

Evidence for the genetic control of immunoglobulin E reactivity to the allergens of *Alternaria alternata*

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Summary

Background The fungus *Alternaria alternata* contains potent allergens, and sensitization to these allergens is associated with a high risk of respiratory disease. The influence of genetic regulation on sensitization to *Alternaria* is unknown.

Objective To determine the influence of genetic factors on IgE responses to specific allergens of *Alternaria*.

Methods The concordance of skin prick test (SPT), radioallergosorbent test (RAST) and IgE-binding profiles of sera were examined from a large cohort of monozygotic and dizygotic twins.

Results Casewise concordance for a positive SPT response was monozygous (MZ) 66%; dizygous (DZ) 40% ($P = 0.002$). Logistic regression confirmed that casewise concordance was significantly stronger between MZ than DZ pairs. Immunoblotting against an *Alternaria* extract revealed 19 allergenic bands. The differences in concordance between the different bands were not significant for either the MZ ($P = 0.97$) or DZ ($P = 0.84$) groups. The pooled MZ:DZ difference in concordance was just significant ($P = 0.049$), suggesting an overall genetic effect on the response to *Alternaria*. This was reinforced by the comparison of the MZ and DZ correlations for total number of bands recognized (MZ $r = 0.65$; DZ $r = 0.37$, $P = 0.015$). Overall, there was a moderate correlation between the individual SPT weal size and RAST score ($r^2 = 0.41$) and a substantial correlation between the number of immunoblotted bands and RAST scores ($r^2 = 0.79$).

Conclusion There is a strong genetic influence on IgE response to the mixture of *Alternaria* allergens and a lesser effect on IgE response to individual allergens.

Keywords allergen, *Alternaria*, atopy, genetic, immunoblotting, twins, IgE

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Introduction

Specific IgE-mediated responses to fungal allergens contribute to the development of allergic disease in exposed individuals [1]. There is a wide range of fungi which are allergenic and the complexity of their diverse allergens is less well understood than for other major allergens. Spores of the genus *Alternaria* are particularly important as they are both common in ambient air and bear potent allergens. Both sensitization and exposure to allergens of *Alternaria* are associated with a high risk of respiratory disease [2, 3]. Studies in inland New South Wales, Australia, identified that 15–23% of school age children showed skin prick sensitization to *Alternaria alternata* and this was associated with increased risk of allergic disease [4, 5]. More recently, increased exposure to *Alternaria* was found to be associated with an increase in risk of airway hyper-responsiveness in sensitized subjects in the same region [6]. A number of separate protein allergens have been identified as targets of the *Alternaria*-specific IgE response [1].

The genetic control of allergic responses may operate at the level of total IgE responsiveness or specific IgE responses to individual allergens [7]. Recent genetic studies have identified the association of the genes for FcεRI on chromosome 11q13 and the IL-4 cluster on chromosome 5q31-q33 [7, 8] with atopy and total serum IgE, in addition to other possible loci [7]. The genetic control of IgE production to specific allergens appears to be variable [9–12]. The strongest associations have been between specific HLA Class II alleles and IgE responses to allergens of ragweed and ryegrass pollen [13, 14] and recently with house dust mite [15, 16].

Comparison of IgE responses in monozygous (MZ) and dizygous (DZ) twins is a powerful tool for dissecting the relative contribution of genetic and environmental influences on responses to individual allergens. Previously, we compared the immunoblotting profiles of sera from MZ and DZ twins to the separated proteins of *Lolium perenne* and *Dermatophagoides pteronyssinus* and found that the genetic control of specific IgE response to individual rye grass allergens [17] and individual dust mite allergens [18] was limited. It appeared that the specificity of the IgE responses to individual components of these allergens was largely determined by environmental factors [19]. The genetic control of responses to *Alternaria* is uncertain,

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although one study has suggested HLA-DR4 was associated with responses to *Alt a* I [20].

Therefore in this study we have utilized a large cohort of twins to investigate the extent to which genetic factors control the production of specific IgE antibodies to individual allergens of *Alternaria*. We examined the concordance of total IgE responses to *Alternaria* as measured by skin prick test (SPT) weal sizes and radioallergosorbent tests (RAST), as well as the concordance of IgE responses to individual allergens determined by immunoblotting. Furthermore, this allowed us to examine the relationship between the three measures of IgE responsiveness to *Alternaria*.

Materials and Methods

Twin cohort

As described elsewhere [21], we recruited a sample of 419 twin pairs where one or both members reported a history of wheeze or asthma. Zygosity testing was performed by genotyping at 10 unlinked highly informative short tandem repeat polymorphism markers. All twins underwent skin prick testing with a panel of allergens including a mixture of *Alternaria* species (Hollister-Stier Laboratories, Spokane, WA, USA). There were 69 pairs of MZ twins and 96 pairs of DZ twins where one or both twins were sensitized to *Alternaria* mixture, defined as having a minimum weal diameter of 3 mm 10 minutes after the prick test. Sufficient serum for blotting was available for 53 MZ and 68 DZ of the skin test positive pairs of twins.

The study was approved by the ethics committees of the University of Queensland and the Queensland Institute of Medical Research.

Preparation of *Alternaria* extracts

A. alternata (strain 28143, New South Wales Department of Agriculture, Australia) was grown for 48 h at 25 °C. The culture was sieved followed by centrifugation to pellet the mycelia. This was suspended 1 : 10 w/v in carbonate buffer containing protease inhibitors (150 mmol/L NaCl, 50 mmol/L NaHCO₃, 5 mmol/L RDTA, 5 mmol/L thiourea, 1 mmol/L PMSF, 0.5% w/v sodium azide), homogenized (Sorvall Omni-Mixer, Newtown, CT, USA) and eluted overnight. The homogenate was centrifuged (26 000 g), dialysed overnight against 20 mmol/L NH₄HCO₃, lyophilized and stored at –20 °C until use.

Electrophoresis of *Alternaria* proteins

Lyophilized extract was reconstituted to 100 mg/mL in 0.125 mol/L Tris (pH 6.8). The extract was separated by electrophoresis on 9–27% gradient vertical slab gels using a discontinuous buffer system [22]. This sample was diluted with an equal volume of 8% w/v SDS, 20% w/v sucrose and 0.005% w/v bromophenol blue, boiled for 5 min, centrifuged at 1000 g and the mixture loaded across the width of a gel at 60 µL/cm. Molecular weights were determined by comparison with low molecular weight markers (Pharmacia, Uppsala, Sweden).

Immunoblotting of electrotransferred proteins of *Alternaria*

Proteins were transferred electrophoretically to nitrocellulose membranes (0.2 µm, Schleicher and Schuell, Dassel, Germany) by the tank transfer method [23]. Protein transfer was performed for 260 Vh at 4 °C. The nitrocellulose was cut into

4 mm wide strips, and blocked for one hour in 0.1% Tween-20 in phosphate-buffered saline (PBS). Each of the 242 sera were incubated overnight diluted to 10% in PBS, then incubated for six hours with I¹²⁵-labelled anti-human IgE (Bioclone, Sydney, Australia) diluted in PBS to give 70 000 c.p.m. per strip. After drying, the strips were exposed to X-ray film (Fuji-RX, Sydney, Australia) kept at –70 °C for 3–7 days, then scored blind to subject zygosity.

RAST and serum IgE levels

Sera from 51 MZ and 67 DZ pairs were tested. Nitrocellulose discs were incubated overnight at 4 °C in 100 µg lyophilized extract reconstituted to 100 µg/mL in PBS. The discs were blocked for one hour in 5% non-fat milk powder (Diploma, Sydney, Australia) in PBS, washed with PBS, then incubated with individual subject serum (50 µL undiluted serum) for six hours, washed, and incubated overnight with 100 µL I¹²⁵-labelled anti-human IgE diluted in PBS to give 20 000 c.p.m. per disc. The amount of anti-IgE bound was measured in a gamma counter and expressed as the percentage of counts retained. Total serum IgE level (tIgE) was quantified by an enzyme immunoassay (Imx Total IgE assay, Abbott Laboratories, Abbott Park, IL, USA).

Statistical analyses

The correlation between members of a twin pair for a dichotomous phenotype (SPT – greater or less than 3 mm weal size; immunoblotting – concordant or discordant twin pairs per allergen band) has been expressed as the casewise concordance:

$$C = 2 N_{AA} / (2 N_{AA} + N_{AU})$$

where N_{AA} is the number of concordant affected pairs, and N_{AU} is the number of discordant (affected, unaffected) pairs. This measure estimates the true recurrence risk, allowing for the ascertainment scheme. Under the usual assumptions of the classical twin study [24], we interpreted a significantly greater MZ than DZ concordance as evidence of genetic determination of the trait.

In addition, we performed equivalent analyses as a logistic regression of twin A's trait value vs. twin B's trait value, including zygosity as covariate in an interaction term. This approach is one that allows the inclusion of quantitative covariates, in this case total serum IgE level, used as a proxy for overall atopic predisposition.

The correlations between RAST score, SPT weal size and the number of immunoblotted bands was determined by Pearson correlation.

To examine the interrelationship between immunoblotted bands of specific allergen, we have performed principal components analysis of the 19 by 19 (binary) correlation matrix.

Analyses were performed using the statistical programs SAS 6.11 (SAS Institute Inc., Cary, NC, USA) and R 1.2.2 [25].

Results

Skin prick test results for *Alternaria*

Treating the *Alternaria* SPT result as a dichotomous variable (Table 1), the casewise concordance for MZ twins was 66%

Table 1. The concordance for *Alternaria* skin prick test weal size between 419 pairs of MZ and DZ twins

Zygosity	Both = 3 mm (N _{AA} †)	One = 3 mm (N _{AU} †)	Both < 3 mm	Casewise concordance
MZ	34	35	124	66%
DZ	24	72	130	40%*

*The difference between casewise concordance between MZ and DZ twins was significant at $P = 0.002$ (Fisher exact test). †N_{AA} is the number of concordant affected pairs, and N_{AU} is the number of discordant (affected, unaffected) pairs.

Table 2. Logistic regression of the likelihood of twin B having a positive *Alternaria* skin prick test against their total serum IgE level and twin A's skin prick test weal size

Term	Odds ratio (95%CI)	P-value
Main effects		
log(total sIgE) of Twin B	3.0 (1.8–5.0)	0.00
Monozygosity of Twin B	0.8 (0.4–1.5)	0.53
Gender of Twin B	1.1 (0.6–2.1)	0.71
Positive SPT result in Twin A	3.9 (2.1–7.3)	0.00
Interactive terms		
Monozygosity × SPT (Genetic effect)	2.7 (1.5–5.1)	0.00
Gender × SPT (Gender effect on concordance)	1.9 (1.0–3.6)	0.04

(SE = 5%) and the concordance for DZ twins was 40% (SE = 6%), a difference which was statistically significant (Fisher exact $P = 0.002$). Including total serum IgE level in the equivalent logistic regression did not alter the conclusion that casewise concordance in *Alternaria* sensitization status was significantly stronger between MZ twins than DZ twins (Table 2).

Immunoblotting of specific allergens of *Alternaria*

SDS-PAGE showed the *Alternaria* extract to be heterogeneous (Fig. 1a). Immunoblotting with a number of sera from *Alternaria* SPT reactive subjects revealed 19 distinct IgE-binding components (Fig. 1b), with relative molecular weights (M_r) ranging from 14 to greater than 94 kDa. The 19 bands have been numbered in decreasing size order.

Concordance between twin pairs was defined as the presence of IgE binding to a specific allergen in both twins of the pair, while discordance was defined as IgE binding to a specific allergen in only one twin of a pair. Pairs where only one twin was blot-reactive thus showed complete discordance, as illustrated by strips o and p in Fig. 2. Of the pairs where both twins were blot-reactive, most showed partial concordance as shown by strips a and b, while few pairs showed complete concordance to all reactive bands, as seen in strips e and f (Fig. 2). Notably, these were pairs where both twins recognized only one or two components.

MZ and DZ twins did not differ significantly in the number of bands recognized (permutation $P = 0.38$, Table 3). A significant difference could only be due to bias in sampling. The 28, 36 and 94 kDa allergens were recognized most frequently. On the basis of molecular weight, the 28 kDa component was probably Alt a 1.

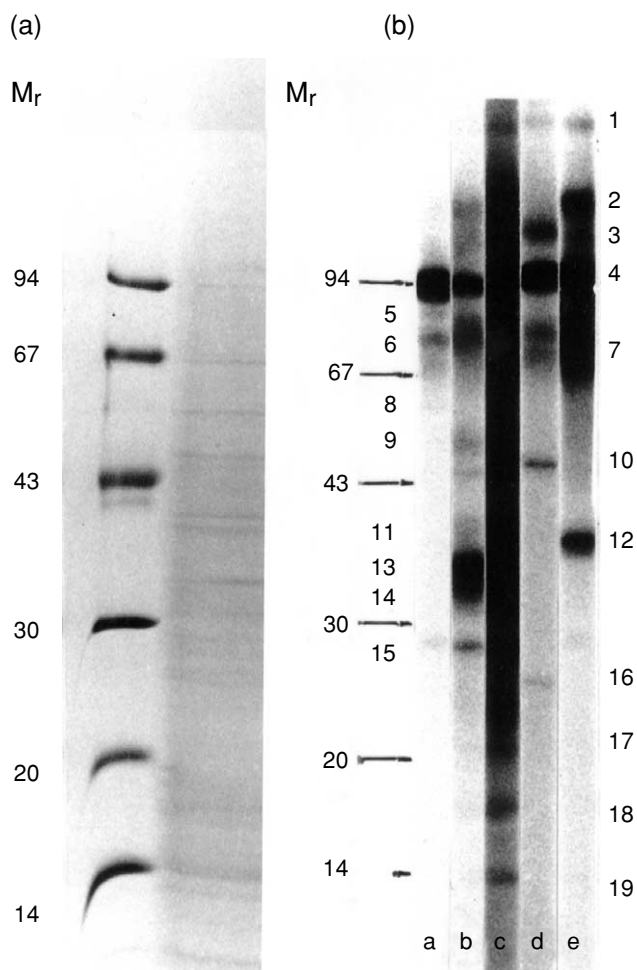


Fig. 1. *Alternaria* extract was separated by 9–27% SDS-PAGE. Molecular weights on the left are shown in kDa. (a) separated proteins stained with Coomassie Brilliant Blue. (b) Location of IgE-binding bands (numbered 1–19, decreasing in size) recognized by 5 sera chosen to demonstrate the entire range of bands identified, shown in relation to molecular weight markers.

Analyses of immunoblotting with twin sera

Most of the subjects tested (69%) did not respond to any of the 19 allergen bands, 11% responded to only one band and 20% responded to more than one band. If there was no correlation between responses to different bands, we would expect 30% responding to no bands, 38% to one band, and 32% responding to more than one band. The principal components analysis showed that a single component explained 34% of the variance of the responses. The pattern of loadings suggested an overall responsiveness to *Alternaria*, and could be summarized by a simple count of the number of positive bands. A second component individually could not explain more than 8% of the variance and represented the effects of the relatively rare bands (numbers 1, 3, 7, 11 and 13).

Analyses of twin concordance for immunoblotting

Since few twins responded to any single band, we applied Fisher exact tests to the data. The MZ casewise concordance for any one band ranged from 0 to 67% (median 50%), and the DZ concordance from 0 to 57% (median 0%; Table 3). The differences in concordance between the different bands were not

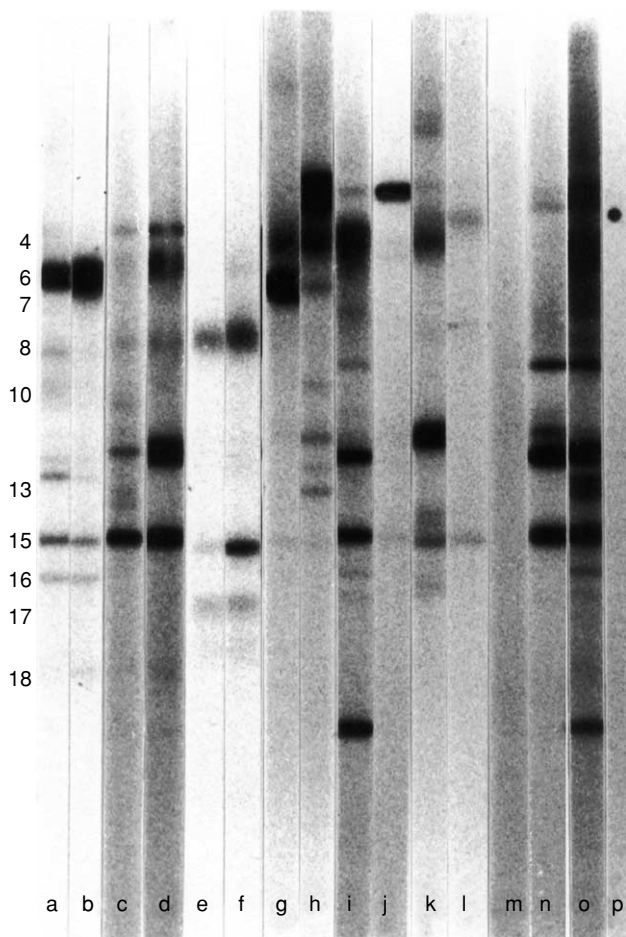


Fig. 2. Immunoblot of IgE binding bands of *Alternaria*, recognized by twin pairs: lanes a, b – monozygous (MZ); c, d – dizygous (DZ); e, f – MZ; g, h – MZ; i, j – DZ; k, l – DZ; m, n – MZ; o, p – DZ.

significantly different within either the MZ ($P=0.97$) or DZ ($P=0.84$) groups. The Mantel-Haenszel chi-square for the pooled MZ:DZ difference in concordance was just significant (exact $P=0.04996$), suggestive of an overall genetic tendency to respond to *Alternaria*. This interpretation was reinforced by the comparison of the MZ and DZ correlations for total number of bands recognized ($r_{MZ}=0.65$, $r_{DZ}=0.37$, $P=0.015$).

RAST correlations

MZ twins tended to have higher average RAST scores, but these differences were not significantly different. The mean intrapair RAST score for MZ twins was 3.5% counts retained ($n=51$, 95% CI 2.1–4.9) compared to 2.9% ($n=67$, 95% CI 2.3–3.5) for DZ twins. The mean percentage difference for MZ pairs was 51.4% (95% CI 39.1–63.8) and for DZ pairs was 73.4% (95% CI 59.0–87.7) which was not statistically different. A trend towards lower difference between RAST scores for MZ pairs was observed.

Relationship between RAST, blotting and SPT

When examining all the twin data, there was a moderate correlation between the individual SPT weal size and RAST score (Table 4). The upper quartile RAST score of those with a SPT

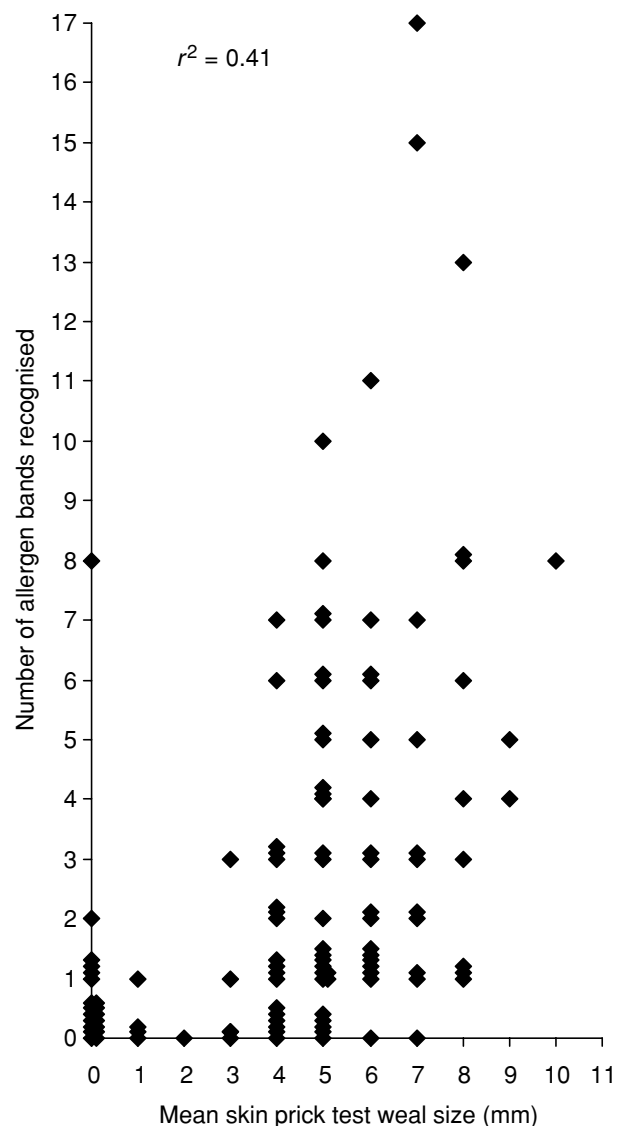


Fig. 3. Distribution of the number of IgE binding bands of *Alternaria* by individual twin sera against their skin prick test weal size.

weal of 0 mm was 1.475 units, and 40% of those with a SPT of 3 mm or greater had a RAST score below this threshold. There was a substantial correlation between the number of immunoreactive bands and RAST scores which used the same extract of *Alternaria* (Table 4), and a moderate correlation between SPT and the number of immunoreactive bands (Fig. 3).

Discussion

Twin and family studies have shown that the heritability for total serum IgE levels ranges from 37 to 84%, while heritability on specific IgE responses to non-purified allergen mixtures is much less, in the range of 10–20% [9]. This study establishes the extent of heritability for specific IgE responses to individual allergens of *Alternaria alternata*. We found that the concordances for specific IgE responses to a mixture of *Alternaria* allergens, as determined by SPT but not RAST, were significantly greater among identical twins than for non-identical

Table 3. Concordance of IgE responses to the individual allergens of *Alternaria* for monozygotic and dizygotic twins

Allergen (kDa)	Monozygotic twins (n = 53 pairs)				Dizygotic twins (n = 68 pairs)			
	% subjects with + recognition	Concordant (N _{AA} *)	Discordant (N _{AU} *)	% concordant	% subjects with + recognition	Concordant (N _{AA})	Discordant (N _{AU})	% concordant
> 200	2.8	1	1	67	0	0	0	0
> 150	5.7	2	1	67	0	0	0	0
115	4.7	1	3	40	2.9	0	4	0
94	13.2	2	9	29	16.2	4	14	36
84	9.4	2	5	40	5.9	1	6	25
77	10.4	3	5	55	5.9	1	6	25
70	6.6	1	5	29	3.7	0	5	0
55	8.5	3	3	67	5.1	2	3	57
48	7.5	2	4	50	3.7	0	5	0
44	6.6	2	3	57	10.3	3	8	43
37	2.8	1	1	67	2.2	0	3	0
36	16.0	5	7	59	11.0	3	9	40
35	4.7	1	3	40	0	0	0	0
32	6.6	2	3	57	0	0	0	0
28	22.6	7	9	58	18.4	7	11	56
26	9.4	2	6	40	2.2	0	3	0
20	0	0	0	0	2.2	0	3	0
18	3.8	0	4	0	6.6	2	5	44
14	4.7	1	3	40	3.7	1	3	40
Median				50				0

*N_{AA} is the number of concordant affected pairs, and N_{AU} is the number of discordant (affected, unaffected) pairs.

Table 4. Pearson correlation values for the relationship between *Alternaria* skin prick test weal size, RAST score, the number of immunoblotted allergenic bands recognized and the total serum IgE level for all twins combined

r ²	SPT	RAST	No. blotted bands	log(tIgE)
SPT	1.00			
RAST	0.43	1.00		
No. blotted bands	0.41	0.79	1.00	
log(tIgE)	0.33	0.36	0.27	1.00

twins. The result remained significant when we adjusted for the effect of total IgE on the responses to *Alternaria*. When the individual allergens of *Alternaria* were examined by immunoblotting, the genetic effect was not significant at the level of individual allergens, but was significant for responses to the total number of immunoreactive bands. Again this provides weak, but significant, support for a genetic effect on the IgE responses to *Alternaria* allergens.

These results extend and are consistent with the findings of two previous studies on house dust mite and ryegrass pollen allergens that used immunoblotting to analyse the specificity of IgE responses for MZ and DZ twins [17, 18]. In both studies the responses of identical and non-identical twins to the different allergens were varied, however, a number of the allergens were recognized significantly more frequently by both pairs of identical twins than non-identical twins. A larger number of twin sera were available for analyses for responses to ryegrass and dust mite allergens than for the *Alternaria*, and thus the

responses to individual allergens of *Alternaria* may have reached significance had more sera been available.

One feature of the immunoblotting patterns were the striking differences between the allergen-specific IgE responses of some identical twins despite their shared genetic inheritance and the likelihood of similar patterns of environmental allergen exposure at least in their early years. Currently, it is unknown what determines the immunodominance of individual allergen bands in a single subject and the variation between twins is multifactorial. Although genetic factors such as variation in the HLA-DR alleles may control responses to individual peptides from an allergen, environmental factors may determine the level of allergen exposure. These factors include the persistence of the allergen on airborne particles, the release of allergens within the lungs and the processing of individual allergens. With time, members of the twin pair may be exposed to different levels of *Alternaria*, or may be exposed to other fungi which share some allergens with *Alternaria* [26, 27]. Moreover, different genetic factors may interact in determining the responses to individual allergenic bands. For example, the genetic predisposition to be a 'high IgE responder' may obscure the genetic regulation of responses to individual allergens. This was evident in a study of the rye grass allergen Lol p 3 [28]. Responses to this allergen in subjects with low total IgE levels were associated with HLA-DR3, but this HLA association was not present in Lol p 3 responders with high total IgE levels.

Several other studies have examined the genetic effects on IgE responses to a purified native allergen in population groups. These include allergens of ragweed, Amb a V [29]; olive, Ole e 1 [30], ryegrass, Lol p 1 [13] and the house dust mite allergens Der p 1 and Der p 2 [31, 32]. It has been considered that specific IgE

response to low molecular weight protein allergens are more likely to be strongly linked with individual HLA-DR alleles. However, the more recent studies demonstrate the complexity of these responses. In a detailed linkage analysis of specific IgE responses to individual allergens of *D. pteronyssinus* in 135 subjects, specific alleles within the HLA-D region were associated with IgE responses to four Der p proteins ranging in molecular weight from 120 to 37 kDa [8]. In a separate study of Venezuelan subjects, a strong association was found between specific IgE responses to Der-p allergens and HLA-DRB1*1101, DQA1*0501 and DQB1*0301. The complexity of the genetic associations at the HLA locus as well as non-HLA loci and environmental factors all contribute to the development of specific IgE responses to individual allergens.

Alternaria allergen extracts are recognized to be heterogeneous and the concordances between the results for SPT and RAST were weaker for *Alternaria* than for other common aeroallergens such as dust mite and cat [33]. The strongest correlations were between the RAST responses of the number of immunoblot positive bands, rather than the RAST and SPT responses. Two different extracts were used for the three measures of the *Alternaria*-specific allergic responses. For the RAST-type assays and immunoblotting, the extract was produced in the laboratory, while the SPT extract was obtained from commercial sources. This is likely to account for some of the variation between SPT and RAST results. Shen et al. showed that a group of sera, classified by a commercial RAST assay and SPT as having no *Penicillium*-specific IgE, had numerous bands on blotting with a laboratory-produced extract [34]. This suggests that even standardized *in vivo* and *in vitro* diagnostic systems can produce different results when used to identify specific allergies. These differences are likely to relate to the stability, binding, denaturation and degradation of the different allergens.

In conclusion, IgE responses to *Alternaria* allergens contribute significantly to the development of atopic disease where environmental conditions favour exposure to this fungus [4, 6]. This study in *Alternaria*-sensitive twins establishes that genetic factors exert a strong influence on responses to a mixture of *Alternaria* allergens and that this effect is apparent, to a lesser extent, on IgE reactivity to individual allergens.

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