Asian Pacific Journal of Allergy and Immunology (1995) 13 : 101-105

Allergens of *Bipolaris* species

SH Lim, FT Chew, SMY Sim, YTG Huang, DYT Goh, HTW Tan[1], TK Tan[1], and BW Lee

SUMMARY **Skin prick tests done previously revealed a significantly higher percentage of sensitization to an extract of *Bipolaris* sp. among atopic individuals (34/147, 23.1%) compared to non-atopic individuals. *Bipolaris*-specific IgE levels were quantified in sera from a representative group of 38 individuals using the Fluorescence Allergosorbent Test (FAST). Results obtained by FAST were found to be comparable to the skin prick test results ($r^2 = 0.60$, $p < 0.001$ for IgE levels vs wheal sizes; $r^2 = 0.44$, $p < 0.001$ for IgE levels vs erythema sizes). Characterisation of the extract's allergenic component by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed 28 protein bands with molecular weights (MW) ranging from 11 kDa to above 100 kDa. Immunoblotting with sera of 10 *Bipolaris*-sensitive (skin prick test, 3+) individuals showed that *Bipolaris* spore extract contained at least 4 IgE binding proteins (MW 11-13 kDa, 16-17kDa, 20-22kDa and 36kDa). All 10 sera reacted to the protein at MW 20-22 kDa, 2 sera with MW 11-13kDa, 3 sera with 16-17kDa and 6 sera with 36 kDa. This study has thus demonstrated that spores of *Bipolaris* sp. contain allergenic components which may elicit IgE-mediated reactions.**

Allergic respiratory diseases such as asthma and rhinitis are common health problems in Singapore.[1-3] Apart from house dust mite allergy[4,5] and air pollution,[6] studies have shown that the components of the airspora (spores found in the air) may also play an important role in asthma exacerbation.[7,8] In an on-going aerobiological survey, it was found that approximately 80% of our local airspora consist of fungal spores.[9-12] Spores of *Bipolaris* sp. made up a high proportion of this total and were detected quite frequently in our atmosphere. Skin prick test using extracts of this spore were found to elicit allergic reactions in approximately 25% of our atopic population.[13]

In this study, we quantified the specific IgE levels in sera of 38 individuals toward spores of *Bipolaris* sp. by the Fluorescence Allergosorbent Test (FAST) and compared them with their respective skin prick test results to confirm that the skin reactions were IgE-mediated. Preliminary characterisation of its possible allergenic components were also carried out using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

MATERIALS AND METHODS

Preparation of *Bipolaris* sp. spore extract

Bipolaris sp. was cultured on synthetic (potato dextrose agar, Oxoid) with daily 9 hours of black light to stimulate sporulation. Sporulating cultures were harvested (spores and mycelia) using a custom made vacuum pump harvester and stored at -70°C until further use. Protein extract was prepared by homogenising the spores in 50 mM ammonium bicarbonate buffer (pH 7.4) at a 1:10 (w/v) ratio. The homogenate was then rotated at 4°C overnight on a rotator and later centrifuged at 2,500 rpm for 20 minutes at 4°C. The supernatant was collected and filtered through Whatman qualitative no. 1 filters

From the Department of Paediatrics and [1]Department of Botany, National University of Singapore, Singapore.

Correspondence : Lee Bee Wah, Department of Paediatrics, National University of Singapore, Singapore 119074.

and lyophilised. The lyophilised extract was reconstituted with distilled water whenever needed and protein concentration was measured according to the method described by Bradford [14]

Patient sera

Sera were collected from 38 randomly selected individuals (24 atopics and 14 non-atopics) who had undergone skin prick tests with the *Bipolaris* spore extract. Table 1 shows the skin prick test reactions and the atopic status of these individuals. Their atopic status was evaluated based on their medical history and skin prick test reactions toward house dust mites and other common indoor allergens. Consent was obtained from each subject prior to skin prick and blood testing

Florescence allergosorbent test (FAST)

FAST was carried out according to the method described by Tsay and Halpern.[15] Lyophilised *Bipolaris* spore extract was dissolved in phosphate buffer (pH 7.5) and coated onto carbon activated wells at 100 μl per well overnight at 4°C at a concentration of 22.5 μg well^{-1}. Sample wells were blocked in 200 μl of phosphate buffer containing 0.5% bovine serum albumin (BSA) for 45 minutes, contents aspirated, and allowed to dry overnight in a sealed container containing silica gel. Four microtitration wells coated with perennial ryegrass reference allergen are used as standards. Reference wells and sample wells were washed 3 times with 200 μl of wash phosphate buffer (IgE FAST-Plus Test kit, BioWhittaker, Inc., USA). Fifty microlitres of calibrated perennial ryegrass specific IgE and individual sera were added to the reference wells and sample wells respectively and incubated for 2 hours at room temperature. After washing with phosphate buffer (3 times), 100 μl of anti-human IgE conjugate were added and incubated

Table 1. Skin prick test and FAST results.

No.	Atopic status	Skin Prick Test Reaction*			IgE levels**
		Wheal size	Erythema size	Degree	(IU/ml)
1	Non-atopic	0	0	0	0.13
2	Non-atopic	0	0	0	0.11
3	Non-atopic	0	0	0	0.11
4	Non-atopic	0	0	0	0.15
5	Non-atopic	0	0	0	0.20
6	Non-atopic	3	3	3+	0.44
7	Non-atopic	0	0	0	0.11
8	Non-atopic	0	0	0	0.19
9	Non-atopic	0	0	0	0.10
10	Non-atopic	3	3	3+	0.28
11	Non-atopic	3	3	3+	0.42
12	Non-atopic	3	3	3+	0.48
13	Non-atopic	3	3	3+	0.53
14	Non-atopic	0	0	0	0.11
15	Atopic	0	0	0	0.36
16	Atopic	0	0	0	0.23
17	Atopic	3	14	3+	0.75
18	Atopic	3	15	3+	0.63
19	Atopic	3	3	3+	0.53
20	Atopic	3	3	3+	0.63
21	Atopic	0	0	0	0.13
22	Atopic	0	0	0	0.22
23	Atopic	3	3	3+	0.66
24	Atopic	3	3	3+	0.72
25	Atopic	3	3	3+	0.64
26	Atopic	3	3	3+	0.78
27	Atopic	3	3	3+	0.57
28	Atopic	3	3	3+	0.53
29	Atopic	3	10	3+	0.44
30	Atopic	3	3	3+	0.51
31	Atopic	3	3	3+	0.49
32	Atopic	4	8	3+	0.67
33	Atopic	4	4	3+	0.40
34	Atopic	4	4	3+	0.45
35	Atopic	0	0	0	0.32
36	Atopic	5	5	3+	0.63
37	Atopic	6	35	3+	0.88
38	Atopic	6	13	3+	0.46

*The wheal and erythema (diametre) sizes are given in millimetre and the reactions were classified according to the Paterson's classification for skin prick test reactions.[17]

In this classification, an erythema size of more than 3mm is considered a positive reaction. The presence of a wheal (>3mm) is classified as a strong reaction.

**Specific IgE levels of above 0.35 IU.ml^{-1} are classified as FAST class I positive levels.

for 2 hours at room temperature. After washing, 100 μl of 4-methyl-lumbelliferyl phosphate (in 2-amino-2-methyl propanol buffer at pH 9.5) were added and incubated at 37°C. Fluorescence was read after 20 minutes using a Fluoro FAST Reader (BioWhittaker) and the specific IgE levels quantified relative to the standard curve. Peanut and cat epithelia coated wells along with their respective known positive and negative sera were used as controls in each experiment.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a 10–15% separating gradient gel and 5% stacking gel according to the method of Laemmli.[16] Forty micrograms well^{-1} of *Bipolaris* spore extract was applied to each lane and the gel was electrophoresed at 200V for 4 hours. Proteins were transferred electrophoretically to a polyvinyldifluoride (PVDF) membrane in a Tris-glycine transfer buffer (pH 8.3) overnight in the cold room (4°C) at 30V. Total protein was detected by either staining the protein bound PVDF membrane in amido black or staining the protein gel in Coomassie blue. Immunoblotting was carried out to the remaining part of the membrane.

Immunoblotting

After the electrophoretic transfer from gel onto a PVDF membrane, unreacted sites were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS), pH 7.5 for 1 hour. The membrane was then washed thoroughly with TBS containing 0.05% Tween 20 and TBS only (twice each) and cut into strips 2 cm wide according to the protein transfered lanes. The strips were incubated for 2 hours in individual sera diluted 1:10 in TBS containing 0.5% BSA. These strips were then washed and incubated

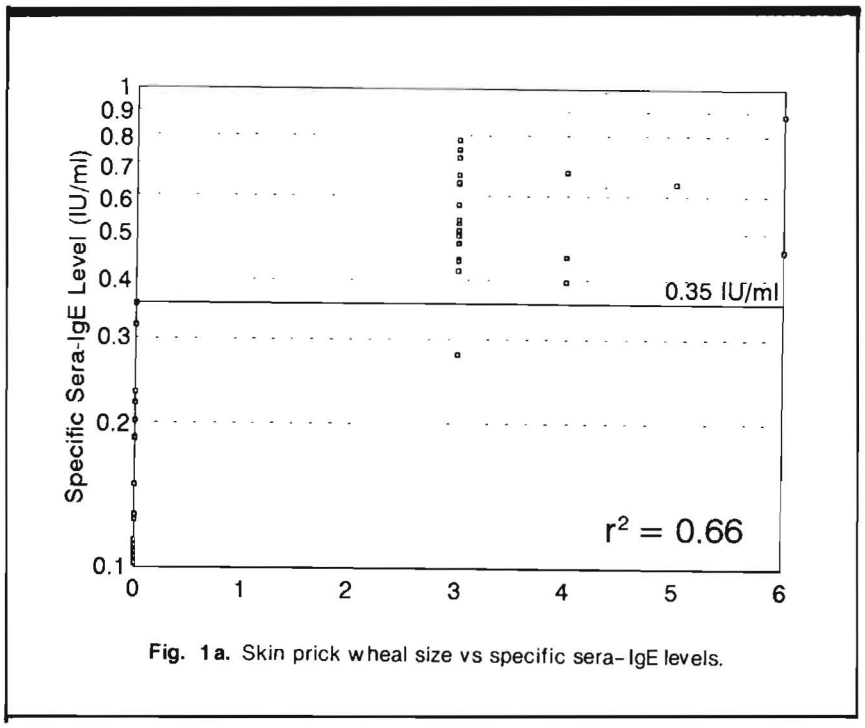

Fig. 1a. Skin prick wheal size vs specific sera-IgE levels.

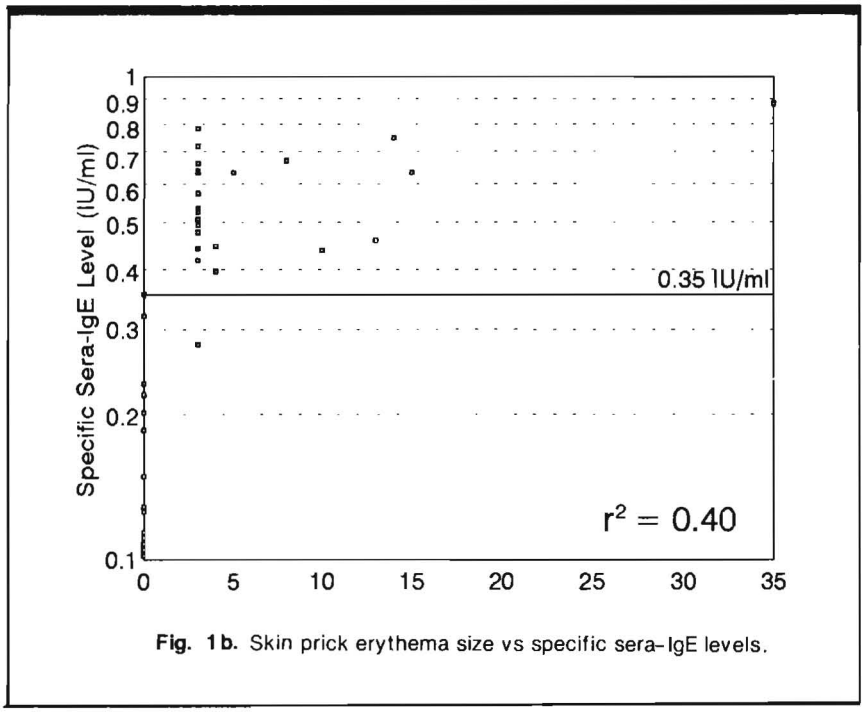

Fig. 1b. Skin prick erythema size vs specific sera-IgE levels.

while its specificity was 13/14 (0.93). (Sensitivity is defined as the proportion of true positive persons who are classified as positive by the test. Specificity is defined as the proportion of true negative persons who are identified as such as the test).

SDS-PAGE and immunoblotting

Via SDS-PAGE, at least 28 protein bands were separated from the crude *Bipolaris* extract with molecular weights (MW) ranging from 11 kilodaltons (kDa) to 100 kDa (Fig. 2). The staining pattern

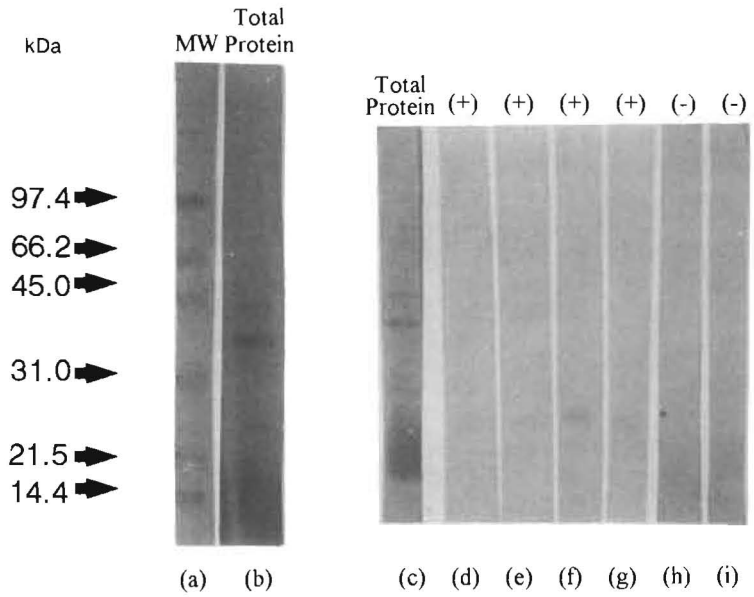

Fig. 2. *Bipolaris* sp. total protein profile following SDS-PAGE and Coomassie blue staining, and its corresponding IgE binding components after immunoblotting (a) low range molecular weight marker; (b) *Bipolaris* sp. total protein profile following SDS-PAGE and Comassie blue staining; (c) *Bipolaris* sp. total protein blot following electroblotting and Amido black staining; (d)-(g) immunoblots of *Bipolaris* sp. with skin prick positive sera showing the IgE binding patterns of the spore protein; (h)-(i) immunoblots of *Bipolaris* sp. with a negative control.

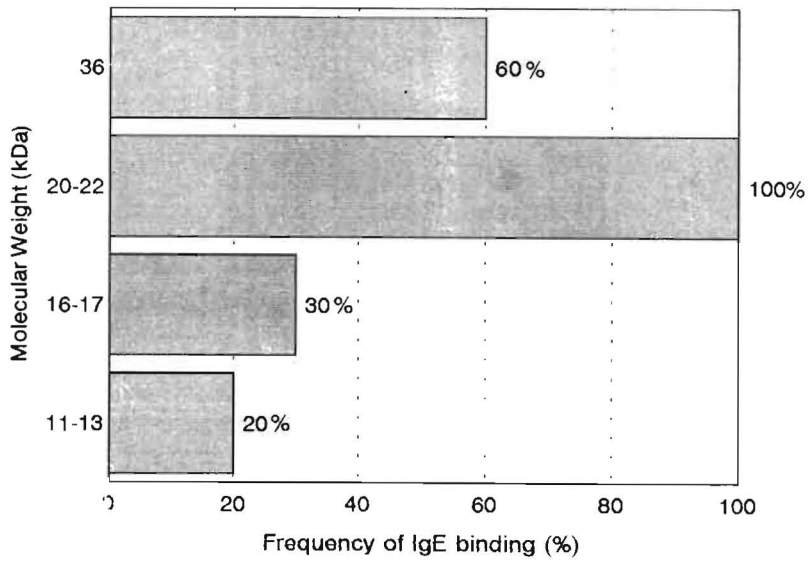

Fig. 3. Frequency of IgE binding.

in gels via Coomassie blue staining and Amido black staining of the for 2 hours at room temperature with horseradish peroxidase labelled anti-human IgE (diluted 1:500 in TBS containing 0.5% BSA). Proteins were visualised using the modified chromogenic detection method of 4-chloro-1-napthol and 3,3-diamino-benzidine. Ten positive sera (skin prick test, 3+) and 5 negative sera were used in this study. In addition, 0.5% BSA in TBS was also used as a negative "blank" control.

RESULTS AND DISCUSSION

Comparison of skin prick test and FAST

The results of skin prick test on the 38 randomly selected individuals (24 atopics and 14 non-atopics) are shown in Table 1. Nineteen of the twenty four atopic individuals reacted positively to an extract of *Bipolaris* sp. spores, while only five of the fourteen non-atopic individuals were skin prick test positive.

When FAST was carried out to quantify the *Bipolaris*-specific IgE levels in sera of these individuals, 20/24 of these atopic individuals were positive (ie 0.35 IU/ml) while 4/14 of non-atopic individuals gave positive results. Results obtained by FAST were found to be comparable to the skin prick test results ($r^2 = 0.60$, $p < 0.001$ for IgE levels vs wheal sizes, Fig. 1a; $r^2 = 0.44$, $p < 0.001$ for IgE levels vs erythema sizes, Fig. 1b). These significant correlation coefficients suggest that higher specific IgE levels in sera of these individuals would elicit stronger skin reactions. The results obtained also suggest that the skin prick test reactions are likely IgE-mediated and thus establish that spores of *Bipolaris* sp. contain components which elicit allergic reactions in sensitized individuals.

Based on these preliminary data, the sensitivity of the FAST,

using the skin prick test as a standard describing the sensitized condition of an individual, was 23/24 (0.96), transferred protein on a PVDF membrane revealed a similar profile. The most intensely stained protein band was at approximately 35–37 kDa and that of the low molecular weights (below 21.5kDa).

Preliminary immunoblotting with 10 positive sera revealed 4 *Bipolaris*-specific IgE binding bands, at 11–13kDa, 16–17kDa, 20–22kDa and 36kDa (Fig. 2). The frequency of reaction of these bands are illustrated in Fig. 3. The five negative sera, along with the BSA control, did not show any IgE binding activity. The 20–22 kDa protein band was the most frequently recognized component (10/10, 100%) with moderately high staining intensity, while the 36 kDa protein band reacted in 6/10 (60%) of the skin prick positive sera tested. The other two components reacted in 20–30% of the sera tested.

This study has thus demonstrated that spores of *Bipolaris* sp. contain allergic components which may elicit IgE-mediated reactions. Preliminary screening of sera of *Bipolaris*-sensitive individuals revealed 4 IgE binding proteins with molecular weights of 11–13kDa, 16–17kDa, 20–22kDa and 36kDa, with that of 20–22kDa likely being a major allergic component of the *Bipolaris* spores.

ACKNOWLEDGEMENTS

This study was sponsored by research grants from the National Science and Technology Board and the National University of Singapore.

REFERENCES

1. Goh DYT, Lee BW, Quek SC, Chew FT and Quek CM. A survey of the prevalence of childhood asthma in Singapore–preliminary findings. J Singapore Paediatr Soc 1994; 36 : 147-52.
2. Ng TP, Hui KP and Tan WC. Prevalence of asthma and risk factors among Chinese, Malay and Indian adults in Singapore. Thorax 1994; 49 : 347-51.
3. Goh DYT, Chew FT, Quek SC and Lee BW. The prevalence and severity of asthma, rhinitis and eczema in Singapore school children. Arch Dis Child (In press).
4. Lee BW, Teo J and Vellayappan K. Role of atopy in bronchial asthma. J Singapore Paediatr Soc 1989; 31 : 53-9.
5. Lee BW, Chew FT, Goh DYT, *et al.* House dust mite allergy–its clinical relevance. J Singapore Paediatr Soc 1994; 36 : 106-111.
6. Chew FT, Goh DYT, Lee BW, *et al.* Asthma exacerbation and air pollution in Singapore. J Allergy Clin Immunol 1995; 95 : 261.
7. Lee BW, Chew FT, Goh DYT, *et al.* Airspora profile and asthma exacerbation in Singapore. XV International Congress of Allergology and Clinical Immunology, Stockholm, Sweden, June 1993.
8. Lim SH, Lee BW, Tan HTW, *et al.* Airspora of the Singapore environment–correlation with asthma exacerbation. The 3rd Congress of the Asian Pacific Society of Respirology, Singapore, July 1993.
9. Tan TK, Tan HTW, Siti Dahlia MD, Lee BW and Chong A. Airborne fungal spores in urban Singapore. The 4th International Mycological Congress, Regensburg, Germany, August 1990.
10. Tan TK, Teo TS, Tan HTW, Lee BW and Chong A. Variation in tropical airspora in Singapore. Myco Res 1992; 96 : 221-4.
11. Tan HTW, Lee BW, Tan TK, *et al.* Airspora of the Singapore environment. The 26th Singapore-Malaysia Congress of Medicine, Singapore, August 1992.
12. Lee BW. Aeroallergens in Singapore. First Asian Pacific Congress of Allergology and Immunology, Bangkok, Thailand, November 1992.
13. Goh DYT. An evaluation of local airspora allergens. Winner of Young Investigator Award. Singapore-Malaysia Congress of Medicine, Singapore, August 1994.
14. Bradford M. A new dye binding method for quantification of protein. Anal Biochem 1976; 72 : 248.
15. Tsay YG and Halpern GM. IgE fluoroallergosorbent (IgE FAST^{TM}) test : concept and clinical applications. Immunol Allergy Prac 1984; 6 : 169-74.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227 : 680-5.
17. Bernard HB. Diagnosis of immediate hypersensitivity. In : Patterson R. ed. Allergic diseases : Diagnosis and management. Philidelphia : JB Lippincott Co. 1985; 102-22.