

Limited genetic control of specific IgE responses to rye grass pollen allergens in Australian twins

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Summary

Background Both genetic and environmental factors are thought to contribute to specific IgE responses, however, the relative contribution of each in the responses to individual ryegrass pollen allergens is largely unknown even though some responses to allergens have been linked to certain HLA complexes.

Objective Using a large group of monozygotic and dizygotic twins, this study was designed to investigate the IgE binding profiles of individual ryegrass pollen (*Lolium perenne*) components to assess the relative contribution of genetic and environmental factors in determining IgE responses to specific allergens.

Methods Ryegrass pollen proteins were separated by electrophoresis and immunoblotted with sera from 191 pairs of twins where at least one sibling had a SPT > 2 mm to perennial ryegrass. Concordance levels for individual ryegrass pollen components were compared between monozygotic and dizygotic twins in a subset group where both twins had SPT > 3 mm to perennial ryegrass.

Results Immunoblotting revealed 23 individual IgE-binding components from ryegrass pollen. Although there was a significantly greater proportion of monozygotic twins with SPT wheals greater than 3 mm when compared with the dizygotic twins, the mean case-wise concordance for the immunoblot components was similar for both groups of twins. The mean case-wise concordance when at least four pairs of sera were involved was 44% for the MZ twins ($n = 11$ components) and 45% for the DZ twins ($n = 12$ components). We found no significant differences in concordance levels for any of the 23 individual components including allergens previously associated with HLA.

Conclusion Evidence for genetic control of allergen-specific IgE responses in a large population sample of twins to individual ryegrass allergens is limited, indicating that the IgE responses to specific ryegrass pollen allergens are determined largely by environmental factors.

Keywords: IgE repertoire, atopy, ryegrass pollen allergy, twins, immunoblotting

Clinical and Experimental Allergy, Vol. 28, pp. 322–331. Submitted 6 January 1997; revised 14 June 1997; accepted 6 September 1997.

Introduction

Atopic allergy, characterized by increased and persistent levels of IgE, is influenced by both genetic determinants [1] and environmental factors [2]. Both family and twin studies have shown a genetic predisposition to atopy although the

pattern of inheritance identified varies between studies. Total IgE levels, but not allergen specific IgE, have been associated with the beta subunit of high affinity IgE receptor gene on chromosome 11 [3] and the cytokine gene cluster, which includes the IL-4 gene, on chromosome 5 [4]. However linkage with the high affinity IgE receptor gene or a closely linked gene to bronchial hyperreactivity has been observed in a large group of sibling-pairs, even in the absence of atopy [5]. Bronchial hyperresponsiveness has also been

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mapped to the cytokine gene cluster [6], although a murine study excluded the IL-4 gene in contributing to the genetic predisposition of allergen-induced hyperresponsiveness [7].

IgE responses to whole allergen extracts, but of one type, appear to be influenced mostly by environmental factors, as revealed by a number of small twin studies [8–10], although both allergen-specific IgE responsiveness [11–14] and non-responsiveness [15–17] have been associated with various HLA complexes. The strongest HLA associations have been with some ragweed allergens and some ryegrass (*Lolium perenne*) allergens [12]. Most often HLA-allergen associations tend to be weak and it has been proposed that HLA restriction is insufficient to account for individual differences in reactivity to common allergens. Therefore it is likely that environmental factors or other genetic loci such as the T cell receptor (TcR) may be involved in determining reactivity to specific allergens [14]. For example significant linkage between the TcR complex on chromosome 14 and highly purified allergens has been observed [18]. This is supported by the non-random use of V α 13 by T-cell clones specific for Lol p 1 [19], a major allergen of *L. perenne*. Since a number of mechanisms may be associated with allergen specific IgE responses it is important to determine the extent of genetic factors in determining such responses.

Immunoblotting is a useful method for examining IgE responses to individual allergens and has been used for a wide variety of allergenic sources, including ryegrass pollen [20]. This paper outlines a unique investigation using a large group of Australian twins [21] to determine the IgE reactivity to specific ryegrass pollen components by immunoblotting.

Materials and methods

Study population

Human sera was provided from a community-based survey of asthmatic twins [21]. Briefly, 5967 pairs of twins registered with the Australian Health and Medical Research Council Twin Registry returned up to two questionnaires screening for asthma and wheezing over the period 1980–1989. The 1989 pairs screening positive for wheeze were resurveyed in 1990 using a longer respiratory questionnaire, with responses being received from 3193 individuals (80%). These twins were also asked, if both were resident in a capital city, to take part in a testing protocol. This included epicutaneous allergen skin prick testing with a panel of 11 allergens common aeroallergens including perennial ryegrass (Hollister-Stier Laboratories, Spokane, WA, USA). The median age of the respondents was 32 years, ranging from 18 to 88 years, with a cumulative prevalence of asthma or wheezing of 13.2% and for hay fever of 32% [21]. For immunoblotting we selected twin pairs (191 in total) where

at least one sibling had a SPT to perennial ryegrass greater than 2 mm, based on a strong correlation between the degree of SPT and immunoblot reactivity for house dust mite [22]. Zygosity of all pairs was initially assigned by two questionnaire items and confirmed by typing with nine micro-satellite markers.

Preparation of ryegrass pollen extracts

Dry ryegrass pollen (2 g) (*Lolium perenne*, Allergon AB, Engelholm, Sweden) was extracted in 0.001 M NH₄HCO₂ (20 mL) overnight at 4°C with constant stirring. The extract was centrifuged (18 000 *g* × 40 min) and the supernatant freeze-dried [20]. Protein content was estimated by the bicinchoninic acid protein determination method (Pierce Chemical Co., Rockford, IL, USA).

Electrophoresis of ryegrass proteins

The components of ryegrass pollen were separated by SDS-PAGE using 9–27% gradient polyacrylamide gels with a discontinuous buffer system under reduced conditions as previously described [23]. Ryegrass pollen extract (1200 μ g) in sample buffer containing 2 β -mercapto-ethanol (5% v/v) was electrophoresed at 400 V for 3.5 h at 4°C. Low molecular weight markers (94, 67, 43, 30, 20.1 and 14.4 kDa; Pharmacia, Uppsala, Sweden) were included on each gel. A side strip of each gel was removed before transfer and stained in Coomassie Brilliant Blue (CBB).

Electrotransfer of separated proteins

Separated proteins were transferred from the gels to 0.2 μ m pore nitrocellulose (NC) (Schleicher and Schuell, Dassel, Germany) as previously described [20]. Proteins were transferred at 80 V for 3.5 h at 4°C using transfer buffer containing 25 mM Tris, 192 mM glycine, 20% v/v methanol and 0.05% w/v SDS [23]. Post transfer NC was stained with 0.005% w/v Ponceau S (Sigma Chemical Co., St Louis, MO, USA) in distilled water containing glacial acetic acid (0.2% v/v) for the presence of protein, rinsed with distilled water containing glacial acetic acid (0.2% v/v) and stored at 4°C until required. Post transferred gels were routinely stained in CBB stain and no remaining protein was detected.

Immunoprobings of transferred ryegrass proteins

Only sera from twin pairs where at least one sibling had a SPT > 2 mm to perennial ryegrass was selected for immunoblotting. NC sheets containing ryegrass proteins were cut into 4 mm strips and blocked in phosphate buffered saline (PBS)-Tween 20 (0.1% v/v) for 60 min, followed by an overnight incubation in sera diluted in PBS (10% v/v). The

strips were washed three times in PBS-Tween 20 (0.05% v/v), incubated with ^{125}I -antiIgE (Bioclone, Sydney, Australia) (70 000 cpm per strip) in PBS for 6 h and washed. Air dried NC strips were incubated for 3 days with Rx photography paper (Fuji, Tokyo, Japan) in Kodak X-Omatic cassettes (Eastman Kodak Co., Rochester, NY, USA) at -70°C . Pairs of twins were probed using adjacent strips and autoradiograms were scored blind to zygosity.

NC strips from each blot were also probed with a rabbit polyclonal serum raised against ryegrass pollen in our laboratory (Tovey, unpublished). NC strips were blocked with PBS-BSA (5% m/v)/Tween 20 (0.01% v/v) for 1 h, washed three times with PBS-Tween 20 (0.05% v/v) and incubated with the polyclonal serum diluted at 1:5000 in PBS-BSA (0.5% m/v)/Tween 20 (0.005%) for 1 h. The strips were washed and incubated with goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate (Silenus, Victoria, Australia) diluted at 1:1000 in PBS-BSA/Tween 20 for 1 h, washed and incubated with 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium substrate for 10 min. All strips were incubated at room temperature and in volumes of 1 mL.

Analysis of data

Each of 23 individual IgE-binding components identifiable on autoradiographs were scored visually as negative, (–) or positive, (+). Positive scores were graded 1+, 2+, 3+, or 4+, however in the analysis to compare concordance only (–) and any (+) was used. Since more DZ twins were likely to be discordant for perennial ryegrass sensitivity by SPT, only twins where both had a SPT score greater than 3 mm were used in the analysis of specific reactivity by immunoblotting. This analysis included a total of 78 pairs of monozygotic (MZ) (40) and dizygotic (DZ) (38) twins. Thus, the absence of a band does not reflect either non-exposure or a non-atopic genotype or phenotype. An immunoblot negative result in this selected sample represents either a level of circulating component-specific IgE undetectable by the present technique or sensitisation to a treatment-labile epitope.

To examine concordance for presence or absence of a given band for each twin pair we compared the concordance levels by Fisher's Exact Test (two-tailed) with $P < 0.01$ considered significant. Case-wise concordance was defined as $2C/(2C + D)$, where C is the number of pairs where both twins exhibit a positive band (+, +) and D is the number of pairs where only one twin exhibits a band (either +, – or –, +).

Different concordances among MZ and DZ twins are expected under different genetic hypotheses. For example, if a character (i.e. IgE specific to a given band) is completely genetically determined, is Mendelian recessive and is present in one twin, then MZ twins are expected to be

completely concordant, and DZ twins concordant in at least one-quarter of cases. If genes are completely unimportant, but early outdoor exposure was critical, then concordance in MZ and DZ twins would be the same and greater than zero. If sensitization is completely random in nature, both concordances would equal the population risk.

Results

IgE-reactivity of twin sera to ryegrass pollen components was determined by immunoblotting in all twins where at least one sibling had a SPT to perennial ryegrass > 2 mm, a total of 382 sera (79 MZ and 112 DZ pairs). In total 23 individual IgE binding components were identified (Fig. 1). Rabbit polyclonal serum raised against ryegrass extract revealed 19 components and was used as a standard to assist in the identification of each component (Fig. 1). Fifty-six per cent (88/158) of the sera from MZ twins bound to at least one component of ryegrass extract while 49% (110/224) of DZ sera bound to ryegrass. Sera from 22% (17/79) and 20% (22/112) of the MZ pairs and DZ pairs respectively bound no components of ryegrass extract in either twin. Fig. 2 illustrates examples of IgE-binding by sera from MZ and DZ pairs. Fetal cord serum was used as a negative control and demonstrated no IgE binding components.

The frequency distribution of the ryegrass components (Fig. 3) for MZ and DZ twins was similar. Components 11, 15, 17, and 18 (M_r 57, 34, 28, and 26 kDa) bound more than 40% of immunoblot positive sera. Six components were only detected once; 1, 2, 3, 4 (all by the same serum), 10 (by a second serum), and 22 (by a third serum).

The distribution of perennial ryegrass SPT positivity among the MZ and DZ twins is shown in Table 1. There was a significant difference in SPT reactivity to ryegrass between the twin groups where both individuals within a pair had a SPT > 3 mm ($P = 0.0207$; chi-square test).

Case-wise concordance for each IgE-binding component was determined for MZ and DZ twins where both twins had

Table 1. Skin-prick test reaction to perennial ryegrass in twin pairs

Zygosity	One > 2 mm	Both > 3 mm ^a	Total ^b
MZ pairs	39	40	79
DZ pairs	74	38	112
Total	113	78	191

^a Number of twin pairs where both members exhibit a SPT > 3 mm for perennial ryegrass.

^b Sera was selected for immunoblotting on the basis of at least one twin exhibiting a SPT > 2 mm.

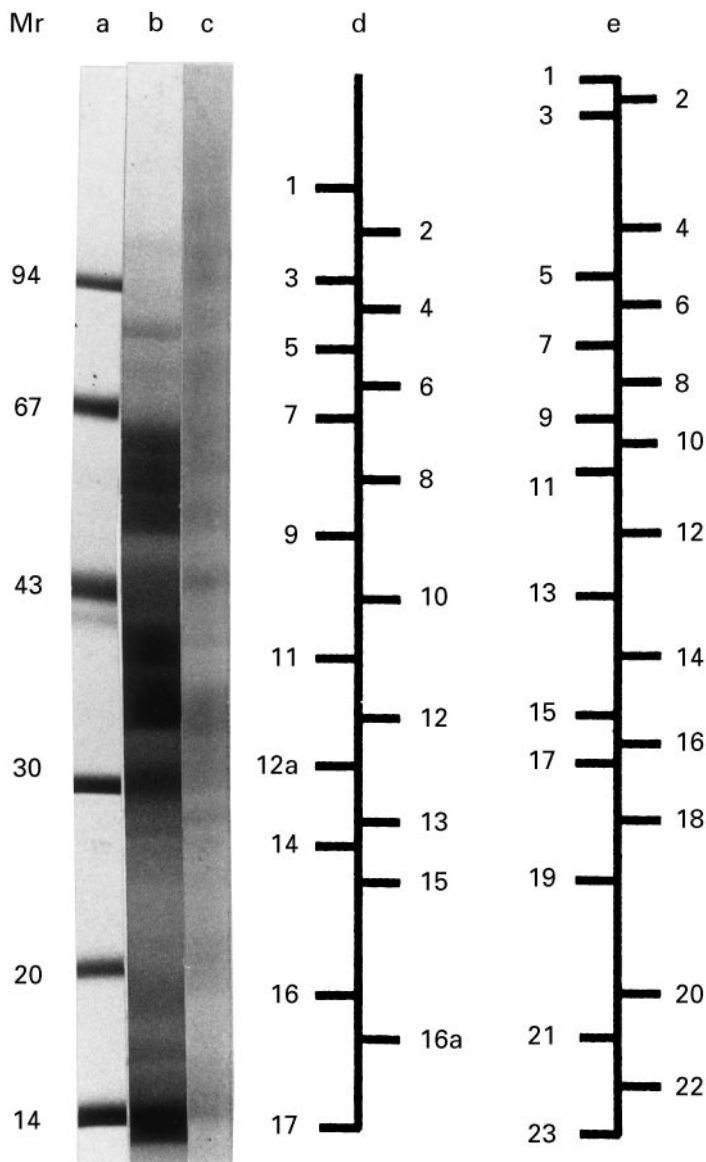


Fig. 1. Proteins staining and immunoprobings of the *L. perenne* extract separated on reducing gradient SDS-PAGE gels. Strips a and b show CBB staining of M_r markers and the resolved ryegrass pollen proteins. Strip c shows the reference pattern produced by the polyclonal rabbit serum run with each blot as a standard. Strip d shows the location of the 19 rabbit immunoglobulin-binding components. Strip e shows the location of the 23 IgE-binding components in human sera.

a perennial ryegrass SPT > 3 mm (Fig. 4). The mean case-wise concordance of components which were involved in at least four pairs of sera was 44% for the MZ twins ($n = 11$ components) and 45% for the DZ twins ($n = 12$ components). The level of case-wise concordance for any individual component when more than four pairs of sera were involved was highest for component 15 with 72% for the MZ twins and 68% for the DZ twins. Statistical comparisons between the MZ and DZ twins for each component revealed no significant differences by the Fisher's Exact Test (Table 2).

Discussion

Individuals who are atopic have an allergic fingerprint to

specific protein components which is determined by genetic and environmental factors [25]. The factors which determine specificity of an individual's response to specific allergen components have not been identified. As an IgE-response does not occur in the absence of exposure, it is likely that this is an important factor. However, twins are likely to have similar exposure. There is some data to suggest that responses to individual allergen components, including ryegrass allergen, may be under HLA control [12,25]. This has not been confirmed in all studies or for all ryegrass allergens, and other studies where reactivity to whole pollen (or other allergens) extracts have been used, have not demonstrated strong evidence of genetic control [9]. In this study we compared the concordance of IgE

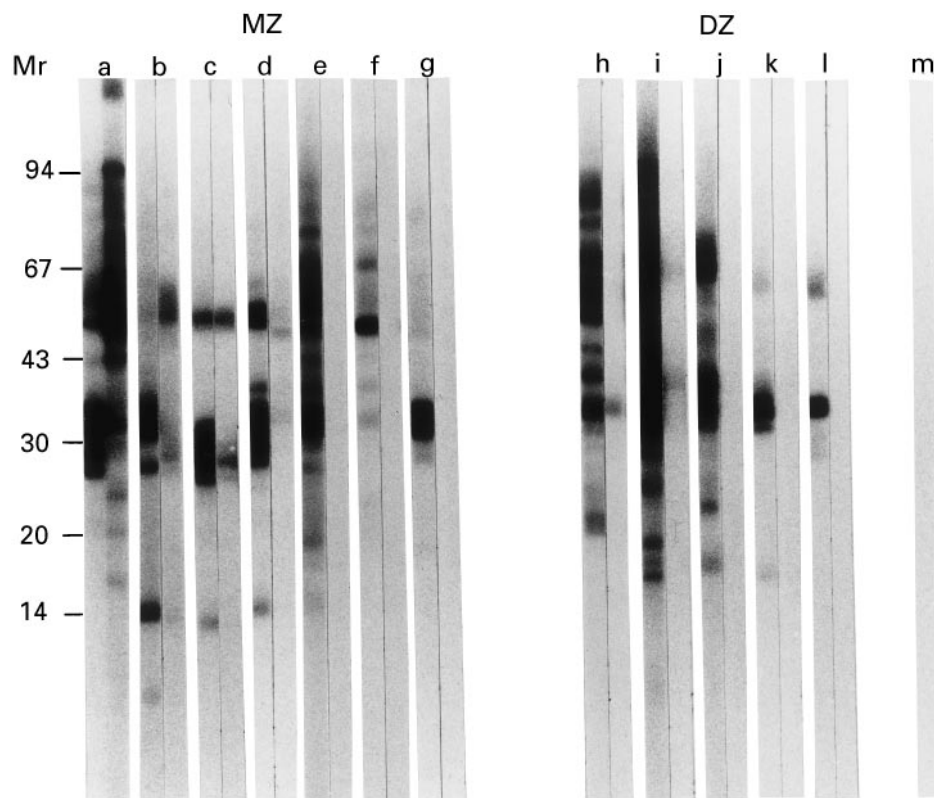


Fig. 2. Examples of the patterns of IgE-binding to *L. perenne* shown by seven pairs of MZ sera (pairs a–g) and 5 pairs of DZ sera (strips h–l). The pairs are from different blots and patterns between pairs cannot be compared precisely. Negative control human fetal cord serum is shown in strip m.

reactivity between MZ and DZ twins to individual components (allergens) of ryegrass pollen. Concordance levels for IgE reactivity to each specific ryegrass pollen component were similar for MZ and DZ twins, although not all components had sample sizes which were large enough for analysis (Fig. 4, Table 1). The similarity in concordance between MZ and DZ twins indicates that the environment is the primary factor in IgE responses to specific ryegrass components. Further support for the role of the environment in specific IgE responses comes from the low level of mean case-wise concordance in the MZ twins. Since MZ twins share an identical genetic background and presumably a similar environment in the early years of their lives, differences in the environment may only need to be slight to affect IgE specificity, as any difference must be due to environmental factors.

Past twin studies [8–10] suggest that total IgE is largely under genetic control, but specific IgE (by SPT or RAST) to individual allergens is predominantly influenced by the environment, however these twin studies did not examine individual IgE-reactivity to each component of specific allergenic sources. A previous study with similarly aged

adult MZ and DZ twins who had been either reared together or apart since early childhood indicated that sensitivity to individual allergen sources, including *Lolium perenne*, may be directed more by the environment than by genetic factors [10]. We did not test the effects of living apart at different times of the subjects' lives, as this would have been difficult to model, as the relevant allergen exposures at these times in the different locations are not known.

The relationship between the IgE-binding components and previously defined allergens [20] was based on their frequency and molecular mass. Components 11, 15, 17, and 18 (M_r 57, 34, 28, and 26 kDa respectively) bound more than 40% of blot positive sera. Component 15 (34 kDa) that bound >80% of sera corresponds to Lol p 1. Lol p 2 and 3, which share the same M_r by SDS-PAGE (14 kDa), correspond to component 23. Component 11 (57 kDa) most likely forms part of the group 4 allergens [20], probably Lol p 4 and/or the high molecular weight basic allergen [26]. Components 17 (28 kDa) and 18 (26 kDa) correspond to the group 5 allergens [27]. Component 22 (16 kDa) which reacted with only one serum may be cytochrome c which has been previously shown to react with IgG but not IgE

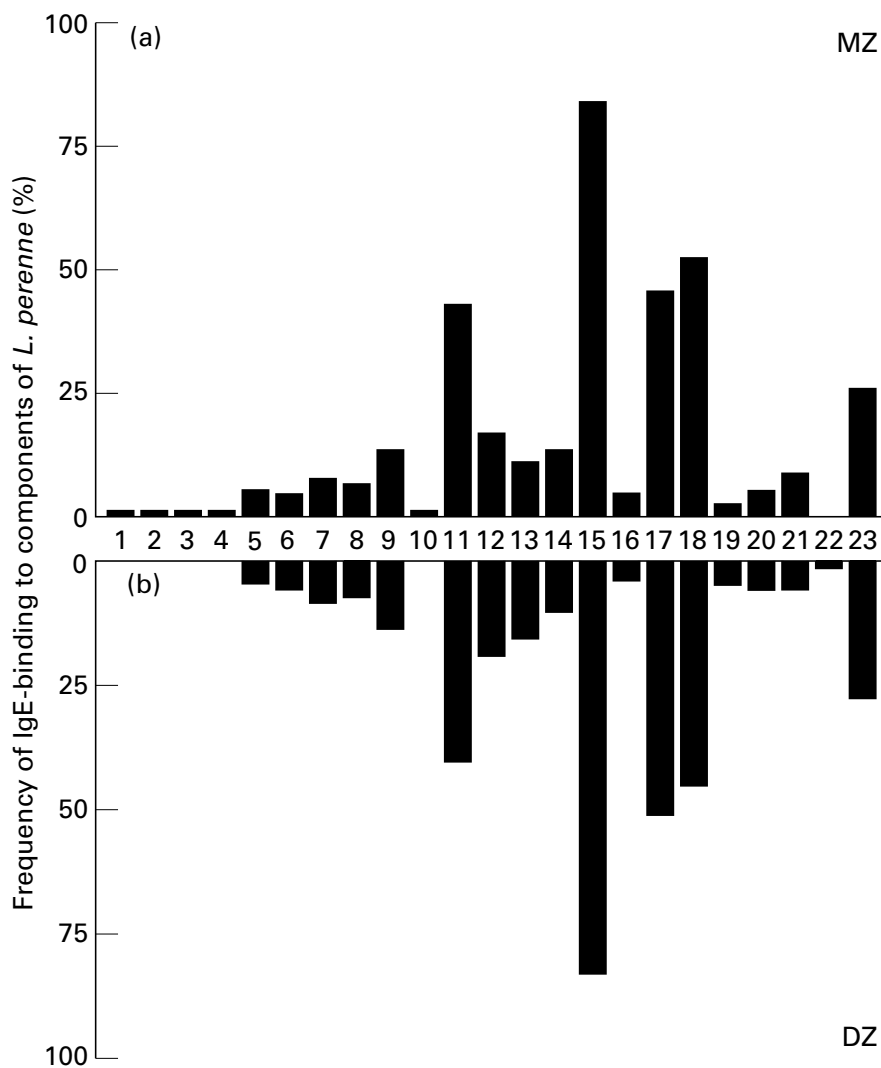


Fig. 3. Frequency of each IgE-binding component of *L. perenne* from sera of MZ twins (a) and DZ twins (b) where at least one sibling had a SPT > 2 mm to perennial ryegrass.

antibodies in a minority (4) of 139 ryegrass sensitive subjects [28].

IgE-reactivity to Lol p 1, 2 and 3 was not significantly different between MZ and DZ pairs suggesting that non-genetic factors, other than association of the HLA, contribute to the specific IgE responses to these components in most individuals. IgE responses to both Lol p 1 and Lol p 2 have been associated with HLA-DR3 [29], however a separate study examining Lol p 1 reactive T cells from ryegrass pollen allergic patients did not find any dominant HLA-DR haplotype usage [30]. IgE reactivity to Lol p 3 has been associated with HLA-DR3 and HLA-DR5 [31]. Interestingly both groups of twins had a high case-wise concordance for Lol p 1 at approximately 70%. The case-wise concordance for Lol p 2/3 was twice as high for the MZ twins (47%) when compared to the DZ twins (24%), however this difference was not significant by Fisher's

Exact Test. The possibility that the process of SDS-PAGE has destroyed all the determinants that may be associated with HLA seem unlikely. While our study can not rule out HLA associations, since the HLA phenotype was not determined, the data do indicate that in most instances of IgE reactivity to the major components of ryegrass pollen the environment is of predominant importance.

It may be possible that individual differences in environmental factors could account for the similar levels of concordance between the MZ and DZ twins observed in this study. Individuals may be co-sensitized to a number of allergens since many of the plant allergens are cross-reactive [32] and pollen reactive IgE antibodies may not even reflect accurately the sensitizing pollen species [33]. It could be expected that there is a high level of diversity in the distribution of different grass species, and although twins may share a common childhood, opportunities for different

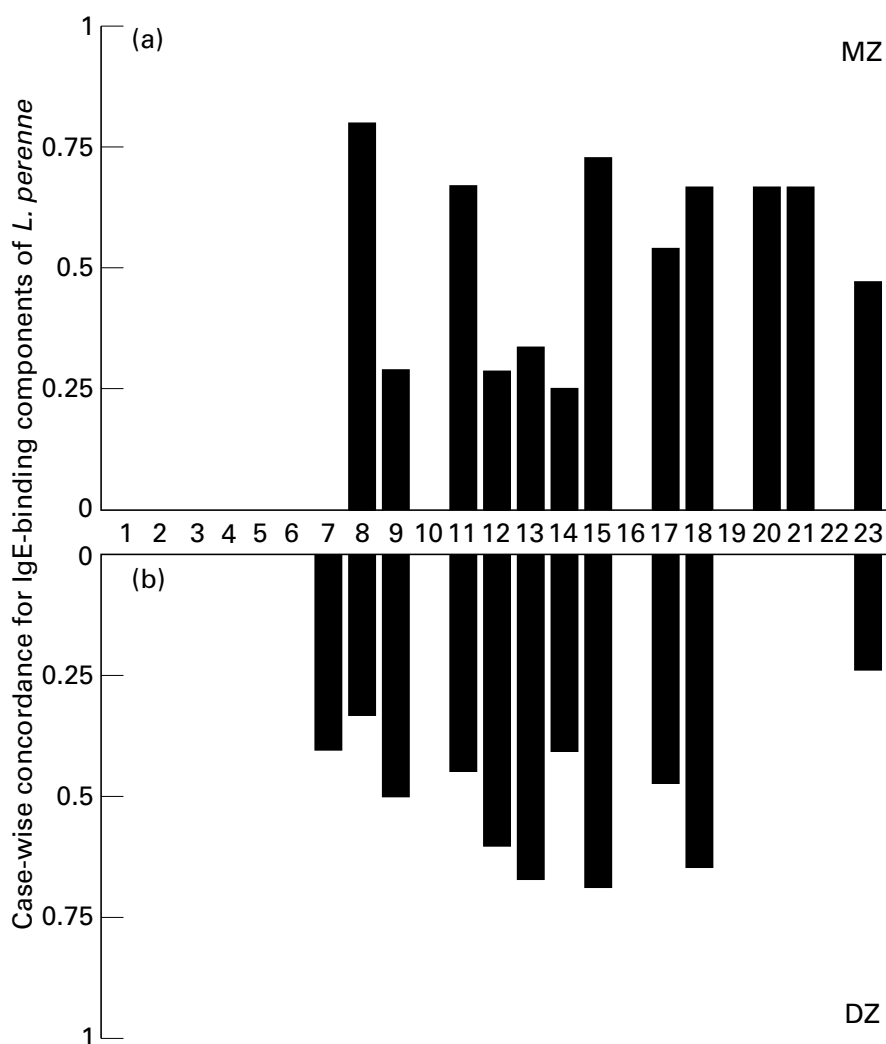


Fig. 4. Case-wise concordance [$2C/(2C + D)$] for each IgE-binding component of *L. perenne* from sera of MZ twins (a) and DZ twins (b) where both twins had a SPT > 3 mm for perennial ryegrass. No significant differences (Fisher's Exact Test) between the level of case-wise concordance for any individual component were observed when more than four pairs of sera were involved.

exposure in adulthood and even in childhood would be high. Although many grass pollens crossreact at a gross level, there are numerous minor differences in allergens and exposure. Furthermore, using this same cohort of twins, we have shown significant higher concordance in IgE binding from MZ twin sera compared with DZ twin sera to two components of the house dust mite, *Dermatophagoides pteronyssinus*, which has very few cross-reactive allergen sources in Australia [22]. Progression to become allergic to other crossreactive antigens may be due in part to TcR determinant spreading [34]. However it is difficult to understand how crossreactivity and/or cross-sensitization could alter concordance levels of IgE to specific allergens, as one would assume if genetic factors were important then MZ twins would still have a greater concordance than DZ twins to components even if crossreactive. The similar levels in case-wise concordance for the twin groups may be in part due to total IgE levels. For instance it was found that the association between Lol p 3 and HLA-DR3 was much more

evident in patients with low, than with high, total serum IgE levels [31]. Furthermore, HLA associations with certain allergens may be more evident in mono-sensitized individuals [13]. Thus, crossreactivity could affect IgE specificity by masking the detection of specific responses as exposure to different allergen sources may broaden the spectrum of IgE-response and increase total serum IgE.

Detection of allergens by immunoblotting is confounded by intra- and inter-species crossreactivity [35–37]. Furthermore a number of isoforms of the group one and five allergens have been identified [37] and results with Lol p 2 and 3 are further complicated by these two allergens sharing the same molecular weight by SDS-PAGE [20]. However, if genetic factors were the main determinant in regulating IgE specificity MZ twins should still show higher levels of concordance as compared with DZ twins.

Genetic control of responses to allergens may occur at the level of the TcR. Past studies with non-atopic twins [38,39] have shown higher levels of concordance for TcR

Table 2. Frequency of concordance and discordance in MZ and DZ twins for each of the 23 IgE-binding components of *L. perenne*

M _r (kDa) ^a	Component	Frequency for MZ		Frequency for DZ		Fisher's Exact P-value ^b
		concordant	discordant	concordant	discordant	
140	1	0	0	0	0	—
130	2	0	0	0	0	—
120	3	0	0	0	0	—
94	4	0	0	0	0	—
88	5	0	1	0	3	—
80	6	0	1	0	3	—
75	7	0	4	1	3	1
68	8	2	1	1	4	0.464
63	9	1	5	2	4	1
62	10	0	0	0	0	—
57(4)	11	9	9	4	10	0.289
51	12	1	5	3	4	0.559
44	13	1	4	2	2	0.524
39	14	1	6	1	3	1
34(1)	15	17	13	15	14	0.796
29	16	0	1	0	3	—
28(5)	17	7	12	7	16	0.748
26(5)	18	10	10	8	9	1
23	19	0	0	0	5	—
20	20	1	1	0	3	0.4
19	21	2	2	0	2	0.467
16	22	0	0	0	1	—
14(2/3)	23	4	9	2	13	0.372

^a Numbers in parentheses indicate defined *L. perenne* allergens as determined by M_r and frequency distribution.^b *P* < 0.01 was considered significant.

usage in MZ than DZ twins. Mohipatra *et al.* [19] have demonstrated restricted usage of V α 13 by the TcR to grass pollen allergens suggesting a limited number of T cell genes may constitute a basis for crossreactivities at the antibody level. It is not known whether responses restricted at the TcR level provide similar restriction at the antibody level. Interestingly studies with birch pollen demonstrated that both healthy and allergic individuals recognise the same T cell epitopes even though they had different genetic backgrounds [40] and a restricted TcR V β gene usage could not be observed for a major allergen of timothy grass pollen [41].

In conclusion, immunoblotting in a large population sample of MZ and DZ twins has revealed limited evidence for genetic control of IgE responses to specific ryegrass components. Similar levels of case-wise concordance were seen for all components including those for which IgE reactivity had been previously associated with the HLA complex. The results indicate that environmental factors are

of primary importance in determining specific IgE responses to ryegrass allergens and that differences in the environment may only need to be slight to influence the response.

Acknowledgments

The authors would like to thank Dr Jenny Peat for assistance with the statistical analysis and helpful discussion. Support for this study was provided by the New South Wales Asthma Foundation and the Australian National Health and Medical Research Council.

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