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Epitope mapping human heat shock protein 90 with sera from infected patients

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Abstract: Epitope mapping with sera from a range of infected patients showed that antibodies are commonly produced which cross-react with a number of epitopes on human heat shock protein 90 (HSP 90). Such autoreactive antibodies were particularly frequent in patients suffering from systemic candidiasis (9 patients), invasive aspergillosis (6 patients), ABPA (2 patients), a patient with aspergilloma and one with malaria. The patient with malaria recognised similar epitopes to those with invasive aspergillosis and candidiasis including the highly conserved epitope LKVIRK and an epitope NNLTGI which was otherwise only recognised by patients with candidiasis. Crossreacting antibodies to relatively few epitopes occurred in patients with *Enterococcus faecalis* and *Corynebacterium jeikeium* endocarditis. This was contrasted with the results from 6 patients with systemic lupus erythematosus (SLE) who were positive on immunoblot against fungal HSP 90. These did not react with the above epitopes but reacted with other areas within human HSP 90.

Key words: Heat Shock Protein 90; Disseminated candidiasis; Aspergillus infection; Malaria; Epitope mapping

Introduction

Recent observations have shown the immunodominance of antibody against heat shock protein 90 (HSP 90) in systemic candidiasis, invasive aspergillosis, allergic bronchopulmonary aspergillosis, patients with an aspergilloma, infection due to *Corynebacterium jeikeium*, and its presence in the sera of patients with malaria and systemic lupus erythematosus (SLE) [1–5]. In all cases the antibody response was dissected by immunoblotting against the corresponding microbial or mammalian antigen and the antigen identified

by its pattern of response with monoclonal antibodies specific to heat shock protein 90. The plethora of diseases and uniformity of the detection technique involved raises the question of whether the antibody detected reacted to the same epitopes or whether the epitopes were disease specific. The antibody response in disseminated candidiasis has already been examined by the Pepscan technique [6]. This delineated the immunodominant epitopes, and subsequent work showed that a monoclonal raised against one of these (LKVIRK) conferred a degree of protection in a mouse model of disseminated candidiasis [7].

Heat shock proteins are highly conserved so that it should be possible to epitope map sera from several conditions where antibody is present

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against a single amino acid sequence. In the present paper human HSP 90 was the test sequence [8]. Variation in epitope can then be demonstrated and correlated with disease state. Sera were first immunoblotted against candidal and aspergillus extracts to ascertain their reactivity with fungal HSP 90.

Materials and Methods

Epitope mapping

Epitopes were mapped by the method of Geyssen et al. [9] with reagents from Cambridge Reasearch Biochemicals, Nantwich, UK. The 732 amino acid residues were synthesised on 716 polyethylene pins as a complete set of overlapping nonapeptides in which peptide 1 consisted of residues 1–9, peptide 2 of residues 2–10, etc. Peptide synthesis was performed with Fmoc-protected amino acid esters and the polyethylene pins themselves were each coupled to Fmoc- β -alanine. Peptides were synthesised by successive cycles of Fmoc deprotection and addition of one amino acid per pin per day, as dictated by the sequence to be synthesised. Hydrobenzotriazole-mediated coupling reactions were carried out overnight in a N,N-dimethyl-formamide solution of each Fmoc-amino acid. After completion of the final coupling reaction and removal of the Fmoc protecting groups the terminal amino group was acetylated in order to remove the unnatural charge of the N terminus of the peptide. Side chain protecting groups were removed by a mixture of trifluoroacetic acid:phenol:ethanedithiol (95:2.5:2.5, v/w/v).

The peptides coupled to the surface of the pins were tested against the sera by enzyme immunoassay (EIA). Pins were pre-coated for 1 h in microtitre plates containing 1% ovalbumin, 1% bovine serum albumin (BSA) in phosphate buffered saline, 0.1% Tween 20 (PBS-T). They were then incubated at 4°C in patients' sera (1/200) washed four times with PBS-T and incubated for 1 h with horseradish-peroxidase conjugated anti-immunoglobulin (1/1000; Sigma, Poole). After further washing the pins were immersed for 30 min in 0.5 mg ml⁻¹ amino-di-3-eth-

ylbenzthiazoline-6-sulphonate in pH 4.0 citrate buffer with 0.03% hydrogen peroxide. Measurements were made with an ELISA plate reader. Pins were cleaned by sonication with fresh buffer each time.

The main problem with this approach is epitope definition. Sera from patients who have recovered from an infection tend to give high background values when examined by the Pepscan technique. In order to circumvent this, epitopes have been defined firstly as a group of at least three consecutive wells where the optical density of each well was at least twice that of the value obtained by an average of 8 wells from the same serum. These were wells A1-A8 on plate one as there was no evidence of an epitope here in any of the sera tested. A mean was calculated of all the positive wells and used as an approximation of the degree of positivity of the individual epitope. This was compared to the corresponding value of a negative control serum obtained by pooling four sera from patients with SLE who were negative on immunoblot. A further control in the case of disseminated candidiasis was the effect in a single patient on the optical density when serum was available before and after seroconversion to candidal HSP 90 and recovery from infection.

Immunoblotting

All patients' sera were immunoblotted against *Candida albicans* and *Aspergillus fumigatus* as previously described [1,2]. Briefly the pressate of *Candida* or *Aspergillus* (prepared in an Xpress, LKB Bromma, Sweden) was boiled for 5 min in cracking buffer (2.6% sodium dodecyl sulphate, 1.3% 2-mercaptoethanol, 6% glycerol, 0.2% bromophenol blue, 50 mM Tris · HCl, pH 6.8) and then run on a 10% acrylamide gel. After blotting onto nitrocellulose the latter was blocked overnight at 4°C in 3% BSA in buffered saline. The blot was incubated for 2 h at room temperature with patients' sera diluted 1:10 in 3% BSA, 0.05% Tween 20. After washing five times in 0.9% saline, 0.05% Tween 20, it was incubated for 1 h in alkaline-phosphatase conjugated goat anti-human immunoglobulin (1/1000). After further washing the membrane was incubated for 10

min with 10 ml of buffer (100 mM Tris · HCl, pH 9.5, 100 mM sodium chloride, 5 mM magnesium chloride) containing 660 μ l nitroblue tetrazolium (NBT) solution (50 mg ml⁻¹ NBT in 70% N,N-dimethylformamide) and 330 μ l of 5-bromo-4-chloro-3-indolylphosphate (BCIP) solution (50 mg per ml BCIP in 70% N,N-dimethylformamide). The reaction was stopped by washing in water.

Patients' sera

Systemic candidiasis. Nine patients were examined, 7 of whom were blood culture positive (4 postoperatively, 2 secondary to candidal peritonitis and 1 neutropenic patient with leukaemia) one had hepatosplenic candidiasis, with yeast seen on biopsy, and one had candidal chorioretinitis and was an HIV antibody-positive drug abuser. The patient with hepatosplenic candidiasis died of other complications. All the other cases of systemic candidiasis survived and were due to *C. albicans*.

Invasive aspergillosis. Six patients were examined from three fatal (necropsy proven) and three surviving cases (bronchoscopy proven).

Allergic bronchopulmonary aspergillosis (ABPA). Two patients were examined.

Aspergilloma. One patient was examined. All as-

pergillus infection was due to *Aspergillus fumigatus*.

Enterococcus faecalis endocarditis. One patient was examined.

Malaria. One patient with *Plasmodium vivax* was examined.

Corynebacterium jeikeium endocarditis. One patient was examined.

Systemic Lupus Erythematosus. Sera from six patients were examined on the grounds of positivity by immunoblotting against fungal HSP 90.

Control. A single pooled serum from four patients with SLE who were negative on immunoblots against fungal HSP 90.

Results

Immunoblotting patients' sera

All nine patients examined with systemic candidiasis had antibody to the M_r 47 000 subcomponent of *C. albicans* HSP 90. The patient with hepatosplenic candidiasis had the weakest antibody to HSP 90 on immunoblotting. All other patients had both IgM and IgG antibodies to the M_r 47 000 band and occasionally to the M_r 40 000

Table 1

Epitopes mapped on human HSP 90: positions (relative to amino end), sequences and reactivity with patients' sera

No. of infected patients recognising each epitope, mean optical densities and standard deviations			SLE			
Epitope			Invasive candidiasis	Invasive aspergillosis		
No.	Position	Sequence		s	d	
1	57	KIRY	0	1 (0.59)	0	4 (0.765 $\pm$ 0.07)
2	101	NNLGTI	7 (0.99 $\pm$ 0.26)	0	0	0
3	210	QFIGYPI	2 (1.4 $\pm$ 0.3)	3 (1.46 $\pm$ 0.57)	1 (0.84)	1 (0.758)
4	271	KKIK	5 (1.1 $\pm$ 0.23)	2 (2.1 $\pm$ 0.47)	3 (0.91 $\pm$ 0.33)	1 (1.05)
5	404	KILKVIRK	7 (0.92 $\pm$ 0.21)	3 (1.48 $\pm$ 0.56)	1 (0.9)	0
6	497	SKEQV	0	3 (1.7 $\pm$ 0.74)	1 (1.3)	3 (0.71 $\pm$ 0.03)
7	549	GLELPE	2 (1.1 $\pm$ 0.2)	3 (1.24 $\pm$ 0.41)	0	4 (0.77 $\pm$ 0.14)
8	580	LDKK	5 (1.0 $\pm$ 0.21)	1 (2.4)	0	4 (0.79 $\pm$ 0.14)
9	607	WTAN	1 (1.16)	3 (1.63 $\pm$ 0.74)	0	0
10	625	NSTMGY	5 (1.05 $\pm$ 0.12)	3 (1.58 $\pm$ 0.8)	1 (1.2)	0
11	642	PIVET	0	0	2 (1.19 $\pm$ 0.1)	3 (0.83 $\pm$ 0.15)
12	655	KN DK	4 (0.98 $\pm$ 1.02)	3 (1.6 $\pm$ 0.6)	2 (0.85 $\pm$ 0.35)	5 (0.84 $\pm$ 0.17)
No. of patients examined:			9	3	3	6

s = survivor.

d = death.

subcomponent as well. Paired sera were available from one of the postoperative patients who developed blood culture-positive systemic candidiasis. On recovery he seroconverted to both the M_r 47 000 and 40 000 bands producing both IgM and IgG. Only two of the nine patients had antibody cross-reacting with *Aspergillus fumigatus* HSP 90 and in each case these were trace amounts of IgM and IgG antibody to the M_r 88 000 and 84 000 bands.

Four of the six patients with invasive aspergillosis, including all three survivors, had antibody to *A. fumigatus* HSP 90 (IgM and IgG to 88 000 and 84 000 M_r bands). None of them had antibody cross-reacting with *C. albicans* HSP 90. Both patients with ABPA had IgM and IgG antibody to *A. fumigatus* HSP 90 (88 000 and 84 000 M_r bands), which did not cross-react with *C. albicans* HSP 90. The patient with an aspergilloma had the same antibody profile to *A. fumigatus* HSP 90 but not to *C. albicans* HSP 90. The patient with *E. faecalis* endocarditis had antibody cross-reacting with both *C. albicans* HSP 90 (47 000 M_r band) and *A. fumigatus* HSP 90 (88 000 and 51 000 M_r bands). Similarly the patient with malaria had antibody cross-reacting with *C. albicans* HSP 90 (92 000 and 47 000 M_r) and *A. fumigatus* HSP 90 (51 000 and 40 000 M_r bands). The patient with *C. jeikeium* endocarditis had antibody cross-reacting only with *C. albicans* HSP 90 (47 000 M_r band). In the cases with SLE, five cross-reacted the *C. albicans* HSP 90, all with the 47 000 M_r band, two with the 40 000 M_r band and one with the 92 000 M_r band. One of these also cross-reacted with the 40 000, 51 000, 84 000 and 88 000 M_r aspergillus bands whilst serum from the last case reacted only with the 88 000 and 84 000 M_r bands of *A. fumigatus*.

Epitope map of human HSP 90

The sera from the patients detected a total of 12 epitopes (Table 1). The control serum produced an optical density for these epitopes which varied from 0.24 and 0.53.

The patients with systemic candidiasis recognised 9 of the epitopes. The most dominant epitopes were (NNLGTI) and (KILKVIRK) which were recognised by 7 of the 9 patients. Epitopes 4

(KKIK), 8 (LDKK) and 10 (NSTMGY) were also recognised by the majority of candidal patients (5 out of 9). The patient who recovered from disseminated candidiasis, seroconverted to the 47 000 M_r band of *C. albicans* and showed a rise in the mean optical density of at least 0.4 to epitopes 2, 3, 4, 7, 8, 9, 10 and 12.

The patients with invasive aspergillosis recognised 11 of the 12 potential epitopes, the exception being epitope 2. Epitopes commonly recognised by the majority of patients with invasive aspergillosis included epitopes 4, 5 and 10 which were also recognised by the majority of patients with deep candidiasis. In contrast, epitopes 3, 6, 7, 9 and 12 were more commonly recognised by patients with invasive aspergillosis than deep candidiasis. Epitope 11 was recognised by patients with fatal invasive aspergillosis but not by patients with deep candidiasis. Single patients with allergic bronchopulmonary aspergillosis recognised epitopes 3 and 7 and both patients recognised epitopes 11 and 12. The patient with an aspergilloma reacted to epitopes 1, 4, 6, 7, 9, 10 and 12. The patient with *E. faecalis* endocarditis recognised epitopes 3, 8, 9, 10 in contrast to the case with *C. jeikeium* endocarditis who was positive for epitope 3 only. The malaria serum reacted with epitopes 2, 4, 5, 8, 9 and 10. The patients with SLE recognised 8 epitopes, most prominently 1, 7, 8 and 12.

The mean optical densities (Table 1) showed that the sera from the patients who had recovered from disseminated candidiasis and invasive aspergillosis had the highest values. The results for the SLE sera were much closer to the control.

Discussion

This paper has epitope-mapped a sequence derived from human HSP 90 against sera from different infections and patients with SLE. All of the sera reacted with fungal HSP 90 on immunoblot and epitope-mapped against the peptides derived from human HSP 90. The infections were heterogeneous ranging from those where the patient had recovered with no evidence of autoimmune disease to others such as ABPA

with a distinct autoimmune component. The results demonstrated twelve different epitopes of which epitopes 1, 6, 7, 8, 11 and 12 were positive for at least half of the sera from the SLE patients. Epitopes 11 and 12 were also detected by both the sera from the patients with ABPA and epitopes 1, 6 and 11 were negative for all the sera from cases of disseminated candidiasis. These results suggest that different epitopes are immunogenic within the same molecule in different disease states.

This can, in part, be explained by differences in the sequence of each individual epitope. When compared to the corresponding *Saccharomyces cerevisiae* [10] and *C. albicans* [11] sequences epitopes 3, 6, 7, 8, 10, 11, 12 are sufficiently different to be likely to be human specific (Table 2). In contrast epitope 5 is conserved between yeast and human HSP 90 and antibody against it was restricted to patients who had recovered from disseminated candidiasis, invasive aspergillosis and malaria. This agrees with earlier work [7].

It has also been suggested that antibody to HSP 90 might be protective against malaria. It has been shown at the Pasteur Institut that squirrel monkeys vaccinated with a protein fraction of *Plasmodium falciparum* developed protective immunity expressed at the humoral level by antibodies against HSP 70 and HSP 90 [3,12]. The puri-

fied IgG from immune monkeys containing antibodies to HSP 70 and HSP 90 cured the blood infection of naive recipient monkeys [13]. The therapeutic effect of gamma-globulin from immune adults in children with severe malaria was demonstrated many years ago in Africa [14].

Schwartz and Mizihami by molecular modelling, suggested that LKVIK occupied a central position in the protein binding site of human HSP 90 [15,16]. This study identifies this as epitope 5 which raises the possibility that antibody against this epitope might neutralise the reactivity of the molecule and form the basis of candidal and malarial immunotherapy.

Antibody against epitope 2 was specific to the patients who had recovered from disseminated candidiasis or malaria. The corresponding candidal sequence is unknown but the sequence from *S. cerevisiae* is identical to that from man. This epitope may thus provide a second target for immunotherapy.

The antibody level in the patients with SLE had a lower mean optical density than the corresponding values from patients with infection (Table 1). This agrees with a recent survey of patients with rheumatic disorders which showed that the incidence of antibodies to human HSP 90 was low, arguing against a direct role in the pathogenesis of such disorders [17].

Table 2

Comparison of the human, *Saccharomyces* and Candidal epitopes

Epitope 90 Number	Human HSP 90 sequence	<i>Saccharomyces</i> HSP 90 sequence	Candidal HSP sequence
1	KIRY	KIRY	—
2	NNLGTI	NNLGTI	—
3	QFIGTPI	EFVAYPI	—
4	KKIK	K-VK	—
5	KILKVIRK	KILKVIRK	KILKVIRK
6	SKEQV	SLKAV	SIKAV
7	GLELPE	FELETD	FELEES
8	LDKK	LGDQ	LDGQ
9	WTAN	WSAN	WSAN
10	NSTMGY	SSMSSY	TTMSSY
11	PIVET	PIIKE	PIIKE
12	KNDK	GAQD	GAED

— = sequence unknown.

Cross-reaction between the monoclonal to LKVIK and specific bands on immunoblots of *E. faecalis* (90 000 M_r) and *C. jeikeium* (86 000 and 52 000 M_r) suggest HSP 90 is an important antigen in these Gram-positive bacterial infections [5]. However, the patients with *E. faecalis* and *C. jeikeium* endocarditis recognised relatively few epitopes on human HSP 90 despite having cross-reactive antibody to fungal HSP 90 on immunoblots.

This study not only confirms that humoral immunity to HSP 90 is common in fungal, parasitic and bacterial infections, but also shows that these antibodies cross-react with a number of epitopes on human HSP 90. In some cases the initial immunogen is probably the microbial epitope which is conserved with an epitope on human HSP 90, such as KILKVIK. In others the immunogen may be quite species-specific but sufficiently similar to the human analogue to induce a cross-reactive antibody. In either case such autoreactive antibodies appear to be of greater benefit than harm to the host, since they have been implicated in protective immunity but not autoimmunity [18].

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