Chapter 6

RESULTS AND DISCUSSION OF EPITOPE MAPPING OF Der f 10 AND Blo t 5 ALLERGENS

6.1 Synthesis of peptides

Epitope mapping of Der f 10 was carried out in an approach summarised in **Fig. 6.1.** Sequence of the *D. farinae* tropomyosin (Der f 10) allergen with 299-AA residues was first derived from the sequence reported by Aki *et al.* (1994). Based on this sequence (**Fig. 6.2**), 73 peptides were synthesised although the sequence reported in the GenBank (Accession No.: Y14906) started from the 15th residue of the sequence below; i.e. from MEAIKK... From this sequence, octamers with an overlap of four amino acids were synthesized. Another allergen from *B. tropicalis*, Blo t 5 (Gen Bank Accession No.: O 96870) **(Fig. 6.3)** was also synthesized as 12-mer peptides.

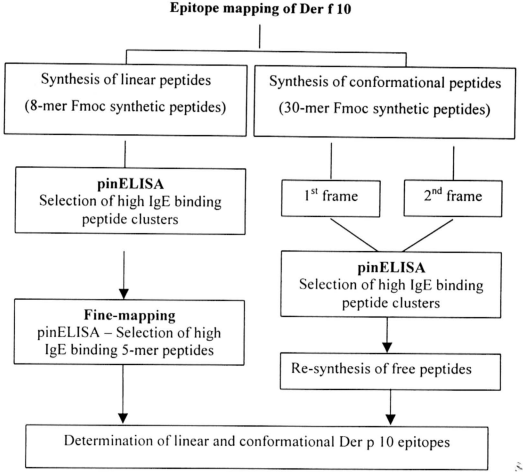


Fig. 6.1: Epitope mapping approach of *D. farinae* tropomyosin (Der f 10)

1:	FFFVAAKQQQ	QPSTKMEAIK	KKMQAMKLEK	DNAIDRAEIA	EQKARDANLR
51:	AEKSEEEVRA	LQKKIQQIEN	ELDQVQEQLS	AANTKLEEKE	KALQTAEGDV
101:	AALNRRIQLI	EEDLERSEER	LKIATAKLEE	ASQSADESER	MRKMLEHRSI
151:	TDEERMDGLE	NQLKEARMMA	EDADRKYDEV	ARKLAMVEAD	LERAEERAET
201:	GESKIVELEE	ELRVVGNNLK	SLEVSEEKAQ	QREEAYEQQI	RIMTAKLKEA
251:	EARAEFAERS	VQKLQKEVDR	LEDELVHERE	KYKSISDELD	QTFAELTGY

Fig. 6.2: Sequence of the 299-AA residues of *D. farinae* tropomyosin (Der f 10)

1:	MKFAIVLIAC	FAASVLAQEH	KPKKDDFRNE	FDHLLIEQAN	HAIEKGEHQL
51:	LYLQHQLDEL	NENKSKELQE	KIIRELDVVC	AMIEGAQGAL	ERELKRTDLN
101:	ILERFNYEEA	QTLSKILLKD	LKETEQKVKD	IQTQ	

Fig. 6.3: Sequence of the 134-AA residues of *B. tropicalis* allergen (Blo t 5)

The Der f 10 and Blo t 5 sequences were appropriately entered into the PepMaker application in the PepMaker software supplied with the Multipin Peptide Synthesis kit. For Der f 10, synthesis schedule was generated for the 73 peptides mite in duplicates, on 146 pins, requiring two blocks. In addition, for each block A and block B, four extra pins were placed at the 1(1,2) and 2(1,2) for the positive (PLAQ) and negative (GLAQ) controls, also synthesized in duplicates (**Appendix 4**). Four peptide syntheses were carried out in this study: (1) Linear peptides of Der f 10 as octamers; (2) Fine mapping of Der f 10 as pentamers; (3) Conformational peptides of Der f 10 as chimeric 16-mer peptides; (4) Linear 12-mer peptides of Blo t 5.

6.2 Determination of quality and sensitivity of synthesized peptides

To determine the quality of peptide synthesis, peptides PLAQ and GLAQ were synthesized simultaneously with the test peptides. Then together with two sets of pre-synthesized control peptide pins supplied in the kit, the reactivity of these peptides with the control antibody supplied achieved in the ELISA assay, served as an indicator of the sensitivity of the synthesised peptides as well as the quality of the synthesis carried out. **Fig. 6.4** shows the values of the reactivity of both the synthesized and supplied control peptide pins. From the absorbance obtained, the synthesized peptides showed reactivity that was relatively comparable to the supplied peptides, thus confirming the success of the synthesis procedure.

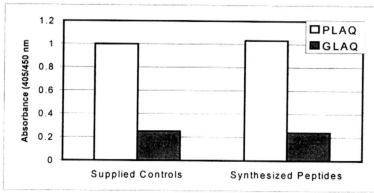


Fig. 6.4: Comparison of the absorbance of the synthesised and the control peptides tested with the monoclonal antibody supplied.

6.3 Pepscan of Der f 10 linear peptides

The 73 peptides were synthesized as octamers overlapping by 4 amino acids on the gears of non-cleavable polyethylene pins as shown in **Fig. 6.5** below;

#1: FFFVAAKQ	#21: AANTKLEE	#41: NQLKEARM	#61: RIMTAKLK
#2: AAKQQQQP	#22: KLEEKEKA	#42: EARMMAED	#62: AKLKEAEA
#3: QQQPSTKM	#23: KEKALQTA	#43: MAEDADRK	#63: EAEARAEF
#4: STKMEAIK	#24: LQTAEGDV	#44: ADRKYDEV	#64: RAEFAERS
#5: EAIKKKMQ	#25: EGDVAALN	#45: YDEVARKL	#65: AERSVQKL
#6: KKMQAMKL	#26: AALNRRIQ	#46: ARKLAMVE	#66: VQKLQKEV
#7: AMKLEKDN	#27: RRIQLIEE	#47: AMVEADLE	#67: QKEVDRLE
#8: EKDNAIDR	#28: LIEEDLER	#48: ADLERAEE	#68: DRLEDELV
#9: AIDRAEIA	#29: DLERSEER	#49: RAEERAET	#69: DELVHEKE
#10: AEIAEQKA	#30: SEERLKIA	#50: RAETGESK	#70: HEKEKYKS
#11: EQKARDAN	#31: LKIATAKL	#51: GESKIVEL	#71: KYKSISDE
#12: RDANLRAE	#32: TAKLEEAS	#52: IVELEEEL	#72: ISDELDQT
#13: LRAEKSEE	#33: EEASQSAD	#53: EEELRVVG	#73: LDQTFAEL
#14: KSEEEVRA	#34: QSADESER	#54: RVVGNNLK	
#15: EVRALQKK	#35: ESERMRKM	#55: NNLKSLEV	
#16: LQKKIQQI	#36: MRKMLEHR	#56: SLEVSEEK	
#17: IQQIENEL	#37: LEHRSITD	#57: SEEKAQQR	
#18: ENELDQVQ	#38: SITDEERM	#58: AQQREEAY	
#19: DQVQEQLS	#39: EERMDGLE	#59: EEAYEQQI	
#20: EQLSAANT	#40: DGLENQLK	#60: EQQIRIMT	

Fig. 6.5: Octamer peptides of Der f 10 as synthesized on the numbered pins. The underlined amino acids were the overlapping residues.

Amino acid coupling of Der f 10 octamers was carried out a cycle per day, which therefore required 8 days for the couplings and another two days for side chain deprotection and drying. A conjugate test was carried out as recommended by the manufacturer to examine the quality of the synthesis. As no primary antibody was used during conjugate testing, the reading observed was low, as expected (**Fig. 6.6**).

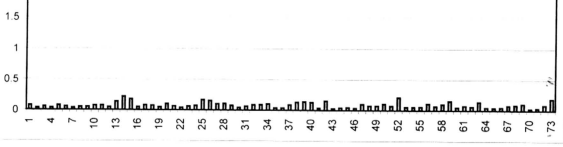


Fig. 6.6: Result of a conjugate test showing IgE binding to the peptides on the pins

These pins, bearing covalently attached octamer peptides were then incubated in primary antibody, secondary antibody, and substrate developer in ELISA plates. The absorbance was measured from the developed plate and the resulting absorbance values were plotted versus peptide number, corresponding to the N-terminal residue number of the peptide in the peptide sequence. A graph was constructed based on results from pinELISA of each patient's Pepscan profile. IgE binding reactivity as detected in a binding assay was considered dependent on the number of IgE epitopes immobilised on the pins which had enough affinity not to be washed out during the washing procedure. The specificity of the antibody binding to each peptide varied for the individual sera. In this study, due to the inherent scarcity of the IgE in the sera, a generally low reactivity was obtained. This made it difficult to pinpoint the exact peptides showing the highest binding reactivity to IgE in the individual sera. The values of absorbance of the individual graphs of IgE-binding reactivity of the sera of 8 allergic rhinitis patients (**Appendix 7a)** were combined in a graph (**Fig. 6.7)** giving a clearer depiction of the antibody-binding profile of the Der f 10 sequence. From this profile the cut-off value was calculated as the (mean + SD) i.e. 0.2217. Therefore any reading above this value would be taken as positive IgE-binding reactivity for the peptides.

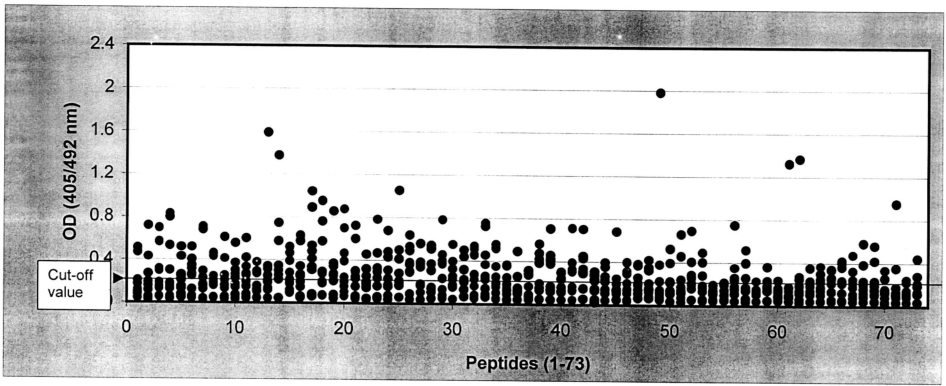


Fig. 6.7: IgE binding reactivities of sera from 8 allergic rhinitis patients to the pin-bound synthetic peptides representing mite tropomyosin Der f 10; as detected by pinELISA. The cut-off value for this pepscan is 0.2217.

These obtained OD values at dual wavelengths of 405/452 nm represented the relative amount of specific IgE in the sera that recognized these octamer peptides. Similarly, for 5 asthmatic patients, the same procedure was carried out to obtain the IgE-binding profiles as shown in **Appendix 7b** and the assembled results as in **Fig. 6.8**. Because there was absence of distinct "peaks" to be regarded as representative of the epitopes on the mite tropomyosin, the plot had to be studied statistically to reveal the potential sites on the mite tropomyosin for antibody binding. Following the recommended algorithm, a cut-off line was calculated by taking the mean added with the standard deviation [Mean + SD], enabling the elimination of non-specific absorbance values along the peptides in the combined plot. Despite the variability in the assay, a few peptides had values above this limit and thus stood out more as "frequently recognised by IgE antibodies". From this analysis, seen here is the frequently recognised linear epitopes, being the common residues of 3 consecutive positive peptides. The percentage of IgE binding to each pin produced so-called "peaks" along the sequence occurred (**Fig. 6.8**), at peptides #14-21 (region I), #25-26 (region II), #29-33 (region III), #41-43 (region IV), #66-68 (region V), and individual peptides; #48, #55 and #63. These peaks were identified as "potential binding sites" whenever a pattern of ascending OD > 0.2 followed by a sharp descent was observed. These regions and the individual peptides from the assay involving the allergic rhinitis patients were determined. The low number of representatives from the asthmatic groups made it pointless to obtain percentage of binding frequency.

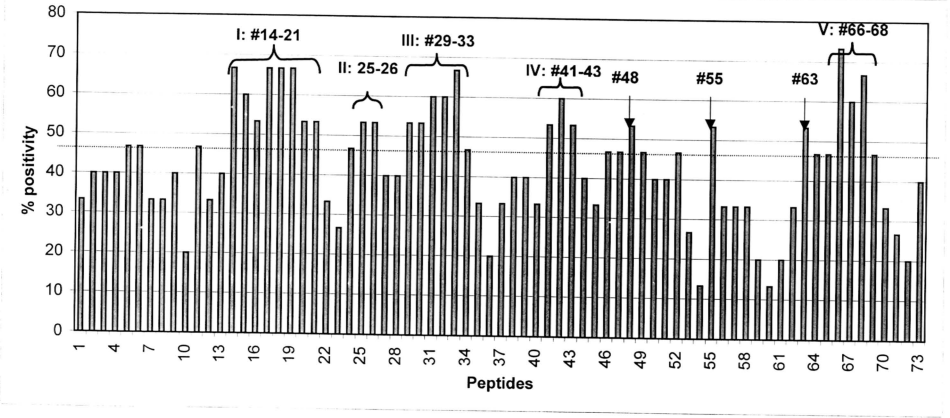


Fig. 6.8: Percentage of positivity of IgE responses to the octamer peptides of Der f 10

Table 6.0: The selected Der f 10 peptide sequences with positive IgE binding reactivities.

N	Peptides/ peptide regions	Start position	Sequence	End Position	No. of residues
1	#14-#21	53	**KSEEEVRALQKKIQQIENELDQVQ-EQLSAANTKLEE**	88	36
2	#25-#26	97	**EGDVAALNRRIQ**	108	12
3	#29-#33	113	**DLERSEERLKIATAKLEEASQSAD**	136	24
4	#41-#43	161	**NQLKEARMMAEDADRK**	176	16
5	#66-#68	261	**VQKLQKEVDRLEDELV**	276	16
6	#48	189	**ADLERAEE**	196	8
7	#55	217	**NNLKSLEV**	224	8
8	#63	249	**EAEARAEF**	256	8

6.4 Fine-mapping of Der f 10 linear peptides

To further analyse the specificity of the peptides for IgE recognition (**Table 6.0**) subsequent re-synthesis of peptide clusters #14-19 and #66-68 in the form of eleven pentamers was done, thereby 'exposing' as much as possible of the suspected major epitopes. Another 44 sera of allergic rhinitis patients were tested and results were also pooled and analysed as was done for the linear Der f 10 peptides. This fine mapping of the #14-19 region showed that 75% of positive sera reacted with the sequence EVRAL while in the #66-68 region, over 80% of the 44 positive sera reacted with three adjacent peptides combined as LQKEVDRLEDELV (**Table 6.1**).

Table 6.1: Percentage of reactivity of the 5-mers against serum samples in the fine mapping following the pepscan of Der f 10

Peptide cluster 14-19	Number of positives (Total = 44)	%
KSEEE	19	43.2
EVRAL	33	75.0
LQKKI	5	11.4
IQQIE	14	31.8
ENELD	14	31.8
DQVQE	19	43.2
EQLSM	20	45.4

Peptide cluster 66-68	Number of Positives (Total = 44)	%
MVQKL	30	68.2
LQKEV	36	81.8
VDRLE	43	97.7
EDELV	35	79.5

6.5 Pepscan of conformational peptides of Der f 10

Peptide synthesis was carried out according to the strategies described in **section 6.4**. These peptides produced in two frame sets (**Fig. 6.9** and **Fig. 6.10**) to increase the periodicity of the synthesized peptides. Inserts from both frames (**Fig. 6.9** and **Fig. 6.10**) were constructed on the individual pins, flanked on both ends with heptads that would coax the 30-mer peptides into a helical structure as described in **section 2.10**. These chimeric peptides were subjected to the same pinELISA with the serum concentration at 1:100; secondary antibody concentration - 1:500. Ten sera from each patient group were tested and the resulting individual profiles of the IgE reactivity throughout the two sets are shown in **Appendix 6a** and **Appendix 6b** (For allergic rhinitis patients) and in **Appendix 7a** and **Appendix 7b** (For adult asthmatics).

I. FIRST FRAME – 38 PINS

1- EAIKKKMQAMKLEKDN
2- QAMKLEKDNAIDRAEI
3-DNAIDRAEIAEQKARD
4-EIAEQKARDANLRAEK
5-RDANLRAEKSEEEVRA
6-EKSEEEVRALQKKIQQ
7- RALQKKIQQIENELDQ
8-QQIENELDQVQEQLSA
9-DQVQEQLSAANTKLEE
10-SAANTKLEEEKEKALQT
11-EEKEKALQTAEGDVAA
12-QTAEGDVAALNRRIQL
13-AALNRRIQLIEEDLER
14-QLIEEDLERSEERLKI
15-ERSEERLKIATAKLEE
16-KIATAKLEEASQSADE
17-EEASQSADESERMRKM
18-DESERMRKMLEHRSIT
19-KMLEHRSITDEERMDG
20-ITDEERMDGLENQLKE
21-DGLENQLKEARMMAED
22-KEARMMAEDADRKYDE
23-EDADRKYDEVARKLAM
24-DEVARKLAMVEADLER
25-AMVEADLERAEERAET
26-ERAEERAETGESKIVE
27-ETGESKIVELEEELRV
28-VELEEELRVVGNNLKS
29-RVVGNNLKSLEVSEEK
30-KSLEVSEEKAQQREEA
31-EKAQQREEAYEQQIRI
32-EAYEQQIRIMTAKLKE
33-RIMTAKLKEAEARAEF
34-KEAEARAEFAERSVQK
35-EFAERSVQKLQKEVDR
36-QKLQKEVDRLEDELVH
37-DRLEDELVHEKEKYKS
38-VHEKEKYKSISDELDQ

Fig 6.9: The first frame of the Der f 10 sequence; fragmented into 16-mers overlapping by 8 residues. These fragments served as the inserts of the whole 30-mer conformational peptides; flanked by 2 heptads on both terminals.

II. SECOND FRAME - 37 PINS

1-KKKMQAMKLEKDNAID
2-KLEKDNAIDRAEIAEQ
3-IDRAEIAEQKARDANL
4-EQKARDANLRAEKSEE
5-NLRAEKSEEEVRALQK
6-EEEVRALQKKIQQIEN
7- QKKIQQIENELDQVQE
8-ENELDQVQEQLSAANT
9-QEQLSAANTKLEEEKE
10-NTKLEEEKEKALQTAEG
11-EKALQTAEGDVAALNR
12-EGDVAALNRRIQLIEE
13-NRRIQLIEEDLERSEE
14-EEDLERSEERLKIATA
15-EERLKIATAKLEEASQ
16-TAKLEEASQSADESER
17-SQSADESERMRKMLEH
18-ERMRKMLEHRSITDEE
19-EHRSITDEERMDGLEN
20-EERMDGLENQLKEARM
21-ENQLKEARMMAEDADR
22-RMMAEDADRKYDEVAR
23-DRKYDEVARKLAMVEA
24-ARKLAMVEADLERAEE
25-EADLERAEERAETGES
26-EERAETGESKIVELEE
27-ESKIVELEEELRVVGN
28-EEELRVVGNNLKSLEV
29-GNNLKSLEVSEEKAQQ
30-EVSEEKAQQREEAYEQ
31-QQREEAYEQQIRIMTA
32-EQQIRIMTAKLKEAEA
33-RIMTAKLKEAEARAEFAER
34-EARAEFAERSVQKLQK
35-ERSVQKLQKEVDRLED
36-QKEVDRLEDELVHEKE
37-EDELVHEKEKYKSISD

Fig 6.10: The second frame of the Der f 10 sequence; fragmented into 16-mers overlapping by 8 residues. As with the first frame, these fragments served as the inserts of the whole 30-mer conformational peptides; flanked by 2 heptads on both terminals.

The IgE binding reactivities of AA-29, AA-27, AA-15 and AA-14 showed distinct peaks at the regions of peptide #18-#25. Peaks were also observed on each profile, almost at the same regions. On the other hand, using sera of AA-18, peaks occurred at the same cluster simultaneously showing 3 reactive peptides (#12, #13, #14).

The collective results were used to construct the consensus plots as shown in **Fig. 6.11-6.14**, each displaying the pepscan of the whole allergen in the first and second frame. **Fig. 6.11** and **Fig. 6.12** show the binding reactivity in 10 allergic rhinitis patients' sera and **Fig. 6.13** and **Fig. 6.14** show the binding reactivity in the sera of 10 asthmatic patients. The upper part of each graph shows the IgE-binding reactivities of the each pin. From these absorbance values, the mean added with the standard deviation (mean ± SD) represents the cut-off value for each graph, thereby resulting in the accompanying figure that shows the number of positive IgE binding reactivities to individual pins carrying the chimeric peptides.

Analysing the IgE binding reactivities of the conformational peptides

Fig. 6.11 shows the profile of IgE-binding in 10 allergic rhinitis patients to the 38 conformational or chimeric peptides. Each column of dots represents the absorbance showing the binding capacity of each peptide to the 10 sera. Only the peptides that were able to capture antibodies at a percentage of above 40% should be considered as the true antibody binding sites. By referring to the first two columns, it is clear that peptide #1 was recognised by 50% of the sera whereas peptide # 2 showed a general low binding of the 10 sera; suggesting that there is a higher likelihood of locating an epitope on peptide #1. Similarly peptide #3, #5, #8, #9, #11, #13, #14, #17, #18, #19 and #21 showed low binding while most peptides like #2, #4, #6, #10 did not show any binding at all. However, the

ALLERGIC RHINITIS PATIENTS

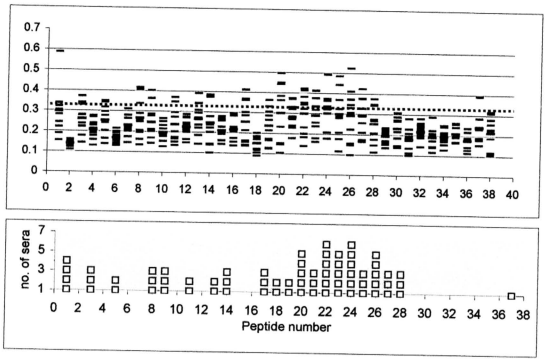


Fig. 6.11: IgE reactivity of sera from 10 allergic rhinitis patients against the 38 peptides from the first frame of the Der p 10 (Cut-off value: Mean+SD = 0.321)

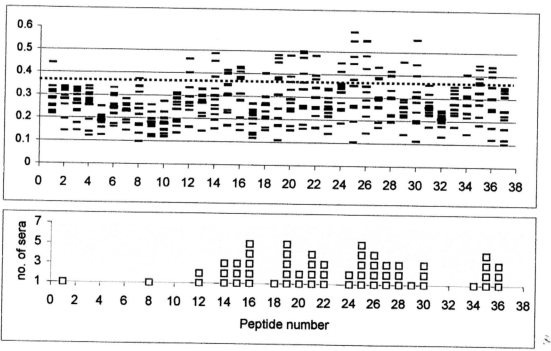


Fig. 6.12: IgE reactivity of sera from 10 allergic rhinitis patients against the 37 peptides from the second frame of the Der p 10 (Cut-off value: Mean + SD= 0.378)

ASTHMATIC PATIENTS

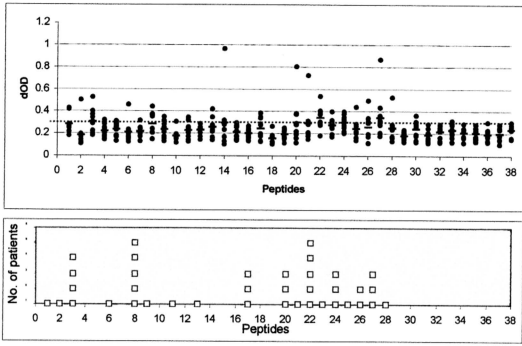


Fig. 6.13:: IgE reactivity of sera from 10 asthma patients against 38 peptides from the first frame of the Der p 10 (Cut-off value: Mean + SD= 0.336)

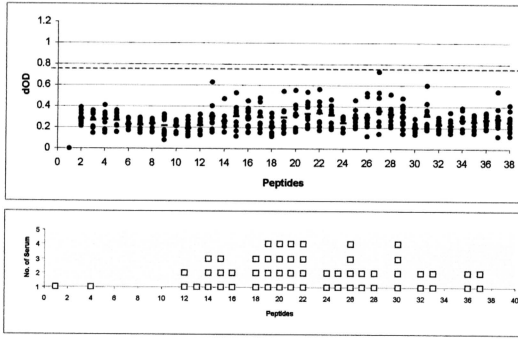


Fig. 6.14: IgE reactivity of sera from 10 asthma patients against 37 peptides from the second frame of the Der p 10 (Cut-off value: Mean + SD= 0.364)

cluster of peptides #20, #22, #24 and #26 showed a strong indication that high binding of IgE had occurred in this region along the Der f 10 sequence. Interestingly, from **Fig. 6.12**, among the second frame of conformational peptides, there is a shift of IgE binding capacity which is strongly related to the shift of synthesis of the conformational peptides (**section 2.9**). Still, the strong IgE binding capacity of the peptides could be observed to occur at the sites within the centre of Der f 10 sequence.

Among asthmatics, as shown in **Fig. 6.13** and **Fig. 6.14**, high binding capacity was observed in the similar regions, but with a lesser degree of intensity especially in the first frame of the conformational Der f 10 sequence. Peptides #3, #8 and #22 in the first frame and #19-22, #26 and #30 in the second frame were more frequently recognized. As observed among the sera of the allergic rhinitis patients, strong IgE binding capacity of the peptides was predominant at the sites within the centre of Der f 10 sequence. This suggests that the central region of the conformational Der f 10 molecule has a propensity to present itself as a site for the location of epitopes.

Combined and analysed as in **Fig. 6.15**, the presentation of IgE-binding reactivities to the pin-bound conformational peptides revealed that there were regions above the cut-off values which could suggest the location of immunodominant sites of the mite tropomyosin. Peptides residues closest to the C-terminal showed the highest binding frequency by the **#22-KEARMMAEDADRKYDE** showed the highest binding frequency (60%). Five other peptides which captured 50% binding frequency were **#20-ITDEERMDGLENQLKE**, **#23-EDADRKYDEVARKLAM**, **#24-DEVARKLAM-VEADLER**, **#26-ERAEERAETGESKIVE** and **#27-ETGESKIVELEEELRV**.

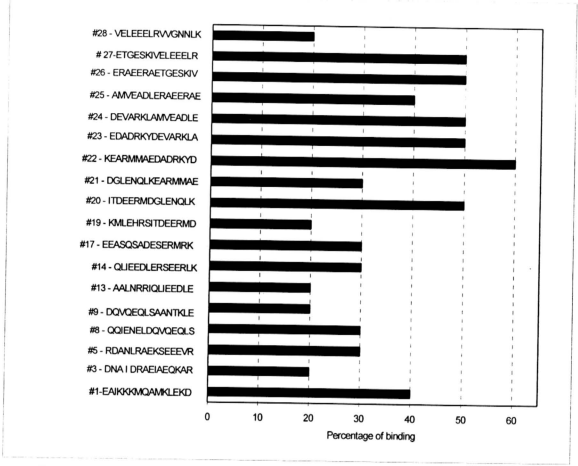


Fig. 6.15: The percentage of positive IgE binding reactivity of sera from allergic rhinitis patients against 16-mers serving as the inserts of chimeric synthetic peptides of mite tropomyosin Der f 10

6.6 ELISA using free chimeric peptides of Der f 10

Synthesis was done in two frames to maximise periodicity. Each peptide was made of overlapping 16-mers and offset by 7 AA, serving as insert in the chimeric peptide. These peptides (kindly provided by Dr J.A. Cooper, QIMR) were synthesized by using an automated synthesizer and did not undergo HPLC purification.

Two sites with consistent highly reactive peptides were identified for further study. Forty HDM-allergic patients were tested with these free conformational peptides, recaptured onto a solid phase (microtitre plate wells) and tested with ELISA method for IgE binding reactivities (**Fig. 6.16**). Peptides CB2-CB4 and CB5-CB7 harboured the

residues that were found to have high IgE reactivity in the linear peptides investigation. Peptides CB8-10 were synthesized based on the result of the pepscan of the conformational peptides at the 25th region or peptide of the first frame. Control peptides were also prepared by constructing the flanking heptads adjacent and repeatedly, without any inserts.

Peptides containing the EVRAL epitope

CB2 – ENKIKQLNLRAEKSEEEVRALQKKIQLENK

CB3 - KQLENKIEKSEEEVRALQKKIQQLENKIKQ

CB4 - ENKIKQLEEEVRALQKKIQQIENEIKQLENK

Peptides containing the VDRLE epitope

CB5 - ENKIKQLERSVQQKLQKEVDRLEDEIKQLENK

CB6 - KQLENKIQKLQKEVDRLEDELVHLENKIKQ

CB7 - ENKIKQLQKEVDRLEDELVHLENKIKQLENK

Peptides of the 25th region

CB8 - ENKIKQLARKLAMVEADLERAEERIKQLENK

CB9 - KQLENKIAMVEADLERAEERAETLENKIKQ

CB10- ENKIKQLEADLERAEERAETGESKIKQLENK

Control peptide

CB11- ENKIKQLENKIKQLENKIKQLENKIKQLEN

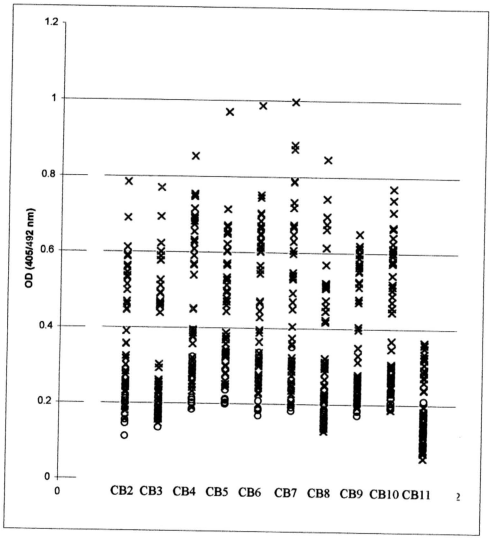


Fig. 6.16: Testing of potential epitope-carrying of Der f 10 peptides after the initial epitope mapping of the full 299 amino acids sequence. Ten peptides were tested out as 30-mer free peptides. Peptides CB2-CB10 were selected from the high IgE-binding reactivity regions (immunodominant regions) while peptide CB11 acted as control. (Red crosses represent IgE-binding reactivity of allergic patients (n=40) and blue circles represent IgE-binding of 5 non-allergic subjects)

As expected, the anti-Der f 10 IgE in the combined profiles of sera, showed a low range of OD, due to its inherent low quantity in the serum. Although not surpassing OD of 0.5, so-called "peaks" occurring along the sequence could still be observed at peptides #12, #14-15, #17-20, #37-40, #50-52, #54 and #57 (**Table 6.2**).

Table 6.2: Peptides of Der f 10 recognized as "peaks", showing high IgE binding reactivities in the pepscan as derived from the consensus plots. These peptides were derived from the sequence in **Fig. 6.2**

Peptides	Start position	Residues	End position	Number of residues
#12	45	**RDANLRAE**	52	8
#14-15	53	**KSEEEVRALQKK**	64	12
#17-20	65	**IQQIENELDQVQEQLSAANT**	84	20
#37-40	145	**LEHRSITDEERMDGLENQLK**	164	20
#50-52	197	**RAETGESKIVELEEEL**	212	16
#54	213	**RVVGNNLK**	220	8
#57	225	**SEEKAQQR**	232	8

6.7 Pepscan of Blo t 5

Blo t 5 is a 134-residue allergen from the house dust mite *Blomia tropicalis*. [GenBank Accession No.: O96780]. Twenty-six 12-mers peptides of Blo t 5, overlapping by 7-mers (**Table 6.3**), with the controls PLAQ and GLAQ were synthesized in duplicates; therefore needing 56 pins on a single block. The whole synthesis procedure was carried out in 14 days.

The peptides were tested on the sera of two groups of patients; a group of 9 allergic rhinitic patients and 12 asthmatic patients; by using the pinELISA method as described in **section 2.11**. Sera were used at 1:50 dilution at 150 µl/well following an hour of pre-coating; and rabbit horseradish peroxidase-conjugated anti-human IgE was used at 1:1000 dilutions.

The individual IgE binding pattern of the Pepscan of Blo t 5 on each serum group is shown in **Appendix 8a** (allergic rhinitis patients) and **Appendix 8b** (asthmatics). The consensus plot for the IgE epitope mapping of the Blo t 5 allergen peptides was constructed for both allergic and asthmatic groups. The same analysis as was carried out for the mite tropomyosin (Der f 10) previously was able to demonstrate a more satisfactory result to pinpoint more definitive regions to be regarded as the epitopes for Blo t 5 IgE binding; thus defining the epitopes. The result of the conjugate test was also compared to these results **(Fig. 5.17)**; showing low background, deemed as appropriate, as no primary antibodies were present. The binding to Blo t 5 showed a repeatable and reproducible pattern among the sera tested, appropriate to be considered as a better pepscan than that shown by Der f 10 peptides. Peaks or region of high antibody binding that stood out on the binding profile of allergic rhinitis patients were located at peptides **#1, #11-#13, #18, #23 and #26**; whereas from the binding profiles of asthmatics, peptides **#1, #3, #12-14, #16, #18 and #26** were indicated (**Fig. 6.17** & **6.18**). From these results, common antibody binding sites of these two allergic group were observed at peptides #1, #11-13, #18 and #26 (**Table 6.4** and **6.5**).

Table 6.3: 12-mer peptides of the Blo t 5 and the corresponding amino acid residues as synthesized in this study

Peptide Number	Residue	Amino acid residues
1	1-12	MKFAIVL IACFA
2	6-17	VL IACFAASVLA
3	11-22	FAASVLAQEHKP
4	16-27	LAQEHKPKKDDF
5	21-32	KPKKDDFRNEFD
6	26-37	DFRNEFDHLLIE
7	31-42	FDHLLIEQANHA
8	36-47	IEQANHAIEKGE
9	41-52	HAIEKGEHQLLY
10	46-57	GEHQLLYLQHQL
11	51-62	LYLQHQLDELNE
12	56-67	QLDELNENKSKE
13	61-72	NENKSKELQEKI
14	66-77	KELQEKIIRELD
15	71-82	KIIRELDVVCAM
16	76-87	LDVVCAMIEGAQ
17	81-92	AMIEGAQGALER
18	86-97	AQGALERELKRT
19	91-102	ERELKRTDLNIL
20	96-107	RTDLNILERFNY
21	101-112	ILERFNYEEAQT
22	106-117	NYEEAQTLSKIL
23	111-122	QTLSKILLKDLK
24	116-127	ILLKDLKETEQK
25	121-132	LKETEQKVKDIQ
26	126-134	QKVKDIQTQGGG

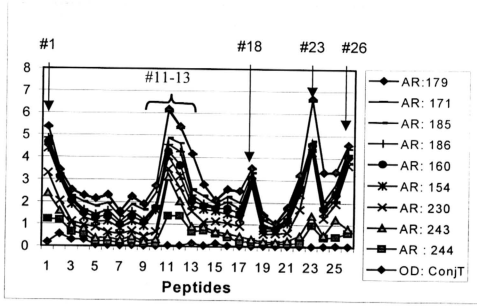


Fig. 6.17: The combined IgE binding reactivities of **9 allergic rhinitis** patients to the 26 linear peptides of Blo t 5. The result of the conjugate test was also included.

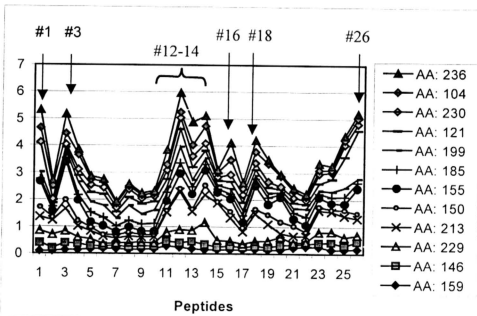


Fig. 6.18: The combined IgE binding reactivities of **12 asthmatic patients** to the 26 linear peptides of Blo t 5.

Table 6.4: Blo t 5 peptides reactive with IgE in the sera of 9 allergic rhinitis patients

Peptide	Residue	Sequence
*#1	1-12	MKFAIVL IACFA
#11	51-62	LYLQHQLDELNE
*#12	56-67	QLDELNENKSKE
*#13	61-72	NENKSKELQEKI
*#18	86-97	AQGALERELKRT
#23	111-122	QTLSKILLKDLK
*#26	126-134	QKVKDIQTQGGG

Table 6.5: Blo t 5 peptides reactive with IgE in the sera of 12 asthmatic patients

Peptide	Residue	Sequence
*#1	1-12	MKFAIVL IACFA
#3	11-12	FAASVLAQEHKP
*#12	56-67	QLDELNENKSKE
*#13	61-72	NENKSKELQEKI
#14	66-77	KELQEKIIRELD
#16	76-87	LDVVCAMIEGAQ
*#18	86-97	AQGALERELKRT
*#26	126-134	QKVKDIQTQGGG

N.B.: * Common epitopes or antibody binding sites: peptides **#1, #12, #13, #18** and **#26**

6.8 Computational analysis of the results

At this juncture, the proposed study to identify epitopes of the Der f 10 and Blo t 5 sequences has located and identified IgE-binding regions through the pinELISA assays by using the non-cleavable synthetic peptides (**Fig. 6.19**).

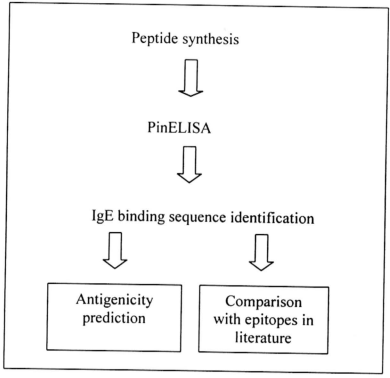


Fig. 6.19: The proposed strategy for identifying potential IgE-epitopes in allergens

This part of the chapter now tries to assess the peptides that were found to be tentative antigenic sites. The exercise in which peptides are examined critically in terms of their strengths and weaknesses complements the whole study of epitope mapping, based on the substantial data collected from past studies used to construct advanced computational programs. For example, Swiss-Prot is a curated protein sequence database that provides a high level of annotations (such as the description of the function of a protein, its domains structure, post-translational modifications, variants, etc.) with high level of integration with other databases. Most often, antigenicity plots are studied and the tentative sequences are further compared with epitopes in literature. One commonly employed algorithm used to

predict antigenicity of a point in a protein sequence is determined by averaging the antigenicity values of this point and the amino acids flanking this point (Hopp and Woods, 1983). Hydrophilic and acidic amino acids, for example, have high antigenicity values. The window size used for the calculation, i.e. the total number of residues that are averaged, can be varied. Hopp and Woods concluded that a window size of six amino acids would be most reliable. The point with the highest score is predicted with high probability to be part of an antigenic determinant of the protein.

6.8.1 Computational analysis for Der f 10

Prediction of the antigenic determinants of the Der f 10 sequence carried out by using MIF Bioinformatics through the http://mif.dfci.harvard.edu/Tools/antigenic.pl revealed the **antigenic propensity** for this protein to be **0.9899**, thus indirectly verifying the potency of Der f 10 as an allergen. From this prediction program, ten antigenic determinants were detected in the sequence as shown in **Table 6.6.**

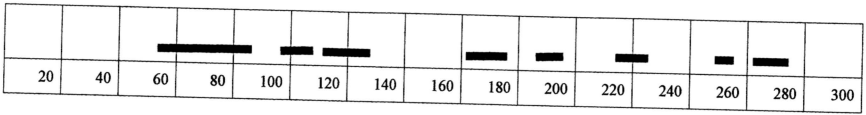
Table 6.6: The predicted sequences of antigenic peptides on the Der f 10 sequence as carried out by using MIF Bioinformatics through the website, http://mif.dfci.harvard.edu/Tools/antigenic.pl

N	Start position	Sequence	End Position	No. of residues
1	4	**VAAKQQQQ**	11	8
2	57	**EVRALQKKI**	65	9
3	71	**ELDQVQEQLSAA**	82	12
4	96	**AEGDVAALNRRIQLIE**	111	16
5	176	**KYDEVARKLAMVEA**	189	14
6	202	**ESKIVEL**	208	7
7	210	**EELRVVGN**	217	8
8	220	**KSLEVSE**	226	7
9	257	**AERSVQKLQKEVDR**	270	14
10	272	**EDELVHE**	278	7

The longest predicted antigenic peptide in the Der f 10 sequence is a 16-mer peptide **AEGDVAALNRRIQLIE** while the shortest are three 7-mer peptides, **ESKIVEL**, **KSLEVSE** and **EDELVHE**. Interestingly, some of the Der f 10 peptides that were found most likely to harbour B-cell epitopes through the pepscan in this study were also predicted as the antigenic sites through computational means. For instance, the peptide found in the linear epitope pepscan, **EVRAL,** was found to be part of peptide No. 2 while another one, **VDRLE**, has its three first residues being part of peptide No. 9 (**Table 6.6**). From the conformational peptide study, chimeric peptides CB8 and CB9 at the 25th region (**section 6.6**), [CB8 - ENKIKQLARKLAMVEADLERAEERIKQLENK and CB9 – KQLE-NKLAMVEADLERAEERAETLENKIKQ] were found to have part of the residues (i.e. **KLAMVEA**) within peptide No. 5 of the predicted antigenic sequences.

Fig. 6.20 shows the comparison of regions showing IgE binding reactivities on the Der f 10 sequence as shown by pepscans using sera from allergic rhinitis (Ist row) and from asthmatics (2^{nd} row). The third row shows the antigenic regions as predicted by http: mif.dfci.harvard.edu/Tools/antigenic.pl. Pepscan using linear peptides detected IgE-binding sites on 8 clusters of the Der f 10 sequence while using the conformational peptides, 7 cluster regions. Through Pepscan, significant IgE binding was not detected at both N- and C-termini but mostly at the central region of the Der f 10 molecule. Although with the linear peptides, IgE-binding occurred almost along 96^{th} – 130^{th} residues, conformational peptides showed no binding at this region. Pepscan using linear peptides traced IgE-binding regions at nearly 42% of the 299-residue Der f 10 sequence while Pepscan using conformational peptides showed IgE binding at only 30% (92/299) of the sequence. Through the computational analysis, predicted IgE-binding regions covered 34% (102/299) of Der f 10 sequence, at ten separate regions with the longest being 16-residues in length. One distinct predicted region that was not detected by Pepscan was the 4^{th}-11^{th} residues, while one region which had significant IgE-binding among the conformational

Analysis using linear peptides:



Analysis using conformational peptides:

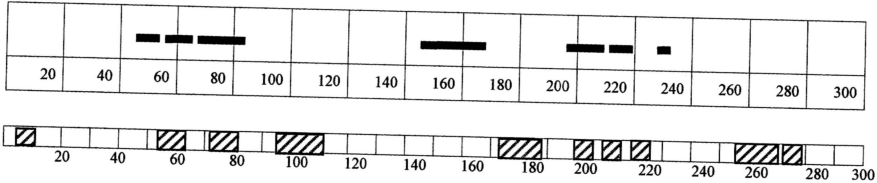


Fig. 6.20: The comparison of regions showing IgE binding reactivities on the Der f 10 sequence as shown by pepscans using sera from linear peptides (from Table 6.0) and conformational peptides (**Table 6.2**). The third row showing truncated shaded blocks, sows the antigenic regions as predicted by http://mif.dfci.harvard.edu/Tools/antigenic.pl (**Table 6.6**)

peptides (at 145th-164th residues of Der f 10) was not among the predicted regions. An estimated 50% of the IgE-binding regions detected through Pepscan using linear peptides were found to be concomitant with the predicted antigenic regions, while with the IgE-binding regions detected by conformational peptides, only about 35% was found to be concomitant with the computational predicted regions.

6.8.2 Computational analysis for Blo t 5

Similarly derived as for Der f 10, the predicted antigenic peptides of the 134-residue Blo t 5 sequence showed a higher antigenic propensity for this protein i.e. 1.0259. Only five antigenic determinants were detected in the sequence as shown below in **Table 6.7**. **Fig. 6.21** shows the comparison of pepscan regions of IgE binding reactivities on Blo t 5 sequence. The third row shows the antigenic regions as predicted by http://mif.dfci.harvard.edu/Tools/antigenic.pl. As shown in **Table 6.4** and **Table 6.5**, through Pepscan, the common IgE binding sites were found on peptides #1, #11-#13, #18 and #26. Therefore, when plotted along the Blo t 5 sequence, the sera from both groups, the allergic rhinitis and asthmatic patients showed almost similar IgE binding sites of the Blo t sequence. Most obvious are the three regions where IgE binding occurred; at both N- and C-termini and the central region of the molecule. Although with the asthmatics sera, IgE binding occurred almost along 55th – 96th residues, allergic rhinitis sera showed a non-binding region at about 72nd-85th residues. Through the computational analysis, IgE binding regions were predicted to occur at nearly 40% of the whole Blo t 5 sequence, at five separate regions with the longest being 15-residues in length. One distinct predicted region that was not detected by Pepscan was the 31st-40th residues. On he other hand, one peptide region showing significant IgE binding in the Pepscans of both groups of sera, but was not among the predicted antigenic region, was the last peptide #26, housing the 126th-134th residues. On the whole, an estimated 75% of the predicted antigenic regions were found to be concomitant with the IgE-binding regions detected through Pepscan.

6.9 DISCUSSION

Synthesis of Der f 10 and Blo t 5 peptides

Unlike the common practice of many researchers, an *a priori* selection from hydropathy plots for hydrophilic regions (for example by Hopp and Woods, 1981, Geysen *et al.*, 1986) was not done in this study. In an earlier allergen characterisation of epitope structure of the codfish allergen M (Gad c 1), an *a priori* selection of potential sites, was recommended (Elsayed and Appold, 1983). However, Shin *et al.*, (1998) proved that hydrophobic residues located in the centre of the Ara h 1 (peanut allergen) epitope appeared to be most critical to IgE binding. This proved that an *a priori* selection to eliminate hydrophobic residues need not be done and the choice to synthesize the whole Der f 10 sequence in this study was right. All 73 overlapping octamers covered the whole sequence of mite tropomyosin Der f 10 to ensure that search for epitopes was focused on all possible promising sites, without running the risk of missing significant epitopes. The difference of species (*D. pteronyssinus* and *D. farinae*) need not be a contention, as the sequence study of both Der p 10 and Der f 10 showed 98.84% homology between the two proteins, with difference only in 3 amino acids (Asturias *et al.*, 1998).

The linear peptides of Der f 10 in this study were synthesized in the size-range of between 5 (for fine mapping) and 8 residues for the primary pepscan. This is based on the estimated length of linear epitopes to be 5-8 residues long whereby scans of octapeptides are expected to detect them all (Kabat, 1970; Schechter *et al.*, 1970). However, Goodman (1969) emphasised that the minimum size of peptides recognised is not solely defined by the dimensions of the antibody-combining site of the amino acid sequence, but also on the minimum size of the peptide necessary for it to assume secondary configurations for antibody binding. This is because specificity is determined by an average of only four to five amino acid residues in an epitope. On the other hand, Rhodes *et al.* (1984) found that the ability to be recognised by antibody also diminishes as peptide length is shortened from

twenty to nine amino acids; due to two effects; (1) deletion of specific amino acid sequences to which the antibody binds and (2) a change in the conformation of the peptide antigen. This further suggests that possible recognition patterns are not mutually exclusive and that observed reactivity may in fact reflect a combination of features such as number of peptides and their folding. Similarly, Beattie and co-workers (1992) pointed out that although reactivities of antisera were detected against octapeptides, the areas of reactivity generally covered 10-13 continuous amino acid residues. Based on their data, they further interpreted that different populations of antibodies may indeed recognize pin-bound octapeptides as true continuous peptides or a smaller core peptide of six or seven residues whose sequence is shared amongst two or three sequential pin-bound peptides. Until 1996, available strategies for reconstructing assembled (discontinuous) epitopes using linear peptides have not proven successful in many cases (Rodda and Tribbick, 1996). In turn, the entire stretch of 10-13 residues may, in fact, represent a single small discontinuous epitope and that antibody reactivity to octapeptides derived from this region may reflect binding to discrete parts of the epitope (Beattie *et al.*, 1992). Moreover, seeking shorter peptide with the same epitope activity is easier and cheaper to synthesize and characterize.

PinELISA of Der p 10 and Blo t 5 synthetic peptides

In this study, the pinELISA results against Der f 10 peptides showed, not the lack of binding, but the lack of a reproducible, expected pattern of a considerably "good" Pepscan. In another synthesis and subsequent pinELISA, linear Blo t 5 peptides showed better performance in terms of more distinct IgE binding. When Blo t-positive sera were tested against Blo t 5 peptides, distinct and repeatable results were observed among the Pepscan conducted. Almost similar patterns of IgE bindings were noticed in the assays, suggestive of good and reliable Pepscan. This could have been the result of a definite allergy to Blo t 5 in most of the Blo t-positive patients selected and tested, since this group of allergen has been found to elicit IgE binding in >50% of the patient *B. tropicalis* as

mentioned in Chapter 5 and as reported by Asturias *et al.* (1998). Furthermore, group 5 HDM allergens have been found to have a high IgE binding strength (Thomas *et al.*, 1998) when compared to the group 10 mite allergens.

The 73 pins bearing Der f 10 linear peptides were not able to show distinct repeatable reactivity profiles, unlike the 26 pins bearing Blo t 5 peptides. The basic reason for this could be the "selective antigenicity" of the patients whose sera were used for the assays. In **Chapter 5**, when recombinant allergens were used on the same serum of a patient, an array of selective antigenicity or more appropriately, "selective allergenicity" was observed among patients. By this, when looking at the IgE-binding capacity of the serum tested on the various recombinant allergens, a patient might be allergic to only a few of the components in the total crude allergen. In the same light, if a patient is not allergic to a particular component allergen, such as Der f 1 or Der f 10, then the serum will not show a distinct pattern of IgE binding for Der f 1 or Der f 10 peptides. Therefore, although Der f-positive sera were used, some did not react to the Der f 10 peptides; simply because the patients were not allergic to Der f 10 or the anti-Der f 10 antibodies were too low to be detected. As this part of the study was carried out earlier than the recombinant allergens study, and rDer f 10 was not available then, the assays involving Der f 10 peptides were tested on "sera with Der f allergy" and not using 'sera with Der f 10 allergy". Undoubtedly, it would have been more precise if the antisera against Der f 10 were used.

Besides having no means to isolate Der f 10-allergic sera, pinELISA using Der f-allergic sera was however utilised on the basis of Der f 10 allergen being a "major allergen" as classified by Aki and co-workers (1995). Logically, if Der f 10 had been detected in 80% of the sera tested in their study, making it a major allergen, then assays involving Der f-allergic patients would have had almost 80% chance of showing positive results. Unfortunately, this was not observed and it was later in the study when recombinant mite tropomyosin (Der p 10; which has at least 98% homology to Der f 10) was tested on

Malaysian HDM-allergic patients that this extrapolation was found not applicable. In Malaysian population, Der f 10 was found to elicit IgE in only about 25-30% of the population, making it a minor allergen as opposed to the Japanese study. This eventually solved the confusion as to why the Der f 10 peptides could not show distinct and similar binding reactivity and subsequently had to be analysed by using the algorithm. It has been an inadvertent step in this study to try out the peptides on "sera with mite allergy" and not "sera with mite tropomyosin allergy" because there were no means to differentiate them then. The spurious sweeping statement, that when one is allergic to HDM, he/she is allergic to all the repertoire of allergens in the mite extract had affected the outcome of the mite tropomyosin pepscan. It would have been more appropriate to screen for individuals who were allergic to mite tropomyosin Der p/f 10 *per se* and only then use their sera for a more specific result. Perhaps, had the recombinant Der p 10 been used to identify the exact sera containing anti-Der p/f 10 IgE, a more definitive epitope would have been established. Another alternative is to test the peptides with a larger population to distinguish Der p 10-positive binding patterns among all the profiles.

The test of reproducibility is the comparison of replicate pins or replicate wells of captured peptide. It has been found that OD shown by replicate pin peptides varies 20% within an assay, where assay-to-assay variability is excluded. Another factor that must be considered in describing reproducibility is the constancy of the "background" of an assay. Due to the extensive nature of whole sets of peptides homologous to a particular antigen, it is feasible to treat some of the peptides as negative controls for the whole set. The test of specificity compares the results of testing sera from allergic individual with sera from non-allergic individual who should be seronegative for the whole allergen. In the pinELISA, successful peptide-antibody binding involves the complementarities between antigen combining sites of antibody, maintained at least partially in both shape and charge (Geysen *et al.*, 1986; 1987b; Tribbick *et al.*, 1989), including stereochemistry of residues in binding peptides, direction of the main chain and adequate peptide length.

The relevance of linear epitopes of Der f 10 described in this study has been confirmed in two ways. First, they reacted with a large panel of sera with allergy. Second, using different antibodies in assays, it was shown that antibody binding to Der f 10 showed different binding pattern for each antibody. Determination of minimal epitope length is another important part of the antibody characterisation procedure. The operational state of the peptide (in solution or immobilized by a certain procedure) plays a major role in determination of the minimal epitope length, already recognised by Van Regenmortel and Pellequer (1994).

Identifying epitopes needs observation of the antibody binding to a peptide. The binding observed may either be irrelevant to the antigen in question, or may be due to antibodies to minor antigen population, such as in denatured antigen. Reasons other than the loss of the epitope, such as stearic interference, long-range conformation changes caused by rapid degradation, may affect the efficiency of epitope mapping. In this indirect test, primary binding of an antibody to peptide is detached by secondary binding of an enzyme-labeled anti-species antibody (conjugate) to the primary antibody, and the detection of the enzyme of the bound conjugate is carried out by substrate conversion. A critical control in such experiments is to show that the conjugate itself does not bind directly to the peptide as a source of artifact.

When serum and overlapping synthetic peptides were used to map the linear, IgE-binding epitopes of Der f 10, not all the sera showed a distinct pattern of IgE-binding reactivities to enable the epitopes to be easily located. As explained by Pomés *et al.*, (2001), allergen dose, route of exposure, and the genetic predisposition of the host are key factors that affect the ability to mount allergen-specific IgE (and IgG) responses. In fact, Reese *et al.* (2001) emphasized that no system for epitope identification will detect **all** epitopes and that the relevance of identified epitopes has to be confirmed with other methods such as inhibition studies, crystallographic analysis or the immunological evaluation of modified whole allergens. For example, in contrast to previous observation

that showed the IgE binding epitopes being distributed evenly along the linear sequence of the molecule, Shin *et al.*, (1998) found that each of the IgE binding epitopes on a homology-based tertiary structure model of Ara h 1 presented themselves in clusters at two main regions. Therefore, although hydrophilicity and hydrophobicity plots provided the first basis of antigenicity prediction for a region of interest in a protein, re-synthesis of a set of truncated versions of a larger epitope peptide allows identification of 'minimal' epitope, important for the antibody characterisation procedure.

Analysis of linear IgE epitopes of Der f 10

The interaction between an allergen and an IgE antibody is highly specific whereby the antibody with its variable site recognizes the ***IgE-binding epitopes***. Epitope analysis has been restricted to the use of polyclonal sera of patients with allergy (Steinberger *et al.*, 1995) because it is still very difficult to isolate intact human monoclonal allergen-specific IgE antibodies. Furthermore, due to the high background as a result of using polyclonal sera of the patients, the antibody binding profile patterns of each serum is therefore not easily amenable to interpretation using conventional statistics. Thus, analysing the Pepscan result is based on the premise that an analyst is able to visually examine the antibody binding to the amino acid sequence profile in the form of a chromatogram and intuitively performs a critical and comprehensive assessment through identification of obviously abnormal levels (as compared to the negative and conjugate test or even with other low binding peptides) or assessment of repeatability or variability of results within the profile.

In this study, peptides #19-#27 demonstrated 40-60% IgE binding reactivity clustered within the chimeric peptides. Among sera of a few asthmatic patients, prominent binding patterns especially at regions between peptide #15 and #65 were observed. The primary scanning of the synthesized tropomyosin peptides, identified the sites of immunodominance at peptides #14-19, #31-33, #46-49 and #66-68 (**Fig. 6.8**). Poor signal to background ratio can be attributed to the lack of a major population of antibodies in the sera which could recognize the linear epitope of the immunodominant region. However, on the other hand, generally high binding among the pins could be an inherent property stemming from the polyclonal sera used. Polyclonal serum contains a variety of antibodies and biological entities, as opposed to the monoclonal serum, containing only specific antibodies raised against it. These variable contents are believed to contribute to non-specific binding which might mask or even append to the binding of antibodies to the putative epitope sites on the peptides. The use of monoclonal antibodies would have made a difference in the resulting pinELISA. Perhaps too, the act of precipitating non-putative antibodies in the serum, prior to the serum incubation of the pin-bound peptides might help in lowering the incidence of non-specific binding. For example, IgG antibodies can interfere with assays for IgM antibodies by competing for substrate binding sites or by forming immune complexes with rheumatoid factor, which can mimic IgM (Doerr *et al.*, 1987).

Epitopes common to the majority of the defining sera or giving the highest titres were selected for further investigation (Geysen *et al.*, 1987a). When an antibody bound more than one adjacent overlapping peptide of a given length, synthesizing and testing sets of shorter peptides usually determined the boundaries of the epitope. The nett absorbance value for the IgE reactivity with each peptide was obtained by subtracting the background values for the individual octapeptides, overlapping four amino acids, corresponding to the mite tropomyosin were tested for IgE binding reactivities using sera from HDM allergic individuals. Peptides which show high OD readings evidently have higher frequency of

antibody binding, therefore suggesting the presence of significant portions of epitopes on it. In most of the assays, sera tested against the peptides were from allergic patients chosen based on their SPT results and only a few controls were used for comparison for each system. However, such systematic test of peptides sets in a bioassay can give a data that is interpretable without recourse to additional controls, because such a set includes many sequences that are unlikely to be reactive sequences i.e. they would automatically act as internal negative controls (Rodda, 1997).

Analysis of conformational IgE epitopes of Der f 10

The delineation of individual patterns of reactivity to epitopes of Der p 1 and Bet v 1 has failed to reveal dominance of specific epitopes. The analysis of epitope-specific antibody response of eight patients to Der p 1 (Greene *et al.*, 1991) indicated that individual sera possessed antibodies toward several of the epitopes defined by a panel of recombinant peptides. Likewise, patients with tree allergy showed that IgE antibodies of individual sera identified several different epitopes of tree Group I allergens (Ipsen *et al.*, 1992). In another study involving epitope mapping of mite faecal allergens Der p 1 and Der f 1 synthesized as overlapping decapeptides by Collins *et al.* (1996), failed to detect specific IgE binding. These failures led to a general conclusion that 'major' epitopes of these allergens are probably discontinuous and require much larger amino acid sequences or even an intact, native molecular conformation to display specific binding activity. This justifies why the conformational Der f 10 peptides were synthesised and tested for more specific IgE binding recognition.

In terms of the sheer number and overall simplicity with which peptides can be simultaneously synthesised and subsequently tested, Maeji *et al.*, (1995) maintained that the pin-bound method proposed by Geysen *et al.*, (1984) is superior. However, reservations about this method of peptide synthesis stem from the inability to assess the purity of the peptides covalently bound to polypropylene pins, directly despite the

consistency of the results in numerous immunological studies (Geysen *et al.*, 1987b). An interesting observation that was made with this small population of test sera, over 40% chimeric peptide #22 representing residue KEARMMAEDADRKYDE was recognised by 6 out of 10 allergic rhinitis patients. This peptide too was recognised by majority of the patients with asthma, making it a common peptide recognised by the IgE antibody.

It is always questionable whether chimeric synthetic peptides used for epitope mapping, are indeed conformational and whether they are stable enough to withstand the rigorous epitope mapping assays. Kammerer *et al.*, (1998) highlighted a rather puzzling and frequently made observation that relatively long heptad-repeat-containing polypeptide chain fragments fail to form coiled-coil structures. This failure cannot be simply explained by instability caused by the type of residues occupying the ***a*** and ***d*** positions of the heptads repeats or by electrostatic repulsion of the two chains. This observation further raises the question whether distinct sites exist within heptad-repeat-containing amino acid sequences to mediate coiled-coil formation. From documentation, a distinct 14-residue "trigger" sequence existing within the cortexillin I oligomerization domain was found imperative to mediate proper assembly of the domain into the parallel homodimeric coiled-coil (Steinmetz *et al.*, 1998). Thus, this suggests that the presence of heptad repeats *per se* is not sufficient for stable coiled-coil formation. In this study, the synthesis of 30-mer peptides was presumed to exhibit helical propensity thereby mimicking the mite tropomyosin, on the basis that there were distinctive binding patterns and not a generally negative outcome during pinELISA.

Considerations for antigenicity

Linear epitopes are conveniently defined in terms of the length of the minimum sequence needed for maximal binding. When so defined, the N- and C-terminal residues of the antibody-binding peptide are essential to the binding, i.e., are "contact" residues. The peptide side chains and the state of the N and C termini determine the overall

hydrophobicity of the peptide. In general, the more charged the groups, the more likely a peptide is soluble in aqueous neutral solution. A common modification is to acetylate the amino terminal, as was done in this study. These modifications eliminate the charge groups on the ends, allowing the peptide to assume a conformation more closely resembling that of internal peptide segments, rather than the protein termini. However, as shown in analogous studies, most linear epitopes may contain one or more residues that are not involved directly in the interaction with the antibody (Geysen *et al.*, 1988). It is also believed that these charged residues of proteins are more likely to be exposed on the surface, while the non-polar and neutral residues stay buried in the interior. In addition, Bosshard, (1995) opines that although one concedes that Pepscan and related methods can reveal only the subset of the sequential epitopes of a protein, one has to keep in mind that many sequential epitopes may not exist on the native protein, but only on its unfolded or partially unfolded isoforms. This knowledge might apply to most aeroallergens, including house dust mites.

Are synthetic peptides representative of antigenic structures of the native protein?

Peptides made or captured on a solid phase are very convenient for repeated testing in simple binding assays. However, difficulties may arise from uncertainties pertaining to the efficiency of synthesis i.e. whether a resulting peptide presents itself optimally to allow interaction with macromolecule such as antibodies. Possible reasons leading to negative outcomes are incomplete peptides missing one or more amino acids; peptides that still retain one or more side-chain protecting group or peptides modified during the synthesis or the cleavage step. Furthermore, the occurrence of false positive or false negative result might occur when using pins and resin, as a result of the significant disadvantage of such passive coating methods compounded with the lack of assessment of quality and quantity. In this study, peptides were not initially raised to be recognised and thereafter to elicit the whole cascade of raising antibodies specific to them. In actual, the question lies in whether the peptides synthesised on the pins could represent part of the antigen mimicking the

invading antigen as recognised by the polyclonal antibodies. This aim not only has to depend on the success of the synthesis, but also on the success of the pinELISA, i.e. the effective binding of antibodies to the peptides.

Is the motive of epitope mapping in this study justified?

Major antigenic/immunogenic determinants (structures which, in the context of the native protein, are naturally recognized by the immune system) seem to correlate well with regions of extraordinary surface exposure and flexibility. Do IgE antibodies from asthma and allergic rhinitis patients recognize peptides differently? Dillner *et al.*, 1987 suggested that the immunological and disease status of patients might influence the responsiveness to different antigenic epitopes, resulting in variable antibody reactivity to different synthetic peptides. The identification of IgE-binding epitopes until now has not answered the question of what makes an antigen an allergen, but in the future it may help to differentiate between patient groups and disease courses (Bufe, 2001). The recognition motifs, i.e. epitopes, for IgE binding are defined by the three-dimensional molecular structure of the allergen. It is thus necessary to determine the molecular structure of the allergen in order to identify and characterize its epitopes. The specificity by which the IgE antibody recognises the allergenic peptides is the basis of mapping the epitopes of proteins. This rests on the assumption that peptides can simulate some of the features of the 'true' epitope recognised by an antiprotein antibody. Different parameters such as the degree of affinity and also binding frequency are to be observed in epitope mapping. While the strength of binding reactivities is reflected in the absorbance of the signals shown by the antigen-antibody complex formed, the frequency of this binding in a series of pinELISA assays would also disclose the pattern of the peptide recognition throughout the sequence. Therefore, when dealing with polyclonal serum that fails to show the exact peptides as the epitopes in a sequence, the consensus plots constructed may provide a clearer path towards identifying or deducing putative immunodominant regions.

Peptide-based assays have identified the antigenic regions of discontinuous sites even to the resolution of single critical amino acids within the sites (Appel *et al.*, 1990; Redlich *et al.*, 1991; Savoca *et al.*, 1991). The term "discontinuous" epitopes refers to epitopes made of distant amino acids brought into proximity by the folding pattern of the protein. Understandably, these conformational peptides are more difficult to mimic than amino acids that are in linear array because of the different relationship between amino acids (Gibbs, 1996). In the case of tropomyosins, as coiled-coil proteins, conformational peptides pinpointed as the antigenic determinant sites for IgE can either represent discontinuous epitopes or can be considered merely as epitopes presenting themselves as closely as possible to the native coiled-coil of tropomyosin. Short synthetic peptides can also give important information about conformational epitopes, since all conformational epitopes contain linear segments (Getzoff *et al.*, 1988). Therefore, the fact that portions of Der f 10 linear epitopes were found to be "embedded" in the later-revealed conformational epitopes, suggests that IgE antibodies recognised the epitopes; for having the right amino acid residues and also the right folding. Indirectly, the epitope mapping carried out in this study fulfilled the target of locating the antigenic sites through both functional and structural features of Der f 10. Thus, this underlines the important use of synthetic peptide strategy to assess polymorphic form-specific epitopes.

Only a small number of defined sequential IgE epitopes on a single molecule were identified, for example, on grass pollen, allergen Lol p 5 (Ong *et al.*, 1995). Ara h 1, the major peanut allergen was found to have a minimal epitope size of 6 consecutive residues (Elsayed and Apold, 1983). Most often too, the greater part of epitopes turned out to be conformational (Schramm *et al.*, 1999; Lombardero *et al.*, 1990). Birch pollen allergen, Bet v 1, lost its IgE-binding capacity when divided in 2 fragments, a C-terminal and N-terminal part (Vrtala *et al.*, 1997). Der p 2, a major mite allergen had no allergenic activity after deletion of a structurally stabilizing disulphide bond, i.e. when the compact structure was destroyed by genetic engineering. However, the disruption of a sequential epitope on grass

pollen allergen Phl p 5b, by site-directed mutagenesis did not lead to a substantial loss of IgE-binding capacity toward the holoallergen. Instead, only a deletion of a region responsible for the stability of the 3-dimensional structure of the allergen induced a significant reduction of both IgE-binding capacity and allergenic activity. Thus, the structural integrity of an allergen is an important prerequisite for binding of polyclonal IgE antibodies and the induction of an allergic response.

Many important allergens from mites, pollens and animal dander, insects and foods have been cloned, sequenced and expressed. In many cases, three-dimensional structures of these allergens have been determined and their epitopes (B-cell and T-cell) have also been mapped. Nearly all these studies stress that allergens have diverse biological functions for example, as enzymes, enzyme inhibitors, lipocalins or structural proteins and that as a rule, the allergen function is unrelated to its ability to cause IgE responses (Chapman *et al.*, 2000). Epitope mapping of allergens have the common aim of determining or pinpointing the exact residues contributing to allergy or the triggering of allergy. In producing malarial vaccine, due to concerns that a protective immune response generated by malaria parasite antigens may be strain- and stage-specific, Cheng *et al.*, (1991) proposed an approach whereby identified epitopes (common to malarial proteins from many stages, strains and even species) were selected to form the basis for a vaccine. Likewise, defined critical and minimal residues representing each component allergen in the HDM can perhaps be assembled for more specific and definite binding, to be used for diagnosis or even be combined as vaccine candidates against HDM allergy. For instance, Fellrath *et al.*, (2003) demonstrated that immunotherapy using allergen-derived long synthetic overlapping peptides mapping the entire major bee venom, PLA2 molecule; was safe and able to induce TH1-Type immune deviation, specific T-cell cytokine modulation marked by increased allergen-specific IL-10 production and IFN-γ secretion, and T-cell hyporesponsiveness. Serum-specific IgG4 response was also enhanced, in contrast to anti-PLA2 IgE. Therefore, long synthetic peptides offer the advantage of covering all possible T-cell epitopes for any

HLA genotype making them suitable as yet another candidate for a novel and safe approach of specific immunotherapy. In addition, Madan *et al.* (2004) discovered that an 11-mer overlapping synthetic peptide (P1) of a major allergen, Asp f 1, significantly inhibited both IgG and IgE binding of the standardized diagnostic antigen. P1 also showed the ability to induce histamine release from sensitized mast cells and a Th2 type of cytokine profile in peripheral blood mononuclear cells of aspergillosis patients, thus proving its antigenicity and also supporting the potential use of synthetic peptides in intradermal testing.

CONCLUSIONS

CONCLUSIONS

This thesis has been a combined study of many aspects of house dust mite allergy. In summary, prevalence of house dust mite sensitivity was first established through skin prick test procedure, followed by the *in vitro* study using ELISA. Emphasis was given to the house dust mites; *Dermatophagoides pteronyssinus*, *D. farinae* and also *Blomia tropicalis.* Mite tropomyosin was selectively produced as recombinant protein and truncated synthetic peptide and subsequently tested with sera from patients with allergic rhinitis and asthma.

I House dust mite sensitivity in Malaysia

The data presented are consistent with previous reports, verifying that *Dermatophagoides* is the major dust mite genus affecting more than 70% adult allergic rhinitis and asthma patients and also asthmatic children in Malaysia. Therefore, house dust mites still prove to be important source of allergy in Malaysia today, as reported about three decades back. *B. tropicalis,* a tropical dust mite emerged as another additional species eliciting allergy with comparable allergenicity with that of *Dermatophagoides* spp. This strongly suggests that a big proportion of susceptible individuals in Malaysia are affected by arachnid species in their surrounding environment causing them to suffer from symptoms of allergy in their daily lives. The fact that mite-specific IgE reactivities have been detected in the sera of young asthmatic children suggests that these three main species of house dust mites could affect the immune system of the young individuals at an early age and start to trigger allergic reactions in them.

Based on the higher concentration levels of Group I allergens, Der p 1 and Der f 1, found in dust samples from mattresses than from the floors of houses of Malaysian schoolchildren, a drive to promote awareness of the role of house dust mites in possibly triggering distressing allergic symptoms (such as asthma, allergic rhinitis and atopic

dermatitis) in children, should be highlighted. Parents of affected children should be guided about allergen avoidance measures and the safe use of inhalants. Data of *Blomia tropicalis* as a novel sensitizing mite is emphasized. No longer playing the role of only a storage mite, the population should be made aware of the invasion of this notorious mite in our household. More studies should be carried out to detect other species of HDMs. For example, the sera of atopic patients as well as the normal healthy individuals should be tested for specific antibodies to implicate any other domestic mites amongst our local fauna. Simultaneously, cross-reactivities among different species of local house dust mites can also be examined.

II Skin Prick Test and ELISA

Allergy complaints often face predicaments in pinpointing the exact allergy triggers. Allergy determination through *in vivo* skin prick tests (SPT) and *in vitro* serological assays such as ELISA testing still face shortcomings; which therefore makes allergy a challenging field in the study of immune system reactions. SPT is not only operator-dependent but also depends on the efficacy of test extracts used, while ELISA too is often plagued by problems such as cross-reactivity. In this study, the correlation of SPT and the presence of specific IgE in the sera of patients were found to be moderate. The evaluation of mite-specific IgE antibodies in the sera and salivary IgA of asthmatic and allergic rhinitis patients, failed to show much correlation in allergy determination, dashing the hope of using saliva as a non-serum sample for simpler diagnostic method. This study also found that severity of asthma symptoms did not have any significant relationship with the age of onset or disease duration. Indeed asthma being a multifactorial disease needs a thorough study involving other various local factors such as food consumption and environmental allergens. Appropriate choice of allergen extracts to determine putative allergen(s) in routine SPT will not only make it cost-effective but will also prevent time-consuming practice of arbitrarily testing out arrays of allergens.

III Tropomyosin and other antigens in the house dust mite

The cloning and expression of the cDNA encoding mite tropomyosin, Der p 10 in the prokaryotic host, *E. coli* and the eukaryotic host, *P. pastoris* yeast carried out in this study were able to produce recombinant proteins which were recognized by IgG and IgE in the sera of patients with allergy, therefore verifying its antigenic property. However, low degree of allergenicity of rDer p 10, affecting less than 30% suggests that Der p 10 is not a major mite allergen among Malaysian patients. This important finding serves to rectify the initial assumption that mite tropomyosin is generally a major allergen. *Pichia*-produced rDer p 10 proved its immunogenicity when it raised IgG antibodies in the mice. In turn, these mice antibodies also recognised tropomyosins from seafood and showed comparable activity with that of rabbit antiserum raised against shrimp tropomyosin. When used in a SPT trial, these recombinants of Der p 10 induced positive *in vivo* reaction, producing wheals and flare. This successful production of functional recombinant mite tropomyosin allergen, therefore suggests that other components of allergens can be locally produced in a desired amount, followed up with their safety and efficacy evaluation in clinical trials and subsequently to be further utilized as reliable diagnostic tools and for safer immunotherapy. Therefore, with over 400 allergen sequences available in the databases today, production of other antigens of interest can be done with greater ease. Isolated antigens or allergens in greater mass would enable *in vivo* immunological tests to be carried out in animal models or even in cells (such as in dendritic cell experiments). Furthermore, cloning of allergens also enables molecular modifications of allergens, for the production of hypoallergenic derivatives which are able to create reduced IgE binding but retain the capacity of T-cell stimulation.

The Der p 10 recombinant proteins produced in this study was aptly utilized together with other recombinant allergens in *in vitro* ELISA assays as reported in **Chapter 5.** This part of the study using recombinant proteins to represent the various allergenic HDM components, has helped to show that allergy to HDM is group selective in nature. Most patients reacted to the Group 1, 2 and 5 allergens of the *D. pteronyssinus* and *B. tropicalis*. This success of distinguishing the IgE-reactivities of separate allergens in the allergic patients reflects the feasibility of utilizing recombinant allergens in an in-house component-resolved allergenicity study. A skin prick test utilizing component allergens shows that one can produce a more precise diagnosis, showing that one can be allergic to a particular allergenic component and not to other(s). Furthermore, a component-resolved diagnosis can also provide useful clinical information for the physician and patients. For example, if a mite-sensitized individual recognizes the group 10 allergen, the patient should be cautioned that he or she might also react to seafood due to cross-reacting tropomyosins. Therefore, a concerted effort at utilizing highly purified, safe and characterized specific recombinant allergens in SPTs in Malaysian allergy clinics would help in pin-pointing the exact or putative allergen(s) that a patient is affected with and subsequently help to establish a program for immunotherapy, through subcutaneous or sublingual methods. Bearing in mind that immunotherapy with whole extracts introduces new antibodies (both IgE and IgG isotypes) to previously-not-recognized B-cell epitopes (Ball *et al.*, 1999), a component-resolved therapy is able to circumvent the problem of introducing sensitivity to new proteins. Therefore, in the event of immunotherapy being introduced in Malaysian hospitals, a battery of well-defined recombinants allergens from local allergen-contributing species will allow tailor-made treatment for allergic patients.

IV B-cell epitope mapping of Der f 10

The concomitant stimulation of the immune system is a principal means by which the body is able to recognise and destroy pathogens. Antibodies act through their ability to bind B-cell epitopes, the highly specific regions of a protein. Epitopes on the allergens can be one or more small continuous linear sequence segments, often having a conformational component, which makes it predictable from knowledge of the primary structure alone and sequence contents. Epitopes of individual members of protein families may be generated by careful selection of unique amino acid sequences and challenged for specific antibody binding. Thus, the scale of antibody responses to a protein bearing such epitopes makes them an important determinant of protein immunogenicity within the immune system. As such, like in most allergy studies, the IgE antibody binding reactivities to the B-cell epitopes of peptides of an allergen, are first observed. In this study, the search for B-cell epitopes on Der p 10 and Blo t 5 allergens has gone through distinct phases of sequence data acquisition, model building in the form of peptide synthesis, *in vitro* investigation (ELISA), and the interpretation of Pepscan results.

Conceptually, the problems of prediction must involve both the immunological question of antigenicity and complex reasoning abilities in order to improve methods for the prediction of antibody-mediated antigenicity. Lastly, this study reveals that the prediction of B-cell epitopes needs improved methods, perhaps with an extensive use of sophisticated and relevant protein prediction softwares, which can enhance the prediction of B-cell epitope localization, and subsequently be extended to the prediction of T-cell epitopes. In order to improve epitope mapping of specific antigens like Der f 10, a reliable selection of sera bearing Der f 10-specific antibodies the putative antigen would have been more appropriate. The presence of specific IgE antibodies in the serum, as ascertained in component-resolved ELISA or SPT, is an important criterion to establish before subjecting a serum to a pinELISA. Conversely, antibodies may be raised to specific areas of a protein

in order to probe biochemical function or to block normal activity. It is also possible to raise antibodies to peptides with sequences conserved within a related family (as in tropomyosins) in order to produce pan-reactive reagents.

General Discussion and Perspectives

The number and range of allergies are often said to be increasing. Although some of this increase in the number of people suffering from allergies is probably because of better diagnosis and understanding of allergy, a proportion is a result of a genuine increase in the number of people suffering from allergic conditions. In the near future, susceptible individuals in Malaysia face the challenge of increased food allergy due to being exposed to a greater number of foodstuffs at a younger age, not to mention the vast number of additives used. In addition, exposure to common inhalants such as mite allergens, animal dander etc., in the following decade, will be compounded with a wide range of chemicals, including artificial ones, in the environment. The increasing evidence of rising incidence of asthma in big cities, including Kuala Lumpur, is a result of the higher levels of air pollution, coming from two main sources: traffic exhausts and industrial emissions. With the worsening quality of our environment, we could well be facing an allergy cascade especially in the younger population. Therefore in Malaysia, future and further studies in the field of allergy should include other aspects such as embarking upon the study of the responses of immune system components or even to explore the genetics of allergy.