effective numbers: 80.4). The 341 unique IgE transcripts pertained to 109 different clonotypes (Shannon index: 4.095; effective numbers: 60.0). Both repertoires were composed of multiple related sequences, but diversity was about one-third higher in IgG4 sequences ($p = 0.0063$). In Fig. 6 we show the phylogenetic trees for IgG4 and IgE sequences from one representative child (patient no. 4 in Table I). From this patient we gained a total of 51 functional IgG4 sequences; among those, 35 sequences were unique, which pertained to just 8 different clonotypes. The most predominant

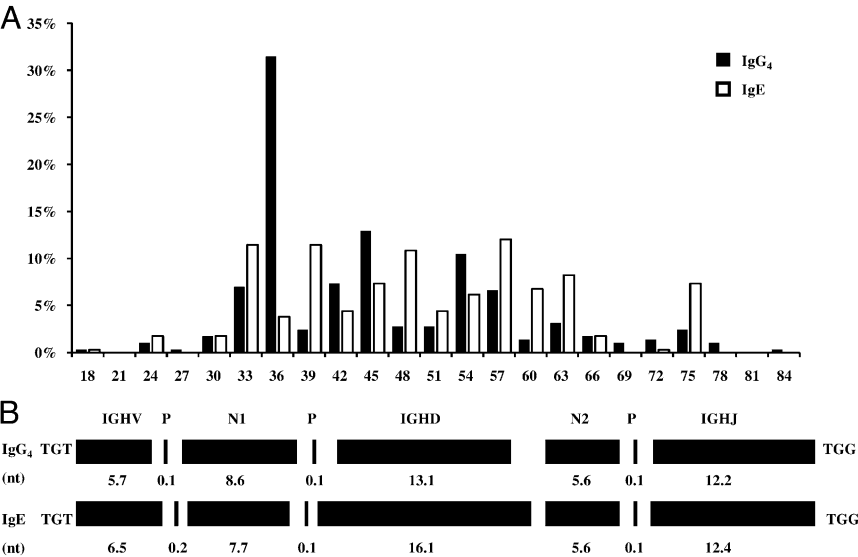


FIGURE 2. Length distribution and CDR-H3 composition. **(A)** CDR-H3 length distribution. On average, CDR-H3 regions were significantly shorter in IgG4 transcripts. **(B)** CDR-H3 composition. Analysis of the average contribution of the VH gene, the D gene, and the JH gene, as well as P and N nucleotides, to CDR-H3. The VH and D genes were shorter in IgG4 transcripts ($p < 0.001$). IgE transcripts from Ref. 35.

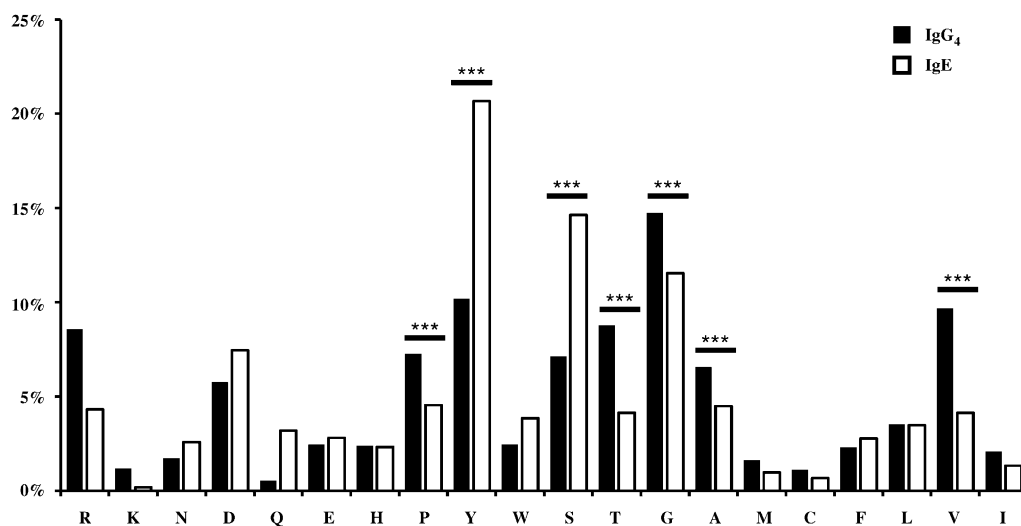


FIGURE 3. Amino acid composition of the CDR-H3 region of IgG4 and IgE. IgE transcripts from Ref. 35. χ^2 test with post hoc analysis; *** $p < 0.001$.

clonotype alone accounted for 8 of the 35 unique sequences. For IgE we found a similar pattern of clonal relatedness: a total of 110 functional IgE sequences, 38 of which were unique, belonging to 15 different clonotypes. Notably, this pattern of distribution between total sequences, unique sequences, and different clonotypes was strikingly similar in every single patient. The diversity plots for each patient are provided in Supplemental Fig. 2.

Discussion

Owing to their unique ability to exchange half-molecules, most IgG4 molecules are heterobivalent (bispecific) in vivo. This characteristic probably enables IgG4 to play a crucial role as a “blocking Ab” during tolerance induction (11, 54–56), but also as a pathogenic Ab in some autoimmune diseases such as pemphigus vulgaris (57). However, it was hitherto unknown if IgG4 production underlies the same selective mechanisms as the other isotypes. Intriguingly, the number and pattern of somatic mutations and the composition of the CDR-H3 region differ significantly between IgG4 transcripts and previously published IgE transcripts from the same patient samples. This finding indicates that the Ag-binding properties of the overall repertoires of IgG4- and IgE-expressing cells might differ even if a (potentially small) subset of both isotypes could be directed against the same epitopes (2). Of interest, the increase in IgG4 production during allergen-specific immunotherapy is only partially directed against allergen (12).

As evidence of Ag-driven selection, both IgG4 and IgE transcripts exhibited a similar degree of clonal restriction, which was also in the range of IgA transcripts (58). However, IgG4 transcripts harbored more somatic mutations than IgE transcripts. The estimation of somatic mutations acquired during each cell division ranges from 1 per 1000 nucleotides (59) to 1 per VH gene. This observation would indicate that, on average, IgG4-producing cells have undergone approximately a third more rounds of cell division than have IgE-producing cells. At first this may appear surprising because IgE-expressing cells can be recruited by secondary class switch from IgG-expressing cells by the so-called “indirect route,” IgM→IgG4→IgE, in humans (31, 60) or IgM→IgG1→IgE in mice (32) and potentially also from a classical germinal-center pathway (61). One could argue that those IgE-expressing cells that originate from the indirect route should harbor at least as many somatic mutations as their IgG4-expressing precursors (unless cells with fewer somatic mutations are preferentially selected for secondary class switch). However, the difference in somatic mutation rate between IgG4 and IgE is congruent with the observation by Erazo

et al. (62) in mice that, once switched to IgE, plasma cells swiftly exit from the germinal center, thus avoiding further somatic mutations. In contrast, IgG4-producing cells that were not selected for IgE class switch remain in the germinal center under the influence of Ag and could acquire more somatic mutations. The lower average somatic mutation rate of IgE transcripts could also be explained by the fact that IgE-expressing cells that evolve from IgM (direct route) might at least initially contain fewer somatic mutations (56). This fact might be the reason that a higher and a lower mutated subpopulation of IgE transcripts in humans (35), and even more pronounced in mice (47), can be distinguished that are not easily accessible for separate transcriptome analyses. We hypothesize that the fraction of IgE-expressing cells with fewer somatic mutations predominantly represents those cells that were recruited from naive IgM-expressing B cells (“direct route,” IgM→IgE) and that the cells expressing highly mutated IgE transcripts originate from the “indirect route” (IgM→IgG4→IgE). Xiong et al. (32) propose that the indirect route results in potentially pathogenic high-affinity IgE and the direct route results in low-affinity, potentially protective IgE. The presence of clonally related IgG4 and IgE transcripts within blood lymphocytes from individual asthma patients suggests that a major proportion of IgE derives from the indirect route (60, 63), although those sequences are not necessarily related by direct switching, as both IgE and IgG4 could separately develop from IgM- or other IgG isotype-expressing cells. Such clonally related sequences were not iden-

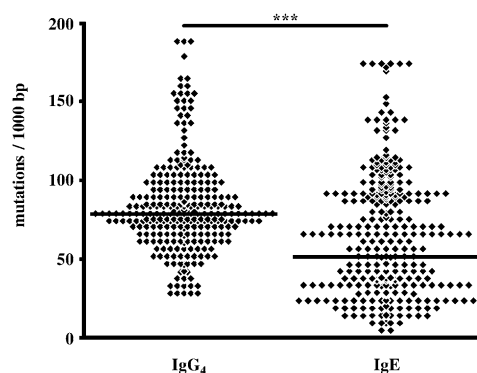


FIGURE 4. Somatic mutation rate. The average mutational rate (mutations per 1000 nt) was 83.1% in IgG4 and 62.1% in IgE transcripts ($p < 0.001$). Bars represent median values. IgE transcripts from Ref. 35.

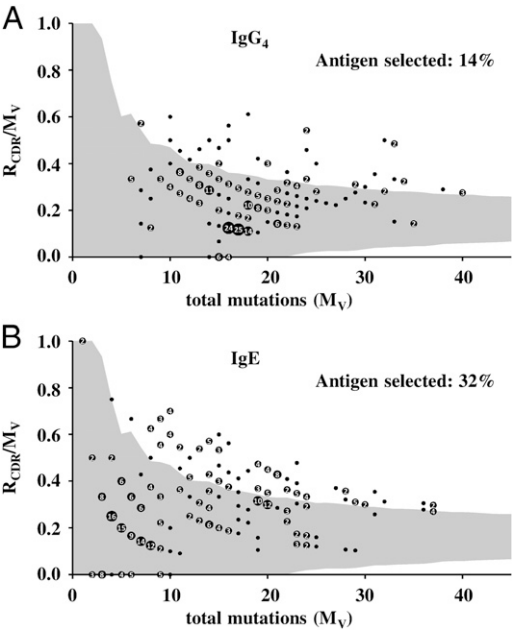


FIGURE 5. Inference of Ag selection in IgG4 (**A**) and IgE (**B**) transcripts. The ratio of replacement mutations in CDR-H1 and CDR-H2 (R_{CDR}) to the total number of mutations in the V region (M_V) was plotted against M_V . The shaded area represents the 95% confidence limit for the probability of random mutations. A data point falling outside these confidence limits represents a sequence that contains a high proportion of replacement mutations in the CDR and was considered indicative of Ag selection. Data points are labeled with their observed frequency. IgE transcripts from Ref. 35.

tified in our relatively large sequence collection (data not shown), indicating 1) that, within the overall repertoire, clonally related IgG4 and IgE transcripts may be less common than anticipated or 2) that the sample volume is too small to harbor a large collection of the available specificities. This idea is consistent with the suggestion by Davies et al. (56, 64, 65) that atopic sensitization is predominantly caused by IgE production through the direct route, as even low-affinity IgE is sufficient to catch allergen and to trigger basophils. Moreover, several studies indicate that maximum Ag affinity neither is achieved by a maximum somatic mutation rate nor is the primary criterion for class switch recombination (66).

Of note, although IgG4 transcripts contained a higher somatic mutation rate, on average they less frequently showed indications of Ag-driven selection than IgE transcripts. Thus, because somatic

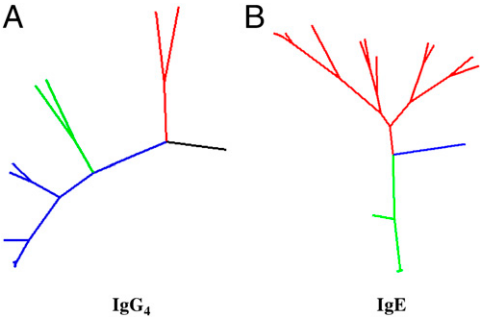


FIGURE 6. Phylogenetic trees showing the clonal relationship among (**A**) IgG4 and (**B**) IgE transcripts from one representative child. In this patient, the 51 functional IgG4 transcripts belonged to 8 different clonotypes. The same degree of clonal relationship was displayed in the 110 functional IgE transcripts that pertained to 15 different clonotypes. Green: IGHV1; black: IGHV2; red: IGHV3; blue: IGHV5. IgE transcripts from Ref. 35.

Table 1. Characteristics of patients

Patient No.	Age, y	Sex	Primary Diagnosis	Sensitization	Functional Sequences	Unique Sequences	Clonotypes (%)	Somatic Mutation Frequency (CDR1-FR3), ‰	Ag Selection, %
1	5	F	AA	HDM, GP, RP, Be, DD	71	14	4 (29)	66.7	0.0
2	14	F	AA (ARC)	Bi, Al, Hz, Be	72	29	8 (28)	92.5	3.4
3	8	M	AA	HDM, GP, RP, DD	47	28	14 (50)	78.7	25.0
4	5	M	AA	GP, RP, MW, Bi, Al, Hz, CD, Rab, CH, Alt, AF	60	41	13 (32)	66.5	2.6
5	5	F	AA (AD, FA)	HDM, CM, HN	42	32	18 (56)	79.5	31.3
6	13	M	AA (ARC)	HDM, GP, RP, MW, Bi, Al, Hz, CD, DD	63	27	6 (22)	90.5	7.4
7	7	M	AA (AD)	HDM, GP, RP, Bi, Be, CD, Ho	64	32	11 (34)	86.2	15.6
8	4	M	AA (AD, ARC, FA)	GP, RP, MW, Bi, Be, DD, HE	41	30	19 (63)	104.5	20.0
9	6	M	AA (AD, ARC)	HDM, GP, CD	58	33	7 (21)	71.1	3.0
10	16	M	AA (AD, ARC)	GP, RP, RW, CD, DD, Ho, Rab, CH	40	26	22 (85)	88.5	23.1

AA, allergic asthma; AD, atopic dermatitis; AF, *Aspergillus fumigatus*; Al, alder; Alt, *Alternaria alternata*; ARC, allergic rhinoconjunctivitis; Be, beech; Bi, birch; CD, cat dander; CH, *Cladosporium herbarum*; CM, cow milk; 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; Rab, rabbit; RP, rye pollen; RW, ribwort.

mutation rate, CDR-H3 length, and amino acid composition differ between IgG4 and IgE repertoires, the selective pressure seems to differ between the two isotypes in children with allergic asthma. It remains unknown whether this is also true for other allergic diseases. Wang et al. (7) amplified IgG1, IgG2, IgG3, IgG4, and IgE sequences from individuals who lived in a rural area in Papua New Guinea with endemic parasitism and compared them against sequences from urban residents of Australia. In agreement with our results, they found that the somatic mutation rate was significantly higher in IgG4 sequences compared with IgE and the other isotypes that were studied. This finding is also in harmony with the temporal model of human IgE and IgG class switching by Collins and Jackson (67), who suggest that class switching predominantly occurs sequentially from IgM to IgG3, to IgG1, to IgG2, and ultimately to IgG4, the last being the IgG subclass with the highest number of somatic mutations; but this model also allows for alternative routes of class switching, as, for example, from IgM directly to IgG4 under the influence of cytokines. In contrast to our results, Wang et al. (7) found that, compared with IgE, a higher fraction of IgG4 sequences from Papua New Guinean subjects showed evidence of Ag selection. This difference could be caused by technical reasons such as different PCR methods, but it is also possible that IgG4 repertoires underlie different selective pressures in allergic asthma and during parasite infections. Using the BASELINE methodology, Jackson et al. found evidence of Ag selection in IgG4 sequences from healthy Australian residents, and similar to findings of Wang et al. (7) and to our results, none of the IgG4 transcripts were unmutated (39). Of interest, in another study, IgE sequences from parasitized individuals contained less evidence of Ag selection than did IgE sequences from healthy individuals (7). Intriguingly, Wang et al. (68) found very few sequences with evidence of Ag selection in IgE transcripts from patients with allergies against bee venom or peanut.

In summary, it must be taken into account that the various mathematical models to identify evidence of Ag-driven selection in Ig transcripts can yield incongruent results (69, 70).

The structure of the Ag binding site has major impact on the function of the Ab (reviewed in Ref. 71). In comparison with IgE, IgG4 sequences contained shorter CDR-H3 regions. Shorter H3 loops protrude less into the solvent and are more likely packed into the space between the other variable loops of the Ag binding site (72). Analyses of Abs with known Ags revealed that CDR-H3 length correlates with the type of the Ag. For example, specific Abs to large Ags (viruses, proteins, and nucleic acids) have longer CDR-H3 regions than do Abs against smaller Ags (peptides, sugars, haptens) (52, 73). Thus, although identical Ags are recognized by some IgG4 and IgE Abs, the overall spectrum of Ag specificities might differ between the IgG4 and IgE repertoires. Moreover, the amino acid composition of the CDR-H3 regions differed between IgG4 and IgE transcripts, which was also the case between Abs directed against various types of Ags (52). This again supports the hypothesis that the Ag-binding properties of the two isotypes' repertoires differ.

One limitation of our study is that it is confined to transcripts from circulating, unsorted PBMCs. It is also possible that, as with IgE, the IgG4-expressing cells consist of two or more subpopulations, such as plasma blasts, plasma cells, and memory cells. However, these subpopulations are too small to be assessed separately in the small blood samples from our pediatric patients. Moreover, circulating B cells do not necessarily have to be representative of the B cells in the inflamed tissue (mucosa).

In comparison with IgM and IgE transcripts that had been previously obtained with the same technique from the same blood samples (35), the VH1 and VH2 families were overrepresented and the VH3 and VH4 families were underrepresented in the IgG4 transcripts reported in this article. However, similar to other protocols, our mixture of family-specific degenerate PCR primers probably amplifies the IGHV genes with variable efficiency (7). Surprisingly, the VH2 gene family was overrepresented in our IgG4 transcripts, in contrast to other reports (e.g., Ref. 74), although our VH2 primer has a mismatch with one of the three VH2 gene segments, VH2-26, at the penultimate position from the 3' end. The detected difference in VH gene usage between IgG4 and IgE transcripts may also be due to different reverse primers and PCR protocols (seminested PCR for IgE and standard PCR for IgG4). With these limitations in mind, it is also possible that some differences in VH gene family usage between IgG4 and IgE transcripts reflect Ag-driven selective pressure.

A subanalysis by VH gene families revealed great heterogeneity between the sequences that use one particular VH gene family and another (Supplemental Table I). For example, IgG4 sequences using VH2 contained fewer somatic mutations than did sequences using other VH gene families and none of the VH2 sequences, but 25% of the VH3 showed signs of Ag selection. Thus, a biased amplification of VH genes might also influence analyses of the somatic mutations and other aspects in the overall repertoire. Yet studies of the VH gene usage require different techniques and larger sample sizes to rule out technical bias and the influence of clonal restriction and interindividual differences on the genetic and transcriptional levels (75).

Hitherto, the tolerogenic role of IgG4 has been studied during specific immunotherapy. However, little is known about the role of IgG4 in untreated or symptomatically treated allergic patients. Therefore, our results may serve as a reference for future longitudinal studies that address the molecular changes of the IgG4 response during specific immunotherapy.

In summary, we found that IgG4 transcripts from children with allergic asthma showed some characteristics of a classical Ag-driven B2 type of immune response. However, the molecular properties of the H chain transcripts revealed that IgG4 and IgE underlie divergent selective forces. A better understanding of the IgG4 repertoire may help to develop new therapeutic strategies to improve the "blocking" activity of IgG4 during specific immunotherapy and to treat IgG4-related disease.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Data

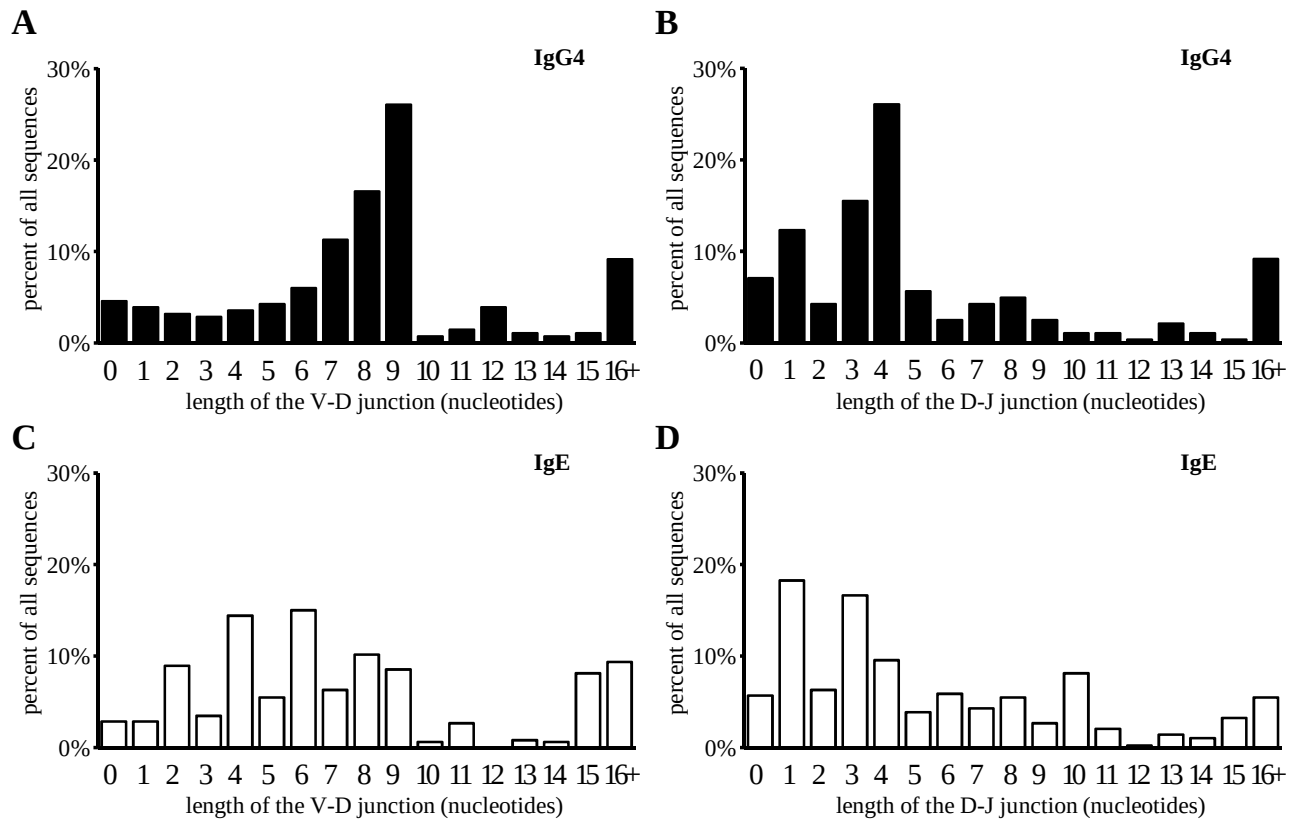
IgG4 and IgE transcripts in allergic children reflect divergent antigen driven selection

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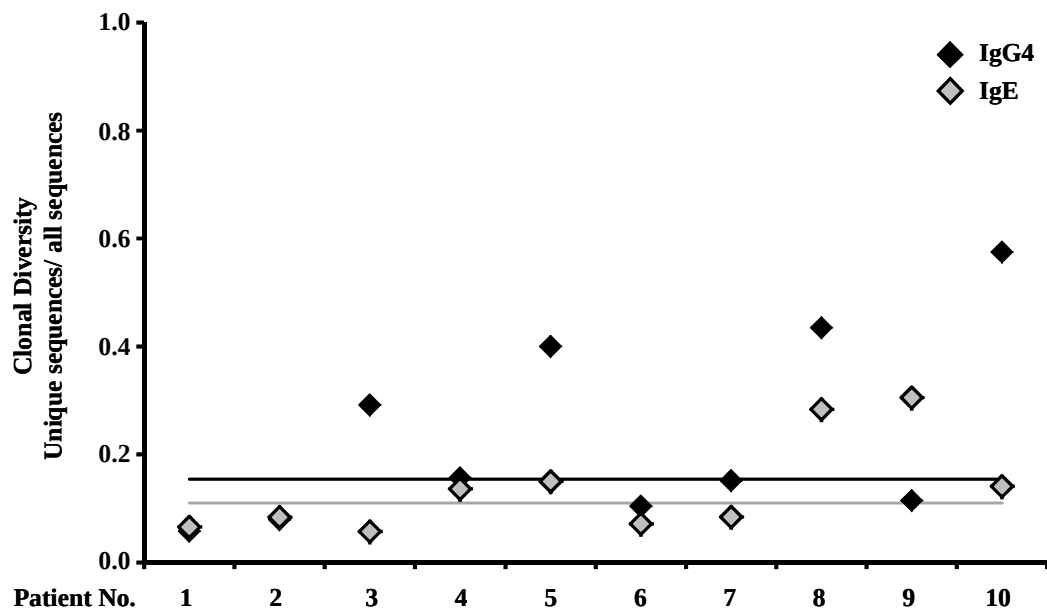
	IgG4*					IgE*				
	VH1	VH2	VH3	VH4	VH5	VH1	VH2	VH3	VH4	VH5
Sequences (n)	78	76	83	0	49	37	42	185	47	30
Sequences (%)	27	27	29	0	17	11	12	54	14	9
Mean CDR-H3 length (nucleotides)	49	37	50		44	41	43	55	44	39
Somatic mutations /1000 nucleotides	97	75	88		66	115	37	62	105	28
antigen selected (n)	12	0	25		3	6	15	66	19	4
antigen selected (%)	15	0.0	30		6	16	36	36	40	13

Supplemental Table 1: Characteristics of IgG4 and IgE transcripts by VH gene family usage. IgE transcripts from (35).

* no sequences using the VH6 and VH7 gene families were observed.



Supplemental Figure 1: Number of N-nucleotides introduced at the V→D junction (A, C) and at the D→J junction (B, D) of the CDR-H3 regions in IgG4 and IgE transcripts. IgE-transcripts from (35).



Supplemental Figure 2: Clonal diversity of functional IgG4 and IgE transcripts from individual patients. Mean diversity of IgE transcripts (Black line) and IgG4 transcripts (grey line). IgE-transcripts from (35).

Reference

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