Vaccine 32 (2014) 3075–3081

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Heterologous protection against alpha toxins of *Clostridium perfringens* and *Staphylococcus aureus* induced by binding domain recombinant chimeric protein

Siva R. Uppalapati, Joseph J. Kingston, Harishchandra S. Murali, Harsh V. Batra *

Defence Food Research Laboratory, Siddarthnagar, Mysore 570011, Karnataka, India

ARTICLE INFO

Article history:
Received 6 February 2014
Received in revised form 3 March 2014
Accepted 5 March 2014
Available online 31 March 2014

Keywords:
Clostridium perfringens
Staphylococcus aureus
Alpha toxin
Heterologous protection

ABSTRACT

Clostridium perfringens and *Staphylococcus aureus* are the two important bacteria frequently associated with majority of the soft tissue infections. The severity and progression of the diseases caused by these pathogens are attributed primarily to the alpha toxins they produce. Previously, we synthesized a non-toxic chimeric molecule r-αCS encompassing the binding domains of *C. perfringens* and *S. aureus* alpha toxins and demonstrated that the r-αCS hyperimmune polysera reacts with both the native wild type toxins. In the present report, we evaluated efficacy of r-αCS in conferring protection against *C. perfringens* and *S. aureus* alpha toxin infections in murine model. Immunization of BALB/c with r-αCS was effective in inducing both high titers of serum anti-r-αCS antibodies after three administrations. Sub-typing the antibody pool revealed high proportions of IgG1 indicating a Th2-polarized immune response. The r-αCS stimulated the proliferation of splenocytes from the immunized mice upon re-induction by the antigen, *in vitro*. The levels of interleukin-10 increased while TNF-α was found to be downregulated in the r-αCS induced splenocytes. Mice immunized with r-αCS were protected against intramuscular challenge with $5 \times LD_{100}$ doses of *C. perfringens* and *S. aureus* alpha toxins with >80% survival, which killed control animals within 48–72 h. Passive immunization of mice with anti-r-αCS serum resulted in 50–80% survival. Our results indicate that r-αCS is a remarkable antigen with protective efficacy against alpha toxin mediated *C. perfringens* and *S. aureus* soft tissue co-infections.

1. Introduction

Acute soft tissue infections like cutaneous abscesses, myositis, necrotizing fasciitis etc. are dangerous bacterial infections with high morbidity and increasing mortality rates, raising serious concerns among medical practitioners [1]. These life-threatening local and systemic complications frequently occur at the body sites that have been injured by a foreign particle or compromised by trauma, ischemia or surgery [2]. Bacterial infection of the soft tissues occurs in a variety of settings *viz.*, soil contamination of the wounds, surgery involving the bowel or biliary system, unhygienic injection of medications/drugs etc. [1]. The severity and progression of these infections are chiefly influenced by polymicrobial aerobic–anaerobic interactions of varied aetiology [3,4]. Many factors like wound type (acute/chronic), depth, location, the level of tissue perfusion and the efficacy of the host immune response influence the diversity of the infecting bacteria [1]. During the polymicrobial infections, the aerobic bacteria utilize the oxygen in the tissue providing satisfactory anaerobic conditions (ischemic muscle) for the rapid growth of anaerobic bacteria [2,4,5]. The most commonly isolated aerobic (facultative) and anaerobic bacteria from soft tissue infections are *Staphylococcus aureus* and *Clostridium perfringens*, respectively [6,7]. The alpha toxins secreted by these pathogens are the key virulence factors involved in causing gangrenous ischaemia, necrotizing fasciitis and myositis in animals and humans [8–10].

The 42-kDa *C. perfringens* alpha toxin (termed αC in this report) is the key virulence factor responsible for gas gangrene in humans and animals [10]. The αC belongs to the zinc-metallophospholipase superfamily with haemolytic, cytotoxic, myotoxic and lecithinase activities besides hydrolysing phosphatidylcholine and sphingomyelin [11]. Crystallographic studies of αC revealed two distinct domains; an N-domain (1–246 amino acids) and a C-domain (256–370 amino acids) connected via a flexible linker (247–255

* Corresponding author. Tel.: +91 9342054402; fax: +91 8212473468.
E-mail addresses: meetsivaram@gmail.com (S.R. Uppalapati), josephkingston@yahoo.com (J.J. Kingston), drmuralihs@yahoo.co.in (H.S. Murali), drharshvardhanbatra@gmail.com, h_v_batra@rediffmail.com (H.V. Batra).

http://dx.doi.org/10.1016/j.vaccine.2014.03.021
0264-410X/

amino acids) [12]. The N-domain is catalytic and the C-domain helps αC in specific and calcium-dependant binding to target cell membranes. Upon binding to the target cells, αC induces carboxyfluorescein leakage and phosphorylcholine release from liposomes and activates sphingomyelin metabolic system through GTP-binding proteins resulting in acute necrosis [13,14]. On the other hand, the 33 kDa *S. aureus* alpha haemolysin (termed αS in this report) is the major cytotoxin responsible for blood, skin, soft tissue, and lower respiratory tract infections by the pathogen [9]. The αS forms heptameric pores by nonspecifically binding onto membranes of many cell types and causes diseases such as dermonecrosis, myositis, necrotizing fasciitis, staphylococcal pneumonia, mastitis etc [9,15]. The three dimensional structure of αS monomer demonstrated three important domains in the toxin *viz*., the cap, stem and rim regions and the heptamer complex is hollow mushroom shaped with a head and stem [15]. The toxin induces arachidonic acid metabolism in mammals, leading to vasoconstriction and cell death resulting in tissue necrosis [16].

C. perfringens and *S. aureus* co-infections progress rapidly and therefore, early diagnosis and proper medical intervention is the cornerstone in the therapy and management. The current treatment regimen as recommended by most authorities involves the use of broad-spectrum empirical antimicrobials associated with fluid therapy, debridation and demotion of necrotic tissue [5]. However, with the emergence of multiple antibiotic resistance, a therapeutic intervention employing multi-component, nontoxic, antigenic domains of the key toxin moieties could be a workable strategy [17,18]. Previously, we synthesized a novel chimeric protein r-αCS, encompassing the truncated regions of αC and αS, and evaluated the detection and *in vitro* toxin neutralizing capabilities of the hyperimmune antisera [19]. In the present study, we assessed the induction of systemic immune response by active immunization of r-αCS and tested for protective immunity against αC and αS induced toxaemia in murine model. Our results show that the r-αCS chimera is potentially an effective vaccine against αC and αS mediated *C. perfringens* and *S. aureus* co-infections.

2. Materials and methods

2.1. Animals

Four to six week old specific-pathogen-free female BALB/c mice were procured from Central Animal Facility, Defence Food Research Laboratory (D.F.R.L.), Mysore, India. The mice were provided with food and water *ad libitum* and acclimatized to laboratory conditions for one week before the experiments. All procedures involving animals were performed according to the standard operating procedures approved by Institutional Animal Ethical Committee at D.F.R.L., Mysore, India.

2.2. Toxins

Purified native wild type αC (P7633) and αS (H9395) were procured from Sigma-aldrich, Bangalore, India. For some experiments, crude exotoxins from culture filtrates of *C. perfringens* ATCC 13124 and *S. aureus* ATCC 6538P were extracted by methanol–chloroform extraction [19,20].

2.3. Cell lines

HeLa cells were procured from National Centre for Cell Science, Pune, India. Cells were grown in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cells were maintained at 37 °C in 5% CO_2.

2.4. Purification of r-αCS

Purification of r-αCS was done according to our previous report [19]. The purified protein was refolded by dialysis against PBS + 10 mmol/l arginine (pH 7.4) for 18 h at 4 °C. The refolded protein was passed through 0.22 μm filter, quantified by Lowry's method and stored at −20 °C until further experiments.

2.5. Immunofluoroscence assay

HeLa cells were grown on poly-L-lysine activated coverslips for 24 h and then fixed with 1% paraformaldehyde for 15 min and 50% ice-cold methanol for 5 min. The coverslips were later blocked in 3% Bovine Serum Albumin solution at 37 °C for 30 min and then placed in a petri dish containing mixtures of r-αCS protein preincubated with either rabbit anti-αC antibodies or anti-αS antibodies for 30 min at 37 °C. Later, the coverslips were gently washed thrice in PBST (PBS (pH 7.4) + 0.05% Tween 20) and subsequently laid over a 200 μl drop of mouse anti-αCS antibodies followed by 1:1000 dilution FITC-conjugated goat anti-mouse IgG solution (Sigma, Bengaluru, India) on parafilm for 30 min at room temperature. After incubation, the coverslips were washed five times in PBST and were mounted onto slides using Fluoroshield (Sigma-Aldrich, India). Slides were visualized with a Nikon Eclipse Ni-E upright fluorescent microscope under 40× objective. Images were captured using NIS-Elements imaging software (Nikon, India).

2.6. Immunization procedures

A total of 24 mice (Four groups of 6 mice each) received subcutaneous injection of a 0.2 ml primary immunizing dose of 50 μg of r-αCS emulsified with Freund's complete adjuvant in 1:1 v/v ratio. On days 7 and 21, each animal received booster i.p. doses consisting of equivalent protein concentrations in Freund's incomplete adjuvant. The control group of mice was sham-immunized with a similar volume of adjuvant in sterile PBS (pH 7.4). Blood samples from one group were drawn periodically (day 0, 14, 28) through retro-orbital sinus and the sera were collected and stored in −20 °C until further use.

2.7. Antibody titers

The serum IgG titer was measured using 2 fold serial dilutions of anti-r-αCS sera by indirect ELISA as described earlier [19]. Endpoint titres were determined as the maximum antibody dilution whose mean O.D. was twice more than or equal to the mean O.D. value of negative sample. The antigenic competition by each of the components in r-αCS was also assessed by Indirect ELISA, where αC and αS were coated onto microtitre plate and probed with anti-r-αCS sera. Absorbance was measured three times at a wavelength of 492 nm in 1 min intervals and the mean O.D. ± SD values were plotted on a graph.

2.8. Antibody isotyping

Serum samples (1:1000 dilution of 28th day sera) from 6 individual randomly selected mice were pooled and assayed for levels of IgG and IgM antibodies against r-αCS by mouse isotyping kit (Sigma, Bangalore, India) as per manufacturer's instructions.

2.9. Splenocyte proliferation assay

Spleens were collected aseptically from r-αCS and sham-immunized mice ($n = 3$). The cells were isolated from the splenic capsule and connective tissue by gently grinding them on a fine wire mesh and flushing with sterile DMEM. Splenocytes were