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Complement activation contributes to the anti-methicillin-resistant *Staphylococcus aureus* effect of natural anti-keratin antibody

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a major public health problem worldwide because of its strong resistance to a variety of antibiotics. Natural immunoglobulin (Ig) M antibodies have been reported to protect against microbial infections. In the present study, the function of a monoclonal natural anti-keratin antibody IgM (named 3B4) in MRSA infection was evaluated. The binding of 3B4 to MRSA was studied using immunofluorescence assay and flow cytometry (FCM). The binding of 3B4 to mannose-binding lectin (MBL) and complement activation were detected by ELISA. For the *in vivo* study, transgenic mice for the V_H gene from 3B4 (TgV_H 3B4) were used. After infection, the bacterial burden was examined in the kidney, spleen and enterocelia. Inflammatory cytokine levels and the neutrophil ratio in peritoneal lavage fluid (PLF) were assessed by ELISA and FCM, respectively. Additionally, the total serum hemolytic activity (CH50) in the early stage of infection was detected by ELISA. The results showed that 3B4 bound directly to MRSA and MBL, and the interaction between 3B4 and MRSA/MBL led to the activation of the classic and the MBL pathway *in vitro*. After 48 h of MRSA infection, the bacterial load in the kidney, spleen and enterocelia was significantly decreased in TgV_H 3B4 mice ($P < 0.05$) compared with wild-type mice. Levels of IL-6, TNF-α, and IFN-γ were increased after MRSA infection. The levels of IL-6 and TNF-α in TgV_H 3B4 mice were decreased by 49.1% and 59.4% compared to wild-type mice. Additionally, the neutrophil ratio in the PLF of TgV_H 3B4 mice was decreased by 65.9%. The CH50 value was significantly higher in TgV_H 3B4 mice than in wild-type mice, indicating that 3B4 promoted the activation of the complement system in MRSA infected mice. The results reveal an important role of 3B4 in the anti-MRSA immune response, and the complement activation contributes to this effect.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an increasingly common pathogen in hospital and community settings [1]. It remains a major public health problem worldwide because of its strong resistance to a variety of antibiotics [1]. Penicillin-binding proteins (PBPs) of bacteria are important for penicillin-like antibiotics to exert their antimicrobial properties. The mechanism leading to methicillin resistance of MRSA is based on the expression of PBP2a, which is a mutated PBP [2]. The expression of PBP2a significantly reduces the binding affinity of β-lactam antibiotics [2]. Since excessive antibiotic use has become one of the top contributors to the development of resistance, new therapies are needed in this ongoing struggle, such as vaccines and antibodies.

Natural antibodies are a type of antibody that are produced without external antigenic exposure [3]. Natural antibodies are mostly of the immunoglobulin (Ig) M isotype and play crucial roles in the immune system [4,5]. IgM plays an important role in enhancing IgG responses and protecting against the development of autoreactive IgG and autoimmune diseases [3]. Natural IgM antibodies have also been reported to protect against microbial infections by recognizing a wide range of different microbial components [6,7]. In a previous study, we reported a monoclonal natural antibody, 3B4, which was isolated from the serum of a unimmunized mice [14]. We demonstrated that 3B4 recognized keratin and a surface antigen located at germ tubes of *Candida*

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; Ig, immunoglobulin; FCM, flow cytometry; TgV_H 3B4, a μ chain transgenic mouse (TgV_H 3B4) using the V_H gene from 3B4; PLF, peritoneal lavage fluid; MBL, mannose-binding lectin; NMS, normal mouse serum; CFU, colony-forming units; MgEGTA, magnesium ethylene glycol tetra-acetic acid; TMB, 3,3′,5,5′-tetramethylbenzidine; HRP, horse reddish peroxidase; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; BSA, bovine serum albumin.

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albicans, and the isotype of 3B4 was IgM [14]. 3B4 inhibited germ tube formation of *C. albicans in vitro* and protected mice from *C. albicans*-induced death *in vivo* [14]. We also established a μ chain transgenic mouse (TgV_H 3B4) using the V_H gene from 3B4, and demonstrated that this mice possessed high level of anti-keratin/*C. albicans* IgM antibodies and were resistant to *C. albicans* infections [14].

The immune system is comprised of two branches: innate and acquired immunity. The innate immune system is the first line of host defense against pathogens, and acquired immunity is involved in the late phase of infection [8]. The complement system plays important roles in both innate and adaptive immune defense by defending against microbial infections, bridging innate and adaptive immunity, and disposing of immune complexes and the products of inflammatory injury [9]. The complement system can be activated by three different pathways: the classic pathway, the mannose-binding lectin (MBL) pathway, and the alternative pathway [10]. It has been reported that natural IgM can activate complement by the classical and lectin pathways under several conditions, such as pathogen infection, ischemia/reperfusion injury and hypoxic stress [11–13]. The effect of IgM in MRSA infection and complement activation needs to be investigated.

In the present study, we evaluated the protective effect of a monoclonal natural anti-keratin antibody IgM (3B4) during MRSA infection and its role in complement activation. The results show that 3B4 can bind to MRSA and activate the classic pathway and MBL pathway *in vitro*. In TgV_H 3B4 mice, the anti-MRSA effect and complement activation were greater than in wild-type mice. These results indicate that complement activation contributes to the anti-MRSA infection effect of the natural antibody 3B4.

2. Materials and methods

2.1. Monoclonal antibody and MRSA

The monoclonal natural anti-keratin antibody IgM was produced as previously described [14]. The clone 3B4, which preferentially recognizes keratin, was used in the present study. The MRSA strain used was obtained from ATCC (Rockville, Maryland, USA).

2.2. In vitro MRSA-3B4 binding assay

For the immunofluorescence assay, 100 μL of MRSA solution (1×10^7/mL) was incubated with 1 μL of 3B4 (1 mg/ml) for 1 h. After washing twice with PBS containing 5% FBS, FITC-labeled anti-IgM (Sigma, St. Louis, MO, USA) was added and incubated in the dark for 30 min. After washing three times, the bacteria were detected by fluorescence microscopy (Olympus, Tokyo, Japan). A purified IgM from murine myeloma (TEPC, 1 mg/ml, Sigma) was used as the isotype control.

Flow cytometry (FCM) was also performed to detect the binding of MRSA and 3B4. Briefly, 1 μL of 3B4 (1 mg/ml) was added to 100 μL of the MRSA solution (1×10^7/mL) and incubated for 1 h. The system was washed twice and the supernatant was removed. FITC-labeled anti-IgM (Sigma) was added to the system and incubated for 1 h. After washing twice, the samples were analyzed by a FACSCalibur instrument (BD Biosciences, San Jose, CA).

2.3. In vitro MBL binding assay

Purified 3B4 and TEPC were coated at different concentrations. The wells were washed three times and blocked for 1 h at room temperature with 0.1% bovine serum albumin (BSA, Invitrogen, Carlsbad, CA, USA). After washing three times, normal mouse serum (NMS, Invitrogen) was added to the incubation system. Following incubation overnight at 4 °C, the wells were washed three times. Biotinylated anti-MBL-A or anti-MBL-C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the wells and incubated for 1 h. HRP-labeled streptavidin (Santa Cruz Biotechnology) was added and incubated for 30 min. Then, the substrate solution was added and incubated for 10 min. The reaction was stopped by adding 0.5 M H_2SO_4 to each well. The plates were read at 450 nm using a BioRad (Hercules, CA, USA) ELISA reader.

2.4. Analysis of complement activation

To elucidate which complement pathways are activated by 3B4, several complement inhibitors were evaluated. The magnesium ethylene glycol tetra-acetic acid (MgEGTA, 5 mmol/l, Sigma) was used to block complement activation. An antibody against C1q (IgG1, Abcam, Cambridge, UK) was used to block the activation of classic pathway. An antibody 20B4 (IgG1, Abcam) was used as isotype control of anti-C1q antibody. D-mannose (200 mmol/l, Sigma), a ligand for MBL which can inhibit the binding of MBL to its ligands, was used for the blockade of MBL pathway. Since the alternative pathway is inactive at serum concentrations below 5%, 2% serum was used to block the alternative pathway. Complement activation was measured by the detection of C3 deposition. The plates were coated with TEPC (1 mg/ml) or 3B4 (1 mg/ml), blocked with BSA, and incubated with MRSA (for classic pathway activation). Subsequently, plates were incubated with 2% or 5% normal mouse serum (NMS, pre-incubated with anti-C1q/D-mannose), with/without the presence of MgEGTA, for 1 h at 37 °C. The plates were washed, then incubated with rat anti-mouse C3 (Abcam) followed by horse reddish peroxidase (HRP)-conjugated rabbit anti-rat IgG (Abcam). Bound antibody was detected using the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB).

2.5. Animal and treatment

The μ chain transgenic mice (TgV_H 3B4) using the V_H gene from 3B4 used in this study was kindly provided by Prof. Yufeng Liu (Fourth Military Medical University, Xi'an, China) [14]. The mice in the infection group were administered 0.5 ml of MRSA (10^8/ml) by intraperitoneal injection. The mice in the control group were injected with the same volume of normal saline. The animals were kept in cages with free access to food and water until they were used. All procedures in this study were approved by the Animal Care Committee of the Fourth Military Medical University.

2.6. Detection of inflammatory cytokines

After 48 h of infection, the mice were subjected to lavage of the peritoneal cavity. The peritoneal lavage fluid (PLF) was centrifuged for 5 min at 200 g. The supernatant was collected for the detection of interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. Levels of these cytokines were detected using ELISA kits (Sigma) according to the manufacturer's instructions.

2.7. Detection of neutrophils ratio

The PLF was washed with PBS containing 5% FBS. The cells in the PLF (5×10^5) were stained with FITC-labeled anti-Gr-1 (Biolegend, San Diego, CA, USA)) for 30 min in dark. After washing twice, the cells were analyzed by FCM.

2.8. Detection of bacterial load

Kidneys and spleens from infected and uninfected mice were surgically removed and homogenized in PBS. Serial dilutions of