

# $\beta_2$ adrenoceptor promoter polymorphisms: extended haplotypes and functional effects in peripheral blood mononuclear cells

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Thorax 2002;57:61–66

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Revised version received 31 July 2001  
Accepted for publication 24 August 2001

**Background:** The  $\beta_2$  adrenoceptor and its 5' untranslated region contain a number of genetic variants. The aim of this study was to investigate the potential for genetic variation at this locus to influence the expression of  $\beta_2$  adrenoceptors on circulating peripheral blood mononuclear cells (PBMCs).

**Methods:** Genotype was determined in 96 individuals with asthma for four polymorphisms at the  $\beta_2$  adrenoceptor locus.  $\beta_2$  adrenoceptor binding and cyclic AMP responses to isoprenaline in PBMCs were determined and the relationship between genotype/haplotype and  $\beta_2$  adrenoceptor expression and response to isoprenaline examined.

**Results:**  $\beta_2$  adrenoceptor promoter polymorphisms were found to be common in white subjects. Strong linkage disequilibrium exists across this locus, resulting in the occurrence of several common haplotypes. No single polymorphism or haplotype was correlated with the level of  $\beta_2$  adrenoceptor expression or cyclic AMP responses to isoprenaline *in vitro*.

**Conclusion:**  $\beta_2$  adrenoceptor polymorphisms, when considered in isolation or by extended haplotypes, do not determine the basal level of expression or coupling of  $\beta_2$  adrenoceptors in PBMCs from asthmatic subjects.

Beta<sub>2</sub> adrenoceptor agonists remain the mainstay bronchodilator agents used in the treatment of asthma. It has recently been suggested that some of the variability observed in response to these agents may be the result of genetic polymorphisms.<sup>1–4</sup> The  $\beta_2$  adrenoceptor locus on chromosome 5q31 contains a number of single nucleotide polymorphisms (SNPs). Within the coding region of the human  $\beta_2$  adrenoceptor gene itself, nine SNPs have been identified,<sup>5</sup> five of which are degenerate. Non-degenerate polymorphisms result in amino acid substitutions in codon 16 (Arg16→Gly), 27 (Gln27→Glu), 34 (Val34→Met), and 164 (Thr164→Ile). In recombinant and non-recombinant cell systems the Gly16 variant shows enhanced downregulation whereas the Glu27 variant is partially protected from downregulation.<sup>6,7</sup> The rare Ile164 variant (allelic frequency ~2% in white populations) reduces the efficiency of receptor coupling with downstream effector pathways.<sup>8</sup> *In vivo* there are data suggesting a functional role in fibroblast cell lines and cultured human airway smooth muscle for the polymorphisms at codon 16 and 164 and possibly also 27 (reviewed by Hall<sup>9</sup>).

Recent studies have also demonstrated the presence of a number of polymorphisms within the 5' untranslated region (UTR) of the human  $\beta_2$  adrenoceptor gene. In a previous study<sup>10</sup> we described eight SNPs within a 1.5 kb region upstream from the ATG start codon. This region is believed to be important for regulation of  $\beta_2$  adrenoceptor gene transcription: it contains the majority of promoter activity for the human  $\beta_2$  adrenoceptor gene and also includes a short open reading frame (sORF) for a 19 amino acid peptide known either as beta upstream peptide (BUP) or the  $\beta_2$  adrenoceptor 5' leader cistron.<sup>11</sup> Using a reporter gene strategy we have shown that a construct containing the most frequently occurring non-wild type haplotype for the four SNPs contained within the 550 bp region of the 5'UTR had reduced luciferase expression in COS 7 cells compared with the wild type.<sup>12</sup> This 550 bp region contains the majority of promoter activity in the

5'UTR of the  $\beta_2$  adrenoceptor gene. McGraw *et al* have also reported reduced  $\beta_2$  adrenoceptor expression with the BUP Cys19→Arg polymorphism in a recombinant cell system where the  $\beta_2$  adrenoceptor was expressed downstream of either the Cys19 or Arg19 form of BUP. More recent work, however, has suggested that when haplotypes—that is, a combination of polymorphisms across this region—rather than single polymorphisms are considered, the most common haplotype containing the Cys19 polymorphism is associated with higher levels of  $\beta_2$  adrenoceptor expression in a recombinant cell system.<sup>13</sup> In contrast, in a preliminary study in primary cultured human airway smooth muscle cells we were unable to detect significant effects of any of four 5'UTR SNPs on expression of firefly luciferase, either when each SNP was studied in isolation or in combination using the most frequently occurring haplotypes across this region.<sup>14</sup> Hence, while functional data in recombinant cell systems suggest a potential role for the  $\beta_2$  adrenoceptor 5'UTR polymorphisms, their importance to responses in subjects with or without asthma remains unclear.

In this study we concentrated on haplotypes and the two 5' SNPs most likely to be functionally important. The first of these SNPs results from a base change 47 bp upstream from the  $\beta$  adrenoceptor gene start codon (–47 T/C) which, as discussed above, substitutes an Arg for a Cys in BUP. The second 5'UTR SNP which appears potentially to be important results from a base change (T/C) at –367 bp from the start codon. This interrupts a putative Sp1 binding site in a region of the promoter containing strong positive promoter activity. To assess the potential relevance of these two 5'UTR polymorphisms and the known common polymorphisms in the coding region of the gene, this study had three aims: (1) to define the allelic frequencies of the 5'UTR polymorphisms in individuals with asthma; (2) to define the extent of linkage disequilibrium between these 5'UTR SNPs and those within the coding region and to determine the most common haplotypes in the white population; and (3) to assess the potential functional

effects of 5'UTR polymorphism on  $\beta_2$  adrenoceptor expression in vivo by studying levels of  $\beta_2$  adrenoceptor expression and cAMP responses to  $\beta_2$  agonist in human peripheral blood mononuclear cells (PBMCs) in relation to genotype. Our hypothesis was that individuals carrying the -47 T/C variant, either in isolation or as part of an extended pro-downregulatory haplotype, would have lower resting levels of  $\beta_2$  adrenoceptors on circulating PBMCs.

## METHODS

### Subjects

Two populations of asthmatic subjects were used for these studies from Dundee, UK and Groningen, the Netherlands.

### Dundee

Fifty eight patients of mean (SD) age 35 (14) years with forced expiratory volume in 1 second (FEV<sub>1</sub>) 2.58 (0.88) l (75.6 (17.9)% predicted) took part in the study; 48% were atopic. All 58 patients were taking inhaled corticosteroids; beclomethasone dipropionate at a mean dose of 522 (456)  $\mu$ g per day in 32, budesonide at a mean dose of 967 (550)  $\mu$ g per day in 18, and fluticasone propionate at a mean dose of 1500 (623)  $\mu$ g per day in eight. Twenty were using long acting  $\beta_2$  agonists (11 salmeterol, eight formoterol, and one bambuterol). All long acting  $\beta_2$  agonist therapy was withdrawn for a washout period of at least 1 week before mononuclear cell tests (see below) were performed. Short acting  $\beta_2$  agonists were withdrawn for at least 8 hours. No patients had received oral steroids for at least 3 months before the measurements. The study was approved by the trial ethics committee.

### Groningen

The Dutch patients were taken from a randomised double blind parallel trial on the treatment of nocturnal asthma.<sup>15</sup> Fifty patients entered the study and DNA was obtained from 38 individuals.<sup>15</sup> The mean FEV<sub>1</sub> was 3.37 (0.10) l (86.9 (16.6)% predicted) and mean PC<sub>20</sub> methacholine was 1.78 mg/ml. Patients were included if they were non-smoking atopic asthmatics aged 18–45 years, reported a history of episodic dyspnoea or wheezing, with bronchial hyperresponsiveness to methacholine bromide (PC<sub>20</sub> <9.6 mg/ml). Inhaled corticosteroids were stopped 4 weeks before the study, oral corticosteroids 2 months before the study, and inhaled long acting  $\beta_2$  agonists for 2 weeks. All data reported here were taken from the baseline measurement before the start of study medication. The genetic part of the study was approved by the medical ethics committee of the University Hospital Groningen and additional consent was obtained from all participants.

### Isolation of peripheral blood mononuclear cells (PBMCs)

30–40 ml of blood was collected into tubes containing EDTA and diluted to 50 ml with phosphate buffered saline (PBS). Equal aliquots of diluted blood were then layered carefully onto 15 ml of Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged. A lymphocyte layer was then removed from each tube and combined before two further washes with PBS and centrifugation. The supernatant was discarded and the lymphocyte pellet resuspended in 5 ml PBS or Tris-buffer (Groningen). The preparation was counted to determine lymphocyte numbers and a sufficient volume was removed to obtain a total of  $2.5 \times 10^6$  (Dundee) or  $\times 10^7$  (Groningen) cells for measurement of cyclic AMP stimulation by isoprenaline. The remainder of the suspension was centrifuged, the supernatant discarded, and the lymphocyte pellet resuspended in the suspension buffer to give a concentration of  $2.5 \times 10^6$  (Groningen) or  $5 \times 10^6$  cells/ml.

### Genotyping

Polymerase chain reaction (PCR) was used to amplify the  $\beta_2$  adrenoceptor regions of interest. Table 1 shows the primers and conditions used for each locus. Coding region polymorphisms at codons 16 and 27 were genotyped using allele specific oligonucleotide (ASO) hybridisation as previously described.<sup>16</sup> The -47 polymorphism within BUP was genotyped using a PCR based restriction length polymorphism (RFLP) assay. A *MspA1* I restriction site is present in the Arg19 (C) form which is not present in the Cys19 (T) sequence. The -367 polymorphism was also genotyped using RFLP; a *Bsu36* I site is present in the sequence containing the T allele while the C allele obliterates the recognition sequence of *Bsu36* I.

### Measurement of cyclic AMP responses and $\beta_2$ adrenoceptor binding in PBMCs

#### Dundee

Lymphocyte  $\beta_2$  adrenoceptor binding affinity (Kd) and maximum binding density (Bmax) were assayed on the prepared cell suspension (see above) after incubation in a waterbath at 37°C in tubes containing (<sup>125</sup>I)-iodocyanopindolol (ICYP) at eight concentrations from 5 to 160 pM. Half the tubes contained CGP 12177A HCl (1  $\mu$ M) to prevent ICYP binding to the receptor sites. After washing with assay buffer the bound and unbound suspension preparations were aspirated onto filter paper using a Brandel cell harvester and the resultant counts determined using a gamma counter. Specific receptor binding was calculated from total binding minus non-specific binding. Receptor density was calculated by Scatchard analysis using the specific and non-specific binding curves plotted for each concentration of ICYP. The intra-assay coefficient of

**Table 1** PCR primers and conditions used to amplify the regions of interest for further analysis. The expected fragment lengths are also indicated

Polymorphism	Primers	Conditions	Fragment length (bp)
Promoter -367	Forward 5' CCTCTGCCTCGAGACCTCAAGCC 3' Reverse 5' CCGTCTGCAGCGCTCGAAC 3'	60°C annealing; 30 cycles	740
Promoter -47 Arg19→Cys	Forward 5' CTTCGGGCTGCCGGCGTG 3' Reverse 5' GACATGGAAGCGGCCCTCAG 3'	68°C annealing; 34 cycles	1031
Coding Arg16→Gly	Forward 5' CCCAGCCAGTGCCTTACCT 3' Reverse 5' CCGTCTGCAGCGCTCGAAC 3'	60°C annealing; 36 cycles	234

**Table 2** Allele frequencies of the β<sub>2</sub> adrenoceptor polymorphisms -367 and -47 in the 5' flanking region, loci 16 and 27 in the coding region (n = 96)

Locus	Allele 1	Allele 2
-367 T/C	T: 0.62	C: 0.38
-47 T/C	T: 0.60	C: 0.40
Arg16→Gly	Arg16: 0.31	Gly16: 0.69
Gln27→Glu	Gln27: 0.48	Gln27: 0.52

variation for analytical imprecision was 5.8% for Kd and 10.3% for Bmax.

Cyclic AMP (cAMP) was determined as follows. The suspension containing 5 × 10<sup>6</sup> cells was centrifuged and the pellet resuspended in PBS containing theophylline 100 μM and bovine serum albumin (10%). It was then stimulated with isoprenaline 10<sup>-4</sup> M during the incubation at 37°C before terminating the reaction by heating to 95°C. After centrifugation the supernatant was removed and cAMP was determined by radioimmunoassay. The intra-assay coefficient of variation for analytical imprecision was 2.0%.

**Groningen**

Samples of 2.5 × 10<sup>6</sup> cells in 900 μl Tris buffer containing 0.5 mM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity were preincubated for 10 minutes at 37°C. After preincubation, 100 μl isoprenaline (final concentration 1 μM) was added to duplicate samples. Basal cAMP levels were determined in duplicate by adding 100 μl buffer solution. For cAMP accumulation the samples were incubated for another 10 minutes at 37°C. The reaction was stopped by adding 100 μl 2 M HCl and 0.1 M EDTA, heated at 80°C for 10 minutes, centrifuged and the supernatants were collected and neutralised with CaCO<sub>3</sub>.<sup>17</sup> After removing excess CaCO<sub>3</sub> the cAMP concentration was measured by immunoassay (Biotrak, Amersham, UK).

The results from the two centres were pooled for analysis by genotype.

**Statistical methods**

Haplotype frequency estimation across the four loci was tested using the Estimated Haplotypes (EH) program developed by Ott and colleagues.<sup>18,19</sup> Expected and observed frequencies were compared using log likelihood methods. Possible associations between genotypes/haplotypes and clinical phenotypes were tested using one way analysis of variance (ANOVA).

**RESULTS**

**Allelic frequencies of the -367 T/C and -47 T/C β<sub>2</sub> adrenoceptor polymorphisms and linkage disequilibrium**

Both the -47 T/C and -367 T/C SNPs were found to be common in the two groups of white subjects studied. The allelic frequencies of these polymorphisms are shown in table 2 and observed haplotype data are shown in table 3. It can be seen that the polymorphisms at -367 bp and -47 bp are in strong linkage disequilibrium and that linkage disequilibrium also exists between these polymorphisms and those at codons 16 and 27 within the coding region of the gene (table 4). Allelic frequencies were not significantly different in the two populations studied so the data were pooled for all subsequent analyses.

**Effect of β<sub>2</sub> adrenoceptor promoter coding region haplotype on β<sub>2</sub> adrenoceptor expression and coupling in circulating PBMCs**

In an attempt to define the potential functional effects of β<sub>2</sub> adrenoceptor polymorphism within the promoter region, we hypothesised that individuals with the Cys19→Arg polymorphism may show reduced levels of β<sub>2</sub> adrenoceptor expression in vivo. Levels of β<sub>2</sub> adrenoceptor expression, affinity, and function in PBMCs obtained from 58 Scottish asthmatic patients and 38 Dutch patients were therefore examined (table 5). These data show that, when either the BUP Cys19→Arg or the -367 T/C SNP are considered in isolation, no clear functional effects of these polymorphisms are evident. As would be predicted, no effect of the Arg16→Gly and Gln27→Glu β<sub>2</sub> adrenoceptor polymorphisms is apparent when considered in isolation (table 5).

Although levels of receptor expression measured by binding provide valuable information, it is possible that receptor coupling may be altered without an accompanying change in the

**Table 3** Individual haplotypes observed in the asthmatic population for which all loci were genotyped (n = 93)

-367 (T/C)	-47 (T/C)	Arg16→Gly	Gln27→Glu	No.	% Total
C/C	C/C (Arg19)	Gly16	Glu27	19	20
T/T	T/T (Cys19)	Het	Gln27	10	11
T/T	T/T (Cys19)	Arg16	Gln27	9	10
T/C	T/C (Het)	Het	Het	8	9
T/T	T/T (Cys19)	Het	Het	8	9
T/C	T/C (Het)	Gly16	Het	7	7
T/T	T/C (Het)	Gly16	Het	4	4
T/T	T/T (Cys19)	Gly16	Glu27	3	3
T/T	T/T (Cys19)	Gly16	Het	3	3
T/C	T/C (Het)	Gly16	Glu27	3	3
C/C	C/C (Arg19)	Het	Het	3	3
T/T	T/C (Het)	Het	Het	3	3
T/C	T/T (Cys19)	Gly16	Glu27	2	2
T/C	T/C (Het)	Het	Gln27	2	2
T/T	T/T (Cys19)	Gly16	Gln27	2	2
T/C	T/T (Cys19)	Het	Het	2	2
T/T	T/T (Cys19)	Arg16	Het	1	1
T/T	C/C (Arg19)	Het	Gln27	1	1
T/C	C/C (Arg19)	Gly16	Het	1	1
T/T	T/C (Het)	Gly16	Glu27	1	1
T/C	T/T (Cys19)	Gly16	Gln27	1	1

**Table 4** Haplotype frequencies determined from the data in table 3 using the Estimated Haplotype program to estimate the haplotype combinations of the double, triple and quadruple heterozygous genotypes, both with and without association

Haplotype				Frequency	
-367 (T/C)	-47 (C/T)	Arg16→Gly	Gln27→Glu	With association	Without association
C	C (Arg19)	Gly16	Glu27	0.32	0.06
T	T (Cys19)	Arg16	Gln27	0.26	0.05
T	T (Cys19)	Gly16	Gln27	0.16	0.12
T	T (Cys19)	Gly16	Glu27	0.13	0.13
T	C (Arg19)	Gly16	Glu27	0.036	0.091
C	C (Arg19)	Arg16	Gln27	0.029	0.023
C	T (Cys19)	Gly16	Glu27	0.022	0.079
T	C (Arg19)	Gly16	Gln27	0.017	0.089
T	T (Cys19)	Arg16	Glu27	0.007	0.058
T	C (Arg19)	Arg16	Gln27	0.007	0.038
C	T (Cys19)	Gly16	Gln27	0.007	0.075
C	C (Arg19)	Gly16	Gln27	0.00006	0.052

Log<sub>e</sub> (I)  $\chi^2$  statistic = 249.08, df = 11 (p = 0.00008) suggests strong linkage disequilibrium across these loci. Four haplotypes did not occur with association.

**Table 5** Mean (SE)  $\beta_2$  adrenoceptor levels of receptor expression (Bmax), receptor affinity (Kd), and the ability of isoprenaline to stimulate cyclic AMP formation (Emax)

Loci	Genotype	Bmax (fmol/mg)	p value	Kd (pmol)	p value	Emax (% above basal)	p value
-367 T/C	T/T	2.75 (0.32)	0.27	21.5 (2.26)	0.71	289 (27.7)	0.01*
	T/C	2.17 (0.17)		19.5 (1.80)		452 (57.3)	
	C/C	2.43 (0.22)		21.3 (1.69)		327 (39.3)	
-47 T/C	T/T	2.78 (0.32)	0.27	20.5 (2.36)	0.98	305 (28.4)	0.17
	C/T	2.38 (0.19)		20.9 (1.89)		409 (57.8)	
	C/C	2.27 (0.18)		20.4 (1.54)		331 (37.5)	
Arg16→Gly	Arg16	2.55 (0.22)	0.73	25.2 (3.24)	0.11	290 (71.6)	0.68
	HET	2.54 (0.17)		21.3 (1.40)		363 (40.2)	
	Gly16	2.33 (0.24)		18.6 (1.65)		339 (31.5)	
Gln27→Glu	Gln27	2.48 (0.23)	0.26	22.4 (1.96)	0.54	329 (31.3)	0.89
	HET	2.22 (0.17)		19.5 (1.73)		353 (31.9)	
	Glu27	2.73 (0.29)		20.5 (1.88)		352 (30.9)	

The data were averaged by genotype groups to test for a particular genotype association with a clinical phenotype (ANOVA). Data were derived from a Dundee population (n = 58) and a Dutch population (n=38). \*p<0.05.

overall level of receptor expression. We therefore examined the ability of PBMCs derived from individuals with different genotypes to generate cAMP in response to isoprenaline. No clear differences in the ability of cells expressing different forms of the  $\beta_2$  adrenoceptor to generate cAMP was evident between genotypes (table 5), but we did observe a small, statistically significant, increase in cAMP responsiveness in those individuals heterozygous for the -367 T/C SNP. However, no trend was observed in homozygous individuals to support a role for this SNP and we believe this to represent a false positive result. In a post hoc subanalysis we also compared the cAMP responsiveness in those receiving inhaled steroid at the time of the study (the 58 subjects in the Dundee study) and those in whom treatment with inhaled steroids had been discontinued (the 38 Dutch patients). Again, no significant differences were observed between groups defined by genotype (data not shown).

#### Haplotype analysis of $\beta_2$ adrenoceptor expression in PBMCs

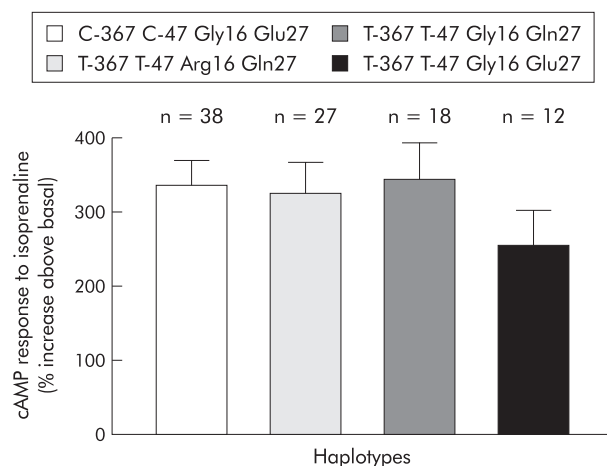
As the in vitro data suggested that effects of  $\beta_2$  adrenoceptor promoter polymorphisms may only be apparent when relevant haplotypes are studied, we attempted to perform a haplotype analysis looking at levels of  $\beta_2$  adrenoceptor expression and coupling in PBMCs studied ex vivo. To maximise the chance of

observing effects we only considered individuals homozygous for all the SNPs or those heterozygous at only a single position. This analysis was inevitably complicated by the strong linkage disequilibrium between these SNPs described above: no individuals with the potentially informative BUP Arg19  $\beta_2$  adrenoceptor Gly 16 Gln 27 homozygous haplotype were identified within our population (table 3). No significant difference was observed between levels of expression or cAMP responses to isoprenaline when considered by haplotype (fig 1), although the numbers are inevitably small for this analysis.

#### DISCUSSION

In this study a structured approach was taken to look for functional effects in vivo of recently described  $\beta_2$  adrenoceptor promoter polymorphisms using PBMCs from patients with asthma. The main conclusions of the study are:

- The 5' UTR  $\beta_2$  adrenoceptor promoter polymorphisms at -367 (T/C) and -47 (T/C) are common in the white population.
- Strong linkage disequilibrium exists across this region which results in the occurrence of several common haplotypes.
- No clear individual effect of  $\beta_2$  adrenoceptor promoter polymorphisms can be seen on the level of expression or



**Figure 1** Percentage change in cAMP formation in response to isoprenaline stimulation of PBMCs with respect to haplotype.

coupling of  $\beta_2$  adrenoceptors in PBMCs isolated from patients with asthma.

- No clear functional effect of any given haplotype on the level of receptor expression and coupling in PBMCs could be identified.

The promoter region for the human  $\beta_2$  adrenoceptor gene (compared with other species such as the rat) has been relatively poorly studied. However, it is clear that promoter activity resides within a region of approximately 1.5 kb and that the majority of promoter activity is found in the first 550 bp upstream of the start codon.<sup>12</sup> This region contains a sORF for BUP which is believed to act as a translational (and possibly transcriptional) inhibition system.<sup>20</sup> Part of the mechanism whereby leader peptides such as BUP exert their effect is believed to depend upon the arginine content of these peptides: the Cys19→Arg polymorphism due to the SNP at -47 (T/C) increases the arginine content of the peptide from 3–4 residues out of the total of 19. This increased number of arginine residues would be predicted to increase its ability to exert translational inhibition. Data to support this suggestion have been published previously.<sup>11</sup> However, preliminary data reported by our group suggest that, at least with luciferase expression as an end point, in primary cultured airway cells the presence of the Cys19→Arg BUP polymorphism is inadequate by itself to cause functional effects.<sup>14</sup> Given these conflicting data, the probable effects in vivo of these SNPs are unclear, and are further complicated by the strong linkage disequilibrium which exists across this region. Four SNPs have been described in the 550 bp region immediately upstream from the ATG which contains most of the promoter activity. However, the polymorphisms at -468 (C/G) and -20 (T/C) seem unlikely to contribute to functional effects, given that they do not alter known transcription factor binding sites. We therefore limited our analysis to the polymorphisms at -367 which is close to an SP1 site, -47 (Cys19→Arg in BUP), and the two common coding region polymorphisms at codons 16 and 27 of the  $\beta_2$  adrenoceptor gene itself.

We then attempted to determine whether  $\beta_2$  adrenoceptor expression ex vivo is determined by  $\beta_2$  adrenoceptor promoter polymorphisms. The known functional effects of the codon 16 and 27  $\beta_2$  adrenoceptor polymorphisms are to alter downregulation profiles, both in recombinant cell systems<sup>6</sup> and in primary cultured human airway smooth muscle.<sup>7</sup> Given the in vitro data, we would predict that the -367 (T/C), Cys19→Arg (BUP), Arg16→Gly, Gln27→Glu  $\beta_2$  adrenoceptor promoter coding region haplotype might lead to reduced in vivo expression of  $\beta_2$  adrenoceptors without agonist exposure, which might increase following agonist exposure, as supported by

McGraw and coworkers.<sup>11</sup> We therefore investigated levels of  $\beta_2$  adrenoceptor expression on circulating PBMCs isolated from patients with asthma. However, when considered in isolation, none of the above polymorphisms altered levels of receptor expression (Bmax) or receptor affinity (Kd). In addition, no difference in the ability of isoprenaline to drive cAMP formation in these cells was observed when different genotypes were considered in isolation. We did observe a small difference in individuals heterozygous for the -367 T/C SNP but, given that no effect was seen in the two relevant homozygous groups, this seems likely to be a false positive result. In a secondary analysis we also investigated whether there was evidence that inhaled steroid therapy (used in the Dundee subjects) may have masked any effect of genotype on cAMP responsiveness. No significant effects were apparent when only subjects without inhaled steroids were analysed, although the power of this analysis is obviously smaller than for the main study. Similarly, downregulation was not more apparent in subsets of the nocturnal asthma group defined by genotype or haplotype. Previously, we have shown that inhaled steroid therapy does not protect against  $\beta_2$  adrenoceptor bronchodilator desensitisation,<sup>21</sup> although effects have been observed on  $\beta_2$  adrenoceptor mediated protection against bronchoconstriction.<sup>22</sup>

One explanation for the lack of effect when each SNP is analysed in isolation is that functional differences are only present in individuals with a particular haplotype across this region. We were able to demonstrate strong linkage disequilibrium across this region, with the most frequently occurring haplotypes being -367 C, Arg19 BUP, Gly16, Glu27, and -367 T, Cys19 BUP, Arg16, and Gln27. We therefore examined the effects of different  $\beta_2$  adrenoceptor haplotypes covering both the promoter and the functional polymorphisms within the coding region on  $\beta_2$  adrenoceptor expression and coupling in PBMCs. Inevitably, this analysis was complicated by the lack of individuals with some potentially informative haplotypes; however, our data suggest that there is unlikely to be a marked effect of  $\beta_2$  adrenoceptor promoter polymorphism on  $\beta_2$  adrenoceptor expression in PBMCs. It is noteworthy that 42% of all the individuals studied in whom the haplotype could be determined carried one of two major haplotypes across this region. Hence, even if a given (rarer) haplotype was important in determining the level of expression and coupling of the  $\beta_2$  adrenoceptor in PBMCs, very large population samples, or samples from ethnic groups in which the SNP frequencies are different, would be required to fully address this issue. While preparing this paper, a further study addressing this issue was published which showed strong linkage disequilibrium across this region in a USA population.<sup>13</sup> This study also suggested that haplotypes rather than individual SNPs might predict bronchodilator reversibility, although the numbers were small.

In order to obtain the number of cells from a reasonable number of individuals necessary for this study we elected to use PBMCs as our assay system. Some studies have suggested that these may not be the best surrogate for studying  $\beta_2$  adrenoceptor expression in the lung. However, desensitisation and/or downregulation has generally been easier to demonstrate in circulating PBMCs than in airway cells, thus it would seem unlikely that marked genotype/haplotype dependent effects would be seen in airway cells but not in PBMCs.

A preliminary assessment of the potential contribution of  $\beta_2$  adrenoceptor promoter/coding region haplotypes to treatment response (defined as  $\Delta FEV_1$  to salbutamol) was able to show differences between groups defined by haplotypes, although the groups with the worst responses and best responses were not those that one would predict from in vitro functional studies.<sup>13</sup> In a post hoc analysis we examined the degree of baseline reversibility in the subjects from Groningen for whom reversibility data and DNA were available (n=30, mean percentage reversibility 16.0%). No significant associations were seen when genotypes were analysed individually (n=30) or as haplotypes (n=13).

One possible criticism of this study is the use of two different patient populations. However, asthma severity was comparable between the groups gauged by FEV<sub>1</sub> and previous medication. No significant differences in genotype distribution were evident between the populations in the two centres, the genotype frequencies being in agreement with previous data on  $\beta_2$  adrenoceptor polymorphism frequencies in other white populations. We therefore believe it unlikely that our results are influenced by the pooling of data obtained in Dundee and Groningen.

In summary, we have shown that  $\beta_2$  adrenoceptor promoter polymorphisms are common in white subjects. Strong linkage disequilibrium exists between these promoter polymorphisms (and those within the coding region of the  $\beta_2$  adrenoceptor), resulting in the occurrence of several common haplotypes. Using PBMC  $\beta_2$  adrenoceptor expression and coupling as functional end points, we were unable to demonstrate marked functional consequences in vivo of any individual SNP or of the most common combinations studied using a haplotype approach. It is therefore unlikely that  $\beta_2$  promoter polymorphisms play a major role in determining basal levels of  $\beta_2$  adrenoceptor expression and coupling in vivo.

#### ACKNOWLEDGEMENTS

This study was funded in part by grants from the National Asthma Campaign (UK), and Stichting Astma Bestrijding. The authors thank Drs E J M Weersink and Y Hoekstra for their contribution to part of the patient selection and cAMP data.

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#### REFERENCES

- 1 **Tan S**, Hall IP, Dewar J, et al. Association between  $\beta_2$ -adrenoceptor polymorphism and susceptibility to bronchodilator desensitisation in moderately severe stable asthmatics. *Lancet* 1997;**350**:995-9.

- 2 **Martinez FD**, Graves PE, Baldini M, et al. Association between genetic polymorphisms of the  $\beta_2$ -adrenoceptor and response to albuterol in children with and without a history of wheezing. *J Clin Invest* 1997;**100**:3184-8.
- 3 **Hancox RJ**, Sears MR, Taylor DR. Polymorphisms of the  $\beta_2$  adrenoceptor and the response to long-term  $\beta_2$ -agonist therapy in asthma. *Eur Respir J* 1998;**11**:589-93.
- 4 **Lipworth B**, Hall IP, Aziz I, et al. A  $\beta_2$  adrenoceptor polymorphism and bronchoprotective sensitivity with regular short- and long-acting  $\beta_2$ -agonist therapy. *Clin Sci* 1999;**96**:253-9.
- 5 **Reihnsaus E**, Innis M, MacIntyre N, et al. Mutations in the gene encoding for  $\beta_2$ -adrenergic receptor in normal and asthmatic subjects. *Am J Respir Cell Mol Biol* 1993;**8**:334-9.
- 6 **Green SA**, Turki J, Innis M, et al. Amino terminal polymorphisms of the human  $\beta_2$ -adrenergic receptor in parts distinct agonist-promoted regulatory properties. *Biochemistry* 1994;**33**:9414-9.
- 7 **Green SA**, Turki J, Bejarano P, et al. Influence of  $\beta_2$ -adrenergic receptor genotypes on signal transduction in human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 1995;**13**:25-33.
- 8 **Green SA**, Cole G, Jacinto M, et al. A polymorphism of the human  $\beta_2$ -adrenergic receptor within the fourth transmembrane domain alters ligand binding and functional properties of the receptor. *J Biol Chem* 1993;**268**:23116-21.
- 9 **Hall IP**. Pharmacogenetics of asthma. *Eur Respir J* 2000;**15**:449-51.
- 10 **Scott MG**, Swan C, Wheatley AP, et al. Identification of novel polymorphisms within the promoter region of the human  $\beta_2$ -adrenergic receptor gene. *Br J Pharmacol* 1999;**126**:841-4.
- 11 **McGraw DW**, Forbes SL, Kramer LA, et al. Polymorphisms of the 5' leader cistron of the human  $\beta_2$ -adrenergic receptor regulate receptor expression. *J Clin Invest* 1998;**102**:1927-32.
- 12 **Scott MG**, Swan C, Hall IP. Differential expression of human  $\beta_2$ -adrenoceptor promoter constructs in COS-7, BEAS-2B and human cultured airway smooth muscle cells. *Br J Pharmacol* 1999;**128**:123P
- 13 **Drysdale C**, McGraw DW, Stack CB, et al. Complex promoter and coding region  $\beta_2$ -adrenergic receptor haplotypes alter receptor expression and predict in vivo responsiveness. *Proc Natl Acad Sci USA* 2000;**97**:10483-8.
- 14 **Swan C**, Scott MGH, Hall IP. Role of 5' CRE and 5' UTR polymorphisms in  $\beta_2$ -adrenergic receptor expression in human airway smooth muscle. *Am J Respir Crit Care Med* 2000;**161**:A726.
- 15 **Weersink E**, Douma R, Postma D, et al. Fluticasone propionate, salmeterol xinafoate, and their combination in the treatment of nocturnal asthma. *Am J Respir Crit Care Med* 1997;**155**:241-6.
- 16 **Hall IP**, Wheatley AP, Wilding P, et al. Association of Glu-27  $\beta_2$ -adrenoceptor polymorphism with lower airway reactivity in asthmatic subjects. *Lancet* 1995;**345**:1213-4.
- 17 **Meurs H**, Kauffman H, Koeter G, et al. Extraction of cyclic AMP for the determination in the competitive binding assay. *Clin Chim Acta* 1980;**106**:91-7.
- 18 **Xie X**, Ott J. Testing linkage disequilibrium between disease gene and marker loci. *Am J Hum Genet* 1993;**53**:1107.
- 19 **Terwilliger J**, Ott J. *Handbook of human genetic linkage*. Baltimore: Johns Hopkins University Press, 1994.
- 20 **Kobilka P**. The peptide product of a 5' leader cistron in the beta 2 adrenergic receptor mRNA inhibits receptor synthesis. *J Biol Chem* 1994;**269**:4497-505.
- 21 **Tan S**, Hall IP, Dewar J, et al. Association between  $\beta_2$ -adrenoceptor polymorphism and susceptibility to bronchodilator desensitisation in moderately severe stable asthmatics. *Lancet* 1997;**350**:995-9.
- 22 **de Jong JW**, van der Mark TW, Koeter GH, et al. Rebound airway obstruction and responsiveness after cessation of terbutaline: effects of budesonide. *Am J Respir Crit Care Med* 1996;**153**:70-5.