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Increasing of Methicillin-Resistant *Staphylococcus aureus* Vaccine Potency, Using a Mixture of Alum-Naloxone: Augmentation of Humoral Immune Responses

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Abstract
Staphylococcus aureus is a Gram-positive bacterium causing septicemia, pneumonia, and endocarditis. In this study, a mixture of naloxone and alum has been used to improve the efficacy of killed methicillin resistant *S. aureus* (KMRSA) as a vaccine. Female Balb/c mice were divided into six groups and the vaccines were either injected alone, with naloxone, alum, or a mixture of naloxone-alum and control group received naloxone and PBS buffer. Total IgG antibody level was measured by ELISA method and finally, the challenge test of this bacterium was performed and the mice were examined regarding the degree of bacteria growth in their kidneys. The stronger immune response was observed when the vaccine was supplemented with a mixture of alum and naloxone and was able to decrease the number of bacteria in the kidney at the lowest level. The mixture of naloxone and alum as an adjuvant with the KMRSA enhances the humoral immunity leading to a high level of protection against MRSA infections.

Keyword: Methicillin-resistant *Staphylococcus aurous*, naloxone, alum, immunogenicity, adjuvant.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is both a human commensal and a frequent cause of clinically important infections, including bacteremia, metastatic abscesses, septic arthritis, pneumonia, and osteomyelitis. [1]. Methicillin has been widely used against this organism since 1940. However shortly after its usage, the first case of MRSA was reported in 1968 [2]. The prevalence of MRSA have been reported to be more than 70% in Asian countries including China, Korea and Taiwan [3], but lower in North America (50%) and in Europe (20%) [4]. Different rates of MRSA, up to 48.5%, have been documented by the Iranian investigators. Such differences are probably due to the application of different testing methods, and quality of antibiotic discs in their studies [5, 6]. One of the major problems in dealing with this pathogen is confronting with its diverse mechanisms for pathogenesis and the lack of knowledge on the effective immune responses against it [2]. In such cases, the use of new adjuvants in vaccines appears to be a promising approach. Alum is a suitable adjuvant which is widely used in vaccinations and its use in human vaccination has been authorized. Furthermore, this adjuvant can appropriately stimulate the immune system; although in some vaccines such as Hepatitis B, a percentage of the population do not show effective antibody responses to this adjuvant [7]. Previous studies have shown that naloxone, an opioid antagonist, has the ability to diverge immune response towards Th1 and is able to enhance immune responses to vaccines [8, 9]. On the other hand, in terms of toxicity or serious side effects, it is completely safe and its use has been approved by the FDA for human drug users. Also, recent studies indicate that naloxone is able to boost the performance of alum adjuvant to induce antibody responses [10, 11]. In this study, the adjuvant effect of a mixture of naloxone and aluminum in the vaccine model of killed methicillin-resistant *Staphylococcus aureus* was evaluated in promoting humoral immune responses and protection in experimental infection with the bacteria.

MATERIALS AND METHODS

Bacterial strain and growth media

The *S. aureus* COL strain (methicillin-resistant *S. aureus*) was obtained from Pasteur Institute of Iran. Luria Bertani medium (LB; Merck, Germany), tryptic soy broth (TSB; Merck, Germany) and Mannitol salt agar (MSA; Merck, Germany) were used for routine cultivation of all bacterial strains.

Preparation of killed MRSA

The *S. aureus* COL strain was cultured in 750 ml of TSB until turbidity of the growth reached to 0.6 at O.D 600 nm. The bacterial cells were harvested by centrifiugation at 4000 rpm for 30 min (Sigma, Germany). Subsequently, PMSF (1mM) was added. The cells were sonicated 3 times at 130 kHz with 10 seconds intervals, each time for 30 second (Hielscher Ultrsound Technology, Germany). Then dialysis was performed in PBS 1x solution (Sigma). Finally, the obtained protein concentration was sterilized via filtration and measured with Bradford method using bovine serum albumin standards.

Mice groups and vaccine administration

The male Balb/c mice, 6 to 8 weeks old were purchased from Pasteur Institute of Iran. They were divided into 6 groups of 24 and kept in separate cages. Study groups were injected with 10 µg of killed methicillin-resistant *Staphylococcus aureus* formulated in naloxone, alum, or a mixture of naloxone and alum. Injections were done on days 0, 14 and 28 subcutaneously. Control groups were received KMRSA without adjuvant, PBS and naloxone with a final volume of 0.5 ml. All animal experiments were authorized by the Ethical committee of Iran University of Medical Sciences.

Evaluation of total IgG level

Two weeks after the final injection, blood samples were taken from the corner of the eyes of mice (sinus retro orbital), and the serum was separated and stored in -20°C to evaluate total IgG level. Total IgG level in the serum of mice was measured using ELISA (Extragen, Korea). 100 µL of 10µg/mL bacterial antigen concentration in PBS (pH=7.5) was added to each well of the micro titer plate and incubated at 4°C overnight. The plates were washed 3 times using PBST buffer (PBS buffer including 0.05% Tween 20), then blocked with PBST including 5% BSA for 2 h at 37°C. The plates were rewashed with PBST 5 times for 1 min, then serum dilutions from 1:100 to 1:12800 were prepared and 100 µL was added to each well. The plates were then incubated for 2 h at 37°C. After four washes, 100µL of HRP-conjugated antibody (rabbit anti-mouse IgG, Sigma) with a 1:10000 dilution was added to each well, and then incubated for 1.5 h at 37°C. After a few washes, 100µl of TMB/H2O2 chromogenic reagent (Razi Institute, Iran) was added to each well. Enzyme activity was stopped using 1 Normal sulfuric acid (Merck) and optical density was measured at 450 nm wavelength (Pharmacia, France).

Evaluating bacterial load in experimental groups

One week after the bacterial challenge, kidney of experimental mice was removed in sterile conditions. After homogenization of the cell suspension, 10 µL of the prepared suspension was cultured in LB agar medium and the next day with the number of grown clones were counted as CFUs (Colony Forming Units) in groups of mice to determine bacterial load.

Statistical analysis of data

SPSS 21 (SPSS Inc, Chicago, Illinois, USA) was used for data analysis and the results of the quantitative data were analyzed based on the average of three repeats of the test using One-Way ANOVA test and LSD method. The data are reported at 95% confidence level with $p<0.05$ considered as significant.

RESULTS

Total antibody response

The total IgG antibody measured in the groups of mice vaccinated with alum, naloxone or a combination of both adjuvants compared to the control groups showed a significant increase ($p < 0.003$) (Figure 1). A mixture of naloxone and alum triggered antibody responses in experimental groups compared to the group that received vaccine plus alum ($p = 0.001$). In addition, administration of the vaccine containing the alum/naloxone showed significant enhancement in total antibody production compared to the group administered with vaccine/naloxone ($p=0.024$).

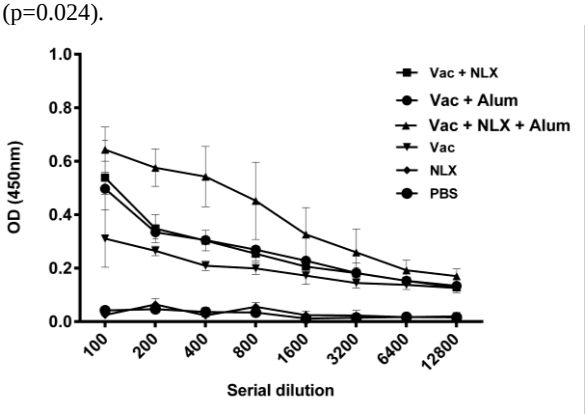

Figure 1. Results of total IgG measurement in various study groups. Total IgG production wash shown to be more in the group administered with killed methicillin-resistant *Staphylococcus aureus* formulated with a mixture of naloxone and alum compared to other experimental groups. Values in represent the mean ± SD of five mice in each group.

Bacterial load in mice experimentally infected with MRSA strain

The average bacterial load in all groups administered with staphylococci vaccine in mixture with the alum/naloxone adjuvant group has significantly decreased in their kidney ($p < 0.05$); but there was no significant difference observed in comparison with the group administered with vaccine/naloxone ($p = 0.001$) (Figure 2).

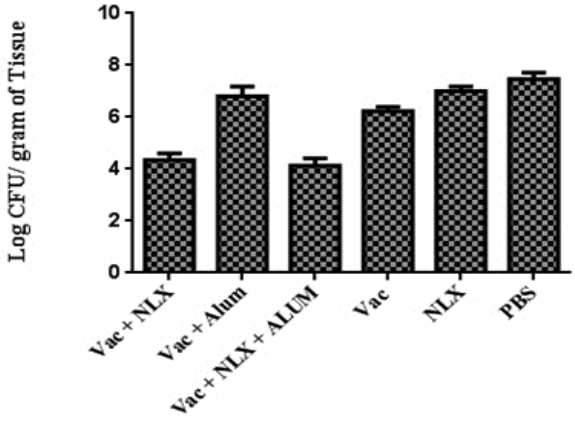

Figure 2. Bacterial load in kidney of mice infected with the MRSA strain.

DISCUSSION

Staphylococcus aureus uses different mechanisms to escape from the immune system [1]. With the emergence of new antibiotic-resistant strains, conventional antibiotic therapies have failed and vaccines may be able to control the disease [12]. Vaccine effectiveness largely depends on the appropriate adjuvant [13]. Naloxone is being used to cure drug addiction with no observed toxicity. It increases the level of IFN-γ cytokine and cellular immunity in HSV-I infections and has been approved by the World Health Organization and the FDA [14]. Therefore, it is presumed that Naloxone may have adjuvant effects [15]. Further studies demonstrated the adjuvant effects of Naloxone as it triggers the level of cellular immune responses via shifting immune responses towards Th1 responses, increases the

level of IFN-γ cytokine and improves proliferative responses of lymphocytes plus cytotoxic effects [16]. It appears that Naloxone acts as an adjuvant due to its function in creating an inflammatory environment [17]. This drug also stops the regulatory effect of T regulatory cells on antigen-presenting cells [18]. Since strains of *Staphylococcus aureus, in* particular MRSA, are multi drug resistant, treatment failure occurs in many cases. Thus in the present study, we have attempted to enhance the effectiveness of vaccines against these pathogens using a mixture of naloxone-alum as an adjuvant.

The results of the present study showed that a mixture of alum-naloxone along with the vaccine enhanced the effectiveness of the vaccine by triggering the humoral immune responses. It also resulted in a decrease of bacterial load in the kidney of immunized animals. Furthermore, this study shows that a mixture of alum-naloxone is more efficacious in inducing humoral responses and inhibiting the spread of bacteria in vivo than alum by itself. Previous studies showed that a mixture of naloxone-alum could enhance humoral immunity [11] which is in agreement with our findings. The function of the alum-naloxone mixture in enhancing humoral responses is probably due to the both alum and naloxone effects [17]. Triggering the immune responses using a mixture of naloxone and alum in order to stimulate humoral immunity [19, 20] can be of great importance in controlling extra-cellular pathogens. With regard to the MRSA, humoral immunity plays a significant role in its controlling. The results of the current study demonstrate a mixture of Alum-naloxone as an adjuvant induce humoral immunity and decrease the bacterial load via antibody-dependent mechanisms in mice more efficiently [11]. It seems that the enhanced humoral immune responses in the naloxone-alum adjuvant group has decreased the bacterial load and this means that compare to alum, which is the conventional adjuvant in humans, the naloxone-alum group has enhanced the efficacy of the vaccine in inducing immune responses. The results of the current study have inspired us to pursue other aspects of immune responses in future studies.

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Conflict of Interest

We declare that there is no conflict of interest among authors of this study or with other authors or research teams.

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