Airway inflammation is a prominent feature of asthma. The pro-inflammatory cytokine Tumour Necrosis Factor shows constitutional variation in its level of secretion, which is linked to polymorphisms within the TNF gene complex and the surrounding MHC. In this study, 413 subjects in 88 nuclear families from a general population sample were examined for association with asthma and TNF polymorphisms. Ninety-two subjects were asthmatic, as defined by questionnaire. Asthma was significantly more common in subjects with allele 1 of the LTαNcoI polymorphism (LTαNcoI*1) \( (p = 0.005) \), and allele 2 of the TNF–308 polymorphism (TNF–308*2) \( (p = 0.004) \). The association was confined to the LTαNcoI*1/TNF–308*2 haplotype, so that it was not possible to differentiate between the effects of LTαNcoI and TNF–308 alleles. The HLA-DR locus was excluded as a cause of this association. The results suggest that genetic influences on inflammation may be important in the pathogenesis of asthma.

INTRODUCTION

Asthma is the most common disease of childhood (1). Most asthma in children and young adults is initiated by IgE mediated allergy (atopy) to inhaled allergens such as house dust mite and cat dander. However, not all asthmatics are atopic, and most atopic individuals do not have asthma, so factors in addition to atopy are necessary to induce the disease (2,3).

Tumour Necrosis Factor (TNF) is a potent pro-inflammatory cytokine that is found in increased concentration in asthmatic airways (4) and in lavage fluid from asthmatic lungs (5). The TNF and Lymphotoxin (LT) \( \alpha \) and \( \beta \) genes are within the human MHC on chromosome 6p (6,7). Increased secretion of TNF by peripheral blood lymphocytes or monocytes has been established in association with the HLA-DRB1*03 genotype (8). More recently, it has been suggested that the level of TNF secretion is associated with allele 1 of an NcoI polymorphism in the LTα gene (abbreviated as LTαNcoI*1) (9), and with allele 2 of a TNF promoter variant at position –308 (abbreviated as TNF–308*2) (10).

We have therefore investigated the role of HLA-DR, LTαNcoI, and TNF–308 alleles in asthmatic and normal subjects. Polymorphisms within the MHC tend to be in strong linkage disequilibrium and form distinct haplotypes, so that it may be difficult to ascribe functional significance to individual alleles within the haplotype. In this study we have therefore studied families rather than individuals, so as to construct haplotypes with alleles from each locus for individual chromosomes. The families were taken from a general population sample, and have been described in detail previously (11).

Many epidemiological investigations have shown that asthma may be effectively recognised by questionnaire, in which responses to questions about a previous diagnosis of asthma discriminate better than questions concerning wheeze or shortness of breath (ref. 12 for review). In this study, asthma was defined by a standard MRC questionnaire which has been in use since 1966.

RESULTS

For the purpose of the following analysis, asthma was defined as a positive response to the question ‘Have you had an attack of asthma on more than one occasion? ’ Similar results were obtained for positive responses to the questions ‘Have you ever had attacks of shortness of breath with wheezing?’ and ‘Has your doctor ever told you that you have asthma? ’.

The sample contained 92 subjects with asthma, and 318 non-asthmatics. Three subjects had not responded to the question, and were classed as unknown asthma status. Thirty-two asthmatics were parents, and 60 were children. Ninety-two percent of the asthmatics were atopic, having either positive skin tests to House Dust Mite or Grass, or elevations of the total serum IgE above 70% of the age and sex matched population (11). The mean age of all the subjects was 24 years (range 5–51).

PCR genotyping failed in four asthmatics and five non-asthmatics. In the remaining subjects, genotypes containing LTαNcoI*1 were associated with asthma in the families \( (p = 0.003) \), as were genotypes containing TNF–308*2 \( (p = 0.001) \) (Table 1). The population attributable risk for LTαNcoI*1 was 0.33 \( (95\% CI = 0.064–0.55) \) and for TNF–308*2 was 0.28 \( (95\% CI = 0.096–0.46) \).

No significant HLA associations with asthma were found, although HLA-DRB1*03 showed a marginal association \( (p = 0.051) \) which was not significant after multiple comparisons were taken into account. This indicated that the HLA-DR locus did not have an important influence on asthma in these subjects.

Seven hundred and forty-four individual haplotypes could be assigned unambiguously by inspection of the family data. LTαNcoI*1 was in strong linkage disequilibrium with TNF–308*2 \( (\text{Odds ratio } 67.0, \text{ 95\% Confidence Interval } 32.0–140.2, p <0.0001) \). The LTαNcoI*1/TNF–308*2 haplotype

*To whom correspondence should be addressed
was in strong disequilibrium with HLA-DRB1*03 (OR 29.1, 95% CI 17.9–47.3, \( p = 0.0000 \), as previously recognised (13–15). Sixty percent of the haplotypes containing LT\(\alpha\)NcoI*1/TNF–308*2 were HLA-DRB1*03 positive, compared with 5% of other LT\(\alpha\)NcoI*/TNF–308* haplotypes. The LT\(\alpha\)NcoI*1/TNF–308*1 haplotype was in disequilibrium with HLA-DRB1*04 (OR 2.2, 95% CI 1.4–3.3, \( p = 0.0003 \)) with 30% of the LT\(\alpha\)NcoI*1/TNF–308*1 haplotypes being HLA-DRB1*04 positive, compared to 16% of other haplotypes. An excess of asthma was exclusively associated with the LT\(\alpha\)NcoI*1/TNF–308*2 haplotype (Table 2), so that it was not possible to differentiate between the effects of LT\(\alpha\)NcoI*1 and TNF–308*2 alleles. Extension of the LT\(\alpha\)NcoI*1/TNF–308*2 haplotype by inclusion of the HLA-DR locus found that the HLA-DRB1*03/LT\(\alpha\)NcoI*1/TNF–308*2 haplotype did not show an excess of asthma over other haplotypes containing LT\(\alpha\)NcoI*1/TNF–308*2, confirming the association with asthma to be independent of HLA-DR genotypes. The loge IgE did not show association with LT\(\alpha\)NcoI or TNF–308 genotypes by analysis of variance, indicating the association of the TNF polymorphisms with asthma to be independent of atopy.

### Table 1. Tumour Necrosis Factor polymorphism and asthma

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>( \chi^2 )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT(\alpha)NcoI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma no</td>
<td>137 (44%)</td>
<td>141 (45%)</td>
</tr>
<tr>
<td>( \text{yes} )</td>
<td>35 (11%)</td>
<td>19 (22%)</td>
</tr>
<tr>
<td>TNF–308</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma no</td>
<td>89 (29%)</td>
<td>12 (4%)</td>
</tr>
<tr>
<td>( \text{yes} )</td>
<td>211 (68%)</td>
<td>44 (50%)</td>
</tr>
</tbody>
</table>

### DISCUSSION

The results show that genotypes known to correlate with increased TNF secretion are associated with an increased risk of asthma. The results are in keeping with functional studies implicating TNF in asthma (4,5,16), and suggest that genetic influences on inflammation are part of the pathogenesis of the disease. The results also imply that treatment strategies directed against TNF may be effective in the management of asthma.

The study employed a simple definition of asthma, based on a standard questionnaire. Answers to the question ‘Have you had an attack of asthma on more than one occasion?’ will depend greatly on the previous medical care and education of individual subjects. Nevertheless, the relevance and reproducibility of the response to this question has been extensively validated, and related to objective measures of airflow limitation and airway reactivity (12). The town from which the subjects were drawn has been the subject of two previous comprehensive studies of respiratory health, in 1981 and 1990 (17,18), in which the diagnosis of asthma by the same questionnaire had been validated (17). In order to fully understand the effects of TNF polymorphisms on the asthma phenotype, it may nonetheless be desirable in future studies to investigate the relationship between genotypes and other indices such as medication use, bronchial responsiveness testing, or bronchial biopsy.

HLA-DR alleles correlate with reactivity to particular allergens (19,20), potentially confounding the relationship between TNF alleles and asthma. The study has confirmed that LT\(\alpha\)NcoI*1, TNF–308*2 and *HLA-DRB1*03 are in linkage disequilibrium (13–15). However, by studying haplotypes we have shown that the association with asthma is not due to the HLA–DRB1 locus. We have not been able to differentiate between the effects of the LT\(\alpha\)NcoI*1 and TNF–308*2 alleles. Identification of novel polymorphisms within the LT\(\alpha\)NcoI*1/TNF–308*2 haplotype and functional studies may be necessary to elucidate this association.

The population attributable risk of asthma in these subjects was of the order of 30%, indicating the presence of other genetic or environmental influences on asthma. Asthma probably consists of several different diseases, the most common of which is atopic asthma. Almost all of the asthmatics in the study were atopic, so that it was not possible to test if the TNF polymorphisms relate to intrinsic non-allergic asthma, which tends to start in middle age. Constitutional variation in TNF secretion might influence other inflammatory airway diseases, particularly chronic bronchitis and obstructive airway disease in smokers. It will therefore be of interest to examine TNF polymorphisms in individuals with non-allergic asthma and chronic obstructive airway disease.

### MATERIALS AND METHODS

#### Subjects

413 subjects from 88 nuclear families were identified from an Australian general population, as previously described (11,21).

#### Phenotypes

A respiratory questionnaire, based on the MRC respiratory questionnaire, was administered as described previously (21). ‘Asthma’ was defined as a positive answer to the questions ‘Have you had an attack of asthma on more than one occasion?’ Other questions included ‘Have you ever had attacks of shortness of breath with wheezing?’ and ‘Has your doctor ever told you that you have asthma?’ Past and present cigarette smoking was recorded. The total serum IgE was determined by Phadezym PRIST (Pharmacia Ltd, Uppsala, Sweden) as described (11,21).

### Table 2. Association of LT\(\alpha\) NcoI/TNF–308 haplotypes and asthma

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>( \text{LT(\alpha)NcoI*1/} )</th>
<th>( \text{TNF–308*1} )</th>
<th>( \text{LT(\alpha)NcoI*2/} )</th>
<th>( \text{TNF–308*2} )</th>
<th>Fisher’s statistic</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma no</td>
<td>94 (17%)</td>
<td>90 (16%)</td>
<td>378 (66%)</td>
<td>8 (1%)</td>
<td>15.2</td>
<td>0.0013</td>
</tr>
<tr>
<td>( \text{yes} )</td>
<td>28 (17%)</td>
<td>\textbf{48 (29%)}</td>
<td>92 (56%)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Genotypes

PCR of the LTαNeo1 polymorphism was carried out using the primers 5′-CCGTGCTCTGTGGTTGGCTA-3′ and 5′-AGA-GCTGTTGGAGACTAIGTCTG-3′ (9), generating a 750 bp product. Two hundred 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 (NH₄)₂SO₄, 0.01% Tween-20, 1 mM MgCl₂ and 0.45 U Taq DNA polymerase. Amplification conditions were 95°C for 6 min followed by 30 cycles of 95°C for 1 min, 64°C for 1 min, 72°C for 1 min. A final extension of 72°C for 5 min was included. Following amplification, 5 μl of PCR product was digested with 5 U of Neo1 (New England Biolabs) at 37°C for 1 h. Resultant products were analyzed on 2% agarose gels. LTαNeo1 allele 1 was identified by 250 and 500 bp fragments, and allele 2 by a single 750 bp band (9). Controls for each genotype were included with each set of digests.

Typing of the TNF–308 polymorphism was by non-radioactive sequence specific oligonucleotide (SSO) probing of PCR products as described previously (22) with a number of modifications. Two hundred ng of genomic DNA was used in each PCR reaction with a final MgCl₂ 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 5 min. After dot-blotting of denatured PCR products, filters were baked at 120°C for 25 min prior to hybridisation with labelled probes. The 3 M TMAC stringent wash was at 62°C for both probes. Controls of known genotype were included on each filter. Accuracy of the method was confirmed by modified direct DNA sequencing (23) of two TNF–308*1 homozygotes, two TNF–308*2 homozygotes and two heterozygotes from each data set. The sequencing primer was 5′-CAACACAGGCTCAAGGACTC-3′.

HLA-DRB1 typing was carried out by SSO probing as described previously (24), except that probes were end-labelled with digoxigenin-ddUTP (Boehringer Mannheim). The HLA–DRB1 types examined included HLADRB1*01–*14. Subtypes were recognised for HLA–DRB1*05 (HLA–DRB1*01 and HLA–DRB1*12) and for HLA–DRB1*06 (HLA–DRB1*06 and HLA–DRB1*14).

Genotypes for all three loci were checked independently by two individuals without knowledge of the phenotype.

Statistical analysis

Examination of the pedigrees allowed haplotypes on individual chromosomes to be constructed for the three loci. Alleles on unambiguous haplotypes were tested for linkage disequilibrium by analysis of contingency tables (SPSS 4.1 for VAX/VMS, SPSS Inc., USA, and STATXACT 2.11, Cytel Corp., USA). Associations between genotypes and asthma were tested by the Mantel-Haenszel χ² test for linear association (SPSS 4.1). As a dose-response relationship across the three genotypes would be anticipated. Associations between the log, serum IgE were assessed by analysis of variance (SPSS 4.1). Associations between haplotypes and asthma were tested by Fisher’s exact test (STATXACT 2.11). The population attributable risk was estimated by standard methods (25).

ACKNOWLEDGEMENTS

The study was supported by the Wellcome Trust and the National Asthma Campaign. WOCMC is a Wellcome Senior Clinical Research Fellow. We thank the Busselton families and our many colleagues who helped in their recruitment.

REFERENCES


