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Antibodies to a Range of *Staphylococcus aureus* and *Escherichia coli* Heat Shock Proteins in Sera from Patients with *S. aureus* Endocarditis

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Antibodies to a range of *Staphylococcus aureus* and *Escherichia coli* heat shock proteins were present in sera from patients with *S. aureus* endocarditis. This suggests the highly immunoreactive nature of a range of heat shock proteins in addition to the GroEL equivalent (common antigen) protein. In one case, antibodies to three proteins unique to the infecting *S. aureus* strain, which were more prominent in heat-shocked cells, were also detected.

The heat shock response in various pathogenic bacteria, including *Borrelia burgdorferi* (5), *Chlamydia trachomatis* (6), *Mycobacterium tuberculosis* and *Mycobacterium leprae* (32), *Escherichia coli* (13), *Neisseria gonorrhoeae* (31), *Pseudomonas aeruginosa* (3), and *Staphylococcus aureus* and *Staphylococcus epidermidis* (20), is now an area of active investigation. Heat shock proteins are produced during in vivo growth of the pathogen, and antibodies against the GroEL-like protein (common antigen) of one species of bacterium cross-react with the equivalent protein in widely divergent bacterial species (9, 17, 19, 21, 24). Nevertheless, the significance of the heat shock response in the pathogenesis of bacterial infections is not fully understood, and the extent of the host's immune response is not fully revealed. According to Young and Elliott (34), it is not yet clear whether other stress proteins in addition to the GroEL equivalent are general targets of the immune response in bacteria.

We have recently reported the basic features of the staphylococcal heat shock response (20). The major *S. aureus* heat shock proteins had apparent molecular masses of 84, 76, and 60 kDa, and other prominent proteins of 66, 51, 43, and 24 kDa were also induced. The proteins were also induced by $CdCl_2$, ethanol, and osmotic stress. Most of the proteins sedimented with the membrane fraction primarily, but the 60-kDa protein was a notable exception.

S. aureus causes 20 to 30% of all cases of infective endocarditis (30). Typically, this is associated with a strong antibody response to a variety of antigens including teichoic acid (28), peptidoglycan (26, 29), and a crude ultrasonic extract (27). It was of interest to observe the antibody response to heat shock proteins in endocarditis.

The index serum studied was obtained 6 days after the diagnosis of *S. aureus* endocarditis, which apparently developed from a cellulitis of the right foot 5 days prior to admission to hospital. The patient presented with severe congestive heart failure, and six cultures grew *S. aureus*. The isolate from the patient, strain ENDO-2, was examined after minimal subculture. In addition, six other endocarditis serum samples were studied, five of which were kindly provided by L. J. Wheat, Indiana University Medical Center, Indianapolis. They have been used in previous studies of the antibody response to cell wall components (7, 27–29).

S. aureus H, NCTC 8325, RN450 (8325-4), and ENDO-2 were studied. Strain H is a well-known laboratory strain with a well-established cell wall structure (18). Strain NCTC 8325 is from the International Phage Typing Set, and RN450 (8325-4) was obtained from strain NCTC 8325 by curing it of three resident prophages (15, 16). The cured strain is the standard background for most *S. aureus* genetic experiments (10). *E. coli* MC4100 (22), a K-12 derivative, was also studied. Growth conditions for *S. aureus* strains were as described previously (20). *E. coli* was grown in LB medium (12) supplemented with 0.2% glucose. The heat shock procedure was as described previously (20). *S. aureus* total cell lysates were prepared with lysostaphin (20), and those of *E. coli* were prepared with lysozyme-EDTA (22). The protease inhibitor phenylmethylsulfonyl fluoride (1 mM) was added to the lysates. Protein was estimated as described by Smith et al. (23), using bicinchoninic acid in a kit (Pierce Chemical Co., Rockford, Ill.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (11), using 1.5-mm-thick gels (5% stacking and 12.5% separating gels). Western immunoblotting was essentially as described by Towbin et al. (25). Gels were electrotransferred to nitrocellulose paper (Bio-Rad Laboratories, Richmond, Calif.) for 20 min, using the Genie Electrophoretic Blotter (Idea Scientific Co., Corvallis, Ore.). The blots were washed twice in distilled water and once in phosphate-buffered saline for 5 min each and then were incubated overnight in blocking buffer (phosphate-buffered saline containing 0.5% gelatin and 0.05% sodium azide) at room temperature. The blots were then washed four times for 5 min each in washing buffer (50 mM Tris-HCl [pH 7.5] containing 150 mM NaCl, 5 mM EDTA, 0.1% gelatin, and 0.25% Triton X-100). Blots were incubated overnight in normal human or patient serum diluted 1:40 in washing buffer at 4°C. After incubation with serum, the blots were washed four times for 10 min each in washing buffer and then incubated for 1 h at room temperature in washing buffer containing 10 μCi of ^{125}I-labeled protein A (specific activity, 30 μCi/μg; ICN Biomedical Inc., Irvine, Calif.). Finally, the

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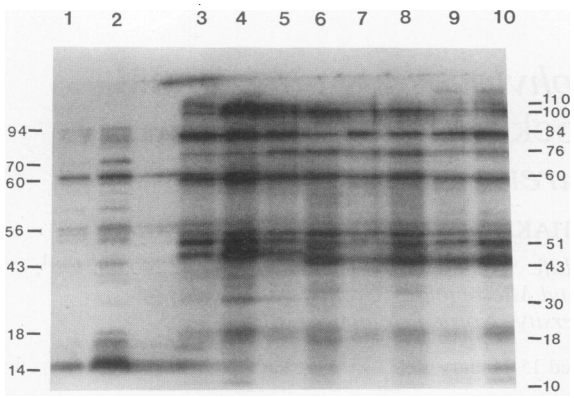

FIG. 1. Western immunoblot of *S. aureus* endocarditis serum against cell lysate proteins of non-heat-shocked and heat-shocked *E. coli* and *S. aureus*. Molecular masses were determined with prestained molecular mass markers, low range, from BRL Life Technologies Inc., Gaithersburg, Md. Numbers on the left- and right-hand sides are molecular masses (in kilodaltons) of the major protein bands. Lanes: 1, *E. coli*, non-heat shocked; 2, *E. coli*, heat shocked; 3, ENDO-2, non-heat shocked; 4, ENDO-2, heat shocked; 5, 8325, non-heat shocked; 6, 8325, heat shocked; 7, RN450, non-heat shocked; 8, RN450, heat shocked; 9, H, non-heat shocked; 10, H, heat shocked.

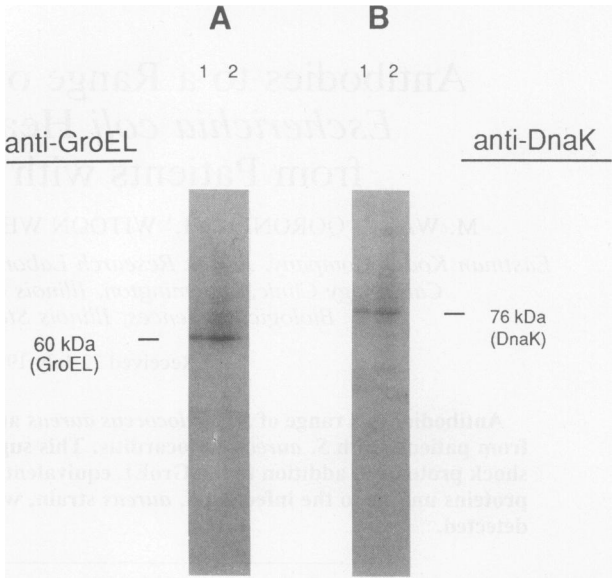

FIG. 2. Western immunoblots of polyclonal rabbit antiserum against purified *E. coli* (A) GroEL and (B) DnaK proteins versus cell lysate proteins of non-heat-shocked and heat-shocked *S. aureus* H. Lanes: 1, H, non-heat shocked; 2, H, heat shocked.

blots were washed three times for 30 min each with washing buffer, dried, and autoradiographed.

The results of Western immunoblots of endocarditis serum versus total cell proteins of *E. coli* and *S. aureus* lysates from non-heat-shocked and heat-shocked cells are shown in Fig. 1. Antibodies were detected versus proteins of 110, 100, 84, 76, 60, 55, 51, 43, 18, 14, and 10 kDa from the various *S. aureus* strains. Most of the bands were more prominent in heat-shocked cells, and several have the same molecular mass (84, 76, 60, 51, or 43 kDa) as previously described *S. aureus* heat shock proteins (20). Additional probable heat shock proteins observed in the present study are the 110-, 100-, 55-, 18-, 14-, and 10-kDa proteins. Higher dilutions (up to 1:2,000) of serum were examined: the more diluted the serum, the lesser number of bands detectable, especially in non-heat-shocked cells. The 1:40 dilution was used to detect a wide range of *S. aureus* and *E. coli* proteins. All of these bands except the 10-kDa protein can be seen on the gels in our previous study of *S. aureus* heat shock proteins (20). Interestingly, we did not note antibodies versus previously identified 66- and 24-kDa heat shock proteins (20).

This serum also recognized proteins of 94, 88, 80, 70, 60, 57.5, 56, 55, 51, 43, 18.4, 16.5, and 14.3 kDa in lysates of heat-shocked *E. coli* cells, which were all more prominent than in non-heat-shocked cells. The bands of 94, 70, 60, and 14 kDa are probably the Lon, DnaK, GroEL, and GroES proteins, respectively, on the basis of their molecular masses and abundance in heat-shocked cells. Also, the *S. aureus* H 76- and 60-kDa proteins reacted in immunoblots with polyclonal rabbit antisera versus the *E. coli* DnaK and GroEL proteins, respectively (Fig. 2). Note the larger band in the lysate from heat-shocked cells. The same result was obtained with *S. aureus* ENDO-2 (data not shown). There was no evidence of *E. coli* infection in the patient. Antibodies to *S. aureus* and *E. coli* proteins were not detected in two batches of normal human serum from different sources at the same dilution.

Antibodies to proteins of 40, 38, and 30 kDa were only detected in lysates of the heat-shocked *S. aureus* endocarditis isolate. These proteins were not detected in the other three strains, which are laboratory strains.

Additional sera from six other endocarditis patients were examined against lysates from *E. coli* and *S. aureus* H and ENDO-2, and the results with four of these serum samples are shown in Fig. 3. All six sera recognized many of the same proteins in *E. coli* and *S. aureus* as those recognized by the index serum. There was a faint band representing antibodies to a 30-kDa *S. aureus* ENDO-2 protein in four of six of the sera. In addition, a band at 48 kDa that was more prominent in heat-shocked *S. aureus* cells was observed in these sera.

Thus, antibodies to a large number of what appear to be heat shock proteins were present in sera from endocarditis patients. The proteins are believed to be heat shock proteins because they were present in larger amounts in heat-shocked cells. Also, their molecular masses corresponded to those described previously for *S. aureus* heat shock proteins (20). Furthermore, the sera contained antibodies that recognized a range of *E. coli* heat shock proteins, yet there was no evidence of *E. coli* infections in the patients. In Fig. 2, it is shown that antibodies raised against purified *E. coli* GroEL and DnaK recognized equivalent proteins in *S. aureus* lysates.

It is well established that the GroEL equivalent protein (common antigen) is an immunodominant antigen in many bacterial infections (9, 17, 21). The present work suggests that many of the other heat shock proteins are also highly immunoreactive antigens. Shinnick (21) and Young and Elliott (34) have suggested reasons for this. These proteins are abundant in bacteria, especially in the stressful conditions of the host environment. Stress proteins might be intrinsically antigenic. Immunological memory for cross-reactive determinants may be generated early in life and be restimulated by subsequent infections. Buchmeier and Heffron (4) have proposed that the expression of *Salmonella* heat shock proteins in the macrophage, which is an antigen-presenting cell, may also contribute to the immunodominance of heat shock proteins. Furthermore, several of the *S.*

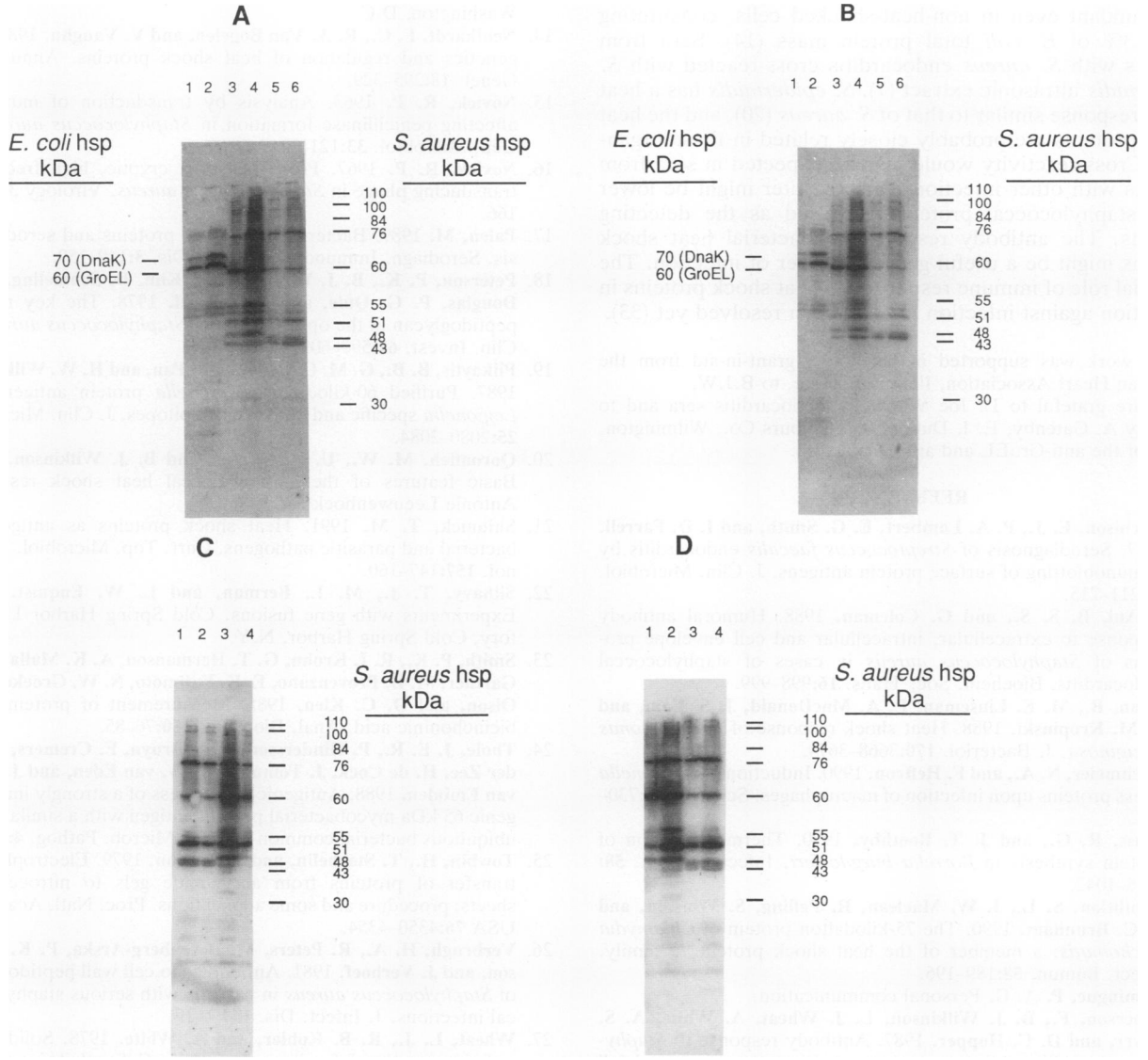

FIG. 3. Western immunoblots of *S. aureus* endocarditis sera against cell lysate proteins of non-heat-shocked and heat-shocked *E. coli* and *S. aureus*: (A) serum 2; (B) serum 3; (C) serum 4; (D) serum 5. Lanes: (A and B) 1, 3, 5, *E. coli*, ENDO-2, and H, respectively, non-heat shocked; 2, 4, and 6, *E. coli*, ENDO-2, and H, respectively, heat shocked; (C and D) 1 and 3, ENDO-2 and H, respectively, non-heat shocked; 2 and 4, ENDO-2 and H, respectively, heat shocked. hsp, heat shock protein.

aureus heat shock proteins appear to be present in the membrane (20), and a cell surface location may also be a factor in immunodominance.

Antibodies were present against three novel, presumed heat shock proteins only present in the strain causing endocarditis. It is not known whether these proteins are a common feature of fresh clinical isolates (Fig. 1). These proteins may be involved in the virulence of the organism, and the genes may be absent or not expressed in the laboratory strains. Avirulent, macrophage-sensitive *Salmonella* mutants failed to synthesize different subsets of proteins usually induced in the macrophage (4).

Al-Ani and Coleman (2) have reported that serum from patients with *S. aureus* endocarditis contained antibodies to a variety of extracellular, intracellular, and envelope *S. aureus* proteins. Aitchison et al. (1) found that *Streptococcus faecalis* endocarditis serum, in addition to recognizing *S. faecalis* antigens, recognized 82-, 70-, 66-, 58-, and 43-kDa *S. aureus* antigens. These molecular masses are strikingly similar to some of those described in this report, even though neither of these groups of authors (1, 2) studied heat-shocked cells. Also, antibodies to *S. aureus* proteins, from non-heat-shocked cells, of 76, 61, 55, 54, 42, 32, and 27 kDa were present in the serum of a patient with *S. aureus* endocarditis (6a). The molecular masses of these antigens are the same as or very similar to those described in this report.

In studies evaluating various antigens for use in the diagnosis of *S. aureus* and *S. epidermidis* endocarditis, an ultrasonic extract which is basically total cell protein, used in radioimmunoassays (7, 27), was more sensitive and specific for serodiagnosis than various purified cell surface antigens. Although the ultrasonic extract was not prepared from heat-shocked cells, presumably antibodies were present against proteins such as DnaK and GroEL, which

are abundant even in non-heat-shocked cells, constituting about 3% of *E. coli* total protein mass (14). Sera from patients with *S. aureus* endocarditis cross-reacted with *S. epidermidis* ultrasonic extract (7). *S. epidermidis* has a heat shock response similar to that of *S. aureus* (20), and the heat shock proteins are probably closely related in these organisms. Cross-reactivity would also be expected in sera from patients with other infections, but the titer might be lower when staphylococcal proteins are used as the detecting antigens. The antibody response to bacterial heat shock proteins might be a useful general marker of infection. The potential role of immune responses to heat shock proteins in protection against infection has not been resolved yet (33).

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