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Analysis of Culture Filtrate Antigens of *Aspergillus fumigatus* Strains and of Antibody Responce in Patients with Aspergillosis by Immunoblotting

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Immunoblotting에 의한 *Aspergillus fumigatus* 菌株의 抗原分析과 이 菌에 感染된 환자의 抗體反應에 관한 연구

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ABSTRACT: Heterogeneity in antigenic composition of *Aspergillus fumigatus* isolates from clinical specimens and in antibody response of patients infected with this fungus was investigated by immunoblotting. A considerable quantitative and qualitative difference was found in composition of the culture filtrate antigens derived from a reference strain (ATCC 13073) and 8 clinical isolates of *A. fumigatus* on SDS-PAGE and immunoblots. The crude CF antigen of a strain AFG7 was selected to identify the serologically reactive and specific components by immunoblotting. Out of more than 36 components separated by electrophoresis, transblotted to nitrocellulose sheet, and reacted with sera that showed a positive reaction to *A. fumigatus* or other fungal antigens on immunodiffusion tests, merely four or so were found useful to serodiagnosis of aspergillosis. An antigen of 82KD was found most reactive and specific component so as to be contained in the standard preparation. Several other components, for example 11KD, 26KD, 30KD and 31KD,also possessed relatively high reactivity and specificity and seemed to be worth while purifying and characterizing. Antibody binding activity (reactivity) of the antigenic components was clearly shown on immunoblots because some were faintly stained with Coomassie blue but darkly stained on immunoblots, while some others behaved contrary to them. A number of components seemed to carry not only species specific but cross reactive antigenic determinants. Immunoblotting proved very useful to identify serologically reactive and specific components that should be present in the antigen to be employed to the serodiagnosis of aspergillosis.

KEYWORDS: *Aspergillus fumigatus*, Antigen, Immunoblotting,Serodiagnosis

Serology is a useful adjunct to the diagnosis of aspergilloma developed in nonimmunocompromised patients or of allergic bronchopulmonary aspergillosis (Coleman and Kaufman, 1978; Kim *et al.*, 1979, 1988; Pepys, 1978; Philpot and Mackenzie, 1976). Patients with invasive aspergillosis, however, often do not produce detectable amount of antibody to the infected fungus, with exception of some cases who are apparently under immunosuppresive state but show a positive serology so as to permit a presumptive diagnosis (Coleman and Kaufman, 1972; Richardson and Warnock, 1982; Young *et al.*, 1972). Despite of a wide use of serological procedures in the diagnosis of aspergillosis there is no standard methods in current use mainly due to lack of the standard antigen (Coleman and Kaufman, 1972; Kim *et al.*, 1978,

1979, 1980; Kurup *et al.*, 1984). Many attempts have been made to produce or to find out appropriate conditions for the production of standard antigen without success. Heterogeneity in composition of *A. fumigatus* antigen, that might have been resulted from strain variation, cultural conditions, and antigen production techniques, and in antibody response of the patients raise a difficulty in production of such materials (Bardana, 1978; Coleman and Kaufman, 1972; Kim *et al.*, 1978; Kurup *et al.*, 1984; Longbottom and Pepys, 1964; de Vries and Cormane, 1969).

This study aimed to investigate antigenic heterogeneity of *A. fumigatus* isolates from clinical specimens and to identify serologically reactive and specific components in the crude culture filtrate antigen for the diagnosis of aspergillosis by electrophoretic separation and immunoblotting. Antibody response of the patients with fungal infection was also investigated in relation with reactivity and specificity of the antigenic components.

Materials and Methods

Patients serum specimens

A total of 79 sera that had shown a positive precipitin reaction against one or more of fungal antigens on immunodiffusion (ID) tests were used in this study. Sera shown a positive ID against *A. fumigatus* antigen were 61 of which 39 reacted only to this fungus. Twelve sera reacted to *Candida albicans*, 3 to *A. flavus*, 1 to *A.nidulans* 1 to *A. niger*, 1 to *Pseudallerscheria boydii*. One patient was infected with both *A. fumigatus* and *flavus*, so reacted strongly to both antigens and another one reacted to *A. fumigatus* and *P. boydii* antigens.

Fungal strains

Strains of *A. fumigatus* used in the produc tion of antigen for the present study were isolated from clinical specimens in this laboratory except for a reference strain AFG1 (ATCC 13073). Certain clinical isolates AFG7 and 8 were morphologically different from reference strain and some indistinguishable but isolated from patients whose sera showed much stronger precipitin reaction against antigens derived from them. Other species of clinical isolates included in this study were *A. flavus* (AFV), *A. nidulans* (AND), *A. niger* (ANG), *C. albicans* (CAD), and *P. boydii*(PAB).

Antigen preparation (Kim *et al.*, 1978; Longbottom and Pepys, 1964)

Fungal spore suspension was prepared with one-week old culture grown on Sabouraud agar medium and inoculated 10^6 spores (or yeasts cells in case of *C. albicans*) per ml of Sabouraud broth medium (dialysate). Inoculated media were incubated at 37°C for one week by shaking at 110 rpm on a gyrotory shaker (Lab-Line Instruments, USA) and then cultured in a stationary phase for 5 successive weeks with an intermittent shaking. The culture filtrate was separated from mycelia by filtration and concentrated by Lab-Cassette concentrator (Ginsco, XX42 YLC KO) using a membrane that has approximate molecular cut-off point of 10KD. Then the concentrated filtrate was exhaustively dialysed against distilled water and the precipitates formed during dialysis if any, were removed by centrifugation prior to freeze-drying.

Immunodiffusion tests (ID) (Coleman and kaufman, 1972; Crowle,1961; Kim *et al.*, 1979)

ID tests were performed in 1% Difco Noble agar contained 1% sodium azide as the electrolyte. The basic technique was described by Crowle (1961) but slightly modified as follows. A 3 mm diameter of wells was made on the agar slide by punching out agar blocks at 3 mm distance between serum and antigen wells. Patient serum and antigen were added in the wells and reacted for 72 hours at room temperature. If precipitin bands appeared, the slide was washed with 5% sodium citrate to dissociate precipitates produced by C-reactive protein if any.

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and immunoblotting

Components of CF antigen were electrophoretically separated by SDS-PAGE described by Laemmli *et al.* (1970) using 4% stacking and 10% separating gels and transblotted to nitrocellulose sheet according to the procedure described by Burnette(1981). Nitrocellulose paper was then stained with Ponceau solution (0.4% Ponceau 2R, 8% trichloracetic acid, 2% acetic acid) and destained with distilled water until transblotted components became visible. Separated antigen lanes were cut-out and stained with Coomassie blue or reacted with patient serum. Mobility of Coomassie blue stained components was compared with those of maker molecules in order to determine relative molecular weight. Unoccupied binding sites on unstained nitrocellulose strips were adsorbed with bovine serum albumin (BSA) in TENT buffer (50 mM tris, 5 mM ethylenediamine trichloracetic acid, 150mM NaCl, 0.25% gelatin, 0.05% Triton X-100, pH 7.5) and then reacted with patient serum diluted to 1:100 with TENT-BSA for 5 hours. After washing, nitrocellulose strips were reacted with goat anti-human IgG peroxidase conjugate (Cappel Laboratories, Inc., USA) in TENT-BSA for $1^{1/2}$ hours and approximately 5 minutes reaction with peroxidase substrate (4-chloro-1-naphtol) followed until dark distinct bands were appeared (Lee *et al.*, 1982; Kadival and Chaparas, 1987).

Results

Most of clinical isolates of *A. fumigatus* have a typical morphology but some isolates show unusual morphology as seen in Fig. 1. Obverse and reverse colonial color of strain AFG7 was different from that of a reference strain (ATCC 13073) and AFG8 sporulated poorly. AFG10, that was not included in antigen analysis, showed a slow growth.

Coomassie blue stained electrophoregrams of CF antigens from 9 different strains of *A. fumigatus* showed some quantitative and qualitative difference in composition one another as seen in Fig. 2. Electrophoretically separated components of AFG2 were numerous and evenly distributed throughout the lane

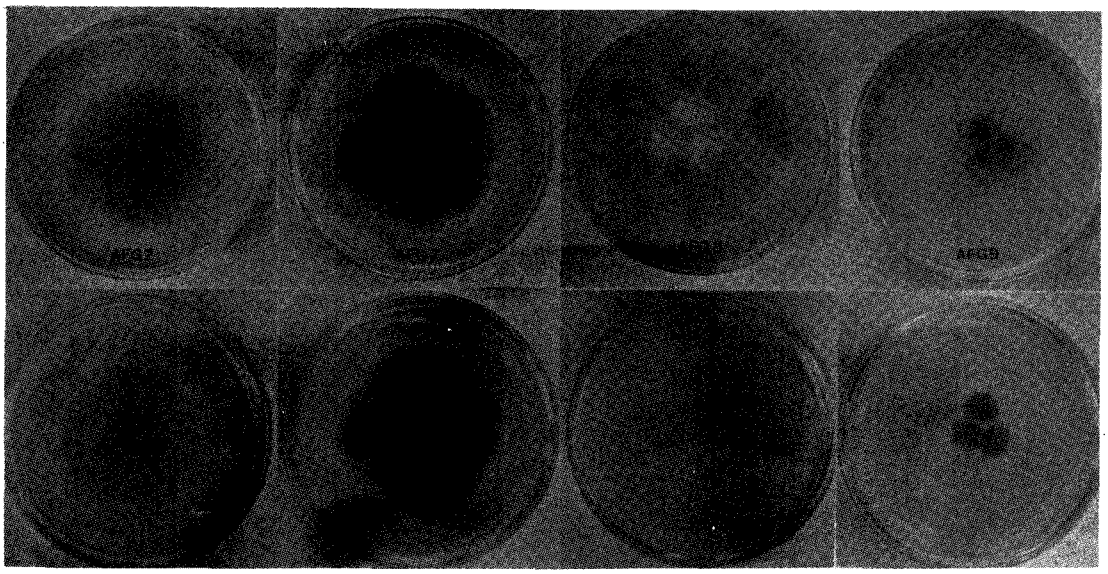

Fig. 1. Colonial morphology of *A. fumigatus* isolates on Czapek agar for 6 days incubation at room temperature.
Upper is obverse and lower is reverse of the same colony.

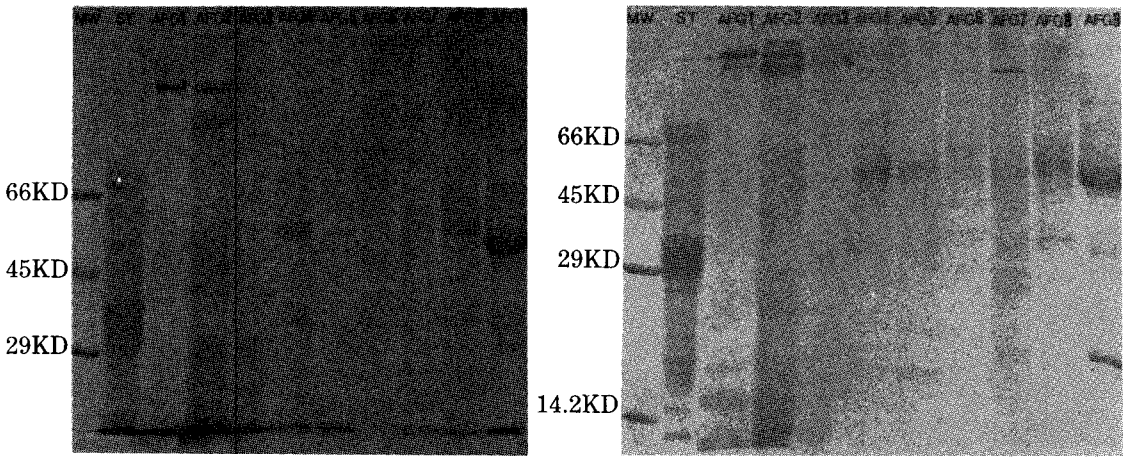

Fig. 2. Coomassie blue stained gels of *A. fumigatus* CF antigens.
Right = 13%T gel. Left = 10%T gel.

but more densely in anodal area, while components of the other preparations were distributed mostly at the zone of 49-59 KD. Electrophoregrams of AFG4, 5, 7, 8 and 9 were somewhat similar although there were quantitative differences of the components having same electrophoretic mobility between them.

Immunodiffusion tests revealed that AFG2, 3 and 7 produced a largest number of precipitin bands with sera from patients (16) infected with *A. fumigatus* as seen in Table I. AFG3 contained antigenic components cross-reactive with

Table I. Immunodiffusion reaction of fungal antigens with sera from patients with aspergilloma or other fungal infection.

Antigens	Sera from *A. fumigatus* infected patients																Sera from mixed or other infections							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
AFG1	2	2	2	2	2	2	2	0	2	3	3	3	2	3	3	2	0	1	0	0	0	0	0	0
AFG2	1	4	3	4	1	3	3	0	2	7	5	4	6	5	4	3	1	2	0	0	0	0	0	0
AFG3	1	3	3	3	2	2	3	1	0	5	5	4	5	3	3	4	1	2	0	0	1	1	0	0
AFG4	3	2	3	2	2	2	1	0	0	3	2	1	3	3	2	2	1	1	0	0	0	0	0	0
AFG5	2	2	3	1	1	2	2	0	0	2	3	1	4	2	3	1	0	1	0	0	0	0	0	0
AFG6	2	2	3	2	2	2	3	0	0	1	2	2	3	3	2	1	0	1	0	0	0	0	0	0
AFG7	4	3	3	1	3	2	3	0	1	1	4	2	3	2	2	2	0	1	0	0	0	0	0	0
AFG8	1	3	2	2	1	1	2	0	0	0	2	1	2	2	3	2	0	0	0	0	0	0	0	0
AFG9	2	3	3	1	2	1	2	0	0	3	2	1	2	1	2	2	0	1	0	0	0	0	0	0
A. flavus	0	0	0	0	0	0	0	0	0	2	3	3	3	3	0	0	3	3	3	0	0	0	0	0
A. nidulans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
A. niger	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
P. boydii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	1
C. albicans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	1	0

Numbers indicate precipitin bands appeared in ID tests.

Table II. Reactivity of culture filtrate antigens of *Aspergillus fumigatus* in immunoblotting assay for IgG.

Molecular weights (KD)	AFG1				AFG2				AFG3				AFG4				AFG5				AFG6				AFG7				AFG8				AFG9			
	PIF		PIO		PIF		PIO		PIF		PIO		PIF		PIO		PIF		PIO		PIF		PIO		PIF		PIO		PIF		PIO		PIF		PIO	
	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W
≧ 120		3				12		1	21	1		1	1	4		1		2								1				2						
115		1								3			1	3		1										2										
105																						7		1	2	9	1		2	2						
100	1	8		3		7				1			2	4		1		1			11	4	1	5	12	4		3	6	2	1	1		1			
96										1			1	5		1					1	8				2			2	4						
94		2			1	12							1	5		1					3	8		3	1	1			2	6			1	1		
90		2				8							1	5		1	2	6			1	6	6		3	1	1									
88	1	1			10	4	1	4					1	5			1	2				5	7		1		2			2	5		1	1	2	
86	1	2											13	3	3	4	2	10			1	3	4		1		11		1	2	4					
82		1			5	7				5			1	14	2	3	4	1	2			6	6		1	12	4		6							
76						4			2	5			1	8	7	1	7	2	3			8	5		3	5	8		2	2	1		1	3	7		1
73						4								10	3	1	5	7	4		1	6	5	1		4	11		1	3	8		2	8	8		6
71														12	3		8	2	4			1				2	5		1	2	1		1	3	7		
69	2	2		1						3	6		1	10	3		2	2	5			4	7			1	12		4	2	3			4	10		6
63										3	6		1	10	5	1	7	3	7			7	5		5	1	13		1	5	8		5	2	8		1
59	7	5		2						2	5		1	1	5			1	3				1			1	12			2			1	9		1
57		8		1						2	3			1	4		1	1				1	8			1	4			2			1	3		
49										4				1	1				1				4			1	2			2			1	1		
47															1				1				1				1			1			1			
44	2	1													1												1									
39	4	6													1							2		1		3									
37						3															1	9		5					2				1		
35						4								2												6							1		
33					5	8		1		6				4							2	3		1		2			2				3		
31	9	4		2	1	10			1	8		1	9	7	1		6	7			9	1		1	9	6	1		5	8		1	7	8		1
30		2		1	3	10	1		6	9	1			9								2				7			1				3		
28				2		1				3				15	1	7		2								8					1				
27	3	9																				2				2							5		
26	1	10		1		9				1				4				2				3			2	11			2			1	10		1
24		7												2																						
21		3				1								1																						
19		3				4																				3							3		
14		6			2	9								9		1	1	4								3									
11														4				2								3									

PIF = immunodiffusion positive sera(16) to *A. fumigatus* antigen(and other fungal antigen)
PIO = immunodiffusion positive sera to *A. flavus*(3), *nidulans* (1), *niger*(1), *C. albicans*(2), or *P. boydii*(1).

sera from patients infected with *A. flavus, A. nidulans* or *A. niger*. AFG2 and 4 reacted with two of 3 sera from patients with infected with *A. flavus*. Patient 8 and 9 reacted with only one CF antigen, for example AFG3 or AFG7. Patient 18 was infected with both *A. flavus* and *A. fumigatus* so as to have reacted to antigens derived from both species. Dual infection with *A. fumigatus* and *P. boydii* was found in one patient 16 whose serum reacted strongly to both species. Sera having precipitin antibody to *C. albicans*(22, 23) or *P. boydii*(24) did not react with *A. fumigatus* antigens except for AFG3.

Immunoblotting analysis revealed that almost all of the components separated by electrophoresis and transblotted to nitrocellulose paper reacted with sera from patients infected with *A. fumigatus* as seen in Table II and Fig. 3. Of the CF antigens compared AFG4 and 7 were found to contain a largest number of components that reacted with most of patients sera. It was of interest to note that AFG2 showed a numerous components on Coomassie blue stained electrophoregram but not many of them reacted with patients sera on immunoblots. Some antigenic components were stained darker than the others on immunoblots despite of paucity in content revealed on Coomassie blue stained electrophoregram, indicating that they were bound with more IgG antibodies so as to possess higher reactivity. Large molecule antigens moved slowly and so retained at the cathodal area were reactive with most of patients sera. Components of 82KD, 86KD, 88KD, 90KD, 94KD, 100KD and 104KD showed a considerable reactivity and were present in the CF antigens of the most of strains studied. Antigenic components of 71-76KD which of reactivity on immunoblots was relatively higher than the others were contained to a considerable quantity in the most of CF antigens except for AFG1, 2 and 3 and highly reactive components of 47-69KD and 26-33KD were also found in all antigenic preparations. Of the CF antigens compared AFG7 appeared being a best candidate to

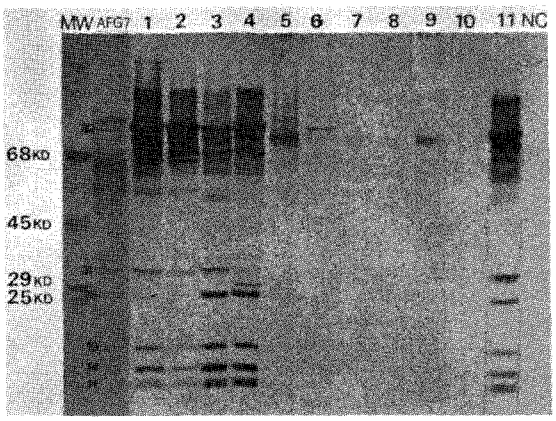

Fig. 3. Immunoblotting patterns of patient sera to *A. fumigatus* (AFG7) antigen.
1, 2 = *A. fumigatus* positive patient
3 = *A. fumigatus* & *A. flavus* positive patient
4 = *A. fumigatus, A. flavus* and other's positive
5 = *A. niger* & *A. fumigatus* (weakly) positive
6, 7 = *A. flavus* positive
8 = *C. albicans* positive
10 = *P. boydii*
11 = *A. fumigatus* & *P. boydii* positive
NC = Normal control

select for production of antigen for the diagnosis of aspergillosis caused by *A. fumigatus*, because it contained largest amount of antigenic components being serologically most reactive and specific on immunoblotting analysis. Reactivity of the components in AFG7 with sera from patients infected with *A. fumigatus* or other fungi was summarized in Table III. A number of electrophoretically separated components at the zones of 31KD, 57KD, 59KD, 63KD, 69KD and 82KD prominently stained with Coomassie blue and reacted strongly with patients sera on immunoblots indicating that they were serologically active and contained to a considerable amount in the crude CF antigen preparation. The components located at the zone of 24KD, 86KD and 88KD were contained to a large or considerable amount but reacted poorly with patients sera on immunoblots. Several components (19KD, 73KD, 76KD and 90KD) were darkly stained on immunoblots indicating high serological reactivi-

Table III. Reactivity of *A. fumigatus* (AFG7) antigenic components with patients sera on immunoblots.

Molecular weights (KD)	Immunodiffusion positive patients[79] to						
	AFG[39]	AFG & others[22]	AFV & others[3]	CAD [12]	AND [1]	ANG [1]	PAB [1]
135	2(1)	8					
130	9	16					
125	1	8					
115	4	6					
105	14	11(1)	1	1			
100	6	15		1			
98	12	16		2			
94	13(1)	16					
90	13(1)	15		1			
88	5	8		1			
86	6	6		1			
82	36(3)	21(1)	1(1)	(2)	(1)	(1)	
76	37(1)	21(1)	(2)	5(4)	(1)	(1)	(1)
73	37(1)	21(1)	2(1)	7(2)	(1)	(1)	(1)
71	31	20(1)	(1)	5			
69	28	18(1)		4			
63	24	18(1)	2	3	(1)	(1)	
59	23	18(1)					
57	26	17(1)		3		(1)	
49	32	21(1)		5			
47	26	17	(1)	2			
44	24	16(1)		3			
39	13	10(1)					
37	9	12(1)					
35	8	12		1			
33	19	14(1)		1			
31	34(1)	17(4)		1			
30	31(2)	19(3)		2			
28	17(3)	15		(2)			
27	9(1)	15(1)	(1)				
26	37(2)	14(1)					
24	2	6					
21	6	10(2)					
19	31(3)	17(5)					
14	32(2)	17(5)					
11	35(4)	21(1)	(1)				

Explanation for the abbreviations, see the text. ( )=weak reactors
[ ]=number of patients sera studied.

ty even though they were faintly stained with Coomassie blue. Serological reactivity of certain components (26KD, 28KD, 31KD and 41KD) varied because they reacted strongly with certain sera from patients infected with *A. fumigatus* but weakly with the others.

Antigenic component of 82KD zone reacted strongly with all the test sera that had shown a positive ID to *A. fumigatus* antigen only (39) or to *A. fumigatus* and other fungal antigens (22), yet reacted with none of sera positive to other fungi except for one of *A. flavus* sera, indicating high reactivity and specificity. Strong reactivity was also found in the components at 70KD and 73KD zones that had shown a considerable reactivity with many of sera positive ID to *C. albicans* antigen. This indicates they carry antigenic determinants common to a wide range of fungal species. The components distributed at the zone of 26-31KD also showed relatively high reactivity and specificity. Out of 61 *A. fumigatus* sera, 56 reacted with 30KD and 31KD, which 2 of *C. albicans* sera reacted with the former and 1 with the latter respectively. Fifty four out of 61 sera reacted with 26KD antigenic component to which none of sera positive to other fungi reacted. Components of small molecules such as 11KD, 14KD and 19KD also showed a comparable reactivity and specificity, so are they worth while investigating further for characterization.

Discussion

Despite of numerous studies for the production of standard antigen, still serodiagnosis of aspergillosis rely on home-made or commercial antigens that are in most cases heterogeneous in composition one another. Heterogeneity of antigenic composition due to a strain variation, different cultural conditions, and different preparation methods exerts difficulties in production of the standard antigen (Bardana, 1978; Coleman and Kaufman, 1972; Fink *et al.*,1977; Kim *et al.*, 1978, 1979; Kurup *et al.*, 1984). This study also revealed a considerable quantitative and qualitative heterogeneity in antigenic composition between strains of *A. fumigatus* isolates from clinical specimens even under the same cultural and production methods. This seems to make it clear that composition of antigens containing mixture of unknown components is not easy to control or to standardize as one may assume. Not all the problems lies in the antigenic composition only but in heterogeneous antibody response of the patients infected with fungi.

In general the standard antigen must contain diagnostically reactive and specific components as many as possible in order to be useful to the serodiagnosis of aspergillosis. In this regard identification of the serologically reactive and specific components among the mixture of unknown components in the crude extract showed be preceded an attempt to produce standard antigen. No one would expect a presence of monospecific antigenic molecules in common antigen products we deal in serodiagnostic laboratory. In fact most of antigenic components present in common preparations carry antigenic determinants both species-specific and common to a wide range of fungi in the same molecule no matter how elaborate techniques have been used to produce them. And antigenecity of components may not be same in different animal species and different individuals of the same species. This situation alludes that it is unlikely to produce monospecific antigen with an omnipotent reactivity. Such situation notwithstanding, it does not rule out a possibility to identify and purify relatively reactive and specific components among the crude mixture to meet minimum requirements necessary for the diagnosis of aspergillosis within a reasonable sensitivity and specificity.

Immunoblotting technique permits to identify such components and has proved very useful in this study. Firstly this technique made it possible to select a strain of *A. fumigatus* whose crude extract comprised as large quantity of diagnostically useful components as possible. The CF antigen of strain AFG7 was found to contain a considerable amount of highly specific and

reactive antigenic components such as 11KD, 26KD, 30KD, 31KD, 69KD and 82KD on immunoblotting analysis. Secondly intensity of stains of electrophoretically separated components on immunoblots reflects amount antibody binding, so makes it possible to compare relative reactivity of antigenic components. In this study several components on electrophoregram, for examples 19KD, 73KD, 76KD and 88KD were faintly stained with Coomassie blue but reacted strongly with patients sera as they showed a very dark staining when exposed immunoblots to peroxidase substrate. On the contrary, some components such as 21KD, 86KD and 88KD showed a weak reactivity on immunoblots despite of that they were contained in a relatively large quantity in the crude extract. Thirdly immunoboltting permits to compare relative specificity of electrophoretically separated components by observing reactivity with sera positive (ID) against one or more of heterologous fungal antigens. Components seen at the zones of 11KD, 26KD, 30KD, 31KD, 69KD and 82KD were found relatively specific which should be present as much as possible in the standard antigen. They are worth while characterizing further.

摘 要

臨床可檢物로부터 分離된 *Aspergillus fumigatus* 菌株의 抗原造成과 이균에 感染된 환자의 抗體反應의 다양성을 immunoblotting으로 관찰하였다. SDS-PAGE와 immunoblots상에 나타난 결과를 보면 비교분석된 培養濾液抗原의 조성에 있어서 菌株間에 定性 및 定量的 차이가 있음을 알 수 있었다. 血淸學的 反應力과 特異性이 비교적 높은 抗原成分을 同定하기 위해 그러한 항원들이 비교적 많이 함유된 AFG7을 선택하였다. 電氣泳動으로 분리하여 nitrocellulose paper로 轉移吸着시켜 *A. fumigatus* 나 기타 眞菌抗原에 陽性沈降抗體(免疫擴散試驗에서)를 가진 혈청과 반응시킨 결과 적어도 36개의 항원성분이 검출되었다. 그러나 혈청학적 진단가치가 있는 항원성분은 4개 정도에 불과했다. 특이성과 반응력이 가장 높아 標準抗原에 반드시 함유되어야 할 것으로 보는 항원성분은 分子量이 82 KD 정도되는 항원이었고 그 외에 11 KD, 26 KD, 30 KD 및 31 KD 등도 우수한 항원성분으로서 앞으로 더 연구검토되어야 할 것으로 본다. 반응력이 큰 항원이면 Coomassie 靑에는 흐리게 염색되어도 immunoblots상에는 짙게 염색되는데 비해 반응력이 약한 항원은 그와 반대현상을 나타내어 immunoblotting 분석법이 항원의 반응력과 특이성 관찰에 매우 유용함을 알 수 있다. 많은 항원성분이 菌種特異 抗原決定基와 함께 광범한 菌群에 분포하는 抗原決定基도 가지고 있었다.

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