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RESEARCH ARTICLE

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Stress proteins of *Clostridium perfringens* type A immunoreact with antiserum from rabbits infected with gas gangrene

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Abstract Various stressors were used to induce stress proteins in *Clostridium perfringens*. Cultures of *C. perfringens* FD-1041 were subjected to cold shock (28°C for 1 h), acid shock (pH 4.5 for 30 min), or heat shock (50°C for 30 min). Cells were lysed and protein samples were analyzed by immunoblotting with antiserum derived from rabbits suffering from gas gangrene. Eight cold shock proteins (approximate M_r 101, 82, 70, 37, 22, 12, 10 and 6 kDa) and also eight heat shock proteins (approximate M_r 101, 82, 70, 27, 22, 16, 12 and 10 kDa) were immunoreactive with the serum. No immunoreactive proteins were detected in samples subjected to acid shock proteins and purified DnaK protein was also non-immunoreactive with the serum. These immunogenic stress proteins may be important in regulating diseases caused by *C. perfringens*. Such proteins could be involved in cell survival mechanisms, serve as targets during infection, or play a role in recognition of the bacteria by the host.

Keywords *Clostridium perfringens* · Immunogenic proteins · Stress proteins · Stress response

Introduction

Clostridium perfringens is a clinically significant bacterium, as it can cause gas gangrene, food poisoning, infectious diarrhea, and several veterinary diseases [3]. The spore-forming ability of this microorganism enables it to survive critical conditions, such as aerobic environments and food processing [3].

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Bacteria and other organisms synthesize various heat shock proteins (HSPs) in response to exposure to heat. Some of these HSPs have been well characterized in certain microorganisms. HSPs are synthesized by vegetative and sporulating cells, and some are required for surviving stressful conditions and can be involved in virulence [1, 4].

A similar response has been observed in cells exposed to other stressors, such as starvation, low pH, cold temperature, etc. The proteins induced in response to various stressors are generally designated as stress proteins. Many of these stress proteins play a role in the defense system used by the host to prevent or limit bacterial infections [7]. During infection, invading microorganisms are subjected to a range of stressful conditions, such as elevated temperature, increased ionic strength and other host defenses, which can induce the cell stress response [6].

Microbial stress proteins can stimulate immune responses to both pathogen-specific and autoreactive epitopes. These molecules bind most efficiently to the major histocompatibility complex (MHC). Thus, some of these are known as major antigens, since they promote presentation of the MHC to immunocompetent cells [8, 9].

Recently, we found that heat shock (50°C for 20 min) induces the expression of seven proteins in vegetative cells of *C. perfringens* [4]. Similarly, acid shock (pH 4.5 for 20 min) induces five proteins [15], and a cold shock (28°C for 1 h) induces six proteins [16] in *C. perfringens*. Currently, no information is available about the nature of the immunogenic properties of these proteins in this microorganism. In this study, we examined the expression of immunogenic stress proteins in response to various stressors, including heat shock, cold shock, and acid shock.

Materials and methods

Culture

C. perfringens strain FD-1041 was obtained from S. Harmon, Food and Drug Administration, Washington, D.C. The strain was

maintained as spore stock cultures in cooked meat medium at −20°C, as previously described [2].

Active cultures were obtained by transferring two drops of the stock culture into test tubes containing 10 ml fluid thioglycollate medium (FTG, Difco, Detroit, Mich.), heat-activated at 75°C for 15 min in a water bath, and incubated overnight (16–18 h) at 37°C. From these cultures, vegetative cells were cultured in 10 ml FTG in test tubes (13×100 mm) at 37°C. When the vegetative cultures reached an A_{600} of 0.8–1.0, they were centrifuged at 10,000 *g* and the cells were resuspended in 1 ml 0.45 M $CaCl_2$. All experiments were performed at least twice.

Gas gangrene development

A modification of the method of Stevens [14] was used to induce gas gangrene in rabbits. Aliquots (150 µl) of the cells suspended in $CaCl_2$ (5×10^5 cfu/ml) were injected in the left rear legs of two white female rabbits, each approximately 1.7 kg. When the characteristic purulent lesion appeared (about 15 days later), the presence of *C. perfringens* in the secreted pus was evaluated. The bacterium was identified as previously described using confirmatory media [12]. Polyclonal sera were obtained from the rabbits both before injection of the bacterium (pre-immune), and after 15 days.

Induction of stress proteins

Cultures (2 ml) that reached an A_{600} of 0.8 were either (1) cold shocked at 28°C for 1 h, (2) acid shocked at pH 4.5 for 30 min, or (3) heat shocked at 50°C for 30 min. After the treatments, the cultures were maintained at 2–5°C in an ice/water bath for 15 min. Cells were pelleted by centrifugation at 10,000 *g* for 10 min at 4°C. The pellet was washed twice with 30 mM Tris-HCl buffer (pH 7.6).

The cells were solubilized as described by Qoronfleh and Streips [10], with the following modifications. The pellet was resuspended in 2 ml of a solution containing 30 mM Tris-HCl (pH 7.6), 500 µg/ml egg white lysozyme, and 50 µg/ml DNAse I (Sigma-Aldrich Quimica, Mexico). The mixture was incubated at 37°C for 30 min, and then frozen at −20°C for 12–14 h to disrupt the cells.

Detection of immunodominant stress proteins

Solubilized cells (0.4 mg/ml) and purified DnaK protein (0.4 mg/ml, Sigma, St. Louis, Mo.) were separately mixed with an equal volume of 4× sample buffer (3% Tris, 20% β-mercaptoethanol, 10% SDS, 0.02% bromophenol blue, and 40% glycerol; pH 6.8), heated at 95°C for 3 min, and then centrifuged to remove any remaining insoluble material. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described [4]. Myosin (molecular weight M_r 205 kDa), β-galactosidase (M_r 116 kDa), phosphorylase b (M_r 97.4 kDa), bovine albumin (M_r 66 kDa), ovalbumin (M_r 45 kDa) and carbonic anhydrase (M_r 20 kDa) were used as molecular weight standards (Sigma-Aldrich Quimica, Mexico).

After electrophoresis, the separated proteins were transferred to nitrocellulose membranes at 360 mA for 4 h at 4°C. Membranes were then blocked overnight with a blocking buffer including 1% (w/v) gelatin in Tris-buffered saline (TBS: 20 mM Tris-HCl pH 7.4, 0.5 M NaCl). The membranes were rinsed three times with TBS containing 0.05% Tween 20 and then incubated for 3 h with either rabbit preimmune serum or polyclonal antiserum obtained after the infection. Each serum sample was diluted (1:500) in blocking buffer. Membranes were rinsed three times and incubated with goat anti-rabbit IgG peroxidase conjugate (diluted 1:2,500 in blocking buffer) for 3 h (Sigma, St. Louis, Mo.). Immunoreactivity was detected by incubating blots with TBS containing H_2O_2 and 4-chloro-1-napthol.

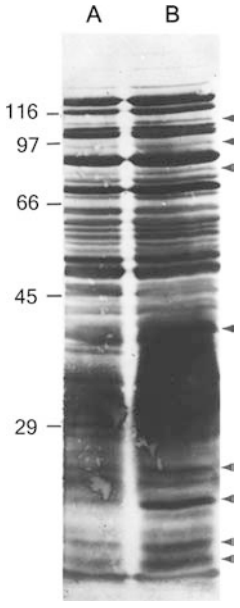

Fig. 1 Effect of cold shock on *Clostridium perfringens*. *C. perfringens* FD-1041 were either cultured under normal conditions or subjected to cold shock at 28°C for 1 h, as described in Materials and methods. Whole cell lysates were subjected to immunoblotting with antiserum derived from rabbits suffering from gas gangrene. Lanes: *A* Control cells, *B* cold-shocked cells. The relative M_r migration (in kDa) of protein standards is indicated by *arrows* on the left. *Arrows* on the right indicate major immunogenic cold shock proteins

To determine if DnaK heat shock protein was an immunodominant protein, purified DnaK heat shock protein derived from *Escherichia coli* (Sigma-Aldrich Quimica, Mexico) was subjected to SDS-PAGE and immunoblotting with the antisera as described above. In a separate experiment, freshly transferred membranes were pre-incubated for 2 h at room temperature with an anti-DnaK antibody (50 µg/25 ml buffer, Sigma, St Louis, Mo.), diluted 1:500 to block the DnaK epitope and then immunoblotted.

Results and discussion

C. perfringens is one of the most important agents causing clostridial myonecrosis (gas gangrene). The infection commences when these bacteria secrete lecithinase to abolish the local defenses of the tissues. Lecithinase may damage or destroy cell membranes, causing cellular lysis, and initiating the typical symptoms of the disease [13]. Rapid onset of myonecrosis, gas production, and sepsis are the hallmarks of this disease. Estimates of incidence of gas gangrene vary. Data from 1975 estimate 900–1,000 cases per year in the United States; however, the incidence is higher in areas or countries with poor access to proper wound care [11]. Thus it is important to find methods to control this disease.

In this work, we identified at least eight immunodominant cold shock proteins (CSPs) with molecular weights of 101, 82, 70, 37, 22, 12, 10 and 6 kDa (Fig. 1). Similarly, eight HSPs were identified with molecular weights of 101, 82, 70, 27, 22, 16, 12 and 10 kDa (Fig. 2). Six of the CSPs and HSPs have

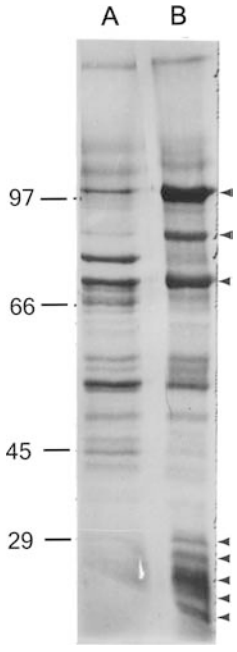

Fig. 2 Effect of heat shock on *Clostridium perfringens*. *C. perfringens* FD-1041 were either cultured under normal conditions or subjected to heat shock at 50°C for 30 min, as described in Materials and methods. Whole cell lysates were subjected to immunoblotting with antiserum derived from rabbits suffering from gas gangrene. Lanes: *A* Control cells, *B* heat-shocked cells. The relative M_r migration (in kDa) of protein standards is indicated by *arrows* on the left. *Arrows* on the right indicate major immunogenic heat shock proteins

similar molecular weights (101, 82, 70, 22, 12 and 10 kDa), and may represent the same proteins. However, further experiments will be necessary to confirm this possibility. No differences in immunoreactivity were observed between control and stressed samples when the preimmune serum was used for immunoblotting (data not shown).

Several reports have shown that a variety of stress proteins are among the major proteins induced in the immune response to various pathogens [6]. Some of these proteins were reported to have molecular weights ranging between 60 to 90 kDa [17], similar to several of the proteins we detected in these experiments.

It has been shown that the DnaK protein is induced in response to heat shock. Previous studies with *C. perfringens* indicated that the M_r of the bacterial DnaK homologue is approximately 82 kDa [4]. It has been documented that this protein could be an immunodominant antigen, and could play an important role in the pathogenesis of infection by other bacteria [5, 17]. In the current study, an 82 kDa protein was induced in response to both heat shock and cold shock. However, neither of these proteins reacted with the DnaK antibodies (data not shown).

Interestingly, no immunodominant proteins were detected in acid-shocked cells (data not shown). It is possible that during the development of the infection, the acid shock response would be insufficient to induce, or to detect, an immunoresponse.

Targeting immunogenic HSPs and CSPs could be important for mediating the illnesses caused by *C. perfringens*. We hypothesize that these proteins may be involved in bacterial survival mechanisms that are activated during infection and in bacterial recognition by the host. Future experiments will be intended to identify these proteins.

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