行政院國家科學委員會專題研究計畫成果報告

探討塵蹣過敏原 Der p2 多株性活化人類 B 淋巴球

Investigating polyclonal activation of human B lymphocytes by house dust mite allergen Der p2.

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中文摘要

近來偶然間發現家塵蹣過敏原 Der p1、Der p2 與 Der p5 可以在加入未經免疫小鼠之脾臟細胞培養 24 小時內引發 B 淋巴細胞多株性活化。諸多現象暗示塵滿過敏原具有類似植物刺激素(mitogen)或超級抗原(super antigen)的特質。

為了研究 B 細胞在個體接觸過敏原時之 B 細胞多株性活化現象在發展過敏性免疫反應的可能重要性，本計畫欲觀查塵滿過敏原對人類 B 細胞是否亦具有多株性活化的能力。然而以轉殖酵母菌產製之塵滿過敏原 Der p2 無法如前地活化小鼠或人類的淋巴細胞。多方證據顯示可能前人實驗中所得觀查結果恐係不明菌體因子干擾所致。由於先前研究亦發現即使前述三種塵滿過敏原蛋白分子結構及功能迥異，此三種過敏原特異性 IgE 抗體產生與 HLA-DPB1*1301、HLA-DPB1*0201 對偶基因具正、負相關，以故探討 HLA DPB1 鄰近之 HLA DMB1 基因多型性變化是否與塵滿特異性 IgE 抗體產生有關聯亦在本計畫工作之列。

關鍵詞：塵滿過敏原、多株性活化、HLA-DM、台灣

Abstract

It was found incidentally that house dust mite (HDM) allergens (Der p1, p2, and p5) were able to polyclonally activate naive murine B cells in an T-cell independent, but accessory cell-dependent manner. The polyclonal activation of naive murine B cells was a phenomenon reminiscent activating T or B cells by mitogens or superantigens.

House dust mites were ubiquitous. However, not all subjects encountering mite allergens develop allergic diseases. In a cross sectional, population study on the association of HLA class II alleles and the production of specific IgE antibodies against the above mentioned mite allergens (hallmark of allergic immune response to given allergens). It was noticed that HLA-DPB1*1301 and*0201 alleles were positively and negatively associated with the IgE responsiveness to these allergens of completely different molecular structure and function.

We proposed that those proteins of allergenic potential were able to activate the naive B cells polyclonally in an accessory cell-dependent manner, while HLA class II molecules might implicate in this process. The activated B cells might in turn direct the immune response Th2-prone and facilitating the IgE antibodies production. With the aim to incorporate the B cell activating process at the HDM exposure stage into the host immune response toward mite allergens, we tested if the HDM allergen Der p2 can also polyclonally activate naive human B cells. However, rDer p2 prepared from transfected Pichia yeast did not show similar capacity in activating both murine and human lymphocytes. To elucidate if genetic polymorphisms on HLA DMB1 locus, physically near HLA-DPB1, plays roles in conferring subjects IgE responsiveness toward HDM allergen Der p2. Two hundred and forty-four subjects were tested for seral Der p2-specific IgE by ELISA and genotyping for HLA-DMB1 by PCR/sequence-specific oligonucleotide probe hybridization(PCR/SSOPH). None of the HLA DMB1 alleles showed association with IgE responsiveness toward HDM allergen Der p2. As HLA DM locus did not show strong linkage disequi-librium with other HLA class II genes, It was postulated that HLA DPB1 alone or genetic locus centromeric to it might plays roles in determining compartment of T helper cell immune response toward mite allergens.

Key words: house dust mite allergen, Der p2, HLA-DM, Taiwanese.

Background and purposes:

The prevalence of allergic disorders was continuously increasing (1) and reached about 30% of general population. House dust mites (*Dermatophagoides spp.*, *D. pteronyssinus* and *D. farinae*) are the most important sources of allergens in Taiwan (2). House dust mites (HDM) are ubiquitous, however, not all subjects encounter HDM develop allergic disorders. Presence of allergen-specific IgE antibodies and a positive skin test are important markers for clinical diagnosis of allergic diseases. It has been well known that the allergic reaction toward mite proteins bias to Th2-type immune responses. The allergen-specific T cell lines derived from asthmatic children were of Th2-type. Th2 lymphocytes enhance the B cells producing allergen-specific IgE antibodies. Activation of IgE-bearing mast cells or basophils through allergen binding trigger immediate hypersensitivity and inflammatory responses. For years, the allergens of HDM were identified consecutively. Those molecules were successfully cloned and recombinant allergen proteins Der p2, p5, and p8 can be produced for clinical and research use. It was also known that not all mite proteins allergenic. Most of the reported HDM allergens are protein mole-

cules of different biochemical properties and biological function (3). Therefore, there are certainly at least two factors determining the development of an allergic response to a given allergen; the genetic background of the subjects encounter the protein, and the allergenic characteristics of the protein itself. The initial phase of immune response compartment into Th2 site was of great interest in studying the development of allergies.

It was found incidentally that the mite allergen Der p5 was able to induce polyclonal activation of naive murine B lymphocytes (4). Such activation was accessory cell-dependent. It was of particular interest that the capability of inducing polyclonal activation of naive murine B cells was a common feature shared by all the mite allergens tested (Der p1, p2, p5, p8) but not by the non-allergenic mite protein HSP. The conformation of the allergens are important for the activity since recombinant fragments or aged preparation of allergens failed to activate B cells as well (data not shown).

In a population study investigating the association of HLA class II alleles with HDM allergens-induced IgE responsiveness, we observed that HLA-DPB1*1301 was positively associated with IgE- but not IgG-responsiveness to HDM allergens Der p1, p2, and p5, whereas HLA-DPB1*0201 was negatively associated with the IgE response (5). As Der p1, Der p2 and Der p5 are proteins of completely different biochemical and biological characteristics, together with the finding mite allergens-induced mu-rine naive B cells polyclonal activation was accessory cell-dependent, we proposed that MHC class II molecules might be implicated in the activation process. Such activation may help to initiate and facilitate allergen-specific Th2-type immune responses by enhancing the B cells function as antigen presenting cells (6).

If the allergen-induced naive B cell polyclonal activation indeed played important roles in determining T cell subset development, we should be able to see similar phenomenon as human B cells were cultured in medium containing HDM allergens. We intended to confirm if HDM allergens also be able to polyclonally activate human B cells.

On the other hand, genetic loci closely linked to HLA-DPB1 gene, such as HLA-DMB1, may determine the Th1/Th2 compartment of immune response toward HDM allergens. As we observed subjects with certain HLA-DPB1 allele were disposed to or protected from rendering IgE responsiveness toward HDM p2 allergen. HLA-DMB1 is a known polymorphic gene physically located between HLA-DPB1 and HLA-DQB1-DRB1 genes. It was also documented HLA-DM molecules are implicated in processing of exogenous antigen and peptide lodging onto HLA class II DR, DQ and DP molecules. We investigate if HLA-DMB1 polymorphism be also associated with IgE responsiveness toward Der p2 allergen.

Material and methods:

1. Preparation of recombinant Der p2 (rDer p2):

Structure gene of HDM group 2 allergen Der p2 was transfected into Pichia yeast. Recombinant Der p2 could be induced secreated into culture medium by adding methanol. Recombinant Der p2 containing sup. was concentrated, dialyzed against PBS. A single band of 15 kD protein was confirmed on 20% SDS-PAGE.

2. animals:

Female Balb/C of 6-8 weeks of age were used to offer splenocytes confirming polyclonal activating capability of rDer p2 on naïve B cells.

3. human PBMC:

Human PBMCs were prepared by Ficoll-Hypaque density gradient centrifigation of heparinized blood. Cell viability was tested by Trypan Blue exclusion

4. Detection of polyclonal activation of murine/human lymphocytes by rDer p2:

$2*10^5$ murine splenocytes or human PBMCs were cultured in 200ul of RPMI 1640/10% FBS on 96 well plates with/without adding different concentration of Der p2 (3,10,30 ug/ml), or LPS (5ug/ml), or PHA-P (10ug/ml for murine cells, 2ug/ml for human cells). LPS and PHA-P were used as positive control. The cells were cultured for 24, 48, and 72 hours. 16 hours before the end of the culture, 1uCi of ^{3}H-Thymidine was added. The cells were harvested and ^{3}H-TdR incorporation was counted by a beta counter. The stimulation index (SI) was calculated by dividing the cpm value of culture containing stimulant with that of the culture without stimulants.

5. Study the possible association of HLA-DMB1 polymorphism with IgE responsive-ness toward Der p2:

a. Sample preparation

EDTA-anticoagulated blood was collected from 244 selected subjects recruited in a student medical check up program, NTU. The plasma was kept at –20°C for detection of HDM allergens specific antibodies. Genomic DNA was prepared from the leukocyte portion by salting-out method, and stored at –20°C for genetic typing of HLA class II genes.

b. Detection of HDM Der p2-specific IgE antibodies by ELISA method:

Recombinant Der p2 protein was coated on the 96-well ELISA plates and blocked in 3% BSA overnight. 1:2 diluted plasma was incubated with the allergen for 12 hours. The specific IgE antibodies were detected by biotin-labelled mouse anti-human IgE followed by Extravidin-ALP conjugate and pNPP substrate (Sigma). The A405nm for each sample was compared to those of the cord blood sera that serve as negative controls. The sample with A405nm 5 times greater than the average absorbance of negative controls was scored as positive.

c. Genetic typing of HLA DMB1 gene by polymerase chain reaction / sequence specific oligonucleotide probe hybridization (PCR /SSOPH).

According to published HLA-DMB1 sequences

(7). There are 5 DMB1 alleles, namely DMB1*0102 to *0105, reported. The allelic variation resides in the exon 3 that encodes beta 2 domain of DMβ molecule. To characterize the genetic polymorphism, primer sets DMB1-L(5'-CGC CCT CCA TCT GAG CAA GTA G) and DMB1-R(5'-ACA GTC ACG AAG GATGGG CTC) were designed to amplify a DMB1-specific DNA fragment of 285 base pairs. Several nucleotides (underlined) were modified to avoid internal structure of the primer. The PCR was set up in 50 ul of reaction mixture containing 10mM Tris.Cl,pH8.3/50mM KCl/ 1.5mM Mg^{2+}/0.5uM of each primer/0.2mM of each dNTP/1u of Taq. pol and 0.2-0.5ug of genomic DNA. Thirty-five cycles, consisted of denaturing at 94°C for 1 min., annealing at 55°C for 1 min. and primer extension at 72°C for 1 min., were conducted. The PCR product was denatured and immobilized onto nylon membranes. Six 5'-biotinylated SSO probes (table 1) were designed to distinguish the polymorphic residues 43 Ala/Asp/Val, 178 Thr and 179 Ile. The membranes were hybridized with SSOP in the presence of HRP-conjugated Avidin and subjected to stringent washing. DMB1 sequence amplified from 10 Taiwanese DNA samples and the genotyping were characterized by the PCR cycle sequencing using BigDye Terminator kit (Perkin Elmer Cetus). DNA samples with confirmed DMB1 genotypes were included in the membrane set-up to serve as control for specificity of SSOPH. Positive hybridization signal was viewed by ECL chemiluminescence (Amersham). The DMB1 genotypes were constructed for all samples.

Table1 Sequence-specific oligonucleotide probes used in HLA DMB1 genetic typing.

MB143A
5'-biotin-CTT GTG CGC ACT GCT
hybridization: 3X, 42°C, 15 min.
washing: 0.1X, 42°C, 15 min.
MB143E
5'-biotin-CTT GTG CTC ACT GCT
hybridization: 3X, 42°C, 15 min.
washing: 0.1X, 42°C, 15 min.
MB143V
5'-biotin-CTT GTG CAC ACT GCT
hybridization: 3X, 42°C, 15 min.
washing: 0.1X, 42°C, 15 min.
MB179I
5'-biotin-AGC CCC AAT GTG CTC
hybridization: 5X, 50°C, 15 min.
washing: 0.1X, 42°C, 15 min.
MB178T
5'-biotin- AGC CCC AGT GTG CTC
hybridization: 3X, 50°C, 15 min.
washing: 0.1X, 42°C, 15 min.
MB1-ALL
5'-biotin-CTC TCC CAT TTA GCC
hybridization: 3X, 42°C, 15 min.
washing: 0.1X, 42°C, 15 min.

Hyb. sln. contained SSPE indicated and 0.5% SDS
Wash sln. contained SSPE indicated and 0.1% SDS

E. Statistics

Subjects were grouped according to presence of detectable IgE specific Der p2 or not. The gene frequencies of each DMB1 alleles were compared between subjects with and without Der p2-specific IgE by Chi-square test. There was little information concerning the possible linkage disequilibrium between HLA-DMB1 and other class II genes such as HLA-DPB1 and HLA-DRB1. We also tried to calculate the HLA DPB1-DMB1 and HLA DMB1-DRB1 two-locus haplotypic frequencies (8) Two genetic alleles with a LD score (linkage diseqilibrium score) higher than 0.001 with a p value less than 0.005 were considered to be in linkage disequilibrium.

Results and discussion:

1. Recombinant Der p2 failed to activate murine and human lymphocytes constantly:

Several batches of rDer p2 were prepared from Der p2-transfected Pichia yeast. The quality of the protein was confirmed on SDS PAGE. However, the recombinant protein prepared from yeast failed to induce action of lymphocyte from both murine and human origin (data not shown). We could not reproduce the phenomenon observed by Chen (4). It was unlikely that the rDer p2 was not in good preparation since the protein preparation worked fine in playing antigen for ELISA detection of Der p2-specific IgE, which implied a satisfactory conformation. We could not exclude the possibility that unknown contaminates contributed to the activation of naïve B cells, as one of our old stock of rDer p2 (kept at 4°C) did showed certain degree of activation on mouse splenocytes. On the other hand, the rDer p2 prepared by Chen was from transfected E. coli in contrast to Pichia yeast in this project. Unknown bacterial or yeast factors might interfere with the experiment thus constant results could not be observed.

Table 2 allelic distribution of HLA DMB1 gene among Der p2 specific IgE(+) and (-) individuals and random Taiwanese sample.

DMB1 allele	Der p2 IgE(+) (n=212) No. (%)	der p2 IgE(-) (n=276) No. (%)	random sample (n=416) No. (%)
0101	99(46.7)	138(50)	198(42.6)
0102	35(16.5)	51(18.5)	67(20.4)
0103	71(33.5)	84(30.4)	149(35.2)
0104	1(0.5)	0(0.0)	0(0.0)
0105	6(2.8)	3(1.1)	2(1.8)

2. There was no association between HLA-DMB1 alleles and IgE responsiveness toward Der p2 found:

Among 244 selected test subjects, 106 were positive for Der p2-specific IgE and 138 were negative. By applying PCR/ SSOPH typing system, HLA DMB1

genotypes could be constructed for all the test subjects. All the five published DMB1 alleles were found in the selected Taiwanese population (table 2). The allelic frequencies of HLA DMB1 locus did not show any difference between Der p2-specific IgE positive and negative individuals (table 2), whereas the allelic distribution was quite similar to that of a random sample of 208 Taiwanese of elder age.

1. Two-locus haplotypes and linkage dis-equilibrium between HLA-DPB1-DMB1 and HLA-DMB1-DRB1 genes:

Six DPB1-DMB1, seven DPB1-DRB1 and four DMB1-DRB1 haplotypes were of statistical significance (table 3). Since linkage disequilibrium between DMB1-DPB1 loci, DPB1-DRB1, and DMB1-DRB1 loci was not as strong and evident as those of the other HLA class II genes (DRB1-DQA1-DQB1) (8), it was hardly to establish extended HLA DPB1-DMB1-DRB1 haplotypes. Although we found that HLA DPB1*1301 was positively associated with IgE responsiveness toward Der p2, the DPB1*1301 linked DMB1*0103 allele did not show similar association. It could be explained by weak linkage between DPB1 *1301 and DMB1 *0103 alleles. It might also be possible that genetic locus that is cetromeric to DPB1 could determine the Th cell compartment in response to house dust mite allergen Der p2.

Table 3 Two locus haplotypes[#] between HLA-DPB1-DMB1, DPB1-DRB1, and DMB1-DRB1.

Two-locus haplotypes	LD score	HF*	P<
DPB1-DMB1			
0202-0105	0.0046	0.0055	0.001
0301-0105	0.0044	0.0055	0.005
0401-0103	0.0299	0.0534	0.0001
4801-0102	0.0034	0.0041	0.005
0301-0101	0.0146	0.0435	0.01
1301-0103	0.0132	0.0392	0.025
DPB1-DRB1			
0201-0403	0.0101	0.0145	0.001
0202-1202	0.0113	0.01614	0.001
0401-0301	0.0379	0.0436	0.00001
0501-0901	0.0235	0.08816	0.005
0901-1301	0.005	0.0020	0.005
0901-1302	0.0058	0.0061	0.0001
1301-0901	0.0185	0.0298	0.001
DMB1-DRB1			
0102-1301	0.0034	0.0042	0.005
0104-1104	0.002	0.0020	0.0000
0104-1401	0.002	0.0021	0.001
0105-1401	0.0048	0.0058	0.001

*HF: haplotype frequency

[#] only haplotypes with LD>0.001 an P<0.0025 were listed.

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