

The glutamine 27 β_2 -adrenoceptor polymorphism is associated with elevated IgE levels in asthmatic families

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Background: The β_2 -adrenoceptor polymorphisms occurring at amino acid positions 16 (arginine to glycine) and 27 (glutamine to glutamate) are known to be functionally relevant and also disease-modifying in subjects with asthma. However, the contribution of these polymorphisms to the development of the asthmatic phenotype or other markers for allergic disease remains to be established.

Objective: This large family study examines the contributions of these polymorphisms in determining the heritable component of markers for allergic disease in asthmatic families.

Methods: Three hundred twenty-four individuals from 60 families multiplex for asthma selected by means of an asthmatic proband were characterized for the following markers of allergic disease: asthma, atopy, and serum IgE. The polymerase chain reaction was used to generate a 234 base pair fragment spanning the region of interest, and the β_2 -adrenoceptor polymorphism was then defined by allele-specific oligonucleotide hybridization. Segregation analysis was then performed.

Results: We found a significant association ($p = 0.009$) between the glutamine 27 β_2 -adrenoceptor polymorphism and elevated levels of IgE, which was supported by the observation of linkage between IgE and β_2 -adrenoceptor polymorphisms at locus 27 ($p = 0.037$). However, there was no association between either the arginine-glycine 16 or the glutamine-glutamate 27 β_2 -adrenoceptor polymorphism and an increased risk of asthma or atopy per se.

Conclusion: The glutamine 27 β_2 -adrenoceptor polymorphism appears to contribute to IgE variability in families with asthma. However, it seems that although both amino acid 16 and 27 β_2 -adrenoceptor polymorphisms are disease-modifying in subjects with asthma, they do not contribute markedly to the development of the asthmatic phenotype. (J Allergy Clin Immunol 1997;100:261-5.)

Key words: β_2 -Adrenoceptor polymorphism, IgE, asthma, atopy, chromosome 5q, genetics of asthma

Abbreviations used

ASO: Allele-specific oligonucleotide

PCR: Polymerase chain reaction

The gene encoding the β_2 -adrenoceptor is located on the long arm of chromosome 5 (5q 31-33) and was first cloned in 1987.¹ Subsequently, nine polymorphisms occurring in this gene have been described.² Five of these are degenerate, but four arise as a consequence of single point mutations resulting in single amino acid substitutions in the β_2 -adrenoceptor. However, only the amino acid 16 and 27 β_2 -adrenoceptor polymorphisms are present with high allelic frequency in both asthmatic and normal populations.² Both in vitro and in vivo evidence suggest that these two polymorphisms are also functionally relevant. The arginine to glycine (Arg-Gly 16) β_2 -adrenoceptor substitution has been shown in vitro to result in increased downregulation of the β_2 -adrenoceptor after agonist challenge in both Chinese hamster fibroblast cells transfected with the human β_2 -adrenoceptor and primary human airway smooth muscle cells in culture.^{3,4} In contrast, the glutamine to glutamic acid (Gln-Glu 27) β_2 -adrenoceptor substitution confers attenuated downregulation in both these experimental systems.^{3,4} Clinical studies have shown that the Gly 16 form of the receptor is associated with nocturnal falls in peak flow rate in subjects with asthma⁵ and with increased airway reactivity.⁶ In contrast, we have shown that the Glu 27 form of the β_2 -adrenoceptor is associated with lower airway reactivity in subjects with mild to moderate asthma.⁷

Since the initial observations of Szentivanyi in 1968,⁸ defects in the β_2 -adrenergic signaling pathway have frequently been proposed to be of importance in the development of asthma.⁹⁻¹² However, although the above studies indicate that β_2 -adrenoceptor polymorphisms are disease-modifying in subjects with asthma, the possible contribution of these polymorphisms to the development of the asthmatic phenotype has not been adequately assessed. Therefore we performed an association study in families multiplex for asthma to determine the possible contribution of β_2 -adrenoceptor poly-

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Supported by the National Asthma Campaign, the National Institutes of Health (USA), and the Medical Research Council.

Received for publication Oct. 1, 1996; revised Mar. 10, 1997; accepted for publication Apr. 4, 1997.

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0091-6749/97 \$5.00 + 0 1/1/82528

morphisms to the development of asthma and allergic disease.

METHODS

Subjects

Three hundred twenty-four individuals from 60 unrelated families selected by means of an asthmatic proband were studied. Of these 60 families, 44 were nuclear in structure. The families were outbred, and all subjects were Caucasian. The probands were recruited from hospital clinics in the Wessex area of Southern England, and all had a physician's diagnosis of asthma. The study was approved by the Southampton University Hospitals Joint Ethical Committee. Informed written consent was obtained from adults, and informed verbal consent was obtained from children.

We looked for an association between β_2 -adrenoceptor polymorphism and the following surrogates for allergic disease: log total serum IgE, asthma score, and atopy score. The total serum IgE was measured by standard methods (ELISA), and the sex- and age-adjusted log IgE was derived for each individual. The distribution of IgE in this population was normal. Asthma and atopy were studied as semiquantitative traits. This method allows reduction to an ordered polychotomy of ranked scores. Asthma was studied by using stepwise principal component regression analysis of bronchial hyperresponsiveness, wheezing, and other features of asthma identified in a previously validated video questionnaire and was dominated principally by bronchial hyperresponsiveness.^{13,14} An asthma score was then obtained by using a slight modification of previously published methods^{13,14}; in brief, the score was derived by using logistic regression of the binary variable "asthmatic" or "nonasthmatic" versus bronchial hyperresponsiveness, the presence of wheezing, and other features of asthma obtained from the video questionnaire. The percentage of parents with asthma in the sample was 44.3%, and the percentage of children with asthma was 71.2%. Similarly, atopy was defined by using stepwise principal component regression analysis of log serum total IgE; skin prick test results; and the presence or absence of eczema, hay fever, and migraine. The atopy score was based on the binary variable "atopic" or "nonatopic" versus eczema, hay fever, migraine, skin prick test positivity, and log IgE^{13,14}; and the main determinant of the atopy score in this sample was skin prick test positivity.

Measurement of airway reactivity and skin prick tests

Skin prick tests were performed with 14 common allergens: Der p 1, cat dander, dog dander, horse dander, mixed grass pollens, mixed tree pollens, *Aspergillus fumigatus*, *Alternaria* spp., *Cladosporium* spp., *Dermatophagoides farinae*, feathers, milk, and egg protein (white plus yolk). Extracts were standardized and obtained from Bayer Corporation (Spokane, Wash.). A 3 mm or greater wheal was considered positive when compared with a negative saline control. A positive histamine control was also used. Bronchial responsiveness to inhaled histamine was determined by the method of Yan et al.¹⁵ using a hand-held de Vilbliss nebulizer. Subjects were asked to abstain from using antihistamines or asthma medication before the challenge. FEV₁ was determined with a dry-wedge spirometer (Vitalograph, Buckingham, U.K.). The higher of two recordings, which were no more than 100 ml apart, was taken as the baseline FEV₁. The challenge did not proceed unless the baseline FEV₁ was greater than 60% of predicted value for height. If the baseline was less than 70% of predicted value, a

bronchodilator challenge with salbutamol was carried out. After an initial saline inhalation, doubling concentrations of agonist were administered until FEV₁ fell by 20% of the postsaline baseline or until the highest concentration of agonist was achieved (2.2 μ mol). Histamine responsiveness was quantified as the provocative dose of agonist that reduced FEV₁ by 20% from baseline (PC₂₀).

Screening for polymorphisms

β_2 -Adrenoceptor genotype was determined by allele-specific oligonucleotide (ASO) hybridization as previously described.^{7,16} Genomic DNA was extracted from 5 ml of whole blood by using the Nucleon extraction system (Scot Lab, U.K.) and resuspended in 500 μ l of water. The polymerase chain reaction (PCR) was then used to generate a 234 base pair fragment from the 5' end of the β_2 -adrenoceptor, spanning the two polymorphisms of interest. A 50 μ l PCR was performed with 1 μ l of genomic DNA, 34 μ l of water, 200 μ mol/L each deoxynucleotide, 5 μ l of PCR 10 $\times$ buffer, 1.5 mmol/L MgCl₂, and 2 μ mol/L each primer. One unit of *Taq* polymerase was added per reaction. The primer sequences were as follows: upstream CCC AGC CAG TGC GCT TAC CT and downstream CCG TCT GCA GAC GCT CGA AC. The PCR consisted of 36 cycles, and the conditions for each step were as follows: melting temperature 94 $^\circ$ C, 90 seconds; annealing temperature 60 $^\circ$ C, 90 seconds; extension temperature 72 $^\circ$ C, 90 seconds. An initial period of 5 minutes at 94 $^\circ$ C was used in the first cycle, followed by 1 minute at 60 $^\circ$ C, after which 1 unit of *Taq* polymerase was added to each reaction. At the very end of the reaction, a 10-minute extension period at 72 $^\circ$ C was used. One microliter of PCR product was then applied to duplicate Hybond N plus filters (Amersham, Buckinghamshire, U.K.) with a dot-blot apparatus. To determine genotype, one filter was hybridized as appropriate with "cold" (unlabeled) ASO probe homologous with the wild-type form of the receptor followed by "hot" (phosphorous 32-labeled) ASO probe homologous with the mutant form of the receptor. The duplicate filter was treated in an identical fashion, but probes were used in the reverse order.

The sequences for the ASO probes used were: Gln27, CACGCAGCAAAGGGACGAG; Glu27, CACGCAGGAAAGGGACGAG; Arg16, GCACCCAATAGAAGCCATG; and Gly16, GCACCCAATGGAAGCCATG. The hybridization conditions used were as follows: hybridization with cold and then hot probe in 5 $\times$ sodium chloride, sodium hydrogen phosphate, ethylenediaminetetraacetic acid (SSPE), 1% sodium dodecylsulfate at 52 $^\circ$ C (60 minutes for each probe). Washes were done with 2 $\times$ SSPE, 0.1% sodium dodecylsulfate at 37 $^\circ$ C ($\times$ 2 for 30 minutes), followed by 5 $\times$ SSPE, 0.1% sodium dodecylsulfate at 52 $^\circ$ C (15 minutes $\times$ 2). For the Arg-Gly polymorphism a 10-fold excess of cold probe was used in the initial part of the hybridization procedure, whereas a 30-fold excess of cold probe was used for the Gln-Glu polymorphism. ASO probes were labeled thus: 0.05 nmol of oligonucleotide probe was added to 5.5 μ l of water, 1 μ l 10 $\times$ polynucleotide kinase buffer, 5 U polynucleotide kinase buffer, and 2 μ l of ³²P γ -adenosine triphosphate (9.25 MBq). The mixture was incubated at 37 $^\circ$ C for 1 hour and then heated for 5 minutes at 70 $^\circ$ C. Probed filters were exposed to x-ray film overnight. An example of a representative blot is shown in Fig. 1. Direct sequencing of a random selection of samples was performed to ensure accuracy. Because of a technical difficulty we were unable to genotype three individuals for the amino acid 16 polymorphism and one individual for the amino acid 27 polymorphism: these individuals were excluded from further analysis.

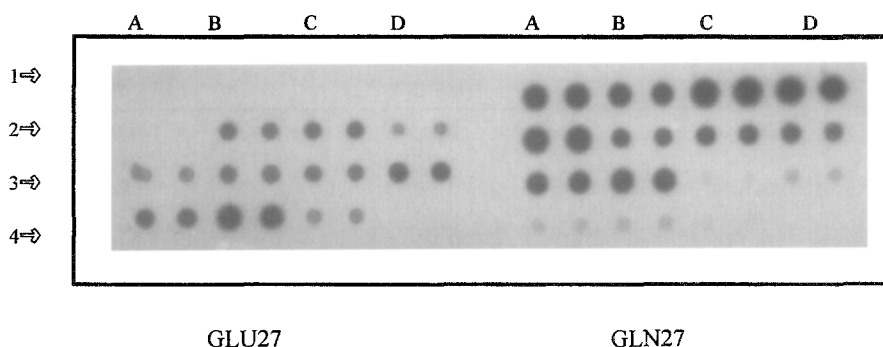


FIG. 1. Determination of genotype by ASO hybridization. For the purpose of this figure, 15 individuals who had previously been genotyped were selected. Duplicate aliquots of PCR product were applied to each filter for five subjects known to be homozygous for the Gln27 form of the receptor (A1-4, B1), five subjects known to be heterozygous (B2-4, C1-2), and five subjects known to be homozygous for the Glu 27 form of the receptor (C3-4, D1-3). The panel at left shows hybridization with an ASO probe directed against the Glu 27 form of the receptor, and the panel at right shows hybridization with the Gln 27 ASO probe. Confirmation of genotype was achieved by direct sequencing (see Methods for full details).

Segregation analysis

Nonparametric segregation analysis and association analysis were performed by using the NOPAR program to detect allelic association and linkage with the β -model, and parametric analyses were performed by using the COMDS program to detect allelic association on an ordered polychotomy of ranked scores as previously described.¹³

RESULTS

We used ASO hybridization^{7,16} to determine the genotype of our subjects (see Methods for details). First, genomic DNA was extracted from whole blood, and a 234 base pair fragment spanning the two polymorphisms was generated by using the PCR. Genotype was then determined with probes specific for each polymorphism. Fig. 1 shows a representative ASO result on samples from 15 individuals who had previously been genotyped by direct sequencing.

In this sample of families multiplex for asthma, the allelic frequencies for the two β_2 -adrenoceptor polymorphisms studied were as follows: Arg 16, 33%; Gly 16, 67% ($n = 321$); and Gln 27, 52%; Glu 27, 48% ($n = 322$). The distribution of β_2 -adrenoceptor polymorphisms within this population is shown in Table I. Both the allelic and genotypic frequencies in our population are in accordance with those obtained in previous studies in the United Kingdom and the United States.^{5,7} The estimates for heritability in this sample were as follows: log IgE, 0.63; asthma score (sex- and age-adjusted), 0.41; atopy score (sex- and age-adjusted), 0.65. The contribution of log IgE to the atopy score was not large, and the major determinant was skin prick test variability.

The association between each polymorphism and log IgE, atopy score, and asthma score, respectively, was analyzed by using the COMDS program; and the results are shown in Table II. There was no significant association of either the amino acid 16 or the amino acid 27 β_2 -adrenoceptor polymorphism with the asthma score or the atopy score examined by semiquantitative trait anal-

TABLE I. Genotypic frequencies of β_2 -adrenoceptor polymorphisms

	Genotype	n	%
Amino acid 16	Arg 16 Arg 16	36	11
	Arg 16 Gly 16	141	44
	Gly 16 Gly 16	144	45
Amino acid 27	Gln 27 Gln 27	84	26
	Gln 27 Glu 27	169	53
	Glu 27 Glu 27	69	21

Distribution of β_2 -adrenoceptor polymorphisms among 324 individuals from 60 families multiplex for asthma.

ysis. However, total log IgE was significantly associated with the Gln 27 genotype ($p = 0.009$). In addition, significant linkage was observed between total serum log IgE and the β_2 -adrenoceptor polymorphism at locus 27 when the NOPAR program was used ($r = 0.24$, $p = 0.037$), but no significant linkage was seen with the asthma or atopy scores. No linkage was observed between the β_2 -adrenoceptor polymorphisms and any of the other clinical parameters studied.

DISCUSSION

The main findings of this study are (1) that the Gln 27 β_2 adrenoceptor polymorphism is significantly associated with elevated levels of serum IgE and (2) that neither the Arg-Gly 16 nor the Gln-Glu 27 β_2 adrenoceptor polymorphism was associated with an increased risk of asthma or atopy in the families studied. The potential contribution of the amino acid 27 polymorphism to IgE variability in this population was further supported by linkage analysis.

An association between total serum IgE and the Gln 27 β_2 -adrenoceptor polymorphism could occur for one of three reasons. First, it is conceivable that this polymorphism is directly involved in altering IgE responsiveness. β_2 -Adrenoceptor agonists have antiinflammatory

TABLE II. Association between each β_2 -adrenoceptor polymorphism and asthma, atopy, and log IgE

Variable	Polymorphism	n	Frequency	r	95% CI	p Value
log [IgE]	Arg 16	228	0.333	-0.077	-0.208, 0.052	0.24
	Gln 27	321	0.465	0.171	0.043, 0.301	0.009*
Asthma score	Arg 16	228	0.333	0.073	-0.054, 0.201	0.26
	Gln 27	321	0.465	0.022	-0.105, 0.149	0.73
Atopy score	Arg 16	228	0.333	-0.041	-0.169, 0.088	0.53
	Gln 27	321	0.465	0.057	-0.070, 0.184	0.38

Association analysis between log IgE (sex- and age-adjusted), asthma score, atopy score, and the Arg-Gly 16 and Glu-Gln 27 β_2 -adrenoceptor polymorphisms. The correlation (*r*) and 95% confidence interval (*CI*) are given for each variable.

*Significant *p* value.

actions in vitro,¹⁷⁻¹⁹ although the relevance of these effects in vivo has been questioned.²⁰ The Glu 27 form of the receptor downregulates to a lesser extent than the Gln 27 form of the receptor after agonist exposure, and hence it is conceivable that individuals with the latter form of the receptor have chronically downregulated airway β_2 -adrenoceptors, resulting from exposure to endogenous catecholamines. This could in turn result in an increased sensitivity to proinflammatory stimuli, thus leading to a higher degree of airway inflammation and a consequently greater IgE response to inhaled allergens. The second possible explanation is that the Gln-Glu 27 β_2 -adrenoceptor, although not involved causally in contributing to the control of IgE production, is in linkage disequilibrium with a locus important in the control of IgE levels nearby on chromosome 5q31. There is certainly further evidence to support the presence of a genetic locus for the control of IgE levels in this region, with linkage being observed between the nearby D5S436 microsatellite marker and total IgE in a recent study of Dutch families²¹: D5S436 is approximately 2-cM from the β_2 -adrenoceptor locus. Chromosome 5q also contains a number of cytokine genes important in the generation of T_H2 responses including those for IL-4, IL-5, IL-9, and IL-13.²² However, these genes are probably too distant (>20-cM) from the β_2 -adrenoceptor gene to explain the association observed, although β_2 -adrenoceptor polymorphisms were not specifically examined in the study by Marsh et al.²² Finally, our results could be the result of a type 1 error, although given increasing evidence suggesting the presence of a major genetic locus for IgE control on 5q together with our previously published data demonstrating the functional relevance of β_2 -adrenoceptor polymorphisms,^{3-5, 7} we consider this unlikely.

Given that we observed an association between the Gln-Glu 27 β_2 -adrenoceptor polymorphism and total IgE and that IgE levels are believed to be a major determinant of the risk of developing allergic disease, why did we not observe a relationship between β_2 -adrenoceptor genotype and asthma or atopy? The heritability of IgE is greater in most studies than that for asthma (0.63 and 0.41, respectively, in our study),²³⁻²⁶ and hence a genetic component for the control of IgE variability would be expected to be more readily appar-

ent in this kind of study. Total IgE is itself believed to be an important contributor to the risk of developing allergic disease²⁷ but even so only explains between 30% and 50% of the risk of developing atopy. We used an atopy score to enable us to examine atopy as a semi-quantitative trait, but IgE was a minor determinant of this score, with the major contribution being that from skin prick testing. The overall variability in IgE, which could be explained by the Gln-Glu 27 polymorphism, is relatively small, and hence it is difficult to exclude a small increase in the risk of developing asthma in individuals with the Gln 27 form of the receptor in this study. Nonetheless, the results of this study indicate that the amino acid 16 and 27 β_2 -adrenoceptor polymorphisms are not a major risk factor for developing asthma in our population.

Previous work has suggested that the Arg-Gly 16 β_2 -adrenoceptor polymorphism may also be disease-modifying in that it is associated with the nocturnal asthma phenotype and with the requirement for oral steroid medication.^{2,5} A preliminary report has also suggested an association with bronchial hyperreactivity.⁶ In addition, in a random population of 600 individuals (Dewar et al. Unpublished data), we have observed linkage disequilibrium between the amino acid 16 and 27 polymorphisms, although this was only partial. However, in this study, we found no relationship between the amino acid 16 polymorphism and markers of allergic disease. The probable explanations for these observations are that the effects of the amino acid 16 polymorphism are related to response to treatment and that because the overall contribution of the amino acid 27 polymorphism to IgE variability is not large, we were unable to observe a correlation with the amino acid 16 polymorphism despite a degree of linkage disequilibrium being present. Given that in our previous study individuals with the Glu 27 form of the receptor had less reactive airways⁷ and that the Gly 16 β_2 -adrenoceptor polymorphism predisposes individuals to nocturnal asthma,⁵ at present it would be safe to conclude that β_2 -adrenoceptor polymorphisms have a potentially disease-modifying role in patients with asthma and are associated with variability in total IgE levels. However, they are unlikely to constitute a major genetic locus involved in determining an individual's risk of developing asthma per se.

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