Association of a lymphotoxin α gene polymorphism and atopy in Italian families

Elisabetta Trabetti, Cristina Patuzzo, Giovanni Malerba, Roberta Galavotti, Laura Carmen Martinati, Attilio L Boner, Pier Franco Pignatti.

Abstract
Tumour necrosis factor (TNF) is a pro-inflammatory cytokine that increases human airway tissue responsiveness and is considered a candidate gene for asthma. Two common polymorphisms (LTuNcoI and TNFα-308) in the TNF gene complex were studied in 600 subjects from 131 Italian families with atopic asthmatic children. Skin prick test (SPT), total IgE levels, atopy (defined as increased IgE levels or SPT positivity or both), bronchial hyperresponsiveness, and clinical asthma were investigated. The observed distribution of the identical by descent alleles at the LTuNcoI locus was different from expected for SPT and atopy (p=0.015). The LTuNcoI genotype distribution for increased IgE levels was different between males and females (p=0.0011), and an association of the 2.2 genotype with increased IgE levels was observed in females (p=0.0032). The results indicate that the LTu gene, or a closely linked locus, is associated with atopy, and suggest a sex difference in the effect of the gene.

Keywords: atopy; asthma; TNF; LTu

Asthma is an inflammatory disease of the lung that results in obstruction of the airways, characterised by airways hyperresponsiveness to various environmental stimuli. Most people with clinical asthma have evidence of increased bronchial responsiveness to methacholine (BHR). The majority of asthmatic children are also atopic, usually with raised total and allergen specific IgE or skin prick test reactivity or both. The aetiology of the disease is multifactorial, with environmental and hereditary determinants. Patterns of clustering and segregation analyses in asthma families have suggested a genetic component. Previous studies have found linkage of asthma and atopy to different candidate genes, among them tumour necrosis factor (TNF) and lymphotoxin α (LTα). TNF is a powerful proinflammatory cytokine that increases human airways responsiveness. LTα and TNFα genes are located on chromosome 6 (6p21.1-6p21.3), between class II/III and class I clusters of the human major histocompatibility complex (MHC). Two polymorphisms of these genes have been described, the LTuNcoI polymorphism, located in the first intron, and the TNF-308 polymorphism, located in the promoter. Recently, these two polymorphisms have been associated with an increased risk of clinical asthma/BHR in two studies in the Australian population.

The aim of the present study was to determine the involvement of the TNF genes in the genetic determination of asthma and allergy in a large series of Italian families.

Materials and methods

PATIENTS AND PHENOTYPES

A panel of 600 subjects belonging to 131 families from the Veneto region in north east Italy was recruited from atopic asthmatic children attending the Allergy and Pulmonology Clinic of the Department of Paediatrics of the University of Verona, as described before. All the subjects were tested for clinical history, total serum IgE level, skin prick test (SPT), and BHR. Clinical asthma was defined according to the American Thoracic Society criteria, including the response to a respiratory questionnaire. Atopy was defined by the presence of one or both of the following criteria: (1) positive SPT to one or more common aeroallergens (house dust mites, cat, dog, Alternaria grass pollen, Parietaria), or (2) raised circulating total IgE (from 0 to 10 years of age: age adjusted standard curve, levels above the 90th centile; above 10 years of age: >200 kU/l). Bronchial hyperreactivity to methacholine was defined as PC20<25 mg/ml.

The total number of patients was 397, of whom 367 were atopic, 329 positive for SPT, 236 with increased IgE levels, 221 with clinical asthma, and 232 with BHR. Seventy subjects with no clinical asthma, negative on SPT and BHR testing, and with known total serum IgE levels were used for LTuNcoI polymorphism case/control genotype frequency comparisons.

GENOTYPE ANALYSIS

Genomic DNA was extracted from whole blood by standard methods. LTu and TNFα polymorphisms were detected after PCR by restriction digestion of the products. For the LTuNcoI polymorphism we used the primers previously described, which generate a PCR product of 740 bp in size (LTuNcoI*2). Restriction of this product with NcoI results in fragments of 545 and 195 bp (LTuNcoI*1). For the TNF-308 polymorphism amplification, we used the primers previously described, in which the sequence of one primer was modified to incorporate a NcoI restriction site. Restriction of the 107 bp product (TNF-308*2) with NcoI results in fragments of 87 and 20 bp (TNF-308*1). Allele denominations for
The two loci were the same as previously reported.14,15 (Moffatt, personal communication).

PCR conditions for both polymorphisms were: 94°C for five minutes, 30 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension step of 10 minutes at 72°C. After amplification, 15 µl of the product were digested with 5 U of NcoI (New England Biolabs) at 37°C for two hours. The DNA fragments were analysed on 1.8% or 3% agarose gels (1:1 NuSieve: SeaKem, FMC Bioproducts). Genotype controls were included with each round of PCR and restriction analysis.

### Statistical Analysis

Affected sib pair analysis was performed with the GAS program (http://www.ebi.ac.uk/biocat/Genetic_tools.html) or with the SIBPAL program, as implemented in the SAGE package. A non-parametric simulation based identity by descent (SimIBD) statistic was performed. Associations were tested by the Extended Transmission Disequilibrium Test (ETDT).14

### Results

We studied 600 subjects belonging to 131 families from the Veneto region of Italy, ascertained through atopic asthmatic children, plus 70 other subjects. All subjects were typed for two TNF gene polymorphisms, LTαNcoI, located in the first intron of the LTα gene, and TNF-308, located in the promoter of the TNFα gene.

Allele frequencies in the Italian families used in this study were determined on founding family members (n=267), irrespective of phenotype. The allele frequencies of LTαNcoI*1 and *2 (number of alleles counted=534) were 0.272 and 0.728, respectively. The allele frequencies (number of alleles counted=368) of TNF-308*1 and *2 were 0.856 and 0.144, respectively.

Linkage analysis between LTαNcoI and TNF-308 alleles, or LTαNcoI/TNF-308 haplotypes, and asthma, atopy, SPT, IgE, and BHR was performed. As shown in table 1, we observed a distortion of expected sharing in affected sib pairs of the LTαNcoI alleles and SPT or atopy (p=0.015). We also observed a distortion of expected allele sharing in affected sib pairs of the LTαNcoI/TNF-308 haplotypes with SPT or atopy (p=0.0075 and p=0.012, respectively), as shown in table 1. Analysis with the SIBPAL program of the estimated proportion of marker alleles shared (not shown in table 1) indicated a significant association with atopy of the LTαNcoI polymorphism (p=0.047) and of LTαNcoI/TNF-308 haplotypes (p=0.029). Simulation analysis on 1000 replicates indicated no significant association of the two polymorphisms, or of the haplotypes, with the five phenotypes considered. Transmission disequilibrium analysis with the ETDT program did not indicate any allele preferentially associated with any of the phenotypes.

When genotypes for LTαNcoI or TNF-308 were examined in the entire sample, no significant association was found with asthma, atopy, IgE, SPT, or BHR, although the LTαNcoI genotype distribution for increased IgE levels was significantly different between affected males and females (p=0.0011, data not shown). Table 2 shows the distribution of genotypes in affected versus unaffected females for total IgE level increase. The total IgE level increase was more commonly present in females with genotype 2.2 compared to those with genotypes 1.1 plus 1.2 (p=0.0032, OR=2.24, 1.29-3.90). No significant association of the LTαNcoI genotypes in affected males (n=338) was observed.

### Discussion

The results obtained on atopic asthmatic subjects by the analysis of polymorphisms for the two TNF loci show a significant allele sharing for the LTαNcoI polymorphism and atopy, but not for the BHR phenotype or for clinical asthma. The LTαNcoI*2 allele is associated with a lower LTα response11 and a higher TNFα level.16 It is possible that the LTα gene intron 1, which contains the NcoI polymorphism, includes a regulatory element which may affect TNFα gene expression.15

Two papers on the south western Australian population reported similar investigations. One analysis was performed on 413 subjects in 88 families from a general population sample from Busselton,14 another was a case-control study on 74 asthmatic and 50 non-asthmatic children from Perth.15 The population allele frequency distribution at the two loci was significantly different in the three asthmatic population samples. LTαNcoI*2 was 0.72, 0.55, or 0.65, in the Italian, in the Busselton, and in the Perth populations, respectively. TNF-308*1 was 0.84, 0.70, or 0.83, in the Italian, in the Busselton, and in the Perth populations, respectively. In the Busselton study,14 genotypes LTαNcoI*1/TNF-308*2, and haplotype LTαNcoI*1/TNF-308*2 were all signific-

### Table 1

**Sharing of LTαNcoI and/or TNF-308 IBD alleles in affected sib pairs**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Markers</th>
<th>Alleles shared</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>SPT</td>
<td>LTαNcoI</td>
<td>18</td>
<td>56.5</td>
</tr>
<tr>
<td></td>
<td>TNF-308</td>
<td>9</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>LTαNcoI/TNF-308</td>
<td>18.5</td>
<td>60.5</td>
</tr>
<tr>
<td>Atopy</td>
<td>LTαNcoI</td>
<td>22</td>
<td>63.5</td>
</tr>
<tr>
<td></td>
<td>TNF-308</td>
<td>11</td>
<td>36.5</td>
</tr>
<tr>
<td></td>
<td>LTαNcoI/TNF-308</td>
<td>23.5</td>
<td>66.5</td>
</tr>
</tbody>
</table>

Allele counts were performed with the GAS program.

*p values were determined on χ² for 3 classes observed. Expected allele sharing: 2 alleles = 0.25, 1 allele = 0.50, 0 alleles = 0.25.

### Table 2

**IgE and LTαNcoI genotypes of the females (and frequency)**

<table>
<thead>
<tr>
<th>LTαNcoI genotypes</th>
<th>Increased IgE</th>
<th>1.1</th>
<th>1.2</th>
<th>2.2</th>
<th>Total</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td></td>
<td>3 (0.03)</td>
<td>27 (0.31)</td>
<td>58 (0.66)</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td>11 (0.05)</td>
<td>98 (0.48)</td>
<td>94 (0.46)</td>
<td>203</td>
<td>0.0088</td>
</tr>
</tbody>
</table>

*p value was determined on χ² in 3 classes observed.

The population allele frequency distribution at the two loci was significantly different in the three asthmatic population samples. LTαNcoI*2 was 0.72, 0.55, or 0.65, in the Italian, in the Busselton, and in the Perth populations, respectively. TNF-308*1 was 0.84, 0.70, or 0.83, in the Italian, in the Busselton, and in the Perth populations, respectively. In the Busselton study,14 genotypes LTαNcoI*1/TNF-308*2, and haplotype LTαNcoI*1/TNF-308*2 were all signific-

### Statistical Analysis

Affected sib pair analysis was performed with the GAS program (http://www.ebi.ac.uk/biocat/Genetic_tools.html) or with the SIBPAL program, as implemented in the SAGE package. A non-parametric simulation based identity by descent (SimIBD) statistic was performed. Associations were tested by the Extended Transmission Disequilibrium Test (ETDT).
cantly associated with clinical asthma. We could not confirm these results in our study population. In the Perth study, an association of LTA(NcoI)*2 with clinical asthma and BHR was observed. The association of this allele with the phenotype is in agreement with our data on the Italian population, and contrary to the allele association in the Busselton population. The Perth study also indicated an association of TNF-308*1 with clinical asthma and BHR. This is the opposite allele to that associated in the Busselton population. In our study we did not observe any association with atopic asthma phenotypes.

HLA-class II typing was not performed. HLA-DR associations with atopy are well recognised, and the TNF locus is in strong disequilibrium with HLA-DR. The discrepancy between the at risk allele among the Busselton, the Perth, and the present study is intriguing and may be attributable to HLA effects on antigen recognition in different environments. Our data (linkage to LTA(NcoI)*2) agree more with those reported in a population living in a similar environment (Perth) than in the one living in a very poor and helmint rich environment (Busselton). This may have a protective effect on the development of sensitisation and subsequent airways inflammation and bronchial hyperresponsiveness.

The finding of a different distribution of LTA genotypes for increased total IgE levels in females, given the lack of correlation with specific IgE (positive response to SPT for common allergens), could be a factor generally involved in the development of sensitisation. In conclusion, the LTA gene, or a closely linked locus, is associated with atopy in the Italian population, even if it does not seem to represent a major gene in the determination of the phenotype.

The results reported in this paper were partially obtained by using the program package SAGE, which is supported by a US Public Health Service Resource Grant (1 P41 RR07655) from the Division of Research Resources. We thank M P Moffatt and W O C M Cookson for providing protocols and controls for polymorphism analysis. We thank Telethon Italy and MURST for support.

1 American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. Am Rev Respir Dis 1988;138:225-44.