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Passive immunization against methicillin resistant *Staphylococcus aureus* recombinant PBP2a in sepsis model of mice: Comparable results with antibiotic therapy

Rojin Zeyaei Naghshbandi[a], Setareh Haghighat[b,*], Mehdi Mahdavi[c,d]

[a] Department of Molecular and Cellular Sciences, Faculty of Advanced Sciences & Technology, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, Iran
[b] Department of Microbiology, Faculty of Advanced Sciences and Technology, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, Iran
[c] Department of Immunology, Pasteur Institute of Iran, Tehran, Iran
[d] Recombinant Vaccine Research Center, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) is a representative pathogen that is responsible for a nosocomial infection and considerable yearly mortality rate. Antibiotic resistance provides a great reason for immunotherapy as an alternative strategy to prevent and/or treat the infection.

Herein, following the preparation of recombinant penicillin binding protein 2a (r-PBP2a), rabbit polyclonal IgG was purified. Specificity of IgG to r-PBP2a was evaluated by ELISA and western blotting. IgG fraction was prepared by sulfate ammonium precipitation. In addition opsonophagocytosis assay confirmed bioactivity of purified IgG. Experimental mice were challenged with lethal dose of MRSA (5×10^8) and mortality rate was recorded in the mice treated with IgG fraction for anti-rPBP2a, normal rabbit IgG, vancomycin therapy, and PBS control group. Bacterial quantity was evaluated by culture of liver, kidney and spleen homogenates. Results showed that passive immunization with anti r-PBP2a resulting in a significant improvement in survival rate as well as vancomycin treatment compared with control groups. Furthermore, anti r-PBP2a IgG enhanced considerably the phagocytosis of the *S. aureus* COL strain, reduced bacterial load, and inhibited the systemic spread of COL strain to the internal organs. These results confirmed that passive immunization by anti-r-PBP2a plays a considerable role in the control of infections caused by *S. aureus* similar to that of antibiotic therapy.

1. Introduction

Staphylococcus aureus (*S. aureus*) is an opportunistic bacterial pathogen, a cause of nosocomial and community-acquired infections such as bacteremia, endocarditis, pneumonia and wound infections [1–3]. Multidrug-resistant *S. aureus* infections continue to increase and some strains respond to few, if any, conventional antibiotic therapies [4–6]. Hence, interest in immunotherapeutic strategies, either passive or active, has seen resurgence in recent years [5,7].

Methicillin-resistant *S. aureus* (MRSA) is resistant to virtually all β-lactam antibiotics [8,9]. Resistance to β-lactams is conferred by the acquisition of a mobile genetic element, the staphylococcal cassette chromosome (SCC *mec*) carrying the *mecA* gene which encodes an altered PBP2a with reduced affinity for β-lactam antibiotics [10–12]. As a result, cell wall biosynthesis in MRSA strains continues even in the presence of otherwise inhibitory levels of β-lactam antibiotics [13].

Today, antibiotic therapy for *S. aureus* infections is limited due to the extensive inherent and acquired antibiotic resistance of the organism, and this has led to a focus on immunoprophylaxis by active and passive immunization [5,14,15].

It is noteworthy that patients with invasive infections die within 7 days and should be quickly treated for infectious agents, indicating that active immunization is not the best choice for the prevention of such infections [15,16]. As time is an important parameter; therefore, it seems that the best therapeutic option for such patients would be passive immunization [15].

In our previous study, we have demonstrated that active immunization of mice with recombinant PBP2a (r-PBP2a) significantly induced specific antibodies, increased opsonophagocytosis and reduced bacterial loads as well as enhanced the survival rate of mice in the sepsis model [10,13]. In this study, it is assumed that the antibody to r-PBP2a might play a key role as a neutralizing agent (opsonin) to limit

* Corresponding author.
E-mail address: Haghighat.s@iaups.ac.ir (S. Haghighat).

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the systemic infection of MRSA and decrease mortality rate of challenged mice. So, herein we hypothesized that application of anti-r-PBP2a antibodies would show therapeutic effects in infected mice. The goal of the present study was to evaluate the therapeutic effect of anti-r-PBP2a IgG as an opsonin for bacterial phagocytosis and to show protective efficacy of passive immunization in infected mice and to further investigate mechanisms that might contribute to reduced bacterial load in the internal organs.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. aureus COL strain (methicillin-resistant *S. aureus*) kindly obtained from Dr. Mohammad Emaneini (Tehran University of Medical Science), was used for the challenge and in vitro experiments. *E. coli* strain BL21 (DE3) (Novagen, Wisconsin, USA) was used for expression of recombinant proteins, Luria-Bertani (LB) broth or agar (Merck, Germany) was used for culture.

2.2. Recombinant PBP2a preparation

The r-PBP2a was expressed and purified as described previously [10,13]. Briefly, the methicillin-resistant *S. aureus* COL strain PBP2a gene (mecA) was cloned into pET-24a and expressed in *Escherichia coli* (BL-21) and purified using nickel affinity chromatography in denaturing conditions. Characterizations of the expressed proteins were assessed by SDS-PAGE and Western blot. The r-PBP2a protein solution was dialyzed against 0.1 M phosphate buffered saline (PBS, pH 7.4) for 72 h to remove urea and refolded form used for immunization.

2.3. Production, purification and characterization of polyclonal anti r-PBP2a

New Zealand female white rabbits (Pasture Institute, Karaj, Iran) were vaccinated subcutaneously (five locations) with 500 μg of purified r-PBP2a emulsified in equal volume of complete Freund's adjuvant (Sigma, USA) at 2 week intervals. Boosting was performed two times with incomplete Freund's adjuvant and blood samples were collected prior to immunization 2 weeks after each immunization. An optimized ELISA was performed on experimental sera and confirmed high level of specific IgG antibodies.

After immunization course, a totally of 30 ml blood samples were collected and incubated at 37 °C for 2 h. Sera were collected from the retracted clot, and clarified by centrifugation (6500*g*). IgG fraction of immunized rabbit samples were precipitated with a saturated solution of ammonium sulfate to a final concentration of 50%. Sera obtained prior to immunization were precipitated in the same method to obtain the control non-immune IgG fraction. Protein concentration in IgG fractions was quantitatively measured using a Bradford protein assay kit (Bio-Rad, USA). Anti r-PBP2a IgG and non-immune IgG were adjusted in sterile PBS to 1 mg/ml and finally stored at −20 °C until use.

The titer of the antigen specific IgG antibodies was determined by ELISA using Extragene microtiter plates that had been previously coated with recombinant protein (1 μg/well) in PBS at 4 °C. The antibody level of purified IgG was defined as the highest dilution with highest absorbance value (OD_{450}).

2.4. Western blot analysis

Purified r-PBP2a protein was separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels and then transferred by electroblot onto polyvinylidene difluoride (PVDF) membrane (Hibond, Amersham Biosciences, USA). The membranes were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at 4 °C under agitation. Following washing with TBST (Tris buffer saline containing 0.1% Tween-20), blots were incubated first with rabbit anti r-PBP2a IgG fraction and then with peroxidase-conjugated anti-rabbit IgG (1:5000 in TBST) antibody (Sigma-Aldrich, St. Louis, MO, USA). Finally, the membrane was washed three times with TBST and developed using DAB solution (0.5 mg/ml diamino benzidine plus 0.1% H_2O_2 in TBS) under dark conditions (Sigma-Aldrich, St. Louis, MO, USA).

2.5. Opsonophagocytic killing assay

The opsonophagocytic assay of IgG fractions was carried out according to the *previously described* procedure [17,18]. The test included *S. aureus* strains COL (OD = 0.2; at 650 nm) (~10^8 CFU/ml in 1% BSA); mouse macrophages (2×10^7/ml); diluted samples (1:2 to 1:16) and 4% baby rabbit serum. Evaluations of opsonic killing activity of the samples were compared with those of the pre-immune one. This test was performed in triplicate for each quantity. Percent of opsonic activity of the samples was calculated as follows:

$$\text{Percentage of killed bacteria} = [1 - (\text{CFU of Immune sample}/\text{CFU of Preimmune sample})] \times 100$$

The data was represented as Mean + SD of three separate experiments.

2.6. Passive immunization and bacterial challenge

The protective efficacy of rabbit anti r-PBP2a IgG fraction against MRSA sepsis infection was investigated by passive immunization. All the animals used in the experiment had the approval of the institutional animal care and ethics committee. Six-to-eight-week-old female BALB/c mice (20–22 g) were divided into four different groups, each containing 16 mice as below:

Group 1: Anti r-PBP2a IgG;
Group 2: Non-immune IgG (control IgG);
Group 3: Witness group (Vancomycin treatment control group); and
Group 4: PBS (control group).

The mice were infected intraperitoneally (i.p) with 100 μl of sub lethal dose of MRSA COL (5×10^8 CFU per mouse) [13,19]. Two hours before and 24 h after infection, mice were passively immunized intraperitoneally with 500 μl of IgG fraction versus r-PBP2a (500 μg) or the same volume of PBS, normal rabbit serum IgG fraction (NRS) for control subjects and vancomycin (15 mg/kg) for witness group(vancomycin treatment control group). The survival rates for all mice were monitored for 30 days after infection.

2.7. Bacterial burden and histopathological analysis

To assess bacterial burden in internal organs, followed by passive immunization, the groups (as mentioned above) of four mice were sacrificed 3 days after infection with MRSA COL strain (approximately 5×10^8 CFU). In aseptic conditions, the blood, spleen, kidneys, and liver were harvested and assessed for bacterial colonization. Briefly, Solid organs were dissected mechanically in cold PBS and then homogenated by vigorous pipetting and after reaching to a homogenous homogenate, a 10 fold serial dilutions were prepared and cultured on LB agar containing 50 μg/ml of oxacillin. The colonies were quantified after 24 h of incubation at 37 °C. Results were calculated as Log CFU per gram of tissue (CFUs/g) (Fig. 1).

Histopathological analysis was conducted on day 3 after infection. Organs were fixed with 10% phosphate-buffered formalin and embedded in paraffin. Four-micrometer-thick sections were prepared and stained with hematoxylin and eosin (H&E) for microscopic examination.