

Original article

Mutations in the high-affinity IgE receptor β -chain are not associated with nonresponder status

Background: Basophils of some individuals do not release histamine upon activation of their high-affinity immunoglobulin E (IgE) receptor ($Fc_\epsilon RI$), but do so if this receptor is circumvented for cell activation. This so-called nonresponder phenomenon is clinically relevant, because in various studies atopy was less frequent or absent in nonresponder individuals. So far, it is unknown if this phenomenon is acquired during adulthood or exists from birth on.

Methods: Histamine release was determined from isolated leucocytes stimulated with anti-IgE or calciumionophore. Also, random primed cDNA was synthesized and the open reading frame (ORF) of the $Fc_\epsilon RI$ β -subunit amplified and sequenced.

Results: In the first part of our study, we examined the role of atopic status, type of atopy, and age in a random population of 95 children of whom we found 22% to be nonresponder. None of these parameters correlated with the nonresponder status. Except for food allergy, no specific type of atopy correlated with histamine release. The mechanism underlying the nonresponder phenomenon is assumed to occur early in the signalling cascade. We hypothesized that mutations in the $Fc_\epsilon RI$ β -chain may be associated with the nonresponder status, and in the second part of our study sequenced the β -subunit in 20 responders and 20 nonresponders. Two conservative and two nonconservative heterozygous one base mutations (Thr179Thr, Asp216Asp, Ile147Leu and Glu237Gly) were found in two nonresponders and one responder. Three of these mutations have not been described so far.

Conclusion: The nonresponder phenomenon is present from birth on and genetically determined. In our population, it was not associated with age or the presence of atopy, and appeared not to be caused by mutations in the $Fc_\epsilon RI$ β -chain.

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Atopy is the most frequent disease within the paediatric population affecting up to 35% of children in industrialized countries. Clinical symptoms of atopy are caused by various mediators released from basophils in response to an allergen cross-linking specific immunoglobulin E (IgE) bound to its high-affinity receptor ($Fc_\epsilon RI$).

Since 1975, it has been known that basophils of some individuals do not release histamine upon activation of their $Fc_\epsilon RI$, but do so if the intracellular signalling cascade is activated by substances such as calciumionophores which circumvent this cell surface receptor (1, 2).

Abbreviations: cDNA, complementary deoxyribonucleic acid; $Fc_\epsilon RI$, high-affinity IgE receptor; $Fc_\epsilon RI\beta$, β -subunit of $Fc_\epsilon RI$; GIT, guanidium isothiocyanate; IgE, immunoglobulin E; mRNA, messenger ribonucleic acid; ORF, open reading frame; PCR, polymerase chain reaction; PIPES, piperazine-*N*-*N'*-bis-2-ethanesulphonic acid.

This phenomenon of nonresponder basophils seems to be of clinical importance, because in various studies atopy has been observed to be less frequent or absent in nonresponder individuals (3, 4). Up to the present, no study has addressed the question of whether or not some children are nonresponders from birth on, or whether this is an acquired characteristic.

Several possible causes for this phenomenon could be excluded in the past: responder and nonresponder basophils have the same amount of IgE on their cell surface and this IgE is effectively cross-linked by anti-IgE (3, 5). In addition, experimental evidence suggests that the underlying cause is genetically determined (6) and manifests itself early in the signalling cascade (3, 7), possibly affecting the functioning of the $Fc_\epsilon RI$ itself. In humans, the β -subunit of this tetrameric receptor is not required for cell surface expression (8), but important for signal transduction (9). It is therefore conceivable that

mutations in this subunit might interrupt the signalling pathway, thus preventing IgE-induced histamine release as it can be observed in nonresponder basophils.

We therefore determined the incidence of nonresponders in a paediatric population, and examined whether or not there is an association between nonresponder status and mutations in the β -subunit of the Fc ϵ RI β .

Methods

Subjects

During a 10-month period, blood was collected from children treated at the University Children's Hospital Munich. Patients with hematological or oncological diseases were excluded. Whenever blood was taken for other reasons, an additional 1.5–3 ml of ethylenediaminetetraacetic acid (EDTA) blood was drawn for this study irrespective of the presence of atopic disease. Medical and family history was obtained from parents after discharge from hospital by telephonic interview, using a standardized protocol specifically designed to search for atopic diseases (Allergopharma, Ganzer KG, Reinbek, Germany). The study was conducted according to the regulations, and with the approval of the local human research review committee.

Cell preparation and histamine release

After separation by 6% dextrane, leucocytes were resuspended in piperazine-*N*'-bis-2-ethanesulphonic acid (PIPES) buffer (10) and divided into four parts for stimulation with 320 IU anti-IgE/ml (Behring, Marburg, Germany), 2 μ g/ml Calciumionophor A23187 (Calbiochem, Bad Soden, Germany), or buffer alone. The remaining fourth was used to determine the total intracellular histamine content by cell lysis at -80°C . After 60 min of stimulation at 37°C , leucocytes were centrifuged, the supernatant used for histamine analysis, and the pellet stored in guanidium isothiocyanate (GIT) at -70°C . Histamine release and histamine standards (Merck, Darmstadt, Germany) were determined by histamine-autoanalyzer (11). Nonresponders were defined as patients with a measurable calciumionophor-induced release, but no anti-IgE-induced release beyond the accuracy of the histamine-autoanalyzer ($<3\%$) (11).

RNA synthesis, PCR and sequencing

Messenger RNA was separated by oligo(dT)cellulose (Quick Prep Micro mRNA Purification Kit, Pharmacia, Erlangen, Germany). Using 25% of the resuspended mRNA, cDNA was synthesized at 42°C for 60 min followed by 45°C for 30 min using 9mer random primer and 400 units SuperScriptRNase H Reverse Transkriptase (Gibco BRL, Karlsruhe, Germany) in a 20 μ l reaction.

For amplification of the β -subunit [–34 to +809 (8), Table 1], 1 μ l of the cDNA was amplified in a Gene Amp PCR System 9600 (Perkin Elmer Cetus, Foster City, CA, USA) employing hotstart technique using 0.625 U Ampli Taq DNA-Polymerase (Perkin Elmer Cetus) and 75 pmol of each primer (22mer) in a 50 μ l polymerase chain reaction (PCR) for 35 PCR cycles ($94^{\circ}\text{C}/15\text{ sec} - 60^{\circ}\text{C}/15\text{ sec} - 72^{\circ}\text{C}/120\text{ sec}$) with one preceding cycle ($72^{\circ}\text{C}/40\text{ min}$). For the three oldest samples, amplification had to be accomplished in two overlapping fragments: –34 to +498 and +338 to +809 (Table 1).

The PCR were separated on 0.8% agarose gels (Sea Kem GTG Agarose, Biozym, Oldendorf, Germany). The appropriate sized

Table 1. Sequence of oligonucleotides used for amplification and sequencing

Name	Orientation	Sequence of primer (5' $\rightarrow$ 3')	Region (bp)
K1	$\rightarrow$	GAAATCCACCAAGTCTCAATA	–63 to –43
SY10	$\rightarrow$	TTCTTTATTCTGGACAGCTCG	–34 to –13
HK961	$\rightarrow$	GCTCTGTACTTGATATTTACAC	239–261
2662	$\rightarrow$	TGTTGTCAATTATATCTGAAAGGA	338–361
HK10	$\leftarrow$	TGATGATCAGGATGGTAATCC	429–451
SY4	$\leftarrow$	TTTCTGGCAACTGTGGATGTG	478–498
K2	$\leftarrow$	GGCTGTGAATCAGAGTGTCT	749–769
SY11	$\leftarrow$	CCAGTAGCCCCCTTAACAAACC	788–809
Not-mutated	$\rightarrow$	CCACTGAAATTGTAGTGATGATGC	533–556
Mutated	$\rightarrow$	CCACTGAATTGGTATTGATGATGC	533–556
Mutated	$\leftarrow$	GCATCATCAATACCAATTCAGTGG	533–556

Shown is the name of each oligonucleotide as well as its orientation 5' to 3' ($\rightarrow$, forward; $\leftarrow$, backward), sequence, and position within the published sequence (8).

band was excised and diluted in 100 μ l water for 1 h. According to the yield of the preceding PCR, 4, 8, or 12 μ l of the supernatant were reamplified for sequencing in a 200 μ l reaction under identical conditions. The reamplification product was separated on low melting agarose gel (Lowmelt DNA, Biozym, Hameln, Germany), the correct fragment excised, and the DNA extracted by GeneClean (Bio101, La Jolla, CA, USA) according to the manufacturers' instructions. After phenol-chloroform extraction, 100 ng of the precipitated DNA were used for sequencing in a 10 μ l reaction ($25 \times 96^{\circ}\text{C}/10\text{ sec} - 50^{\circ}\text{C}/5\text{ sec} - 60^{\circ}\text{C}/4\text{ min}$) with PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and 4.2 pmol primer (Table 1). After purification (Centri-Sep Spin Columns, Perkin Elmer Cetus), automated analysis was performed on an ABI Prism 377 DNA Sequencer (Perkin Elmer Cetus). In all samples with questionable base pairs, the same and the opposite strand was repeatedly sequenced using different primers as appropriate until all ambiguities could be resolved ($n = 30$).

Negative controls

In addition to the usual negative controls, mimicry samples were used to exclude contaminations that might otherwise mask mutations, thus reducing the overall number of detected mutations. These samples consisted of 0.9% NaCl instead of EDTA blood and were taken through the complete procedure.

In order to detect invisible, but amplifiable contaminating material, agarose gel slices of negative controls were excised at the position where an appropriate band would have to be expected and reamplified together with positive controls. All these controls remained negative.

Positive controls

In order to confirm that homozygous and heterozygous mutations could be detected, PCR-fragments with artificial mutations were synthesized using appropriate primers (Table 1). All point mutations were detected.

Statistics

Mann–Whitney *U*-test, Spearman rank correlation, or Fisher's exact *t*-test were performed for statistical analysis as appropriate, using STATVIEW 4.0 for Macintosh and SPSS 10.0.5 for Windows.

Results

Histamine release was determined from 95 children aged between 6 months and 18 years. Sixty patients were male and 35 female with a similar age distribution for both sexes. The median cell count of the leucocyte suspension used for histamine release was 3000 cells/ μ l independent of sex and age (Table 2).

The absolute histamine content was between 1.3 and 16.8 ng histamine/ml leucocyte suspension, of which 9% was released spontaneously. Calciumionophor-induced release (A23187) was 75% and the only parameter significantly higher in girls than in boys (Mann–Whitney *U*-test, $P = 0.04$). None of these parameters correlated with age (Fig. 1).

Twenty-one of the children (22%) were nonresponders, while 74 (78%) were responders with an anti-IgE-induced median histamine release of 24% (Table 2). There was no correlation between responder or nonresponder status and any of the following parameters: age, sex, family or medical history, leucocyte cell count, absolute histamine content, spontaneous histamine release, calciumionophor-induced histamine release and medication. Nonresponders were 1.5 times more often atopic than responders. However, due to small patient numbers, this difference did not reach statistical significance. Except for food allergy, no specific type of atopy correlated with the amount of histamine released. The group of five patients with food allergy had a very small anti-IgE-induced median histamine release of 0%.

The full-length open reading frame (ORF) of the $Fc_\epsilon RI\beta$ -subunit was amplified and sequenced in 20 nonresponders and 20 responders (Table 2). In all 40 patients, a PCR fragment of appropriate size could be amplified. In addition to automatic sequence analysis, the results were compared manually for peak uniformity to detect base substitutions (homozygous mutation) or reduced peak height with accessory peaks (heterozygous mutation). In three patients, four different heterozygous mutations were found: the two base changes 537 T to T/A and 648 T to T/C were without consequence for the amino acid sequence (Thr179Thr and Asp216Asp). By contrast, the base change 439 A to A/C resulted in the amino acid substitution Ile147Leu and 710 A to A/G resulted in a Glu237Gly substitution (Fig. 2).

Discussion

Up to the present, no definite cause of atopy has been established, but several associated factors including the so-called nonresponder phenomenon have been described. The latter is defined as basophils that show no histamine release upon the activation of their $Fc_\epsilon RI$, but do so if this receptor is circumvented for cell activation (1, 2). The phenomenon of nonresponder basophils appears to be clinically important, because in various studies atopy has been reported to be less frequent or absent in nonresponder individuals (3, 4).

Table 2. Characteristics of the study population

	All children	Responder	Nonresponder	<i>P</i> -value
Histamine release				
Number of children [<i>n</i> (%)]	95 (100)	74 (78)	21 (22)	NA
Age (years)	9.3 (6.2–12.0)	9.0 (6.0–12.1)	9.6 (6.5–11.3)	0.895
Male [<i>n</i> (%)]	60 (63)	44 (59)	16 (76)	0.610
Atopic [<i>n</i> (%)]	24 (25)	17 (23)	7 (33)	0.627
Number of leucocytes [μ l]	3000 (2100–4250)	2800 (2000–4100)	3600 (2850–4550)	0.250
Absolute histamine content (ng/ml)	5.0 (3.6–7.2)	5.4 (3.6–8.0)	4.4 (3.6–6.0)	0.109
Spontaneous histamine release (%)	9.2 (5.9–13.7)	8.6 (5.1–13.7)	10.3 (6.8–13.4)	0.268
Anti-IgE-induced histamine release (%)	14.3 (3.6–35.5)	23.8 (9.8–43.6)	0.0	0.000
Calciumionophor-induced histamine release (%)	75.0 (61.7–81.9)	76.0 (62.1–82.8)	71.4 (58.7–77.8)	0.210
PCR/sequencing				
Number of children [<i>n</i> (%)]	40 (100)	20 (50)	20 (50)	NA
Age (years)	9.3 (7.2–10.6)	9.0 (7.4–9.8)	9.7 (6.4–11.5)	0.423
Male [<i>n</i> (%)]	22 (55)	11 (55)	11 (55)	1.000
Atopic [<i>n</i> (%)]	15 (40)	9 (45)	6 (35)	0.587
Number of leucocytes [μ l]	3100 (2575–4200)	2800 (2350–3150)	3600 (2775–4575)	0.202
Absolute histamine content (ng/ml)	4.6 (3.7–6.1)	5.3 (4.4–8.2)	4.3 (3.4–5.9)	0.077
Spontaneous histamine release (%)	9.4 (6.3–13.4)	7.7 (4.8–11.5)	10.6 (7.0–14.4)	0.101
Anti-IgE-induced histamine release (%)	6.6 (0.0–33.4)	34.8 (28.5–53.9)	0.0 (0.0–0.3)	0.000
Calciumionophor-induced histamine release (%)	75.0 (60.5–82.3)	78.5 (70.3–85.7)	68.1 (57.7–77.9)	0.059

Shown are demographic, clinical and histamine release data for all children as well as for the subgroup in which amplification and sequencing of $Fc_\epsilon RI\beta$ was performed. Leucocytes count was available for 74 children, status of atopy for 64. In the upper and lower part of the Table, the percentages of the 'number of children' as well as of 'male' and 'atopic' children were calculated on the basis of the overall number of children included in the study and in the subgroup, respectively. If not indicated otherwise, median (interquartile range) is given.

IgE, immunoglobulin E; $Fc_\epsilon RI\beta$, β -subunit of high-affinity IgE receptor; NA, not applicable; PCR, polymerase chain reaction.

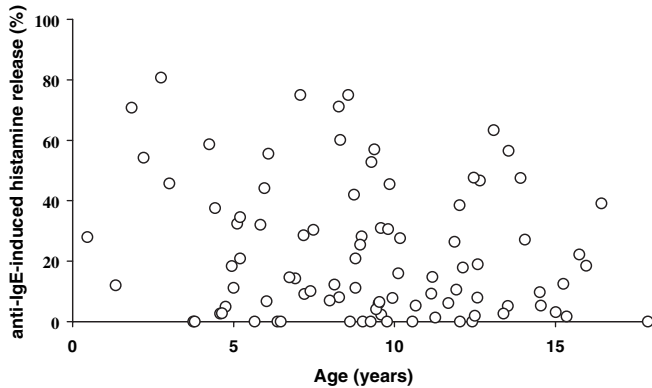


Figure 1. Anti-immunoglobulin E (IgE)-induced histamine release in relation to the age of each child.

Nonresponders and responders in our study were of all ages, and the age distribution was similar in both groups. The youngest infant was 6 months old at the time of blood letting. In this paediatric study population, the percentage of nonresponders was equal to that previously published for the adult population (3). This finding is in accordance with the fact that, similar to other authors, we observed no association between anti-IgE- or calcium-ionophor-induced histamine release and age (4, 10), as well as the fact that the nonresponder status has been found to be reproducible over long periods of time (7).

In contrast to other studies, we found nonresponder children to be slightly more often atopic than responder ones. The other way round, it is interesting to realize that many allergic children are basophil nonresponders in contrast to the finding in adults where nonresponders are virtually all nonallergics. One might speculate that such a genetically determined nonresponder status is by itself independent of the allergy status but over time inhibits a pathway leading to signs and symptoms of allergy.

Because the underlying cause is assumed to occur early in the signalling cascade (3, 7), we analysed the first molecule in the signal transduction pathway leading to histamine release, the Fc_εRI. This tetrameric receptor consists of three subunits of which the α-subunit is solely responsible for IgE binding not altered in nonresponders (3, 7) whereas the β- and the γ-subunit are both involved in signal transduction (9). The γ-subunit is part of several other important immune receptors such as the T-cell receptor and at least three of the IgG receptors: Fc_γRI, Fc_γRII, and Fc_γRIIIA. Thus, functionally relevant mutations of the γ-subunit should result in obvious signs of immunodeficiency not seen in any of our nonresponder patients. Consequently, mutations in the α- or γ-subunit are not a likely cause for the nonresponder phenomenon. On the contrary, the β-subunit solely functions as a critical amplifier of Fc_εRI-mediated signalling (9), but is not required for receptor cell surface expression in humans (8). In particular, it has been hypothesized that mast cells and basophils may not only express the

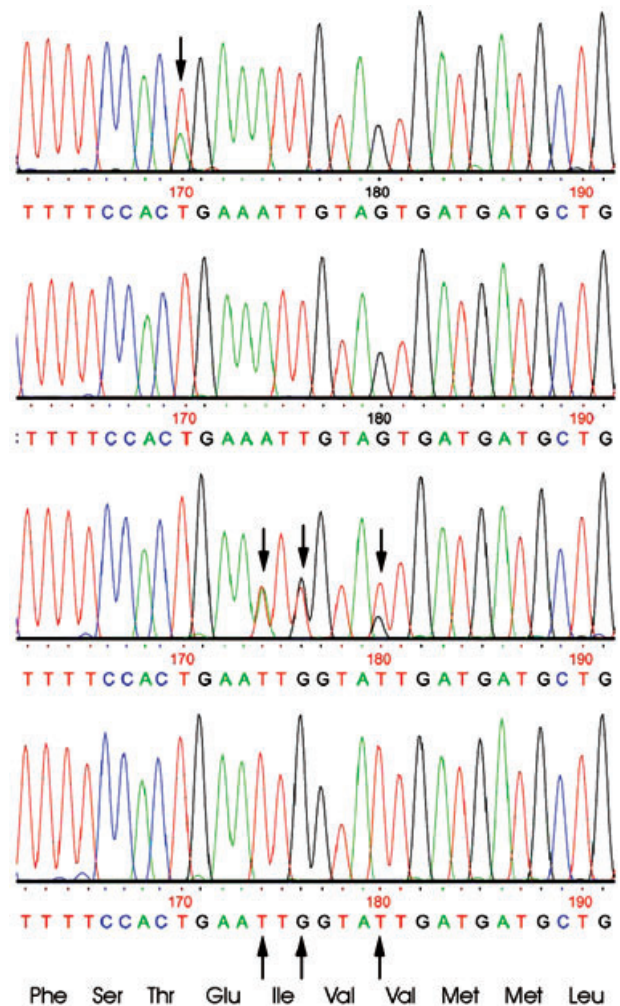


Figure 2. Mutations in the β-subunit of high-affinity immunoglobulin E (IgE) receptor (Fc_εRIβ)-subunit. Depicted are four electropherograms showing part of the Fc_εRIβ-subunit [base pair 529–558 of the open reading frame (ORF)]. Lane 1 is the sequence of a child with a heterozygous mutation at base pair 537 (A to A/T) (arrow from above), lane 2 the wild-type sequence without mutations, lane 3 a sequence with three artificial heterozygous mutations (arrows from above) and lane 4 a sequence with three artificial homozygous mutations (arrows from below). The bottom line gives the amino acid sequence of the wild type in three-letter code. Note the uniformity of the peaks between the four different sequencing reactions.

tetrameric αβγ₂-complex on their cell surface, but also the trimeric αγ₂-isoform (12). In addition to the central role of the β-subunit in signal amplification (13), it has also been described to amplify the cell surface expression of the Fc_εRI (14). The combination of these two distinct amplifier functions, the signal and the expression amplification, lead to an estimated 12- to 30-fold amplification of downstream events (14).

It is therefore conceivable that structural changes of the β-subunit due to point mutations could lead to reduced

intracellular signalling, thus resulting in impaired releasability. To search for such changes, we sequenced the β -subunit of the IgE receptor, concentrating on that part of the gene that is translated into protein. In order to examine the allele actually translated into mRNA and expressed as protein on the cell surface, explicitly cDNA instead of genomic DNA was used for sequencing.

In 37 of the 40 patients, the wild type sequence of the β -subunit was obtained. In three patients, four different heterozygous mutations were found: two without effect on the amino acid sequence, one conservative exchange, and one nonconservative close to an ITAM-motif. Both exchanges concern amino acids conserved between human, mouse, and rat. Of the three patients involved, one was a 6.4-year-old male nonresponder, the second a 10.6-year-old female nonresponder, and the third a 9.9-year-old male responder. Family and allergy history showed no correspondence to the mutations found.

As of our knowledge, three of the mutations observed in our study have not yet been published. Conversely, the Ile181Leu mutation described by others to be present in up to 17% of a nonselected population (15) was not detected but a new one two amino acids upstream, instead. Interestingly, a strong association was reported between the Ile181Leu variant and atopy (15) as well as histamine release from basophils to anti-IgE stimuli (16). However, none of the three published β -chain variants Ile181Leu, Val183Leu, and Glu237Gly neither correlated with total IgE levels and skin test responses to aeroallergens (16) nor exerted any direct effect on the two amplifier functions of the β -chain, namely amplification of receptor expression and signalling (17). These contrasting results call for additional studies to specifically look for the relevance of this mutation hot spot in well-defined subpopulations like basophil nonresponders.

In conclusion, our findings appear to indicate that mutations in the ORF of the Fc ϵ RI β -subunit do not make up a significant cause for the nonresponder phenomenon as such mutations should have been found more frequently in our study population. Therefore, other reasons have to be considered. These might be mutations found in the untranslated region of the β -chain leading to reduced

transcription, mRNA-stability, or translation. In addition, it has been suggested that the variants found to be associated with clinical or laboratory effects are in linkage disequilibrium with other more pertinent polymorphisms or could influence hitherto unknown functions of the β -chain (17). Intriguingly, results published (18, 19) indicate a central role for the tyrosine kinases lyn and syk in Fc ϵ RI-mediated basophil degranulation. Syk was reported to be absent, and levels of Lyn to be reduced in nonreleasing basophils but mRNAs of both proteins were detectable at normal levels. Moreover, maximum histamine release correlated with the expression of both kinases. In contrast, looking at calcium response the same group was able to artificially mimic only one of the two defects in nonreleasing basophils: while a specific inhibitor of both lyn and syk kinase increased the timelag similar to that seen in basophils of nonresponders the ultimate response was unchanged and similar to that of responder basophils (20). Therefore, the underlying defect of nonresponder basophils must consist of more than altered lyn and syk kinase levels. In addition, it seems unlikely that a mutation exists in both proteins to specifically suppress translation or normal protein transport in basophils in such a similar manner for both proteins as described (19). In conjunction with the above discussed clinical observation of the nonresponder status to effect the allergy status in adults but not during infancy one could speculate that the nonresponder status might influence a so far unknown factor leading among other effects to a change in translation or processing of lyn and syk kinase over time.

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