Heat Shock Protein 88 and *Aspergillus* Infection

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Immunoblotting was used to dissect the antibody responses in the sera of 50 patients with proven invasive aspergillosis, 28 patients with suspected invasive aspergillosis, 35 patients with allergic bronchopulmonary aspergillosis, and 10 patients with an aspergilloma. This demonstrated the immunodominance of antigenic bands at 88, 84, 51, and 40 kDa. Monoclonal antibodies against the heat shock protein 90 complexes of *Candida albicans* and the water mold *Achlya ambisexualis* identified these four antigenic bands as homologous proteins. Similar antigens have been described in humans, mice, *Saccharomyces cerevisiae*, chickens, and *Drosophila* species. The antibody against *A. ambisexualis* has previously been shown to cross-react with antigens belonging to the human heat shock protein 90 complex. *Aspergillus* heat shock protein 90 was extracted from the sera of patients with invasive aspergillosis by affinity chromatography. This was done with both a rabbit hyperimmune antiserum raised against an extract of *Aspergillus fumigatus* NCPF 2109 and a monoclonal antibody against the heat shock protein 90 of *C. albicans*. In vivo expression of the antigen was demonstrated in an aspergilloma surgically removed from a patient. The role of the antigen as an allergen in allergic bronchopulmonary aspergillosis is also discussed.

Previously, we analyzed by immunoblotting the serological responses of 14 patients with invasive aspergillosis and 2 patients with an aspergilloma (23). The results showed that 10 patients produced antibody to a component of *Aspergillus fumigatus* with an apparent molecular mass of 88 kDa and that 13 patients produced antibody to a component of 40 kDa. Subsequent studies (3, 4, 12–14) examining patients with allergic bronchopulmonary aspergillosis (ABPA) or an aspergilloma confirmed this, but one study (15) produced conflicting results. None of those studies identified either the nature or the function of the antigen. In this study, we examined the antibody responses in the sera of 50 patients with proven invasive aspergillosis, 28 patients with suspected disease, 35 patients with ABPA, and 10 patients with a proven aspergilloma. Sequential serum samples, when available, were examined so that changes in antibody levels against individual antigenic bands could be delineated and correlated with any alteration in the disease state. The pattern of cross-reactivity of an immunodominant 88-kDa antigenic band was determined by its reaction with monoclonal antibodies raised against the heat shock protein (HSP) 90 of *Candida albicans* (21) and *Achlya ambisexualis* (32). The expression of *Aspergillus* antigens in vivo was also studied both in patients with invasive aspergillosis and in a patient with an aspergilloma.

MATERIALS AND METHODS

Patients. (i) **Controls.** Sera were examined from 100 pyrexial neutropenic patients with no evidence of invasive aspergillosis and 71 nonneutropenic patients with either asthma (34 patients) or an abnormal chest X ray and pneumonia but no evidence of *Aspergillus* infection (37 patients). Sera were also available from six patients whose sputum was positive for *A. fumigatus* in the absence of chest X-ray changes and any evidence of clinical disease.

(ii) ***Aspergillus* infection.** Sera from 35 patients with ABPA

were examined. All had a history of recurrent pulmonary infiltrates, asthma, blood eosinophilia, a positive serum precipitin test for *A. fumigatus*, and a positive sputum culture for *A. fumigatus*.

Sera from 10 patients with an aspergilloma were examined. Aspergilloma was demonstrated by chest X-ray changes showing a discrete lesion with a halo and a positive culture for *A. fumigatus* obtained either repeatedly from sputum culture or by bronchoscopy.

A total of 126 serum samples were collected from 50 patients with proven systemic invasive aspergillosis. The sera were taken at the start of infection and at irregular intervals during it until the patient either died or no longer had evidence of invasive aspergillosis. A total of 48 cases of aspergillosis were due to *A. fumigatus*, 1 was due to *Aspergillus niger*, and 1 was due to *Aspergillus terreus*. Invasive aspergillosis was diagnosed by one or both of the following: (i) cultural and histological evidence from the postmortem examination of the lung (41 patients) and (ii) cultural and histological evidence from a deep-organ biopsy specimen during life (10 patients). In nine of these patients, the primary organ involved was the lung, and in the remaining patient it was the liver. In one patient, a postmortem examination confirmed *Aspergillus* endocarditis following aortic valve replacement. Six patients survived infections caused by *A. fumigatus*. Multiple sequential serum samples were available from 32 of the patients, including 5 of the survivors. For one patient, a single serum sample obtained late in the infection was examined. Twenty patients also had positive sputum cultures, and 40 patients were neutropenic with acute myeloid leukemia.

A total of 93 serum samples from 28 patients with suspected disease were examined. The criterion for inclusion in this group was either of the following: (i) a pyrexial neutropenic patient with an abnormal chest X ray who recovered with antifungal therapy (12 patients) or (ii) a pyrexial neutropenic patient with an abnormal chest X ray who died while on antifungal therapy and on whom an autopsy was not performed (16 patients). For six patients, the sputum culture was positive. All had detectable *aspergillus* antigen (titer

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greater than or equal to 1 in 4) by reverse passive latex agglutination (RPLA). Fifteen patients were neutropenic with acute myeloid leukemia. Twelve patients on antifungal therapy survived, and sequential serum samples were available from all 12 patients.

Antigen. *A. fumigatus* NCPF 2109 was grown at 37°C for 3 days in Sabouraud broth (18). The mycelial growth was harvested, washed, filtered, and disrupted three times in an Xpress (LKB Instruments, Bromma, Sweden) at a pressure of 200 mPa at -20°C. The disintegrated cells were removed from the press and centrifuged at $38,000 \times g$ for 1.5 h at 4°C. The supernatant (pressate) was used for raising rabbit antiserum to *A. fumigatus* and as antigen in the immunoblot analyses. The same protocol was used to prepare a pressate from the strain isolated from the aspergilloma.

RPLA. *A. fumigatus* antiserum was prepared in three New Zealand White rabbits (weight, 3 kg). Pressate (25 mg) was mixed with 0.75 ml of Freund complete adjuvant, and the mixture was injected subcutaneously. Additional injections of the same mixture were given at 14, 28, and 42 days. Serum samples obtained at 56 days were pooled for use in immunoblots, affinity chromatography, and the RPLA test. Latex particles (diameter, 0.8 μ m; Difco Laboratories, Detroit, Mich.) were sensitized with rabbit antiserum that was previously diluted 1 in 10 in glycine-buffered saline (pH 8.4). The mixture was incubated for 30 min at room temperature and spun at $2,500 \times g$ for 10 min, the supernatant was discarded, and the pellet was resuspended in glycine-buffered saline (6). Patient serum (30 μ l) was mixed with sensitized latex (30 μ l) on a clean glass slide. The slide was rotated for 2 min at room temperature. Serial doubling dilutions of sera were made in glycine-buffered saline to determine the titers of the positive serum samples. A latex control for nonspecific agglutination was included for each serum sample.

Immunoblotting of patient sera. After heating with cracking buffer (2.6% sodium dodecyl sulfate [SDS], 1.3% 2-mercaptoethanol, 6% glycerol, 0.2% bromophenol blue, 0.05 M Tris hydrochloride [pH 6.8]) at 100°C for 5 min, 25 μ l of *A. fumigatus* pressate and 25 μ l of buffer were loaded into each well on a 10% polyacrylamide gel. Electrophoresis and transblotting were performed as described previously (25). The gel was transferred onto a nitrocellulose membrane in an LKB transblotter (LKB Laboratories). The buffer contained 20% methanol, 25 mM Tris, and 192 mM glycine at pH 8.3; and transfer was allowed to proceed at 25°C for 45 min. The nitrocellulose paper was blocked in 3% bovine serum albumin in buffered saline (NaCl 0.9% and 10 mM Tris [pH 7.4]) at 4°C overnight. The nitrocellulose was incubated at 25°C for 2 h with the human serum diluted 1 in 10 in buffered saline containing 3% bovine serum albumin and 0.05% Tween 20. After washing five times for 30 min in 0.9% saline-0.05% Tween 20, the nitrocellulose was incubated for 1 h at 25°C with alkaline phosphatase-conjugated goat anti-human immunoglobulin M (IgM), IgG, or, in the case of 10 of the serum samples from the patients with bronchopulmonary aspergillosis, IgE (Sigma Chemical Co.). After another washing, the membranes were incubated for 15 min at 25°C with buffer (100 mM Tris hydrochloride [pH 9.5], 100 mM NaCl, 5 mM MgCl₂) containing a mixture of 66 μ l of nitroblue tetrazolium (50 mg/ml in 70% *N,N*-dimethylformamide) per 10 ml and 33 μ l of 5-bromo-4-chloro-3-indolylphosphate (50 mg/ml in 70% *N,N*-dimethylformamide) per 10 ml. The reaction was stopped by washing the membranes in water. Immunoblots were divided, on the basis of reflectance densitometry (20), into those with a trace response

TABLE 1. Details of the sera used in the affinity chromatography

Patient no.	RPLA titer	Clinical status ^a	Detection of antigenic band with the indicated molecular masses (kDa) on the following:		
			Polyclonal column 84-88	Monoclonal HSP 90 column	
				84-88	50-52
1	Nil	Control	-	-	-
2	Nil	Control	-	-	-
3	Nil	Control	-	-	-
4	1:2	Inv Asp	-	-	-
5	1:4	Inv Asp	-	-	-
6	1:4	Inv Asp	-	-	-
7	1:4	Inv Asp	-	-	-
8	1:4	Inv Asp	-	-	-
9	1:4	Inv Asp	-	-	-
10	1:4	Sus Asp	-	Trace	-
11	1:8	Inv Asp	-	+	+
12	1:10	Inv Asp	Trace	+	+
13	1:20	Inv Asp	+	+	+
14	1:20	Sus Asp	Trace	+	+
15	1:40	Sus Asp	+	+	+

^a Inv Asp, proven invasive aspergillosis; Sus Asp, suspected invasive aspergillosis.

(less than 40 mm) and those with positive responses (greater than 40 mm).

Molecular mass markers were Prestained Rainbow Markers (code RPN 756; Amersham International), as follows: myosin, 200 kDa; phosphorylase *b*, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.3 kDa.

Antigen identity. Nitrocellulose strips loaded with *A. fumigatus* NCPF 2109 pressate prepared as described above were probed with the monoclonal antibodies raised against the HSP 90 complex of *A. ambisexualis* (monoclonal antibodies AC16, AC19, and AC88) at a dilution of 1 in 100. These were kindly provided by David Toft. The nitrocellulose strips were also probed with a rabbit antiserum (1 in 20) and a monoclonal antibody, CA-Str-7 (1 in 100), both of which were raised against the synthetic peptide epitope LKVIKRNIVKMIE of *C. albicans* HSP 90 as described previously (22, 24). The reaction was repeated with both the polyvalent antiserum and the CA-Str-7 monoclonal antibody, each previously cross-absorbed at 4°C overnight with the synthetic peptide (50 μ g/ml).

Affinity chromatography. The IgG fraction from the rabbit antiserum to *A. fumigatus* prepared by protein A chromatography was coupled to a 5-ml, CNBr-activated Sepharose 4B column (Pharmacia, Uppsala, Sweden) containing 1 mg (dry weight) of cyanogen bromide. Then, 2.5-ml serum samples from each of nine patients with proven invasive aspergillosis, three patients with suspected invasive aspergillosis, and three control sera were run down this column (Table 1). The antigens were eluted with 0.1 M glycine-hydrochloric acid buffer (pH 2.5) at a flow rate of 1 ml/min. Each 1-ml fraction was collected in 0.2 ml of 1 M Tris hydrochloride buffer (pH 9.0). The column was equilibrated and thoroughly washed with the coupling buffer (0.1 M sodium bicarbonate, 0.5 M sodium chloride [pH 8.3]) between samples.

A second column was prepared with the mouse monoclonal antibody CA-Str-7 raised against HSP 90 of *C. albicans* at an IgG concentration of 1 mg/ml. Sera from the same

patients were passed down the column and eluted as described above.

Each eluate was desalted in a PD10 column (Pharmacia) before it was heated at 100°C for 5 min with cracking buffer and electrophoresed on a 10% SDS-polyacrylamide gel. Immunoblotting was performed against a 1:20 dilution of the rabbit antiserum to *A. fumigatus* and a 1:100 dilution against the monoclonal antibodies against HSP 90 of *C. albicans* (CA-Str-7) and *A. ambisexualis* (AC16, AC19, and AC88).

Aspergilloma. The aspergilloma was removed from the right lung of a patient in whom it had developed following the successful resection of a carcinoma of the bronchus. Culture demonstrated *A. fumigatus*. A pressate of the mold was made as described above. The aspergilloma was loaded into an Xpress and crushed at a pressure of 200 mPa at -20°C. The disintegrated material was removed from the press and centrifuged at $38,000 \times g$ for 1 h at 4°C. The supernatant was examined by immunoblotting with monoclonal antibody CA-Str-7 against HSP 90 of *C. albicans* (1:100).

RESULTS

Immunoblotting. (i) Control sera. Of the 100 serum samples from the pyrexial neutropenic patients, 93 had no detectable antibody response. Two produced IgM and two produced IgM and IgG against a band at 29 kDa. One produced IgM and one produced IgG against a band at 220 kDa. The serum of another patient produced IgM and IgG against bands at 51 and 63 kDa. In sera from the patients with asthma, 31 had no antibody; 1 produced IgM and IgG against bands at 88, 84, and 82 kDa; 1 produced IgM and IgG against a band at 101 kDa; and 1 produced IgG against bands at 63 and 88 kDa.

Of the 37 patients with an abnormal chest X ray, pneumonia, and no evidence of aspergillus infection, no antibody was demonstrated in the sera of 36 of them. The serum from the remaining patient had IgM against bands at 51 and 220 kDa and IgG against the 220-kDa band. The six patients with a positive sputum sample for *A. fumigatus* and no evidence of clinical disease had no antibody.

(ii) Aspergillus infection. In the sera from patients with ABPA, 19 antigenic bands were identified ranging in molecular mass from 29 to 220 kDa. The cluster of bands at 88, 84, and 82 kDa was the most immunogenic, with the sera of all patients producing IgM against the band at 88 kDa. Other highly immunogenic bands were at 220, 101, 63, 51, and 40 kDa (Table 2). Results for five of the patients are illustrated in Fig. 1. In terms of the total antibody response, the IgM class dominated in 25 patients, the IgG class dominated in 4 patients, and in six patients the production of IgM and IgG was approximately the same. IgE was measured in 10 of the patients. This was negative in four patients. In the sera from the remaining six patients, trace reactions were detected most commonly against the bands at 88, 84, 82, 63, 51, and 40 kDa.

In the sera from patients with aspergilloma, 14 antigenic bands were identified ranging in molecular mass from 29 to 220 kDa. The group of bands at 88, 84, and 82 kDa was the most immunogenic, with the sera of nine patients producing IgM and the sera of five patients producing IgG against the band at 88 kDa. The band at 40 kDa was also an immunodominant antigen. All patients produced both IgM and IgG class antibody. In the sera of seven patients, antibody production was approximately the same, while the IgM response predominated in the remaining three patients (Table 3).

TABLE 2. Distribution of the antibody responses in sera of patients with ABPA

Apparent molecular mass (kDa)	No. of patients				
	IgM (<i>n</i> = 35) ^a		IgG (<i>n</i> = 35)		Trace of IgE (<i>n</i> = 10)
	Trace	Positive	Trace	Positive	
200		10	1	6	
200		3	1	4	
101	2	12	2	9	
88	3	32	5	22	6
84	2	28	5	17	4
82	3	22	3	14	3
77	2	3	1	4	
75	2	3	2	4	1
73	2	3	1	3	
67		6		5	2
65		3	1	2	
63	3	13	3	7	3
51	3	6		6	4
47				1	
40	5	14	6	10	4
37		2		3	
35		1		1	
31		4		1	
29		1			

^a *n* is total number of patients.

In the sera from patients with proven invasive aspergillosis, 17 antigenic bands were immunogenic, with apparent molecular masses ranging from 29 to 220 kDa. Twenty-four patients who died, including those with infections caused by *A. niger* and *A. terreus*, produced no detectable antibody response. The remaining 26 patients were subdivided into 6 who survived and 20 who died. For six of the patients who died but who produced antibody, only a single serum sample was available for examination. In the sera of the remaining 14 patients who died, constant or fading antibody responses occurred to the bands at 220, 88, 63, and 51 kDa (Table 4).

Among the six survivors, one had only a serum sample from late in the course of the infection, and this showed an IgM and IgG response to the band at 40 kDa and an IgM response to the band at 63 kDa. The sera of the remaining five patients all showed antibody rises against one or more *Aspergillus* bands, with a rise in the IgM response to bands at 40 and 88 kDa. Results for one of the patients are

FIG. 1. IgM (M) and IgG (G) antibody responses in five patients with ABPA (lanes 1 to 5 represent each patient). Molecular masses are given on the sides, in kilodaltons.

TABLE 3. Distribution of the antibody responses in sera of patients with aspergilloma

Apparent molecular mass (kDa)	No. of patients			
	IgM (<i>n</i> = 10) ^a		IgG (<i>n</i> = 10)	
	Trace	Positive	Trace	Positive
220	1	2	1	2
200		1		1
88	1	8		5
84		8		5
82		6		4
67		3		2
65		3		2
63		2		1
51		2		1
47		2	1	
40		8	1	6
35			1	
33		1	1	1
29		1		1

^a *n* is total number of patients.

illustrated in Fig. 2. The five sequential serum samples show a trace IgM response to the bands at 88, 84, 82, and 75 kDa, which declined with successful treatment. This was accompanied by an increase in the IgG response to bands at 88, 84, 82, 77, 75, 65, 63, 51, and 40 kDa. This patient was 17 years old and had chronic granulomatous disease and short bowel syndrome. He had pulmonary aspergillosis which was successfully treated by resection of the lung and prolonged therapy with amphotericin B and itraconazole.

Sera from patients with suspected invasive aspergillosis reacted to the same range of antigenic bands as the sera from patients with proven invasive aspergillosis did. Sera from 10 of the patients who died produced no detectable antibody. Sera from the remaining six patients who died produced only a low-level IgM response (Table 4).

Sequential serum samples were available from all of the 12 patients who survived with treatment. Sera from all 12 patients produced IgM responses to the band at 40 kDa, and the sera of 9 patients produced IgM responses to the band at

FIG. 2. Sequential IgM (lanes 1 to 5 on the left) and IgG (lanes 1 to 5 on the right) in a patient with proven invasive aspergillosis who recovered. Molecular masses are given on the right, in kilodaltons.

88 kDa. The IgM responses were much greater than the IgG responses (Table 5).

Identity of the antigen. The monoclonal antibody AC88 against *A. ambisexualis* reacted strongly with the bands at 88, 63, 47, and 40 kDa and produced a weak reaction with a band at 84 kDa (Fig. 3, lane 1). Monoclonal antibodies AC16 and AC19 produced indistinguishable patterns with bands at 88, 84 (trace), and 63 kDa (Fig. 3, lane 2). The rabbit serum against HSP 90 of *C. albicans* cross-reacted with the bands at 88 and 51 kDa, and the monoclonal antibody CA-Str-7 reacted with the band at 88 kDa (Fig. 3, lanes 3 and 5, respectively). In each case, these reactions disappeared when the antibody was cross-absorbed with the immunizing synthetic peptide (Fig. 3, lanes 4 and 6).

Affinity chromatography. The results of affinity chromatography are summarized in Table 1. No band was detectable in the sera of the three controls or in the sera from patients 4 to 9. As determined by RPLA, the sera with the highest titers were the ones most likely to give positive results by affinity chromatography. The column sensitized by the rabbit *A. fumigatus* antiserum extracted a band with an apparent molecular mass of 84 to 88 kDa. The eluates

TABLE 4. Distribution of the antibody responses in patients with fatal proven and suspected cases of invasive aspergillosis^a

Apparent molecular mass (kDa)	No. of patients											
	Proven cases (<i>n</i> = 20)										IgM suspected cases (<i>n</i> = 6)	
	IgM					IgG						
	C		F			C		F			C	
	Trace	Positive	Trace	Positive		Trace	Positive	Trace	Positive		Trace	Positive
220	2			1	3			1				2
101										1		
88	3	2		1	3	1				1		
84												
82												
63		1	1	1	1	1						
51		3		1				1		1		
40	2	4			4	1				1		1
37		3			1	1						
35		3			1							
33		3			1							
29												1

^a C, constant antibody response; F, fading antibody response either from trace to nil or positive to trace or nil; *n* is the total number of patients.

TABLE 5. Distribution of the antibody responses in the sera of surviving patients with proven and suspected cases of invasive aspergillosis^a

Apparent molecular mass (kDa)	No. of patients											
	Proven (n = 5)				Suspected (n = 12)							
	IgM		IgG		IgM				IgG			
	I		I		C		I		C		I	
	Trace	Positive	Trace	Positive	Trace	Positive	Trace	Positive	Trace	Positive	Trace	Positive
220		1		1			1	1		2		2
101					2							
88	1	4	1	1	2		1	6			2	3
84		3		1				4			1	2
82		3		1				4			1	
77				1	1						1	1
75		1		1	1			1			1	1
67		1						1				
65		2		1	1			1				
63		2	1	1	1			4			2	2
51		2	1					5			1	
40	1	4	2	1	2	1	1	8			2	4
37		1			1		1					1
35	1	1										
33					1	1		1		1		
29					1	1						1

^a C, constant antibody response; I, increased antibody response either from nil to trace or nil or trace to positive. *n* is the total number of patients.

reacted on immunoblotting with the rabbit hyperimmune antiserum; the monoclonal antibody to HSP 90 of *C. albicans* (CA-Str-7); and monoclonal antibodies AC16, AC19, and AC88 raised against HSP 90 of *A. ambisexualis*. The eluates from patients 12, 13, and 15 stained by the monoclonal antibody against HSP 90 of *C. albicans* are illustrated in Fig. 4, lanes 1 to 3.

The CA-Str-7 monoclonal antibody column was more sensitive, extracting antigenic bands at 50 to 52 kDa as well as 84 to 88 kDa. These were detected on immunoblotting by the monoclonal antibody itself and monoclonal antibodies AC16, AC19, and AC88. Figure 4 illustrates the results for

the eluate from the serum of patient 13 stained with AC16 (lane 4), AC88 (lane 5), and CA-Str-7 (lane 6).

Aspergilloma. Immunoblots of the patient's serum against pressates of *A. fumigatus* NCPF 2109 and the strain from the patient demonstrated conserved bands at 220, 88, 84, 82, 63, 51, 40, and 33 kDa. A pressate of the aspergilloma itself probed with monoclonal antibody CA-Str-7 revealed a bright band at 50 to 52 kDa.

DISCUSSION

Antibody responses to *Aspergillus* infections were dissected by immunoblotting sera from patients with invasive

FIG. 3. Pressate of *A. fumigatus* immunoblotted with monoclonal antibodies against HSP 90 of *A. fumigatus* (AC88 [lane 1] and AC16 [lane 2]), rabbit hyperimmune antiserum against the synthetic peptide epitope of HSP 90 of *C. albicans* (lane 3) and after cross-absorption (lane 4), and the mouse monoclonal antibody against the same epitope of HSP 90 of *C. albicans* (lane 5) and after cross-absorption (lane 6). Molecular masses are given on the sides, in kilodaltons.

FIG. 4. Affinity chromatography. The eluates were from sera from patients 12, 13, and 15 (lanes 1 to 3, respectively) from the column sensitized by rabbit *A. fumigatus* antiserum immunoblotted against the monoclonal antibody CA-Str-7 against HSP 90 of *C. albicans*. The eluate from serum from patient 13 off the column sensitized with monoclonal antibody CA-Str-7 was immunoblotted against AC16 (lane 4), AC88 (lane 5), and the *C. albicans* CA-Str-7 monoclonal antibody (lane 6). Molecular masses are given on the right, in kilodaltons.

aspergillosis, aspergilloma, and ABPA. Antibodies were largely negative in control groups, with there being detectable levels in only 6.5% of patients overall. Sera from all patients with ABPA produced detectable antibody, which is in agreement with previous observations (26, 33). Immunoblotting demonstrated the common occurrence of IgM and IgG to a cluster of bands at 88, 84, and 82 kDa. IgE was detected by a relatively insensitive colorimetric method in the sera of 6 of 10 patients. It was predominantly against the bands of 88, 84, 51, and 40 kDa, suggesting that these antigens may play a role in the pathogenicity of ABPA. The sera from patients with an aspergilloma also detected multiple bands, and similarly, the bands at 88, 84, 82 and 40 kDa were immunodominant.

In 1983, Piechura et al. (30) described a fraction, CS2, that they isolated from *A. fumigatus* Ag-507 cell sap by gel filtration. This was conserved between three isolates and had an apparent molecular mass of 150 kDa. On an SDS gel, it broke down into three components with apparent molecular masses of 81, 53, and 40 kDa, which are similar in size to the bands of 82 to 88, 51, and 40 kDa described here. Immunoelectrophoresis, indirect enzyme-linked immunosorbent assay, and radioimmunoassay confirmed the presence and relative specificity of both IgG and IgE against the antigen in the sera of patients with ABPA and aspergilloma (4, 13, 14). Harvey and Longbottom (9) have described a 38-kDa allergen that they designated Ag 7. Brouwer (3) recognized, by immunoblotting, bands at 36, 50, and 100 to 120 kDa by comparing strongly precipitin positive, weakly precipitin positive, and precipitin-negative sera from patients. The gels contained between 11 and 13% polyacrylamide, and molecular masses above 80 kDa were estimated by extrapolation. Taking this into account, the band with an apparent molecular mass of 36 kDa may be equivalent to the 40-kDa band, 50-kDa band may be equivalent to the 51-kDa band, and the 100- to 120-kDa may be equivalent to the 88-kDa band. Baur et al. (2) analyzed 28 patients with ABPA at various stages of the disease. They showed that IgE levels increased as the disease progressed and that the bands at 80, 51, and 40 kDa were immunodominant. They also identified antigens at 23 and 18 kDa which they thought were identical to Ag 3 (17) and antigen 40 (36), respectively. A band at 55 kDa was also described when the sera of patients with cystic fibrosis and an aspergilloma were immunoblotted (31). Cross-reactivities between the allergens of *A. fumigatus* and those of *Alternaria* species (>100, 80, 39, and 32 kDa) and *Cladosporium* species (39, 35, and 23 kDa) have also been demonstrated (35).

These results differ from those of Leung et al. (15), who described, by immunoblotting sera from 22 patients with ABPA, an immunodominant antigen with an apparent molecular mass of 70 kDa.

The measurement of antibody in the sera of patients with invasive disease has previously been shown to be disappointing (33). For example, Young and Bennett (38) failed to demonstrate an elevated level of antibody in the sera of 15 patients with hematological malignancies who had invasive aspergillosis. In the present study, no antibody was detectable in the sera of any of 24 patients who died. In the sera of the remaining 20 patients who died, the level of antibody was generally low and either remained constant or faded terminally (Table 4). Survival was associated with an increase in antibody levels, and the immunodominant antigens were those of 88 and 40 kDa. The data from the suspected cases of invasive aspergillosis confirmed the importance of the antigenic bands at 88 and 40 kDa (Table 5).

The identities of these immunodominant antigens were established by the patterns of cross-reactivity with the antibodies raised against the HSP 90 proteins of *A. ambisexualis* and *C. albicans*. All of them reacted with the 88-kDa antigen, which, in the case of the candidal antibodies, was neutralized by cross-absorption with the immunizing peptide. The *A. ambisexualis* monoclonal antibodies also reacted weakly with the band at 84 kDa, suggesting the existence of a second similar HSP. Two HSP 90 proteins occur in *Saccharomyces cerevisiae*, *C. albicans*, mice, and humans (7, 11, 21, 24, 28). The antibodies against *A. ambisexualis* detected additional antigenic bands at 63, 47, and 40 kDa. In the case of *C. albicans*, it has been known that HSP 90 disrupts on SDS gels to produce several bands, including one at 47 kDa and one at 40 kDa (22). The sequence of the carboxy-terminal epitope of the candidal protein is species specific, and antibody raised against it recognizes antigenic bands at 92 and 40 kDa (22, 24). The rabbit hyperimmune antiserum and monoclonal antibody raised against the conserved peptide LKVRKNIVKMIE detected bands at 92 and 47 kDa. This suggests that the species-specific carboxy end of the protein is on the 40-kDa fragment, while the conserved epitope is on the 47-kDa fragment. An analogous situation may occur in *A. fumigatus*, in which rabbit antibody against the conserved peptide recognizes a band at 51 kDa that is analogous to the 47-kDa band of *C. albicans*. The 40-kDa antigen would be a further breakdown product of HSP 90 containing the carboxy end of the molecule. This is more species specific in all cases sequenced so far, including humans, mice, chickens, *Drosophila* species, and *S. cerevisiae* (7, 11, 16, 28).

The 47-kDa antigen of *C. albicans* must be differentiated from the 48-kDa cytoplasmic antigen (34). The latter has been identified as enolase (19).

Candidal hyperimmune serum raised against a pressate of *C. albicans* in rabbits detects circulating HSP 90 and its breakdown products (6, 20, 25). This serum was shown by epitope mapping to be reactive mainly against the species-specific carboxy epitope, which explains how RPLA can distinguish between candidal and aspergillus antigens (6, 22). Both the monoclonal antibody to the HSP 90 of *C. albicans* and the rabbit antiserum against *A. fumigatus* were used to purify antigen directly from the sera of patients by affinity chromatography. The hyperimmune rabbit antiserum extracted an antigen with an apparent molecular mass of 84 to 88 kDa in sera from four of the patients. This reacted with all four monoclonal antibodies against HSP 90, indicating that it is the HSP 90 of *A. fumigatus*. It is likely that this antigen was extracted by its species-specific carboxy epitope. The monoclonal antibody against the conserved epitope of *C. albicans* HSP 90 extracted this 88-kDa band and a second band with an apparent molecular mass of 50 to 52 kDa. The 50- to 52-kDa antigen could be analogous to the 47-kDa antigen of *C. albicans* in containing the conserved sequence LKVRKNIVKMIE and being a breakdown product of HSP 90. Direct examination of the antigenic extract from the aspergilloma also demonstrated a high level of expression of the 50- to 52-kDa antigen in vivo. This was detected by the same monoclonal antibody against HSP 90 of *C. albicans*. This monoclonal antibody was thus capable of both extracting the 50- to 52-kDa antigen from the sera of patients and identifying it in an aspergilloma. An antigen with an apparent molecular mass of 58 kDa (52 to 62 kDa) has subsequently been determined by immunoblotting to be immunodominant in 38 patients with invasive aspergillosis (8). This was done

with a 12.6% polyacrylamide gel and was compatible with it being part of the same antigenic complex.

An 80-kDa antigen was identified in the sera of 6 of 15 experimentally infected rabbits (29). In experimentally infected rats, SDS-electrophoresis and immunoblotting of serum and urine samples demonstrated strongly reacting antigenic materials in the 88-, 40-, 27-, and 20-kDa regions (39). These results are consistent with the hypothesis that HSP 90 is a key circulating antigen in the sera of patients with invasive aspergillosis, both as the native molecule and as breakdown products such as those of 51 and 40 kDa. Haynes et al. (10) examined the urine from patients with invasive aspergillosis and found predominantly low-molecular-mass antigens, but these may represent additional smaller breakdown products of HSP 90.

The HSP 90 of the mold *A. ambisexualis* has been implicated as part of the *Achlya* steroid receptor complex (5). There are at least two mating types of *A. ambisexualis*, designated male and female. The female strains produce antheridiol (1), and this induces HSP 90 production. The role of HSP 90 for *A. fumigatus* is obscure, but it could be involved in fungal replication. The effect of circulating aspergillus HSP 90 during invasive aspergillosis is also unknown. Antibody against the 47-kDa breakdown product of the HSP 90 of *C. albicans* in sera from patients who have recovered from disseminated candidiasis protected mice from a lethal dose of the yeast (24).

A. fumigatus induces an allergic response in patients with ABPA. In the sera of patients with this disease, there were elevated levels of IgM, IgG, and IgE against the fungal HSP. Immunofluorescence has shown the expression of a protein antigenically related to HSP 90 on the surface of macrophagelike cells obtained by bronchoalveolar lavage from patients with interstitial pulmonary fibrosis or asthma and from the synovial fluid of patients with rheumatoid arthritis (37). Antibody against human HSP 90 occurs in patients with systemic lupus erythematosus (27). This has been shown to cross-react with candidal and aspergillus HSP 90 (unpublished data). This suggests that fungal HSP 90 is an important example of molecular mimicry, being involved in both protective immunity and autoimmunity.

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