Research Paper Biomedical

Extraction of M-Protein From Streptococcus Pyogenes Isolated From the Wound Infection and Controlled Release in Mice

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ABSTRACT

This study was designed to identify the predominant serotypes of group A streptococci (GAS) responsible for fatal wound infection. The wound infection swabs were screened for the pathogens that play a main role in the infection. The microbial flora that are present in the wound swab comprised of several facultative anaerobe, Gram Negative and Gram Postive bacteria. Of those species, S.pyogenes were identified to contribute more than any other bacteria. The Streptococcal cell contains the surface protein that serves as the majotr contributor for the pathogenesis. Hence the surface protein, M protein was extracted and purified to trigger the immune response in mice in a controlled release technique. This was done by limited pepsin digestion and purification by ion-exchange chromatography on DEAE-Sephadex, followed by gel filtration. Purified M protein was found to be homologous on SDS-PAGE with the molecular weight of 28 KDa.

Keywords : Bacteremia, Facultative anarerobe, M-protein, Pathogenecity,Wound infection

Introduction

Group A streptococci (GAS) cause a variety of superficial infections, such as impetigo, invasive diseases, including bacteremia, necrotizing fasciitis, and myositis (Fiona et al, 2004). The Group A Streptococcus carries the terms “pyo-,” pus. “-gen-,” forming. It is known as flesh eating microorganism which causes severe wound infections in many cases. The specific type of disease is known as “Necrotizing fasciitis” (Stevans, 2000). Once the bacteria enters the skin through a small niche, the infection spreads rapidly inside the flesh at a rate as rapid as one inch per hour.

Materials and Methods

Collection of Isolates

One-hundred samples along with negative controls were taken for the study. The isolates were studied by standard procedures (Rotta et al, 1971).

Diabetic wound infection isolates were procured from the Institute of Microbiology, Madras Medical College, Chennai. Specimens were obtained either by swabbing from deep areas of primary lesions or by needle aspiration. The swab was placed into anaerobic transport medium and was generally inoculated within 2 h after collection. Needle aspiration was performed with an 18- or 20-gauge needle attached to a 5- or 10-ml syringe. The syringe was immediately sealed and was generally transported to the laboratory within 30 min of specimen collection.

Sheep blood, chocolate, and MacConkey agar plates were inoculated for the isolation of aerobic organisms. The plates were incubated at 37°C aerobically (MacConkey agar) and under 5% carbon-di-oxide (blood and chocolate agars) and were examined at 24 and 48 h. For the isolation of anaerobes, specimens were plated onto prereduced vitamin K1-enriched brucella blood agar, anaerobic blood agar plates containing kanamycin and vancomycin, and anaerobic blood plates containing colistin and nalidixic acid and were then inoculated into enriched thioglycolate broth. The plated media were incubated in GasPak jars and were examined at 48, 96, and The isolates were cultured on to a basal medium and subsequently the biochemical tests were carried out to identify the potential inhabitants.

Processing of the samples

Microbiological analysis was performed by swab wound culture. According to results of microbiological cultures, a significant percentage of cases have been polymicrobial. The most common isolated gram-positive organisms were Streptococci and Staphylococcus. The most common isolated gram-negative organisms were Escherichia coli along with other microorganisms.

Isolation of Streptococcal antigenic Protein

Crude M protein was extracted by limited pepsin digestion (Manjula et al, 1980). Cells were resuspended in 67mM PB, pH 5.8The cell suspension was warmed at 37°C and pepsin was added to a concentration of 1 mg/IO g of bacteria and incubated for 45 min at 37”C, with slow stirring. At the end of the process of digestion, the flask was transferred to an ice bath and solid sodium bicarbonate was added to raise the pH to c. 7.4. The bacteria were sedimented by centrifugation at 10 000 rpm for 20 min. Supernate was sterilized through a 0.2 pm filter (Millipore) and concentrated to c. one-fifth of its original volume by pressure filtration with a UM 10 membrane. Concentrated protein was dialysed against 50 mM ammonium bicarbonate buffer and lyophilised. The sediment is then filtered using Millipore filtration.

Purification of M protein

Crude M protein was purified by DEAE Sephadex A-25 ion-exchange chromatography. Lyophilized crude M protein was dissolved in 10 mM PB, pH 8.0, loaded on a DEAE-Sephadex A-25 column (0.9 x 26 cm) and equilibrated with the same buffer. The protein was first eluted with the eluting buffer and then with a NaCl gradient 100 mM, 200 mM up to 1 M in 10 mM PB.

SDS-PAGE
Peak fractions of ion-exchange chromatography were checked on polyacrylamide gel 12% in the presence of SDS 0.1% with a Tris glycine buffer system (Maizel, 1971). Protein standards (14-66 kDa, Sigma) were run to enable estimation of the mol.wt.

Immunogens in Delivery systems
Biodegradable microspheres of poly lactide co glycolide (PLGA, 50: 50) were tested for their efficacy as slow release microparticle with different proteins in generating high titer and long lasting antibodies. These are polymers of lactic acid and glycolic acid and excreted during normal metabolic pathways. The microspheres product is a free-flowing powder of spherical particles which can be produced in a size range from <1 mm to as large as 3 mm in diameter. They exhibit adequate immunostimulating properties for weakly immunogenic (synthetic) antigen and mimic booster doses after single dose administration thereby serving as single shot vaccine delivery vehicle.

Preparation of Microspheres for encapsulation
Microspheres entrapped with the protein were prepared using PLGA (50:50) by double solvent evaporation method (W1/O/W2 method) described by Jeffery et al., 1991.

Protein entrapped in PLGA is used as a microsphere that serves as a protein delivery vehicle.

Controlled release of the Delivery systems
Female outbred mice of age group 6-8 weeks were procured from King's Inventive Medicine Institute, Chennai. All experimental groups consisted of six animals. All the animals were provided food and water ad libitum. All experiments were conducted in accordance with the guidelines of CPCSEA (for the care and use of laboratory animals), New Delhi.

The Intramuscular route of immunization was followed for our study. For intramuscular immunization, 50µl of microsphere containing protein in PBS was injected on the thigh region with a primary dose of 50µg on day 0 followed by a booster of 50µg on day 45. Booster was given on day 45 with microsphere containing 50µg of protein.

The blood samples were collected from the mice through venous puncture. The samples were allowed to stand for an hour at room temperature and another hour at 4º C; it was centrifuged at 10,000 rpm for 10 minutes. The prepared serum was stored in 0.5 ml polypropylene microcentrifuge tubes at -20º C until use.

Enzyme Linked Immunosorbent Assay (ELISA)
96 well microtitre plate was coated with 100ng/well of protein (l00ml/well) in coating buffer (0.05M, pH 9.6) and kept overnight at 4°C. Nonspecific binding sites were blocked with 5% milk powder for 2 hours at 37°C. After washing thrice with PBS containing 0.05% Tween 20 (PBS-T), test samples (l:100 dilution for serum IgG) 100µl/well were added and incubated for 2 hours at 37°C. All the samples were taken in duplicates. After incubation, the plates were washed thrice with PBS-Tween and peroxidase conjugated goat-anti mouse IgA (Sigma; 1:1000 for lavages and sera), goat anti-mouse IgG (l:l000 dilution for sera) was added and incubated at 37°C for 1hour. The color was developed using OPD as chromogen and H2O2 as substrate in citrate buffer (pH 5.0). The reaction was finally stopped using 8N H2SO4 and absorbance read at 492 nm. The absorbance of control wells were subtracted from the test reading and expressed as Mean + S.D and estimated.

RESULT
The swab of the wound infection was anlalysed and the bacteria which is prevalent in the sample was isolated and characterized by the basic biochemical tests and cultural techniques. The microbial flora was identified as Streptococcus pyogenes, Staphylococcus aureus, Pseudomonas sp, Proteus, etc..

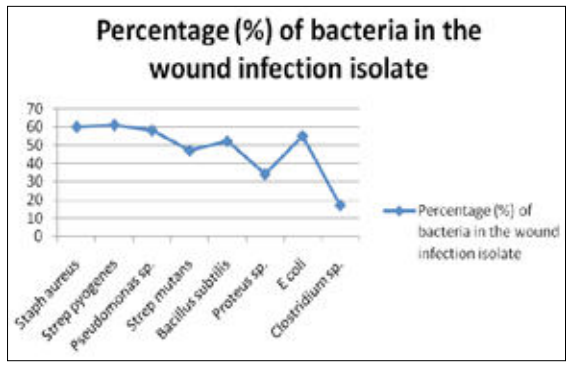

Fig 1: Potential inhabitants of the wound infection sample
Peak fractions of protein were checked by SDS-PAGE along with the standard mol.wt markers, to estimate the mol.wt of proteins in peaks eluted with 200 mM NaCl in 10 mM PB that showed immunoreactivity. One peak was found to be homogeneous on SDS-PAGE, with a mol.wt 28kDa while a second peak showed a major band at 28kDa and minor low mol.wt bands. Other peaks showed multiple bands in the range 15-26 kDa.

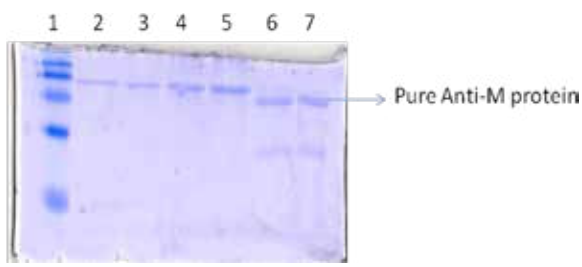

Fig. 2: SDS PAGE- Pure Anti-M Protein

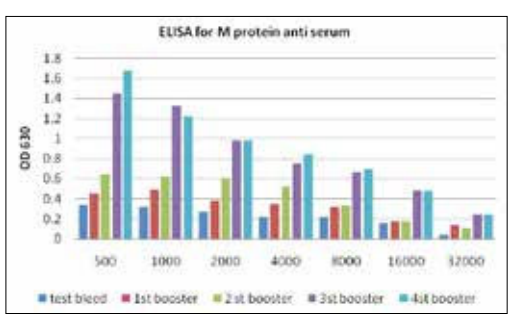

Fig 3: ELISA

DISCUSSION
The wound infections are more common in the post surgery cases, diabetic patients which range from mild to severe in the intensity. In such cases the wounds are more susceptible for the invasion of the microorganism as it provides the necessary growth atmosphere. The delayed wound healing is the major challenge for these cases. These cases in the worst forms are known as Necrotising Fascitis. This means the flesh eating disease. This has the highest morbidity and mortality in the current scenario. This is more common in the patient with diabetes.

Several microorganisms are involved in these infections like Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas sp., Proteus sp, Bacillus subtilis, Clostridium, etc. From those wound isolates, the most prominent species were identified as Streptococcus sp. Streptococcus sp were identified as spherical, Gram-Positive bacterium typically produce large zones of beta-hemolysis on blood agar plates. They are Catalase-negative. When it is compared with the MTCC strain of S.pyogenes procured from the Institute of Microbial Technology (IMTECH), Chandigarh.

The strain is then cultured and purified to isolate the surface protein known as M protein by the standard techniques like affinity and ion exchange methods. The M protein is the major virulence factor for the bacteria that acts as a barrier to the host immune system which tries to engulf the antigen (Vohra et al, 2005).

The M-protein is released into the mice in a controlled manner with repeated boosters. The serum can be collected from the mice and purified for vaccine development as it has the strong virulent factors.

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