BACTERIAL PATHOGENICITY

Expression of heat-shock proteins in *Streptococcus pyogenes* and their immunoreactivity with sera from patients with streptococcal diseases

J. A. C. LEMOS, M. GIAMBIAGI-DEMARVAL and ANGELA C. D. CASTRO

Medical Microbiology Department, Microbiology Institute, CCS, Federal University of Rio de Janeiro, 21491-590, Rio de Janeiro, RJ, Brazil

The heat-shock response of *Streptococcus pyogenes* following exposure to elevated growth temperatures, and the immunological reactivity of heat-shock proteins (HSPs) in streptococcal infections were studied. Two major proteins of 65 and 75 kDa were expressed when a *S. pyogenes* strain was shifted from 37°C to heat-shock temperatures of 40, 42 and 45°C. Such proteins are members of the GroEL and DnaK families recognised in a Western blot assay with polyclonal antibodies against *Escherichia coli* GroEL and *E. coli* DnaK, respectively. Two-dimensional autoradiograms of polypeptides labelled at 37 or 42°C showed an increased intensity of three spots at 42°C. A monoclonal antibody (MAb) against HSP 63 of *Bordetella pertussis* also recognised the 65-kDa inducible protein, although MAbs against *Mycobacterium tuberculosis* HSP 65 failed to recognise this protein. Immunoblot analysis of sera from individuals with rheumatic fever or uncomplicated streptococcal diseases revealed seven major immunogenic protein bands, two of which also reacted with anti-*E. coli* GroEL and DnaK polyclonal antibodies. Furthermore, antibodies to the GroEL and DnaK proteins were also detected in sera from patients with either rheumatoid arthritis or systemic lupus erythematosus. These results demonstrated a heat-shock response of *S. pyogenes*, and indicated the presence of an immune response against HSPs in streptococcal diseases.

Introduction

Streptococcus pyogenes (Lancefield group A *Streptococcus*) is an important human pathogen causing various infections from mild sore throat to fatal toxic shock-like syndrome; and may also be involved in non-suppurative sequelae (e.g., rheumatic fever, RF). Since the mid-1980s, particularly in the USA and Europe, there has been an increasing resurgence of severe group A streptococcal diseases [1].

Heat-shock proteins (HSPs) or stress proteins are a family of proteins whose synthesis is induced or accelerated when cells are exposed to sublethal heat shock or to various other stress factors. HSPs are ubiquitous in nature and are grouped according to their apparent mol. wt. They represent a group of proteins with a highly conserved structure [2]. HSPs have been implicated in the pathogenesis of several autoimmune diseases, including rheumatoid arthritis (RA) [3]. One of the major symptoms in the

pathogenesis of RF is arthritis and it is possible that RA and RF may be similar in their pathogenic pathways. However, few reports are available that correlate HSPs with streptococci [4–8] and to our knowledge this is the first characterisation of this group of proteins in *S. pyogenes*.

The heat-shock response in one strain of *S. pyogenes* (145/93) is reported, in addition to the identification of two HSP homologues to *Escherichia coli* DnaK and GroEL, based upon immunological reactivity, mol. wt and induction by heat shock. Furthermore, this study examined the reaction of *S. pyogenes* protein homologues to GroEL and DnaK with human immune sera from patients with RF, mild streptococcal diseases (MSD), RA and systemic lupus erythematosus (SLE).

Materials and methods

Bacterial strains and culture conditions

S. pyogenes (145/93), M non-typable, isolated from blood culture was used for the HSP induction studies

and Western blot analysis. Whole-cell proteins of strains of *E. coli* (ATCC 2592), *Mycobacterium tuberculosis* (1850) and *Bordetella pertussis* (165) belonging to our laboratory collection were used as positive controls, with monoclonal and polyclonal antibodies against HSPs and also with the human sera described below. The strains were cultivated in Brain Heart Infusion Broth (BHI; Difco) overnight at 37°C, except for *M. tuberculosis* which was grown in Middlebrook 7H9 medium for 2 weeks at 37°C.

Human sera

Human sera collected from patients with RF (12 patients), MSD (6), SLE (4) and RA (4) were also used in a Western blot assay (diluted 1 in 100). Eight sera from healthy individuals (anti-streptolysin $0 < 125$ IU/ml) were included as controls.

Isotopic labelling and bacterial proteins analysis

Overnight cultures were diluted in BHI to 10^7 cfu/ml and incubated for 30 min at 37°C. Cells were harvested by centrifugation at 5000 *g* for 10 min and resuspended to 10^8 cfu/ml in a methionine-free essential medium (Gibco) with ^{35}S -methionine (Amersham) 100 $\mu\text{Ci}/\text{ml}$. Portions of these cultures were heat-shocked (40, 42 and 45°C) for 30 min whilst controls were left at 37°C. Cells were collected by centrifugation at 5000 *g* for 10 min, washed in NaCl 0.85% w/v and incubated at 37°C for 2 h with lysozyme (Sigma) 10 mg/ml and mutanolysin (Sigma) 500 U/ml.

SDS-PAGE

Cells were lysed by boiling in one volume of sample buffer (0.5 M Tris, SDS 4% w/v, β -mercaptoethanol 10% v/v, glycerol 20% v/v, bromophenol blue 0.1% v/v). Cellular extracts containing 10^5 cpm, from each set of samples, were loaded on to each gel as described by Ausubel *et al.* [9]. Gels were exposed to X-ray film (X-OMAT, Kodak) and processed with MC-110 developer (Kodak) and fixed (X Dental, Kodak).

Two-dimensional gel electrophoresis

Cells were precipitated with TCA 10% w/v, washed once in ice-cold absolute ethanol and once in chloroform:methanol (1:1). After centrifugation at 5000 *g* for 10 min, cells were resuspended in lysis buffer comprising 9.5 M urea (Gibco); nonidet P-40 v (LKB Pharmacia) 2% v/v; ampholytes pH gradient 5–8 (LKB Pharmacia) 1.6%; 0.4% ampholytes pH gradient 3.5–10 (LKB Pharmacia) 0.4%; β -mercaptoethanol 5% v/v. Two-dimensional electrophoresis was performed as described previously [9]. The isoelectric focusing (IEF) gels were pre-run for 30 min each at 200, 300 and 400 V. Cellular extracts, containing 10^6 cpm from each set of samples were loaded on to each gel, electrophoresed for 16 h at 400 V and for an additional 2 h at 800 V. A

polyacrylamide 10% gel was used for the second dimension. Radioactive gels were exposed to X-ray film and processed as described above.

Western blot analysis

Whole-cell proteins separated by SDS-PAGE with 10% gels were transferred on to nitrocellulose membranes (Immobilon-P, Millipore) as described previously [9]. After electrophoretic transfer, membranes were blocked with TBS buffer (20 mM Tris, 137 mM NaCl, pH 7.6) with skimmed milk 4% w/v and Tween 20 0.1% v/v. After blocking, membranes were incubated with *E. coli* anti-GroEL or anti-DnaK polyclonal antibodies or with anti-HSP 63 *B. pertussis* (54G8) and anti-HSP 65 *M. tuberculosis* monoclonal antibodies (MAbs: IT-13, IT-56 and IT-64) or with human sera for 1 h at room temperature. After three washes in the blocking buffer the membranes were incubated for 1 h with peroxidase-conjugated secondary antibodies. Bound antibodies were detected by the ECL system (Amersham) with MAbs and by 3.3 diaminobenzidine (Sigma) 0.5 mg/ml in TBS with polyclonal antibodies and human sera. Antisera were diluted 1 in 1000 in TBS buffer with the exception of the antibodies against *M. tuberculosis* (1 in 100).

Results

Cultures of *S. pyogenes* that were heat shocked from 37°C to higher temperatures (40, 42 and 45°C) showed a progressive increase in the synthesis of two proteins (65 and 75 kDa) (Fig. 1). A 40% reduction of whole-cell protein synthesis at 45°C was demonstrated by radiolabelled counts. Two-dimensional autoradiograms of polypeptides labelled at 37°C or 42°C showed an increase in the intensity of three spots at 42°C with apparent molecular masses of 82, 75 and 65 kDa and with estimated isoelectric points of 6.5, 5.5 and 5.3, respectively (Fig. 2).

Western-blot analysis with *E. coli* anti-GroEL (Fig. 3a) and anti-DnaK (Fig. 3b) polyclonal antibodies revealed bands at 65 and 75 kDa, respectively, in heat-shocked (40 and 42°C) and control cells (37°C) of *S. pyogenes*. These bands were identical to the major bands found in the autoradiograms. The 65-kDa heat-induced protein was also recognised by a MAb produced against a 63-kDa HSP of *B. pertussis* and did not react with the three MAbs against HSP 65 of *M. tuberculosis* (data not shown). Immunoblot analysis of the proteins subjected to two-dimensional electrophoresis showed only one spot that was immunologically related to each antibody, indicating only one isoform to each of the protein families (data not shown). Seven major streptococcal proteins (75, 65, 54, 48, 46, 38 and 29 kDa) were detected by Western blot assays with 18 human sera from individuals with RF or MSD (Fig. 4). Differences in band patterns

Fig. 1. Autoradiograms of [35 S]methionine-labelled proteins of *S. pyogenes* (145/93) under non-heat-shocked and heat-shocked conditions for 30 min. Total cell proteins of *S. pyogenes* at: 1, 37°C; 2, 40°C; 3, 42°C; 4, 45°C, were separated on SDS-PAGE 10% gels; 10^5 cpm were loaded on to each lane. Arrows indicate the position of the heat-shock proteins, and mol. wt standards are indicated on the left in kDa.

were not observed between the RF and MSD sera. The 75- and 65-kDa protein bands corresponded in molecular mass to the *S. pyogenes* DnaK and GroEL homologues. Furthermore, human sera from individuals with RA (four) and SLE (four) reacted with whole-cell proteins from *S. pyogenes* whether or not these cultures had been subjected to heat shock and failed to react with whole-cell proteins from *E. coli* and *M. tuberculosis*. Also, the 75- and 65-kDa heat-induced proteins of *S. pyogenes* reacted with the RF, RA and SLE sera (Fig. 5). In all cases, the reactivity of immune sera tested against heat-shocked and non-heat-shocked preparations appeared to be more prominent in heat-shocked preparations. None of the eight normal human sera tested showed any significant reactivity against the *S. pyogenes* proteins (one of these sera is shown in lane 5 of Fig. 4).

Fig. 2. Two-dimensional autoradiograms of [35 S]methionine-labelled proteins of *S. pyogenes* (145/93) at: **a**, 37°C, **b**, 42°C, for 30 min; 10^6 cpm were loaded on to each gel. Total cell proteins were separated by IEF in the first dimension (pH 5–8) and by SDS-PAGE in 10% gels in the second dimension. Arrows indicate heat-inducible proteins.

Discussion

Despite current knowledge concerning the autoimmune response in RF [10] and the possible role of HSPs in autoimmune diseases [3], the heat-shock response has not been characterised in *S. pyogenes* to date. The present study demonstrated that *S. pyogenes* exhibited a heat-shock response when exposed to high growth temperatures by a decrease in general protein synthesis and an increase of two protein bands (75 and 65 kDa), which belong to the DnaK and GroEL families, respectively. In concordance with these results, Jayaraman and Burne [7] observed two heat-inducible protein

Fig. 3. Identification of GroEL and DnaK homologues in solubilised cellular extracts of *S. pyogenes* by Western blot. Proteins from unstressed cells and heat-shocked cells of strain 145/93 were probed with polyclonal antibodies against (a) GroEL, (b) DnaK. Lanes contain proteins from heat-shocked cells at (1) 45°C or (2) 42°C and control cells maintained at (3) 37°C. Mol. wt standards are indicated on the left in kDa.

Fig. 5. Western blot analysis of sera from patients with rheumatic fever (RF), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) against protein lysates of *E. coli*, *M. tuberculosis* and *S. pyogenes*. Lanes contain proteins from: 1, *E. coli*; 2, *M. tuberculosis*; 3, 5, 8, *S. pyogenes* non-heat-shocked cells; 4, 6, 7, *S. pyogenes* heat-shocked cells. Arrows indicate GroEL and DnaK proteins.

Fig. 4. Western blot analysis of sera from patients with rheumatic fever (RF) or mild streptococcal diseases (MSD). Total cell proteins from *S. pyogenes* (145/93) were separated on SDS-PAGE 10% gels and probed with human sera. Lanes: 1 and 2, MSD; 3 and 4, RF; 5, normal serum. Arrows indicate major immunogenic bands, asterisks indicate bands with the same mol. wt as GroEL and DnaK homologues and mol. wt standards are indicated on the left in kDa.

bands in *S. mutans*; these proteins also belonged to the DnaK and GroEL families. Eight HSPs were found in *Staphylococcus aureus* by two-dimensional electrophoresis when this micro-organism was subjected to heat shock at 46°C [11] and in *Leuconostoc mesenteroides*, cells subjected to heat shock, cold shock or ethanol treatment showed the presence of two major HSPs of 60 and 70 kDa [12]. Two-dimensional analysis of *S. pyogenes* revealed an increase in the intensity of three spots at 42°C, but only one isoform of each GroEL and DnaK protein was observed. In some bacteria, e.g., *Borrelia burgdorferi* [13], the presence of more than one isoform of these proteins has been demonstrated. Therefore, the possibility that additional members of HSP 60 and HSP 70 families exist in *S. pyogenes* which are not cross-reactive with the polyclonal antibodies used cannot be disregarded.

The induced *S. pyogenes* 65-kDa protein also reacted with the MAb anti-*B. pertussis* HSP 63, but failed to react with MAb against HSP 65 of *M. tuberculosis*. Quinn *et al.* [8] showed in an immunoblot assay that the streptococcal GroEL failed to react with the mycobacterial HSP 65 polyclonal antibody, although it reacted with the *Legionella* HSP 60 antibody. These results suggest that these anti-mycobacterial antibodies were directed against epitopes not shared by *S. pyogenes*.

Many bacterial HSPs have been shown to be

immunogenic proteins. Epitopes shared among immunodominant antigens of infectious agents may be important in the development of immunity against diverse pathogens [3]. It is currently thought that HSPs from bacteria are involved in the rheumatic diseases, as seen in RA [14]. It has been demonstrated that pre-treatment of rats with *M. tuberculosis* HSP 65 protected them from developing both *M. tuberculosis*-induced arthritis and streptococcal cell wall (SCW)-induced arthritis [4].

The early phase of RF, characterised by polyarthritis and erythema marginatum [15], may represent pathogenic pathways similar to those found in RA. Several unanswered questions remain, despite many advances in the understanding of the pathogenesis of RF [15]. In 1990, Bahr and colleagues [5] examined the sera from 53 RF patients in an ELISA against members of the HSP 60 and HSP 70 families of *E. coli*, *M. tuberculosis* and human cells. They found that sera from these patients did not differ from normal control sera. In this study, Western blot analysis of *S. pyogenes* whole-cell proteins probed with human sera from patients with RF or with MSD demonstrated at least seven major immunogenic proteins. Two of these proteins corresponded in molecular mass to the *S. pyogenes* DnaK and GroEL homologues. Furthermore, it was observed that sera from patients with RA and SLE showed a specific reaction with *S. pyogenes* proteins and not with *E. coli* or *M. tuberculosis* proteins. The 75- and 65-kDa heat-inducible proteins were present in the SLE and RA sera. Thus, antibodies to what appeared to be HSPs were present in sera from RF, RA and SLE patients. Auto-antibodies against HSPs 65, 70 and 90 have been detected in various rheumatic and autoimmune disorders, including SLE and RA [14] and these results may support the hypothesis of the involvement of HSPs in *S. pyogenes* infections and their sequelae.

The current state of knowledge indicates that some auto-immune disorders manifest T-cell responses to HSPs, as do rats with adjuvant arthritis. The B-cell response to HSPs has been observed in many infectious and auto-immune diseases; however, the frequency of these antibodies varies amongst studies and is dependent upon the detection methods. Studies evaluating the sensitivity and specificity of these antibodies have not been described. Therefore, the humoral response against HSPs should be interpreted with caution.

In conclusion, this study demonstrated that *S. pyogenes* exposed to high temperatures shows increased production of two HSP homologues to DnaK and GroEL proteins and a reduction in general protein synthesis. The clinical significance of *S. pyogenes* HSPs in streptococcal infections is unknown, but antibodies against these proteins were observed in sera from patients with RF and MSD. Also, Quinn *et al.* [8] demonstrated that antibodies produced against myco-

bacterial HSP can react with *S. pyogenes* M protein. It has been suggested that antibodies to M protein may play a role in the pathogenesis of severe group A streptococcal disease in general and of RA and RF [16]. Thus, the role of *S. pyogenes* HSPs in the pathogenesis of streptococcal diseases should also be considered.

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