Expression of cell surface markers on methicillin resistant
Staphylococcus aureus stimulated lymphocytes

Isil Fidan, Emine Yesilyurt, Berna Erdal, Sultan Yolbakan & Turgut Imir

Gazi University, Faculty of Medicine, Department of Medical Microbiology, Ankara, Turkey

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Background & objectives: Methicillin-resistant Staphylococcus aureus (MRSA) has gradually been increasing, new strategies in the treatment of MRSA infections are required. This depends on the understanding of the infection pathogenesis and the immune response. This study was therefore designed to determine the immune response which develops during MRSA infection and the role of chemokines in this response, and also to compare the results with the changes occurring after MSSA infection.

Methods: The expression of the surface markers of human lymphocytes stimulated by heat-killed MRSA or MSSA was analysed by flow cytometry. The chemokine levels in the lymphocytes culture supernatants stimulated or not stimulated by microorganisms were determined by ELISA.

Results: Human peripheral blood mononuclear cells (PBMCs) stimulated by MRSA the levels of CD4+CD25+ regulatory T cells, CD69 expressions in the activated T lymphocytes, CD3−CD16−CD56+ NK cells and the levels of MIP-1α, MIP-1β, MCP-1 chemokines increased as compared to the cells not stimulated by MRSA. Although stimulation by MSSA caused an increase in CD25 expression after 24 h, the increase was found to be lower than the one caused by MRSA stimulation. The increase in CD69 expression was statistically significant compared to the cells stimulated by MRSA. Different from the cells stimulated by MRSA, no change was observed in the expressions of CD54 and CD3−CD16−CD56+ NK cells in the cells stimulated by MSSA.

Interpretation & conclusions: Our findings showed that cellular as well as humoral immunity are critical in MRSA infection and that T cell activation and the increase in chemokines may play a role in the regulation of immune response.

Key words Flow cytometry - immune response - MRSA

Staphylococcus aureus (S. aureus) causes severe infections such as suppurative diseases, toxic shock syndrome, and pneumonia. Over the past two decades, methicillin-resistant Staphylococcus aureus (MRSA) is occurring with increased frequency not only in hospitalized patients, but also in the community. MRSA infections have also been associated with increased morbidity and mortality.

Lymphocytes are an important part of the adaptive immunity against infections. Humoral immunity is the
principal protective immune response against extracellular bacteria such as *S. aureus*. The protein antigens of extracellular bacteria activate CD4+ T helper cells. These cells produce cytokines which stimulate antibody production, induce local inflammation and enhance the phagocytic and microbicidal activities of macrophage.

Chemokines are produced and released by a wide variety of leukocytic and nonleukocytic cell types. Inflammatory chemokines control the recruitment of effector leucocytes at inflammation sites and they are chemotactic primarily for monocytes and T cells.

Chemokines are induced by bacterial cell wall components and related to pathophysiologic changes in the bacterial infections. The production of chemokines stimulates migration of lymphocytes to the site of inflammation and switches initial immune reactions to the antigen-specific mechanisms of the cellular immune response.

Although the immune response mechanisms that develop during MRSA infection are known, the effective factors have not yet been clarified well. Determining the immune changes during *S. aureus* infection and the factors leading to such changes will make it possible to prevent the spread of the infection and to eliminate the pathogens resistant to many antimicrobial agents.

We carried out this study to examine the immune response against MRSA in an *in vitro* experimental system using heat-inactivated MRSA-stimulated human peripheral blood mononuclear cells (PBMCs), and to determine the role of cell mediated immune response and the effective factors. We also investigated whether cultured human PMBCs after stimulation by MRSA produce chemokines such as Monocyte chemotactic protein 1 (MCP-1), Macrophage inflammatory protein 1α and 1β (MIP-1α and MIP-1β) and RANTES (regulated upon activation of normal T cell expressed and secreted). We compared the results with those of the PBMCs stimulated by Methicillin-sensitive *S. aureus* (MSSA) and of control PBMCs not stimulated by microorganisms.

**Material & Methods**

The study was carried out in Gazi University, Faculty of Medicine, Department of Medical Microbiology (Ankara, Turkey) between January 1, 2006 and April 20, 2006. Heparinized blood was collected from 12 blood donors. Donors had no past history of any illness. They have to be healthy, and under the age of 60. Twenty ml of blood was collected from each donors. The informed consent was taken prior to the sample collection.

**Preparation of human PBMC:** PBMCs were separated from blood by Ficoll-hypaque density gradient centrifugation (Sigma, UK). After centrifugation, buffy coats were collected and washed in phosphate buffered saline (PBS, Gibco, Germany) three times and resuspended at a concentration of 2x10⁶ cells/ml in complete RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.05 mM 2 ME and supplemented with 10 per cent foetal calf serum (Gibco, Germany). Cell viability was 95 per cent by the trypan blue exclusion test.

**Microorganism:** MRSA and MSSA strains were isolated from cultures of blood. To identify *S. aureus*, Gram stain, coagulase test, pigment production, haemolysis, oxacillin susceptibility were used. After isolation of microorganisms, bacteria were inoculated into brain heart infusion broth (BHI) (Oxoid, UK) for 12 h at 37°C. At the end of the incubation, bacteria were washed and resuspended in PBS. Bacterial suspensions were adjusted at a concentration of 10⁸ cfu/ml (colony forming unit) in PBS, and were heated at 100°C for 30 min to obtain heat-killed bacterial suspensions.

**Preparation of cell culture:** Human PBMCs (2x10⁶) were delivered in culture flasks. Heat-killed bacteria (10⁸ cfu/ml) were added to the same culture flasks to stimulate PBMCs. Control culture flasks did not contain bacteria. Each assay was done in duplicate. Control culture flasks and culture flasks containing heat-killed bacteria were incubated for 0, 24, 48 h in 5 per cent CO₂ at 37°C. At the end of each selected time period, contents of culture flasks were transferred into tubes and centrifuged. The culture supernatants were removed and stored at -30°C until used in ELISA (BioTek, USA). The pelleted cells were analyzed by flow cytometry.

**Flow cytometric analysis of PBMCs:** The pelleted cells were washed in PBS and stained with antihuman antibodies for 30 min at 4°C. PBMCs (10⁶ cells) were incubated with monoclonal antibodies specific for human CD antigens. Antibodies used were anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD69, anti-CD54, anti-CD16, anti-CD56 (e-bioscience, USA). Flow cytometric analysis was done using a Coulter FC500 flow cytometer (Coulter, USA).

**ELISA:** The lymphocytes culture supernatants stimulated or not stimulated by microorganisms were
used for determination of chemokines. Levels of chemokines such as RANTES, MIP-1α, MIP-1β, MCP-1 were determined by specific ELISA techniques according to the manufacturer instructions (Biosource, California, USA). The concentration of chemokines was determined spectrophotometrically. The absorbance was read at 450 nm. A standard curve was constructed using chemokine standards. The chemokine concentrations for unknown specimens were calculated according to the standard curve.

Statistical analysis: The data were analysed using the one-way analysis of variance (ANOVA). The Bonferroni test was used as Post Hoc analysis, \(P<0.05\) was considered to be significant.

Results

Expression of cell surface markers on lymphocytes: In vitro stimulation of PBMCs by heat-killed MRSA or MSSA did not affect the percentage of CD4+ T lymphocytes, CD8+ T lymphocytes compared to control groups. There were no significant differences between specimens with MRSA or MSSA or control groups.

While CD25 (IL-2R) surface antigen of which expressions increased on the surface of activated T lymphocytes, was found to have increased in the specimens with MRSA in 24 h compared to the control groups. There were no significant differences between specimens with MRSA or MSSA or control groups.

CD69 expression on activated lymphocyte surfaces was found to have increased significantly on both CD4+ T and CD8+ T lymphocyte surfaces when the PBMCs stimulated by MRSA were compared to PBMCs not stimulated by MRSA after 24 and 48 h. When the cells stimulated by MSSA were compared to the control specimens, the same results were found as in the specimens stimulated by MRSA, but after 48 h CD69 expression in CD8+ T lymphocytes was lower than that in control specimens and in the specimens stimulated by MRSA. It was observed that while CD69 expression increased following the stimulation by MSSA compared to the control specimens, the values without any heat-killed MRSA; however, no significant increase was observed in 48 h in comparison with the control groups. After 24 h CD25 expression was found to be significantly higher in CD4+ T cells stimulated by MSSA than that in the control specimens. After 24 h CD25 expression was significantly higher in the cells stimulated by MRSA compared to the specimens stimulated by MSSA \((P=0.001)\) (Fig.1). After 48 h no difference was observed among the three groups.

In our study, CD54 (ICAM-1) expression increased on T lymphocyte surfaces in the specimens with MRSA in 24 h compared to the control groups, but it was statistically not significant. There was no significant difference between PBMCs stimulated by MSSA compared to the control specimens not stimulated by Staphylococcus aureus.

Fig. 1. Flow cytometric analysis of CD4+CD25+ T cell expression in the three groups in 24 h.
were statistically low compared to PBMCs stimulated by MRSA ($P<0.05$) (Figs 2-5).

CD3^+CD16^-CD56^+ natural killer (NK) cells did not show any significant differences with the presence of MRSA compared to the control group in 24 h, there was a significant increase after 48 h ($P<0.05$). The stimulation of PBMC by MSSA did not cause any change in the levels of CD3^+CD16^-CD56^+ NK compared to the control cells.

**Chemokines levels:** The chemokine RANTES decreased significantly in the specimens stimulated by MRSA or MSSA in 24 h compared to the control group whereas it increased significantly in the specimens stimulated by MRSA or MSSA in 48 h compared to the control group. MIP-1$\beta$, MIP-1$\alpha$ and MCP-1 were not increased in the control group, they were found to be at significantly high levels in PBMC supernatants stimulated by MRSA or MSSA ($P<0.05$) (Fig. 6).

![Fig. 2. Flow cytometric analysis of CD4/CD69 T cell expression in the three groups in 24 h.](image1)

![Fig. 3. Flow cytometric analysis of CD4/CD69 T cell expression in the three groups in 48 h.](image2)
Discussion

Our study revealed that there was no difference between PBMCs stimulated by MRSA or MSSA and the specimens not stimulated by *S. aureus* in relation to the percentage of CD4⁺, CD8⁺ and total T-lymphocytes. This may be attributed to the fact that we did our studies under *in vitro* conditions. Holub *et al*¹² reported that the numbers of these cells significantly decreased in patients with Gram-positive sepsis.

While we observed a statistically significant increase in the adhesion molecules CD54 (intracellular adhesion molecule -1, ICAM-1), one of the markers of which expressions we investigated in CD4⁺ and CD8⁺ T cells, in the specimens stimulated by MRSA after 24 h compared to the control group, there was no such effect in the specimens stimulated by MSSA. However, different from our observation, Numani *et al*¹³ reported this adhesion molecule might increase the surface expression.

Fig. 4. Flow cytometric analysis of CD8/CD69 T cell expression in the three groups in 24 h.

Fig. 5. Flow cytometric analysis of CD8/CD69 T cell expression in the three groups in 48 h.
The expression of CD69, which is an early T cell activation antigen, increased significantly on the surface of both CD4+ and CD8+ T lymphocytes after stimulation by MRSA compared to the control group. Since CD69 does not exist in resting T cells, the increase in the expression of CD69 in our study suggests CD69 mediated activation of CD4+ and CD8+ T cells. Stimulation by MSSA caused a significant increase in CD69 expression in CD4+ and CD8+ T cells compared to the control cells in 24 h. However, after 48 h there was not any increase in CD8+ T compared to the control specimens. This could be due to *in vitro* PBMC culture. The increase in CD69 expression caused by MSSA was observed to be significantly lower than the increase caused by MRSA.

Our study showed that CD25 (IL-2R) surface antigen, increased significantly in the specimens with MRSA in 24 h compared to the control groups. Stimulation by MSSA caused a similar increase in CD69 expression in CD4+ and CD8+ T cells compared to the control cells in 24 h. However, after 48 h there was not any increase in CD8+ T compared to the control specimens. This could be due to *in vitro* PBMC culture. The increase in CD69 expression caused by MSSA was observed to be significantly lower than that by MRSA.

Our investigation of CD3 - CD16 + CD56 + NK cell expression revealed that there were more NK cells in the specimens stimulated by MRSA in 48 h than in the control group. NK cells may play an important role in the bacterial infections, especially in the infections due to more virulent strains, just as it happens in viral infections. Tran *et al.* have reported that there is an early increase of NK cytotoxicity in PBMCs exposed to *S. aureus*. Ami *et al.* have also remarked that human NK cells may be associated with staphylococcus enterotoxin A-induced immune response.

We also studied the role of lymphocyte-specific chemokines in the regulation of cell-mediated immune response in PBMCs stimulated by MRSA or MSSA. RANTES levels decreased significantly in the specimens stimulated by MRSA or MSSA in 24 h, while it increased significantly after 48 h compared to control. Like most other chemokines, RANTES is expressed transiently by a variety of cell types. But, RANTES differs from other cytokines and it is a ‘late’ expressed gene in activated T cell activation, they may control T cell-mediated inflammatory response in *S. aureus* infections. Chang *et al.* have indicated that CD4+CD25+ regulatory cells may help to reveal the immune regulatory system of the host infected by *S. aureus*.

In our study, CD25 and CD69 lymphocyte activation markers were found to have increased during stimulation by *S. aureus* compared to the cells not stimulated. Yet, these increases were determined more clearly after stimulation by MRSA. Just one factor may not be enough to explain the difference seen in the lymphocyte responses of MRSA and MSSA strains. Presumably there will be many factors leading to the differences in responses such as virulence factors, adherence factors and *mecA* gene. The fact that MRSA isolates are more virulent and more resistant to antimicrobial agents and that their infections develop clinically more severely than MSSA may provide an explanation for this situation. Moreover, another factor that could be an explanation to our observed data, is the fact that the clinical response to patients with MRSA infection is slower than that of patients with MSSA infection. Since methicillin sensitive isolates were more phagocytized and more sensitive to killing by neutrophils and monocytes than MRSA, we suggested that this situation may be other possible reason for the differences in the stimulation of the lymphocytes by MSSA and MRSA.

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24 h. MIP-1α, MIP-1β and MCP-1 levels were observed to have increased significantly in the specimens stimulated by MRSA or MSSA compared to the control group. There is a chemokine gene expression during S. aureus infection and this can be prevented by dexamethasone and prostaglandin E2 (PGE2); such therapeutic interventions may lessen the tissue damage.

Jedrzkiewicz et al25 have reported that MCP-1 and RANTES expressions increase in S. aureus enterotoxin B-activated immune cells and that chemokines may have a role in T cell recruitment following the immune activation of bacterial antigens. Tekstra et al26 have also indicated that MCP-1 production increases in the endothelial cells infected by S. aureus and the amount of increase is associated with the virulence of S. aureus and also that the increase of MCP-1, which is the most potent chemoattractant, in the cells infected by S. aureus causes the emigration of monocytes to the inflamed tissues.

Megyéri et al27 reported variable results about the immune response level and pattern in Staphylococcus infections and there were specific differences in types and strains. Our study also revealed the differences between MRSA and MSSA especially in their effects on lymphocyte stimulation.

In conclusion, our study showed that after stimulation by S. aureus, T lymphocyte activation and the levels of lymphocyte-specific chemokines increased remarkably especially in the methicillin-resistant strains and there were some other factors different from MSSA infection that played a role in MRSA infection pathogenesis and virulence. In the light of these findings it may be considered that cell-mediated immunity plays an important regulatory role during the stimulation by MRSA and MSSA and there is T cell activation during this process. The understanding of the mechanisms of humoral and cell-mediated responses taking place after S. aureus infection is very important for the pathogenesis and this may provide useful information for developing new strategies to treat S. aureus infection, especially MRSA infection, and also for vaccination.

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Reprint requests: Dr Isil Fidan, Gazi University, Faculty of Medicine, Department of Medical Microbiology, Dekanlik Binasi 2 Kat Besevler/Ankara 06500, Turkey
e-mail: isilfidan@yahoo.com