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Raising Antibody Against the Purified Streptococcal M-Protein in Mice

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ABSTRACT

The Streptococcal M protein was found to be on the receptors of Streptococci cells. They are the important protein that acts as a barrier for the phagocyte activity of the host cell. The M-protein resists the ingestion and killing of the host immune system because of its virulent receptors. In this study the M-protein are extracted from the Streptococcus pyogenes and subjected to purification. The purified form of the protein is used to raise the immune response in mice model.

Keywords : Pathogen, M-protein, Microsphere, Affinity, Virulent factors, Receptors

Introduction

The wound infections normally comprises of various micro organisms such as Staphylococcus aureus, Streptococcus pyogenes, Vibrio sp, Aeromonas sp, etc.(Edwards et al, 2004). Streptococcus pyogenes (Group A) as the name suggest 'pyo' means 'pus' and 'genes' means 'forming'. It is a Gram-postive bacterium which causes Impetigo, scarlet fever, rheumatic fever, Necrotisiing fasciitis, streptococcal toxic shock syndrome, etc. (Stevans et al, 1989). Group A Streptococci (GAS) is a common pathogen which can cause from mild to serious or life-threatening diseases. It contains several virulence factors that are implicated by the surface proteins. Of those proteins, R28 and M-protein plays a major role in the virulence mechanism. S. pyogenes is composed of one or more M-protein which acts as a barrier for the phagocytic activity expressed by the host cell. The main reason for the study of M-protein is the ability of these proteins to confer protective immunity that lasts for a long time. (John et al, 1992) Hence these proteins can be employed for vaccine development against many diseases caused by the GAS strains.

Materials and Methods

Isolation of Streptococcus pyogenes from the wound infection isolates

Diabetic wound infection isolates were procured from the Institute of Microbiology in Madras Medical College. The Potential inhabitants in the sample were identified by the basic techniques. As per the references the potential inhabitants in the wound infection were Streptococcus sp, Staphylococcus aureus, Vibrio sp, Clostridium sp, E.coli, etc. (Basak et al, 1992).

Preparation of Wound Infection Isolates

The wound infection isolates were procured from the Madras Medical College, Chennai. The sample collected from 100 patients in different time intervals were subjected to the microbiological examination for the predominant species (Lifetek Biosciences laboratory, Chennai). Gram Staining, Culture media preparation, Biochemical tests were performed for the identification of the predominant micro organisms in the sample. The most predominant species, Streptococcus sp was identified and cultured (Leigh et al, 1974).

Bacterial Culturing (Sutcliffe et al, 1996)

Streptococci cells were grown in 37 L batches of Todd-Hewitt Broth 74g/lit (Himedia) supplemented with yeast extract 0.2%, was auto clave at 121° C , for 36 hrs the OD 0.627, The Cell pellet 2g/lit were harvested by centrifugation and washed twice with 20 mM PBS, pH 7.4, and once with 67mM phosphate buffer (PB), pH 5.8.

Extraction of the M-protein

Cells were re-suspended in 67mM PB, pH 5.8, to a final concentration of 1 g of bacteria in 2 ml of buffer. The cell suspension was warmed at 37°C and pepsin (Himedia) was added to a concentration of 1 mg/l0 g of bacteria and incubated for 45 min at 37C, with slow stirring. At the end of the process of digestion, the flask was transferred to an ice bath and solid sodium bicarbonate (Merck) was added to raise the pH 7.4. The bacterial cells were sedimented by centrifugation at 10,000 rpm for 20 min. Supernatant was sterilized through a 0.2 pm filter (Millipore) and concentrated to one-fifth of its original volume by pressure filtration with a UM 10 membrane. Concentrated protein was dialysed (Himedia) against 50 mM ammonium bicarbonate buffer and lyophilised (Beachey et al, 1974).

Crude M-protein was purified by DEAE Sephadex A-25 ion-exchange chromatography. Protein was contents and dialysed, the peaks were measured by broad ultraviolet (280 nm).

Gel filtration was done using a Sephadex G-25 (2 x 150 cm) column which was equilibrated and the sample was loaded 0.5% of bed volume. Then the sample was eluted with 10 mM ammonium bicarbonate buffer, pH 8.2. Each 5ml of sample was collated and the flow rate was measured as 6.8ml Cm and Hr-1. Column fractions were monitored for protein by measuring the absorbance at 280nm.

Peak fractions of ion-exchange chromatography were checked on polacrylamide gel 12% in the presence of SDS 0.1% with a Tris glycine buffer system. Protein standards (14-66 kDa, BD) were run to enable the evaluation of the molecular weight.

Preparation of Microsphere as Delivery system

Microspheres prepared with poly (lactic-*co*-glycolic acid) (PLGA) have been extensively studied for controlled-release drug delivery. (Fu et al, 2000) .Microspheres entrapped with the protein were prepared using PLGA (50:50) by double solvent evaporation method (W1/O/W2 method) (Jeffery et al., 1991). Protein (5mg) dissolved in 100ml of PBS (0.01M, pH 7.2) and emulsified in 5ml of DCM containing 15% PLGA (750 mg) to form the primary water-in-oil (W1/O) emulsion. The primary emulsion was then added slowly to 60 ml of an aqueous solution of polyvinyl alcohol (10% PVA, W2) and ho-

mogenized to produce a stable water-in-oil-in-water (W1/O/W2) emulsion for 5 min using a Silverson homogenizer (Silverson Machines Ltd). The resulting W1/O/W2 emulsion was then stirred over night at ambient temperature to allow solvent evaporation to proceed, with resultant microspheres formation. Following preparation, microspheres were collected by centrifugation, washed three times with double distilled water to remove un-entrapped protein and excess PVA. The final product was freeze-dried and stored in a desiccator at 25°C till use.

Humoral Response in Mice

Female wistar albino 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.

Immunization

50µl of microsphere containing protein in PBS was injected through intramuscular route of immunization on the thigh region with a primary dose of 50µg on day 0 followed by a booster of 50µg on day 45. Another five groups of mice were immunized by the intranasal immunization route by priming on day 0 with 50µg of each protein in microparticle formulation in PBS. 25µl of microsphere containing protein was immunized intranasally using a micropipette while the mice were under isoflurane anesthesia. Booster was given on day 45 with microsphere containing 50µg of protein.

Production of polyclonal antibodies (pAbs)

Three 7-week old female wistar albino mice were injected intraperitoneally with 4 × 10 live, M protein in PLGA according to the method described by prior to immunization, blood was collected to serve as a control.

Preparation of Serum

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. The antibody titer of the serum was assayed using ELISA as described below. For the priming immunization, each mouse was injected intraperitoneally with M-protein with the PLGA antigen emulsion. Throughout the process, the mixture was chilled on a bed of ice from time to time to keep the mixture as close to 4º C as possible to prevent denaturation of the protein. Blood was collected and the serum was prepared and assayed, 10 days after the priming immunization. Four weeks after the priming immunization. The mice were boosted again after two additional weeks. The mice were bled 10 days after each booster immunization and the serum was prepared assayed and estimated using standard ELISA protocol.

The serum was then purified to isolate the pure IgG antibodies by Affinity Chromatography technique.

Results

Streptococci cells were harvested 37 L batches of Todd-Hewitt Broth for 36 hrs the OD 0.627, The Cell pellet 2g/lit were used in M- Extraction. The ion-exchange chromatography profile at 280 nm revealed seven major peaks of protein. The ammonium sulfate (30% saturation) precipitated Proteins were dialysed against ammonium bicarbonate buffer, pH 8.0, followed by lyophilisation. Only peaks eluted with 200 mM NaCl in 10 mM PB showed immunoreactivity. Most of the typespecific immunoreactive material was located in a broad ultraviolet (280 nm) absorbing area with a peak OD 1.521. The final yield of M protein was 0.8 mg/ liters of streptococcal culture

The peak eluted from ion-exchange chromatography was further subjected to Sephadex G-25 gel filtration to separate the major protein from the lower mol.wt (Fig.1) components present after the DEAE ion-exchange chormatography. However, the final yield of the purified protein, 2.5mg/l6 mg of crude protein, after gel filtration Active peak was Fra -4 to Fra-7. The peak fractions were again checked for purity and estimation of the mol.wt by SDS-PAGE. The major peak showed a 28 kDa band.(Fig.2)

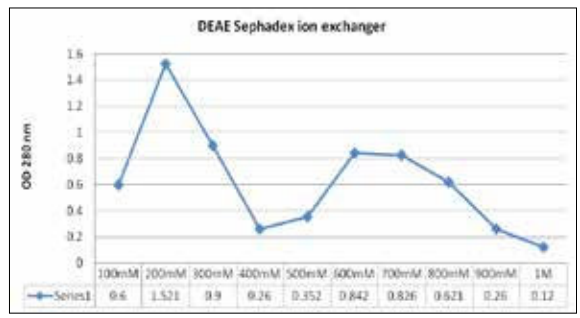

FIG. 1: Stepwise elution of pep M from DEAE Sephadex A-25. 50 mg Samples were eluted with stepwise increases in NaC1 concentrations as indicated. Fraction volume 2.5 ml; flow rate 1 ml/2min.

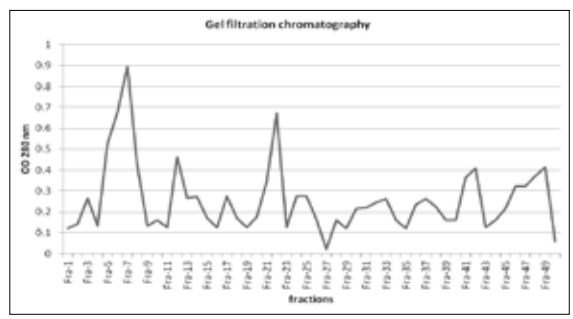

Fig. 2. Gel filtration chromatography of crude DEAE Sephadex pure M protein, eluted with 10 mM ammonium bicarbonate buffer, pH 8.2. each 5ml of sample was collated, Flow rate was 6.8ml Cm,Hr-1

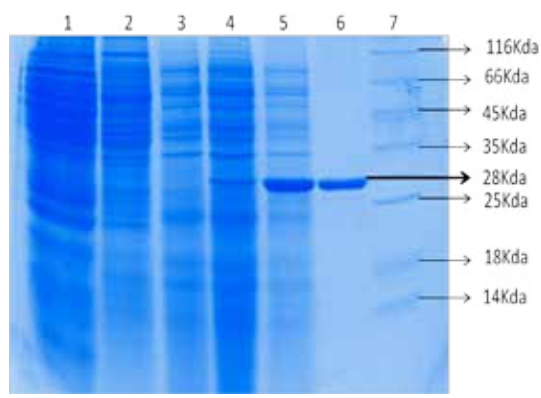

Fig. 3: Coomassie Blue Staining SDS-PAGE Gels - 12%
Lane 1: Cured bacterial pellet; Lane 2:Cured M Protein; Lane 3: ion-exchange flow through; Lane 4: lyophilized M protein; Lane 5: ion-exchange chromatography pure M Protein; Lane 6: gel filtration chromatography pure; Lane 7: molecular weight marker

Discussion

The wound infections are more common and they are caused by bacteria, fungi, etc. Infections in the surgical wound are most significant and serious as it causes major morbidity and mortality. This infection, which is usually induced by virulent, toxin producing bacteria, can occur in any region of the body but it is predominantly located in the abdominal wall, perineum and extremities (Green et al, 1996; Singh et al, 2002).

The most Invasive infections caused by the group A streptococci including streptococcal toxic shock syndrome and necrotizing fasciitis, are associated with considerable morbidity and mortality despite the use of appropriate antibiotics (Hoge at al, 1993; Davies et al, 1996).

The principal virulence factor of the group A streptococcus known to be M- protein and type-specific antibodies against this cell-wall antigen confer protective immunity. The purified antibodies can thus be employed in the vaccine development. The effect of these antibodies can be tested in the animal models like mice or rat. Antibodies provide protection against the infections by several different mechanisms.

Antibodies bind to the adhesion on the surface of any antigen such as bacteria and block adhesion of the bacteria to host cells or neutralize the function of the virulence factor of the organism. Thus the antibody confers the immunity to the host.

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