Original articles

Extended tumour necrosis factor/HLA-DR haplotypes and asthma in an Australian population sample

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Abstract

***Background*—Tumour necrosis factor (TNF) is a potent pro-inflammatory cytokine which is prominent in asthmatic airways. TNF shows genetic variations in secretion which are linked to polymorphisms in the TNF gene complex and the surrounding major histocompatibility (MHC) locus. These polymorphisms do not seem to be themselves functionally important. In these circumstances, the identification of disease associated haplotypes (combination of alleles on individual chromosomes) may narrow the search for polymorphisms which alter gene function.**

***Methods*—TNF-308, LTα *Nco*I, and HLA-DRB1 polymorphisms were investigated for association with asthma, bronchial responsiveness, and medication use in 1004 subjects in 230 families from a general population sample.**

***Results*—The common LTα *Nco*I*1/TNF-308*2/HLA-DRB1*03 haplotype, which was present in 11% of unrelated individuals, was weakly associated with asthma (OR = 1.38, p = 0.016, corrected for familial correlation). The rarer LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02 haplotype, which was found in 0.6% of unrelated subjects, was more strongly associated with asthma (OR = 6.68, p = 0.002). This haplotype also showed association with bronchial hyperresponsiveness (OR = 21.9, p = 0.0000) and the use of inhaled or oral steroids (OR 8.0, p = 0.04).**

***Conclusions*—The results of this study show only two extended TNF/HLA-DR haplotypes to be associated with asthma. The search for functional alleles responsible for an increased risk of asthma should concentrate on the LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02 haplotype.**

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Atopic asthma is the most common disease of childhood.[1] Asthma and atopy are strongly familial, indicating a genetic predisposition to the disease. Although asthma in children and young adults is usually initiated by atopic allergy to inhaled allergens, most atopic individuals do not have asthma. Inflammation of the airway wall is a principal feature of the disease[2 3] and it may therefore be of interest to identify factors that could directly influence the intensity of airway inflammation.

Tumour necrosis factor (TNF) is an inflammatory cytokine that is found in increased concentrations in asthmatic airways[4] and in lavage fluid from asthmatic lungs.[5] The TNF and lymphotoxin (LT) α and β genes are within the human major histocompatibility (MHC) locus on chromosome 6p.[6 7] Constitutional variation in the level of secretion of TNF by peripheral blood lymphocytes or monocytes has been established in association with polymorphism in the TNF gene cluster and the HLA-DRB1 locus.[8–10]

We have previously shown that the bi-allelic TNF promoter polymorphism TNF-308 is associated with asthma.[11] We have now sought to extend these studies in a larger Australian population sample. In addition to examining various questionnaire responses indicative of asthma, we have tested for effects on bronchial hyperresponsiveness and medication use.

The MHC region is complex and contains many genes which might influence asthma. In particular, the HLA-DR gene products have been associated with specific Immunoglobulin E responses to several allergens including house dust mite.[12–14] Discriminating between the effects of neighbouring genes is made difficult because distinctive alleles of individual loci will show non-random association with alleles of neighbouring polymorphisms, a phenomenon known as linkage disequilibrium. The combination of individual alleles on a chromosome is known as a haplotype. In general, in the genome, linkage disequilibrium occurs over 50–500 kilobases (kb) of DNA.[15] However, disequilibrium may extend over several megabases within the MHC and "ancestral haplotypes" are recognised that predate the formation of many modern human populations. When the alleles which actually cause disease are not known, the identification of disease associated haplotypes may narrow the search for functional polymorphisms. In addition, haplotypes may show stronger associations

than discrete alleles. In the present investigation we have therefore studied families rather than individuals to allow direct construction of individual haplotypes.

Complex loci such as the HLA-DR system contain many alleles, leading to multiple comparisons and loss of statistical power when individual alleles are tested for association with various phenotypes. This may be dealt with by multiple regression analysis, in which simultaneous analysis of all alleles takes place.[14 16 17] This approach has been used in the present study and has been followed by tests of association which take into account familial association of markers and phenotypes.

Methods

SUBJECTS

The subjects were from the rural coastal town of Busselton in south-western Australia. The aim was to recruit young nuclear families. Children under five were excluded because they could not complete respiratory testing. Families were identified through adults aged 55 years or under, from an alphabetical electoral roll of approximately 9000. Families were recruited serially until a predetermined target of 1000 individuals was reached. The final sample consisted of 1004 subjects in 230 nuclear families. All families contained both parents and at least two children, and all were Caucasian. Subjects knew the respiratory interest of the investigation before agreeing to participate. It was emphasised that normal individuals were important to the study.

CLINICAL PROTOCOL

Testing took place in the winter months of May, June, and July 1992 to minimise the seasonal effects of pollen exposure. A respiratory questionnaire, based on the 1966 Medical Research Council questionnaire, was administered. This questionnaire shares key questions with the American Thoracic Society questionnaire. "Asthma" was defined as a positive answer to the questions "have you had an attack of asthma?" and "if yes, has this happened on more than one occasion?". "Attack of asthma in the last month" was defined as a positive answer to the questions "have you ever had an attack of asthma?" and "if yes, has this happened in the last month?". "Wheeze" was a positive answer to the questions "has your chest ever sounded wheezing or whistling?" and "if yes, has this happened on more than one occasion?". "Attacks of shortness of breath with wheezing" was a positive answer to the question "have you ever had attacks of shortness of breath with wheezing?". "Doctor diagnosed asthma" was a positive answer to the question "has your doctor ever told you that you have asthma?". "Ever smoked" was defined as having smoked as much as a cigarette a day for a minimum of a year. The current use of inhaled β agonists and inhaled and oral steroids was recorded.

Skin prick testing to *Dermatophagoides pteronyssinus* (HDM), mixed grass pollen, cat and dog dander, *Aspergillus fumigatus*, *Alternaria alternata*, and a negative control (Dome-Hollister-Steir, Spokane, USA) was carried out as previously described[18]; weal diameters were calculated minus the negative control. A positive skin test was considered to be a weal of diameter ⩾3 mm larger than that of a negative control.

Bronchial responsiveness to methacholine was measured as previously described by the rapid method of Yan *et al*[18 19]; the maximum dose administered was 12 μmol. The slope of the dose-response curve was calculated as (pre-dose forced expiratory volume in one second (FEV_1)—last FEV_1)/final cumulative dose of methacholine. To allow log transformation, negative slopes and slopes of 0 were coded as 0.001. As in other studies[20–22] $\log_e$ transformation gave a normally distributed variable which was informative in all subjects.

The top 15% of values for slope were classified as bronchial hyperresponsiveness. This corresponded to a PD_{20} of 4 μmol methacholine.

Blood was taken by venipuncture as a source of peripheral blood lymphocytes for DNA studies. DNA was extracted by standard phenol-chloroform techniques.

GENOTYPES

Polymerase chain reaction (PCR) of the LTα *Nco*I polymorphism was carried out using the primers 5'-CCGTGCTTCGTGCTTTGGACTA-3' and 5'-AGAGCTGGTGGGGACATGTCTG-3'[11] generating a 750 bp product. 200 ng of genomic DNA extracted from venous blood was added to a 15 μl reaction mixture containing 0.5 μM of each primer with 200 μM of each dNTP, 67 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1 mM $MgCl_2$, and 0.45 U *Taq* DNA polymerase. Amplification conditions were 95°C for six minutes followed by 30 cycles of 95°C for one minute, 64°C for one minute, and 72°C for one minute. A final extension of 72°C for five minutes was included. Following amplification, 5 μl of PCR product was digested with 5 U of *Nco*I (New England Biolabs) at 37°C for one hour. The resultant products were analysed on 2% agarose gels. LTα *Nco*I allele 1 was identified by 250 and 500 bp fragments and allele 2 by a single 750 bp band.[11] Amplification failed in 1.5% of subjects.

Typing of the TNF-308 polymorphism was by non-radioactive sequence specific oligonucleotide (SSO) probing of PCR products.[11] 200 ng of genomic DNA was used in each PCR reaction with a final $MgCl_2$ concentration of 1 mM. 1.5 U of *Taq* DNA polymerase were added prior to amplification with the initial denaturation of 95°C being decreased to five minutes. Amplification failed in 1% of subjects. After dotting of denatured PCR products, filters were baked at 120°C for 25 minutes prior to hybridisation with labelled probes. The temperature of the 3 M TMAC stringent wash was 62°C for both probes. Controls of known genotype were included on each filter. The accuracy of the method was confirmed by modified direct DNA sequencing[23] of two TNF-308 allele 1 homozygotes, two TNF-308 allele 2 homozygotes,

Table 1 Characteristics of the study subjects

Mean (SD) age of adults (years)	40.2 (14.5)
Mean (SD) age of children (years)	12.6 (4.7)
No. (%) of asthmatic children (% of all children)	113 (21%)
No. (%) of asthmatic adults (% of all adults)	66 (14.5%)
Total no. of asthmatic subjects (% of population)	179 (18%)
No. (%) of subjects with "attacks of wheezing and whistling"	211 (21%)
No. (%) of subjects with physician diagnosed asthma	206 (20%)
No. (%) of subjects taking inhaled bronchodilators	115 (11%)
No. (%) of subjects taking inhaled or oral steroids	41 (4%)
No. (%) of subjects who ever smoked	240 (24%)
No. (%) of asthmatics who ever smoked	38 (21.5%)
Geometric mean IgE (kU/l) in asthmatics	130.3
Geometic mean IgE (kU/l) in non-asthmatics	37.3

and two heterozygotes from each data set. The sequencing primer was 5'-CAAACACAGGCCTCAGGACTC-3'.

HLA-DRB1 typing was carried out by SSO probing of PCR products using probes end labelled with digoxigenin-ddUTP (Boehringer Mannheim) as described.[24] The HLA-DRB1 types examined included HLA-DRB1*01–*14. HLA-DRB1*02 was subdivided into HLA-DRB1*1501, DRB1*1601, and DRB1*1602. DRB1*1601 and DRB1*1602 were rare, and for analysis DRB1*02 was confined to the DRB1*1501 subtype. Subtypes were recognised for HLA-DRB1*05 (HLA-DRB1*11 and HLA-DRB1*12) and for HLA-DRB1*06 (HLA-DRB1*13 and HLA-DRB1*14). Typing failed in 1.5% of subjects.

All genotypes were checked independently by two individuals without knowledge of the phenotype. When there was disagreement about genotype the samples were retested until agreement was reached.

STATISTICAL ANALYSIS

For statistical analysis "asthma" and the various categorical phenotypes were coded as 1 = absent and 2 = present. Inhaled bronchodilator use was similarly coded. Inhaled and oral steroid use were considered together as 1 = absent, 2 = present. Sex was coded as male = 1, female = 2, age was in years, and smoking was coded as 1 in the presence of a positive answer to the question "have you ever smoked as much as a cigarette a day for as long as a year?", and coded as 2 for a negative response.

Haplotypes were created for the three loci (LTα *Nco*I, TNF-308, and HLA-DRB1) by inspection of the family data. For each of the 56 possible haplotypes a variable was created ("haplotype class") in which 1 = absent and 2 = present.

Multiple logistic regression analyses were carried out with "asthma", bronchial hyperresponsiveness, and medication use as the dependent variables and haplotype classes, age, sex, and smoking included as independent variables (SPPS for OSF 1, SPSS Inc, USA). The significance of the likelihood ratio test for the full model was assessed before interpreting the regressions for significant effects of individual independent variables.

As the regression analysis did not take into account familial aggregation of asthma or particular phenotypes, the effect of familial correlation on positive results was examined by use of the ASSOC routine of the SAGE program (Release 22, Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio, USA).[25]

Results

The sample contained 1004 subjects who were able to give complete clinical information and blood samples for laboratory testing (table 1). The mean age of the subjects was 25.2 years (range 5–55). One hundred and seventy nine subjects (18%) answered yes to the questions "have you ever had an attack of asthma?" and "if yes, has this happened on more than one occasion?". Sixty six of the parents (14.5%) and 113 (21%) of the children were asthmatic by this criterion. One hundred and twelve subjects (11%) were taking inhaled bronchodilators and 44 (4%) were taking inhaled or oral steroids; 24% of the whole population and 21.5% of the asthmatics had ever smoked. The geometric mean total serum IgE was 130.3 kU/l in the asthmatic subjects and 37.3 kU/l in the non-asthmatics (range 1–2000 in both groups). PCR and genotyping of the alleles was successful in more than 98% of subjects.

Haplotypes were constructed from the alleles at each of the three loci; 41 of 56 possible haplotypes were recognised (table 2).

Logistic regression was then carried out with asthma and other categorical phenotypes as the dependent variables and the various haplotype classes as predictor variables. The odds ratios (Exp(B)) for positive phenotypes are reported from the regressions (table 3). In general there was little difference between the p values from the regressions and those generated by ASSOC when familial correlations were taken into account (table 3). p values from ASSOC are given both in the tables and in the text.

The overall model with asthma as the dependent phenotype was highly significantly different from random (χ^2 = 57.3, 30 df, p = 0.0000), so associations between asthma and

Table 2 Haplotype frequencies (parents only)

*Haplotype**	*n*	*%*	*Haplotype*	*n*	*%*
1/1/01	17	1.9	2/1/01	51	5.6
1/1/02	7	0.8	2/1/02	117	12.6
1/1/03	8	0.9	2/1/03	17	1.9
1/1/04	44	4.8	2/1/04	131	14.4
1/1/07	10	1.1	2/1/07	115	12.6
1/1/08	5	0.5	2/1/08	18	2.0
1/1/09	3	0.3	2/1/09	7	0.8
1/1/10	1	0.1	2/1/10	8	0.9
1/1/11	7	0.8	2/1/11	34	3.7
1/1/12	2	0.2	2/1/12	10	1.1
1/1/13	31	3.4	2/1/13	47	5.2
1/1/14	4	0.4	2/1/14	13	1.4
1/2/01	8	0.9	2/2/03	2	0.2
1/2/02	6	0.6	2/2/04	1	0.1
1/2/03	102	11.0	2/2/07	2	0.2
1/2/04	10	1.1	2/2/13	2	0.2
1/2/07	6	0.7	2/2/14	1	0.1
1/2/08	3	0.3			
1/2/11	8	0.9			
1/2/12	7	0.8			
1/2/13	14	1.5			
1/2/14	5	0.5			

*Haplotypes coded as LTα *Nco*I*/TNF-308*/HLA-DRB1*
Twenty eight individual haplotypes (3.1%) contained rare HLA types (DRB1*1502, 1601, 1602 or 0103) and were not included separately in the analysis.

Table 3 Association between asthma and extended haplotypes: logistic regression analysis

Variable	*B (SE)*	*p value*	*pASSOC*	*Exp (B)*
LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02	1.900 (0.618)	0.002	0.002	6.683
LTα *Nco*I*1/TNF-308*2/HLA-DRB1*03	0.323 (0.177)	0.068	0.016	1.381
Age	−0.018 (0.004)	0.0000	—	0.982
Sex	−0.391 (0.120)	0.001	—	0.676
Constant	−3.955 (0.876)	0.0000	—	

Table 4 Association between bronchial hyperresponsiveness and extended LTα NcoI/TNF-308*/HLA-DRB1* haplotypes: logistic regression analysis*

Variable	*B (SE)*	*p value*	*pASSOC*	*Exp (B)*
LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02	3.087 (0.697)	0.0000	0.0000	21.9
Age	−0.038 (0.005)	0.0000	—	0.96
Constant	7.216 (14.11)	0.61	—	

individual haplotypes were sought by stepwise regression. A highly significant positive association was found with the LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02 haplotype (OR = 6.7, p = 0.002; table 3). This haplotype was rare in the population, being carried by 0.6% of unrelated subjects (the parents). A weaker association with LTα *Nco*I*1/TNF-308*2/HLA-DRB1*03 was found (OR = 1.38, p = 0.016). This haplotype was common, being found in 11% of unrelated individuals. Neither haplotype was significantly associated with a positive skin test to HDM.

Similar results were found with the LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02 haplotype when asthma was defined as "attacks of shortness of breath with wheezing and whistling" (OR = 11.6, p = 0.0003), or as "physician diagnosed asthma" (OR = 8.0, p = 0.001).

Multiple logistic regression was then performed with bronchial hyperresponsiveness as the dependent variable. The overall model was highly significantly different from random (χ^2 = 111.6, 30 df, p = 0.0000). The LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02 haplotype was highly correlated with bronchial responsiveness (OR = 21.9, p = 0.000; table 4), but other haplotypes were not associated with the trait.

Significant associations were not found between bronchodilator use and any of the haplotypes. However, inhaled or oral steroid use was again positively associated with the LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02 haplotype (OR = 8.0, p = 0.04).

Discussion

The results show that the two haplotypes containing the TNF-308*2 allele (LTα *Nco*I*1/TNF-308*2/HLA-DRB1*03 and LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02) are associated with questionnaire diagnosed asthma. Although the LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02 carried a high risk of asthma, the haplotype was rare and did not account for much of the population attributable risk of asthma in these subjects. Nevertheless, the haplotype was also associated with bronchial hyperresponsiveness to methacholine and with steroid usage.

Asthma is usually recognised epidemiologically by questionnaire, in which responses to questions about previous diagnosis of asthma discriminate better than questions concerning wheeze or shortness of breath.[26] In the present study, asthma and related symptoms were identified by a standard questionnaire based on the MRC and ATS questionnaires which have been extensively validated.[26] The population from which the subjects were drawn has been the subject of two previous major studies of respiratory health, in 1981 and 1990,[27 28] in which the diagnosis of asthma by similar questionnaire had also been validated.[28] The prevalence of asthma in the study population is consistent with other investigations of asthma prevalence in Busselton[28] and other Australian populations.[29]

The difficulty of multiple comparisons when examining complex allelic systems for association has been dealt with in the present study by the use of logistic regressions.[14 16 17] The calculations of risk from regressions assume independence of observations. Whilst association between phenotypes and alleles may be tested in families, familial aggregation of the trait under study means that individuals in a family may not be fully independent.[30] We therefore examined the results with the ASSOC program which includes familial correlation in tests of association between quantitative traits and genotypes.[25 30]

In vitro studies of peripheral blood leucocytes suggest that the LTα *Nco*I*1 and TNF-308*2 alleles correlate with increased TNF secretion[8 9] consistent with the present findings. Other studies give conflicting results and it seems unlikely that either polymorphism itself alters function.[31] Our results show that the LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02 haplotype is more strongly associated with asthma than other LTα *Nco*I*1/TNF-308*2 containing haplotypes. Replication of the result in other populations would indicate that the search for functional polymorphism should be concentrated on this particular haplotype.

The association between the LTα *Nco*I*1/TNF-308*2/HLA-DRB1*02 haplotype and steroid use is consistent with observations that compounds directed against TNF may be effective in the treatment of asthma.[32] Stratification of subjects by 5-lipo-oxygenase (5-LO) genotype has been shown to differentiate 5-LO antagonist responders and non-responders,[33] and it will be of interest to stratify steroid responsiveness by TNF genotype.

Tumour necrosis factor is a strong mediator of inflammation that has been apparent in asthmatic airways.[4 5] This study suggests that constitutional upregulation of TNF secretion is part of the genetic predisposition to asthma and has narrowed the search for the functional sequences that may alter TNF secretion. However, the human MHC is highly complex and contains many genes which may influence asthma. These need to be further studied in detail before any clinical value can be realised.

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