Antigen selection in the IgE response of allergic and nonallergic individuals

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Background: Affinity maturation within germinal centers should usually lead to an accumulation of replacement mutations in complementarity-determining regions (CDRs) of Ig genes as a result of antigen selection. A number of studies have suggested, but not statistically demonstrated, that antigen selection might not guide such an accumulation of replacement mutations in allergic IgE sequences. This has been suggested to reflect the nature of allergens themselves or of the allergic response.

Objective: We sought to investigate the role of antigen selection in the evolution of the IgE response by mean of analysis of Ig sequences derived from both allergic and nonallergic individuals.

Methods: IgE sequences were amplified from peripheral blood of allergic and nonallergic individuals by using seminested RT-PCR. Additional IgE and IgG sequences were obtained from public databases. Analysis considered replacement mutations in the CDRs as a proportion of total mutations and compared IgE sequences with IgG sequences.

Results: The nonallergic IgE sequences were significantly less mutated than both the allergic IgE ($P < .001$) and IgG ($P < .01$) sequences. There was a low proportion of replacement mutations in the CDRs of both nonallergic and allergic IgE sequences and a significantly increased proportion of such mutations in IgG sequences ($P < .001$).

Conclusions: IgE antibodies in both nonallergic and allergic individuals appear to accumulate few somatic point mutations as a consequence of antigen selection.

Clinical implications: Allergic and nonallergic IgE responses might often develop along a common pathway that is distinct from the conventional germinal center reaction through which the IgG response develops. (J Allergy Clin Immunol 2006;117:1477-83.)

Key words: *Human, B cells, IgE antibodies, allergy, repertoire development, antigen selection*

Abbreviations used
CDR: Complementarity-determining region
FR: Framework region
IGHV: Immunoglobulin heavy chain variable
IMGT: ImMunoGeneTics
M_V: Total number of IGHV gene mutations
R_{CDR}: IGHV replacement mutations in the CDR

A hallmark of allergic disease is the development of IgE antibodies directed against innocuous environmental antigens, and it is generally accepted that the IgE response emerges in parallel with that of the more abundant IgG and IgA isotypes; that is, within the germinal center, some antigen-selected B cells undergo class switching to the various isotypes. During expansion of the responding cell population, these switched cells are subjected to the targeted mutation process that gives rise to an accumulation of somatic point mutations within the variable regions of the Ig genes.[1] This mutation process leads to the production of higher affinity antibodies as a consequence of selection of mutations that lead to improved antigen binding.[2]

It has been argued that this process of selection should favor replacement mutations rather than silent mutations within the complementarity-determining regions (CDRs).[3] In contrast, the need to select against mutations that compromise the structural integrity of Igs should lead to selection pressures against many replacement mutations in sequences encoding the framework regions (FRs). Analysis of the distribution of replacement and silent mutations along immunoglobulin heavy chain variable (IGHV) gene sequences of antigen-selected B cells should therefore show higher than expected replacement/silent mutation ratios in the CDR and lower than expected ratios in the FR. Such selection pressures have generally been assumed to act on Ig genes in cells expressing antibodies of all isotypes.

The possibility that mutational features of antigen selection are absent from IgE sequences has been raised in a number of studies.[4-6] These claims have been made, however, after consideration of very few IgE sequences, and statistical confirmation of the claims have not been provided in any of these reports. In fact, the suitability of the analytic approach taken in these studies has been challenged.[7] In this study we report a rigorous approach to the statistical inference of antigen selection from the distribution of somatic point mutations. In addition, we

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report sensitive techniques for the study of the IgE sequences that have allowed us to investigate IgE sequences from nonallergic individuals for the first time. We present results suggesting that the IgE response in both allergic and nonallergic individuals is fundamentally different in character from the IgG response. Although biases in the distribution of replacement mutations are readily apparent in sets of IgG sequences, neither allergic nor nonallergic IgE sequences show a similar degree of mutational evidence of antigen selection.

METHODS

Patient samples

After informed consent, peripheral blood was collected from 2 individuals with no clinical symptoms or history of allergy or parasitic disease. They were determined to be nonallergic on the basis of negative serum allergen-specific IgE (RAST) assay and skin prick test results to an extensive array of important local allergens (house dust mite, cockroach, cat epithelia, dog epithelia, *Alternaria alternata*, *Aspergillus fumigatus*, *Plantain lanceolata*, *Lolium perenne*, couch grass, cod, potato, sesame, peanut, hazelnut, soybean, wheat, yeast, milk, and egg), and they had total serum IgE concentrations of 21 IU/mL and 93 IU/mL, which are within the reference range (<190 IU/mL). Blood was also collected from an individual with atopic dermatitis who had multiple severe allergic sensitivities and a total IgE concentration of greater than 5000 IU/mL. RAST assays demonstrated IgE reactivity to the house dust mite *Dermatophagoides pteronyssinus* (>100 kUA/L), rye grass pollen (>100 kUA/L), cat dander (34.1 kUA/L), *A alternata* (4.9 kUA/L), peanut (24.4 kUA/L), shrimp (5.8 kUA/L), egg white (8.6 kUA/L), and egg yolk (7.1 kUA/L). All work performed was approved by the University of New South Wales Human Research Ethics Committee.

RNA extraction, cDNA synthesis, and PCR amplification

Mononuclear cells were prepared from peripheral blood samples by means of density gradient centrifugation, and total cellular RNA was extracted by using the method of Chomczynski and Sacchi.[8] Synthesis of cDNA was performed with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, Calif), according to the manufacturer's protocol. In brief, 1 μg of total RNA, 500 ng of oligo(dT)$_{12\text{-}18}$ primers (Promega, Madison, Wis), 0.2 mM dNTP Mix (Promega), 5 mM dithiothreitol, 40 units of RNase Inhibitor (Promega), and 100 units of Superscript II were incubated for 2.5 hours at 42°C and then for 15 minutes at 70°C. IgE heavy chain gene rearrangements were then amplified by using a seminested PCR. For the first PCR (PCR1), specific primers for each IGHV gene family (IGHV1-IGHV6) were used as forward primers in separate reactions, together with an outer IgE constant region 1 (CH1) reverse primer (Cε1 reverse, GCT ACT AGT TTT GTT GTC GAC CCA GTC).[9] The gene family–specific primers were as follows: IGHV1 forward, CAC TCC CAG GTG CAG CTG GTG CAG TCT GG; IGHV2 forward, CAG GTC ACC TTG AAG GAG TCT GG; IGHV3 forward, GTC CAG TGT GAG GTG CAG CTG GTG GAG TCT GG; IGHV4 forward, GTC CTG TCC CAG GTG CAG CTG CAG GAG TCG GG; IGHV5 forward, GTC TGT GCC GAG GTG CAG CTG CTG CAG TCT GG; and IGHV6 forward, GTC CTG TCA CAG GTA CAG CTG CAG CAG TCA GG. In the second PCR aliquots of PCR1 products were amplified by a combination of the same IGHV gene family forward primers and an inner IgE CH1 reverse primer (GGG GAA GAC GGG TGG GCT CTG TGT GG). PCR amplification was performed with approximately 40 ng of cDNA, 0.6 μM of each primer, 1.5 mM $MgCl_2$, 0.2 units of *Taq* polymerase (Promega), and a buffer supplied by the manufacturer. In a control PCR the β-actin gene was amplified by using β-actin forward (CCA ACT GGG ACG ACA TG) and reverse (CAG GGA TAG CAC AGC CT) primers. Cycling in both PCRs was 94°C for 5 minutes, followed by 94°C for 30 seconds, 54°C for 50 seconds, and 72°C for 1.5 minutes for 35 cycles and then 72°C for 10 minutes on a Tpersonal 48 cycler (Biometra, Gottingen, Germany). PCR products were purified by means of agarose gel electrophoresis and a gel extraction kit (Qiagen, Chatsworth, Calif) and then ligated into the pGEM-T Easy Vector (Promega) and transformed into *Escherichia coli* JM109 cells (Promega), according to the manufacturer's protocol. Random colonies were picked, their plasmids were prepared (Qiagen), and the inserts were sequenced.

V gene sequencing and V gene sequence alignment

Cloned IgE heavy chain inserts were sequenced with a Dye Terminator Kit (Applied Biosystems, Foster City, Calif) with 200 ng of plasmid and 3.2 pmol of M13 forward (GTT TTC CCA GTC ACG ACG) or M13 reverse (CAT GAT TAC GCC AAG CTA TT) primer. Sequences were analyzed on an ABI 3730 automated sequencer (Applied Biosystems) by using the following cycling conditions: 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes for 25 cycles.

IgG sequences and additional IgE sequences were identified from the ImMunoGeneTics (IMGT) Ig sequences database.[10] One hundred fifty-two IgG sequences were selected after exclusion of disease-related sequences, nonproductive sequences, and sequences including ambiguous nucleotides. One hundred twenty-eight IgE sequences from allergic individuals were identified in the IMGT[10] and Genbank databases. The IgE sequences were isolated from individuals with allergic rhinitis,[11,12] dermatitis,[4,13] peanut allergy,[14] and asthma.[5,15,16] All IgE and IgG sequences used in this study are detailed in Tables E1 and E2, respectively, which are available in the Online Repository at www.jacionline.org. All sequences were complete for the region beginning at codon 10 and ending at the 3′ codon of the FR3 region (codon 104) by using the IMGT numbering system.[17]

IgE and IgG sequences were aligned with the IMGT VQUEST program,[10] and all silent and replacement mutations in the sequences, from codon 10 to codon 104, were noted. Where more than one mutation affected a codon, each mutation was treated as an independent event, and all possible mutational pathways to the final sequence were computed. The number of replacement mutations scored for that codon was then calculated as the mean of the number of replacement mutations seen in the various pathways. For analysis of antigen selection, CDRs were defined to encompass codons included in the CDRs of both the IMGT[17] and Kabat[18] definitions. The CDR1 region was therefore defined to include nucleotides from codon 27 to codon 40, and the CDR2 region was defined to include those from codon 55 to codon 74.

Statistical analysis of somatic point mutations was conducted by using Pearson goodness-of-fit tests, with P values calculated by using a simulation-based method for testing the level of total mutations in the different groups. We also examined the way that the probability of replacement mutations in CDR depends on group (IgG, allergic IgE, or nonallergic IgE) and on the total number of mutations by fitting a quasibinomial model.[19] The distribution of replacement mutations was also compared with mutations seen in a model of the mutation process in which patterns of mutation are determined entirely by the location of mutational hotspots. These hotspots were determined by reference to trinucleotide mutability scores, as previously described,[20] and the relative mutability of the FR and

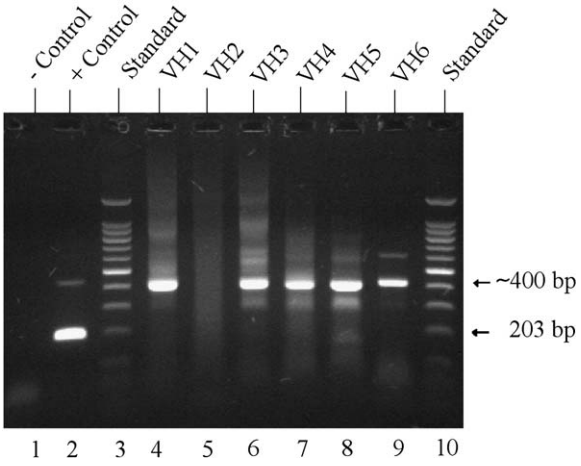

FIG 1. Representative 1.5% agarose gel showing the amplification by seminested PCR of IgE heavy chain cDNA from a healthy nonallergic donor, giving principal PCR products of approximately 400 bp as marked. − *Control*, DNA-free negative control; + *Control*, β actin–positive control showing a 203-bp PCR product.

CDR regions of representative germline sequences was determined from the sum of overlapping trinucleotide mutability scores.[20] The probability that a random mutation in the CDR would result in a replacement mutation was then determined by using the method of Chang and Casali,[3] and these estimates were then used to model the random accumulation of mutations by using the binomial distribution.

RESULTS

Screening of heavy chain gene libraries

A representative gel of an amplification by means of seminested RT-PCR of mRNA from rearranged Ig heavy chain genes is shown as Fig 1. PCR products were cloned and sequenced, and the sequences* were aligned by using the IMGT VQUEST program.[10] A summary of IGHV gene use in these sequences, as well as in database-derived sequences, is shown as Table I. Sequences were identified that used IGHV gene segments from all 6 IGHV gene families that were investigated. Nonallergic IgE sequences showed an essentially stochastic gene use, whereas the allergic IgE sequences included a high proportion of IGHV4 gene sequences. The IgG dataset and the database-derived IgE sequences showed a biased use of the small IGHV5 gene family, and the IgG sequences also showed an overrepresentation of IGHV6 sequences. This probably reflects the focus of some of the studies that generated the sequences. Certainly many of the IgE sequences were reported from studies that deliberately targeted IGHV5 transcripts.[4,15]

Analysis of somatic point mutations

The extent of mutations in allergic IgE, nonallergic IgE, and IgG sequences are shown in Fig 2. The nonallergic IgE sequences included a very high percentage of sequences (55%) having fewer than 10 mutations in the IGHV gene segment. In contrast, just 28% of allergic IgE sequences and 27% of IgG sequences had fewer than 10 mutations. Pearson goodness-of-fit tests showed significant differences between the frequency of total mutations and sequence type ($P < .001$). Comparisons between datasets showed significant differences between the nonallergic and allergic IgE groups ($P < .001$) and between the nonallergic IgE and IgG groups ($P < .01$) but not between the allergic IgE and IgG groups ($P > .05$). Thus the nonallergic IgE sequences had significantly fewer mutations than either of the other 2 groups.

*AY781805–AY781891; DQ005253–DQ005305

TABLE I. Frequency of use of IGHV gene families in rearranged IGH gene sequences from nonallergic and allergic IgE sequences and from IgG sequences

	IGHV1	IGHV2	IGHV3	IGHV4	IGHV5	IGHV6	Total
Nonallergic IgE	10	2	25	19	2	2	60
Allergic IgE*	3	0	21	48	2	2	76
Allergic IgE†	15	0	30	10	70	2	127
IgG	20	2	62	32	18	18	152

*Sequences produced as part of this study.
†Sequences obtained from public databases.

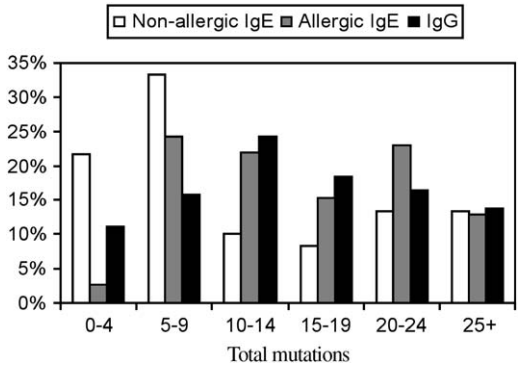

FIG 2. Frequency of human nonallergic IgE, allergic IgE, and IgG sequences seen with varying numbers of M_V. Allergic IgE sequences include sequences first reported here, as well as database-derived sequences.

Modeling random mutations of Ig heavy chains

The probability of mutations in the CDR was determined by reference to trinucleotide mutability scores to model a mutation process that was uninfluenced by antigen selection and was determined only by sequence hotspots.[20] The relative mutability of the FR and CDR regions of representative germline sequences was determined from the sum of overlapping trinucleotide mutability scores, and as a consequence, the probability that a random mutation would appear in the CDR was estimated to be 0.34. The method of Chang and Casali[3] was then used to estimate the probability that a mutation in the

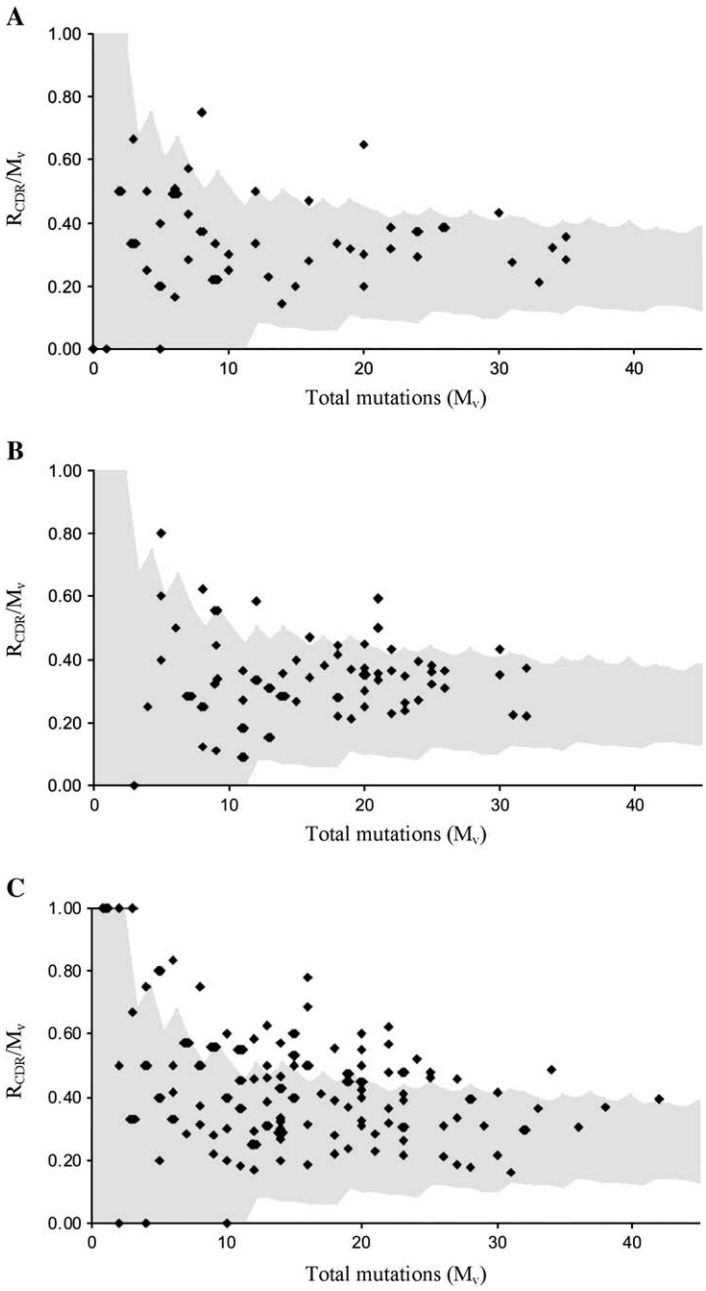

FIG 3. Ratio of R_{CDR} to M_V plotted against total numbers of mutations in nonallergic IgE **(A)**, allergic IgE **(B)**, and IgG **(C)** sequences. Allergic IgE sequences include sequences first reported here, as well as database-derived sequences. The *shaded areas* represent the 95% confidence limits for the R_{CDR}/M_V ratio in a model of random mutation, where $P(R_{CDR})$ equals .26. Data points have been adjusted to highlight clusters of overlaid values.

CDR would be a replacement mutation. This probability was estimated to be 0.78. The probability that a random mutation would therefore introduce a replacement mutation in the CDR was estimated to be 0.26. This estimate was then used to model the random accumulation of mutations by using the binomial distribution to establish 95% confidence limits for the ratio of replacement mutations in the CDR (R_{CDR}) to the total number of IGHV gene mutations (M_V). The 95% confidence limits were determined for sequences having total mutations numbering from 0 to 45, and the results are included in Fig 3.

Inference of antigen selection

The difference in the distribution of mutations of the IgG and IgE sequences is shown in Fig 3, which shows plots of the R_{CDR}/M_V ratio for nonallergic IgE (Fig 3, *A*), allergic IgE (Fig 3, *B*), and IgG (Fig 3, *C*) sequences. The allergic IgE sequences include both database-derived

sequences and sequences produced as part of this study. These data points are shown against a shaded region that represents the 95% confidence limits of the R_{CDR}/M_V ratio in our model of random mutation. Many IgG sequences fall outside these confidence limits, having high proportions of replacement mutations in the CDR. This would be expected if the IgG sequences evolved under the influence of antigen selection. On the other hand, fewer allergic and nonallergic IgE sequences appear to fall outside the confidence limits. We analyzed the distribution of replacement mutations in CDR in the different groups by fitting a quasibinomial model[19] to the data. We modeled the log odds of replacement mutations in CDR as an intercept plus indicator variables for the 2 IgE groups and the IgG group and an indicator variable for whether total mutations were greater than 20. After model selection by stepwise deletion of terms, we arrived at a model with a significant effect for the IgG group and an interaction between the IgG group and total mutation numbers. This model is consistent with the IgG group having a distinctly different distribution of replacement mutations with respect to the IgE sequences ($P < .001$). This striking difference between the groups was not seen with conventional mutation analysis.[3] All groups showed reduced numbers of replacement mutations in FR, as is to be expected for sets of productive immunoglobulin sequences. The ratio of R_{CDR} to IGHV silent mutations in the CDR was 2.7 for nonallergic IgE, 2.7 for allergic IgE, and 3.2 for IgG sequences. These differences were not significantly different between groups.

DISCUSSION

To better understand the nature of the allergic response, we have examined IgE heavy chain gene rearrangements from both allergic and nonallergic individuals and compared them with IgG sequences obtained from the IMGT Ig gene database.[10] Analysis of the distribution of somatic point mutations within the sequences showed that the nonallergic IgE sequences were significantly less mutated than either the allergic IgE or the IgG sequences ($P < .001$). In addition, there was a strikingly reduced proportion of allergic IgE sequences with less than 5 mutations, although allergic IgE sequences that totally lack mutations have been seen in a number of previous studies.[6,11,13,15,21]

Statistical analysis of the distribution of replacement and silent mutations between the FRs and the CDRs has been used to infer whether antigen selection has played a role in the evolution of Ig sequences[3] and has usually been applied to individual Ig sequences. We believe the inference of antigen selection is more appropriately arrived at from analysis of sets of sequences rather than from individual sequences, given the underlying randomness of the mutation process.

In this study we have adopted an approach that is different than the usual method of mutation analysis in a number of ways. In some studies sequences of interest have been compared with nonproductive Ig rearrangements,[22,23] and in such circumstances useful comparisons can be made of mutations in both the FR and CDR. We wished to compare productive IgE sequences with productive IgG sequences, and on the reasonable assumption that all such sequences have been subject to the same selection pressures for structural integrity, we were able to simplify the analysis by ignoring FR replacement mutations as a separate category. In our analysis we dichotomize mutations between R_{CDR} and all other mutations. To reduce the consequences of errors in the partitioning of mutations between the CDRs and FRs, we redefined the CDRs for this analysis to include all nucleotides that fall within the CDR according to either the IMGT[17] or Kabat[18] definitions. We considered these mutations with respect to the M_V because this measure best represents the extent to which a particular sequence has been subjected to the mutation process.

The standard method of mutation analysis[3] did not show significant differences between the IgE and IgG datasets in our study. Using our modified analysis and a model-fitting approach, however, we arrived at a model with a significant effect for the IgG group and an interaction between the IgG group and total mutation numbers. We only found an effect of the total number of mutations on the proportion of replacement mutations in CDRs for the IgG group. This reflects the fact that mutations in IgG sequences were distributed differently than IgE mutations in less mutated sequences but were similarly distributed in highly mutated sequences. Fig 3 shows that all highly mutated sequences have a relatively restricted range of the R_{CDR}/M_V ratio. This is to be expected because high R_{CDR}/M_V ratios in highly mutated sequences are likely to require more replacement mutations than can be tolerated in the short CDRs.

Seminested PCR can involve relatively high, random PCR error rates. Monitoring of the error rate in the sequencing performed as part of this study showed there to be 1 error in approximately 800 nucleotides or 1 error in approximately 2 to 3 IgE sequences. Simulation of the introduction of random errors suggests that PCR errors do not contribute significantly to the number or distribution of mutations seen in these IgE sequences and that PCR errors cannot explain the differences seen between these IgE sequences and the IgG sequences. No differences were seen in the number and distribution of mutations in these IgE sequences and in the database-derived IgE sequences that were also included in this study. Although we cannot be certain of the extent of sequencing errors in the database-derived IgE sequences, this suggests that such errors have not substantially contributed to the differences that were seen between the database-derived IgE sequences and the IgG sequences. The data demonstrate a greater role for antigen selection in the evolution of IgG sequences. By contrast, the significantly lower R_{CDR}/M_V ratio in both the allergic and the nonallergic IgE sequences compared with the IgG sequences and the similarities between the distributions of IgE mutations and the model of random mutation suggests that antigen selection has relatively little influence on the evolution of the allergic IgE or nonallergic IgE sequences.

Our study is not the first to propose a reduced influence of antigen selection in the evolution of allergic IgE sequences, although we believe it is the first to demonstrate this statistically. Other studies that have made similar claims have generally been based on few sequences and have involved little or no analysis of the data. For example, in one of the largest previous studies of which we are aware, a replacement/silent mutation ratio of greater than 2.9 for CDR mutations was taken as indicative of antigen selection.[4] Only 6 of the 19 IgE sequences in that study were reported to have replacement/silent mutation ratios of 3.0 or more, but no analysis was conducted to determine the expected number of sequences with high replacement/silent mutation ratios. As our analysis of IgG sequences shows, a majority of sequences evolving in response to antigen will have a distribution pattern that is indistinguishable from patterns seen in models of random mutations. This is particularly true for sequences with relatively low numbers of total mutations.

It has previously been suggested that an absence of mutational evidence of antigen selection in allergic IgE sequences could result from superantigen-like activation of B cells by some allergens.[15,24] It has also been suggested that low allergen concentrations, the absence of danger signals, or both could lead to the formation of immature germinal centers, which might favor the development of IgE antibodies.[25] If this was the case, unusual patterns of replacement and silent mutations seen in allergic IgE sequences might result from different selection processes in such immature germinal centers. However, the similarity reported here in the frequency and distribution of replacement mutations in nonallergic and allergic IgE sequences challenges this view, as well as the superantigen hypothesis. Unless the IgE response in nonallergic individuals is principally targeted toward allergens, a proposition for which there is no evidence, then the nature of allergens themselves cannot be invoked to explain the accumulation of essentially random mutations in both allergic and nonallergic sequences. The results we report are consistent, however, with the development of both allergic and nonallergic IgE responses in the absence of the antigen selection pressures of the conventional germinal center reaction. Although allergic IgE sequences include a greater number of mutations, these would appear to accumulate through similar processes to those that act on IgE-committed B cells in nonallergic individuals. Extrafollicular development of the IgE response could perhaps be occurring at mucosal sites, where class switching to IgE and somatic point mutation are now known to occur,[12,21] or it could be occurring at other extrafollicular sites, even as a consequence of CD40 ligand–independent, T cell–independent B-cell activation.[26,27]

Among the allergic IgE sequences in particular, some sequences were seen with higher proportions of replacement mutations in the CDR. It is possible that antigen selection has influenced the development of these sequences, and in different conditions IgE sequences could be developing in different ways. Further analysis of allergic IgE sequences from individuals with particular allergic conditions will be required to resolve this issue.

The reduced selection for replacement mutations in the CDRs of IgE sequences suggests that the IgE response might be generally of lower affinity than IgG antibodies, although there are certainly convincing studies that have demonstrated the existence of high-affinity IgE antibodies.[28,29] Certainly there are theoretic grounds for believing that affinity maturation could be less important for the function of IgE than IgG antibodies. Because IgE antibodies operate to sensitize cell surfaces, they might be functionally multivalent. Although there is no direct *in vivo* evidence for this proposition, we have demonstrated in an *in vitro* model that low-affinity IgE antibodies are able to effectively sensitize mast cells against multivalent antigen.[30] It might be that some IgE antibodies serve a similar role to natural IgM antibodies, contributing to protective immunity against a broad array of antigens, even at low antibody concentrations, and without the need for affinity maturation.[31] Whatever the normal role of IgE in our defenses, the observations reported here suggest that the derangement of the IgE response that characterizes allergic disease might occur at sites and in ways that have not previously been considered.

The design and completion of the experimental work sequencing IgE gene rearrangements was performed by Dr Isabell Dahlke, and the sequence analysis was performed by Dr Andrew Collins.

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Correction

With regard to the April 2006 supplement entitled ''Use of intravenous immunoglobulin in human disease: A review of evidence by members of the Primary Immunodeficiency Committee of the American Academy of Allergy, Asthma and Immunology'' (2006;117:S525-53): Between the dates of March 30, 2006 and April 5, 2006, an inaccurate version of the supplement was available online at ScienceDirect (www.sciencedirect.com). In this version, there was an error in the ''Dose'' section on page S543. An incorrect unit of measure appeared in the dose information in 3 instances in this section. The unit of measure was given incorrectly as milliliters per kilogram; the correct unit is milligrams per kilogram. The unit of measure for this dose information should have appeared as ''0.5 to 1.0 mg/kg per minute,'' ''1.5 to 2.5 mg/kg per minute,'' and ''4 mg/kg per minute.'' A corrected version of the supplement was posted online on April 6, 2006 and now appears online at ScienceDirect and the Journal's Web site (www.jacionline.org).