Pulmonary immune response during (myco)bacterial infection
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Lipoteichoic Acid and Peptidoglycan from \textit{Staphylococcus aureus}

Synergistically Induce Neutrophil Influx into the Lungs of Mice

Jaklien C. Leemans, Mirjam Heikens, Kok P. van Kessel, Sandrine Florquin, Tom van der Poll
**ABSTRACT**

*Staphylococcus aureus* is an important pathogen in nosocomial pneumonia. Lipoteichoic acid (LTA) and peptidoglycan (PepG) are part of the staphylococcal cell wall. To determine the effects of and a possible interaction between these bacterial components in the pulmonary compartment, mice were intranasally inoculated with purified LTA, PepG or both. Both LTA and PepG induced a rapid recruitment of polymorphonuclear cells (PMNs) to the bronchoalveolar space. PMN influx was significantly greater after the combined administration of LTA and PepG than the additive effect of the two components alone. Although LTA and PepG also elicited the release of tumor necrosis factor-α and CXC chemokines into bronchoalveolar lavage fluid, the effect was not synergistic. These data suggest that LTA and PepG may act in synergy to cause PMN recruitment in the early phase of *S. aureus* pneumonia.
**INTRODUCTION**

*Staphylococcus aureus* is a common pathogen in hospital-acquired pneumonia (1). Peptidoglycan (PepG) and lipoteichoic acid (LTA) are components of the cell wall of gram-positive bacteria, including *S. aureus*. PepG is a large polymer that contains long sugar chains and is predominantly responsible for the protective and shape-maintaining properties of bacterial cell walls. LTAs are phosphate-containing polymers that mediate the attachment of certain bacteria to host cells. Both membrane components can stimulate the generation of pro-inflammatory cytokines and activate leukocytes *in vitro* (2-5).

Recently, we demonstrated that the intranasal administration of LTA or PepG from *S. aureus* to mice resulted in acute pulmonary inflammation characterized by influx of polymorphonuclear cells (PMNs) into the alveolar compartment and local production of proinflammatory cytokines and chemokines (6). Interestingly, lung inflammation elicited by LTA or PepG appeared to be regulated by different mechanisms, considering that interleukin (IL)-6 deficient mice displayed enhanced PMN recruitment after local instillation of LTA, but a reduced PMN influx after exposure to PepG (6). Intravenous administration of LTA and PepG into rats has been found to induce synergistic systemic inflammation when compared with the infusion of either one bacterial product alone (7, 8). Together, this prompted us to examine the combined effects of LTA and PepG in the mouse lung.

**MATERIAL & METHODS**

Female BALB/c mice (Harlan Sprague Dawley Inc., Horst, the Netherlands; 8 weeks of age) were intranasally inoculated with sterile saline (controls), *S. aureus* LTA (50 µg; Sigma, St. Louis, Mo.), *S. aureus* PepG (50 µg), or LTA and PepG (both 50 µg; final volume 50 µl in sterile saline) according to methods described previously (6). PepG was prepared from *S. aureus* according to the method of Peterson *et al.* (9). Each experimental group consisted of 5 mice. After 4h., mice were anesthetized by intraperitoneal injection of Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdecht, the Netherlands), and sacrificed by bleeding from the vena cava inferior. Lungs were lavaged with two aliquots of 0.5 ml saline via a catheter inserted
into the trachea. The 4 h time point was chosen since it is representative for studying inflammatory responses to bacterial products in the lung (6, 10, 11). Total leukocyte counts were determined by using a hemocytometer. Numbers of alveolar macrophages (AMs), PMNs and lymphocytes were calculated from these totals, using cytospins from bronchoalveolar lavage (BAL) cells stained with Diff-Quick (Baxter, McGraw Perk, IL). The BAL fluid (BALF) was then centrifuged for 10 min at 750 g, and stored at -20°C. Cytokines and chemokines were measured in duplicate in BALF by specific ELISAs according to the manufacturers’ instructions (R&D Systems, Minneapolis, MN). The detection limits of these ELISAs were 31 pg/ml for tumor necrosis factor-α: (TNFα), 8 pg/ml for KC and 46 pg/ml for macrophage inflammatory protein (MIP)-2. For histopathological investigations, lungs were removed and fixed in 4% paraformaldehyde in PBS for 24 hours. After embedding in paraffin, 4-μm-thick sections were stained with eosin hematoxylin-eosin, and analyzed by a pathologist. The Animal Care and Use Committee of the University of Amsterdam, the Netherlands, approved all experiments. All values are expressed as mean ± SEM. Differences between groups were analyzed by Mann-Whitney U test. To examine a possible synergistic effect of the individual bacterial components, P values were calculated by linear regression analysis. P ≤ 0.05 was considered statistically significant.

RESULTS

To study synergism between LTA and PepG in vivo, we intranasally inoculated mice with either LTA, PepG, or a combination, and sacrificed them after 4 h. Inoculation with either LTA or PepG induced a profound increase in total leukocyte numbers in BALF, which was predominantly due to a rise in PMN numbers (both P < 0.05 versus saline; Table I). PepG, but not LTA, induced a modest increase in AMs in BALF (P < 0.05 versus saline). Interestingly, the combined administration of LTA and PepG resulted in a synergistic effect on PMN influx (P < 0.05 versus the effect of LTA and PepG together). Additionally, mice treated with LTA or PepG showed an increase in TNFα, MIP-2 and KC concentration in BALF when compared to BALF of saline-treated animals (Fig. 1). Although the concentrations of TNFα, MIP-2 and KC in BALF were highest after simultaneous administration of LTA and PepG, the effect of LTA and PepG was not synergistic. Histopathological examination of lung tissue of mice administered with LTA
**Table I** Effect of the administration of LTA, and PepG or a combination on cell subsets in BALF.

<table>
<thead>
<tr>
<th>Cells x10⁴/ml</th>
<th>Leukocytes</th>
<th>AMs</th>
<th>PMNs</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3.0 ± 1.0</td>
<td>2.9 ± 1.0</td>
<td>0.04 ± 0.04</td>
<td>0.07 ± 0.07</td>
</tr>
<tr>
<td>LTA</td>
<td>24.0 ± 2.8 *</td>
<td>2.2 ± 0.3</td>
<td>21.1 ± 1.3 *</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>PepG</td>
<td>15.3 ± 1.3</td>
<td>8.4 ± 1.2 *</td>
<td>8.1 ± 1.4 *</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>LTA + PepG</td>
<td>51.8 ± 5.1 * †</td>
<td>10.1 ± 1.8 *</td>
<td>39.0 ± 4.4 * †</td>
<td>1.4 ± 0.4 *</td>
</tr>
</tbody>
</table>

* Mice were intranasally inoculated with LTA (50 µg), PepG (50 µg) or a combination of these components and sacrificed after 4 h after which leukocyte influx was analyzed in BALF. Data are mean and standard error of the mean (SEM) of five mice. *P<.05 compared to saline, † P<.05 compared to the effect of the individual components together.

showed a dense granulocytic inflammatory infiltrate (Fig. 2A). After administration of PepG numerous well-defined collections of leukocytes (abscesses) together with a slight interstitial inflammatory infiltrate were found (Fig. 2B). The combined administration of LTA and PepG resulted in an increased accumulation of leukocytes in small clusters (Fig. 2C).

**DISCUSSION**

Previous studies have documented synergistic effects of intravenously administered LTA and PepG to induce septic shock and multiorgan failure in rats (7, 8). We here demonstrate that intrapulmonary delivery of LTA and PepG from *S. aureus* elicits recruitment of PMNs to mouse lungs in a synergistic way, whereas the induction of TNFα, MIP-2 and KC by the combined administration of LTA and PepG was additive at best.
CXC chemokines with an ELR motif near the N-terminal end play a pivotal role in the recruitment of PMNs to sites of infection and inflammation (12). MIP-2 and KC are the most prominent ELR positive CXC chemokines in the mouse. Both chemokines have been found to contribute to influx of PMNs to the alveolar compartment in various models of lung infection and inflammation (13-16). Recently, our laboratory reported that administration of recombinant MIP-2 and KC into the cisterna magna of rats elicited leukocyte influx into cerebrospinal fluid in a synergistic way (17). It is therefore conceivable that the modestly elevated levels of MIP-2 and KC in BALF of mice treated with both LTA and PepG played a role in the synergistic effect of the two staphylococcal cell wall components on PMN recruitment. In addition, the locally elevated concentrations of TNFα may have contributed to this response (18, 19).

It remains to be established why LTA and PepG did not synergistically induce TNFα and CXC chemokines in mouse lungs in vivo. In human whole blood in vitro, LTA and PepG do induce TNFα release in a synergistic way [our own unpublished data]. Nonetheless, the clear synergism regarding PMN recruitment between LTA and PepG could mean that different receptors and signaling pathways are simultaneously activated by the two agents. Indeed, the signal transduction pathways that are used by PepG and LTA likely are at least in part different. Toll-like receptor 2 (TLR2) has been reported to be the signaling receptor for PepG from S. aureus (20, 21). Although TLR2 has also been implicated as a signal transducer for LTA from Bacillus subtilis, Streptococcus pyogenes, and
Streptococcus sanguis (22), TLR4 has been found to be required for signal transduction by S. aureus LTA (20, 23, 24). The concept that synergism is caused by the use of different receptors is supported by several observations. Indeed, bacterial DNA, that signals via TLR9 (25), synergistically acts with LPS, that signals via TLR4, for induction of inflammatory cytokine production (26, 27). In addition, bacterial lipopeptides and lipoprotein, or mycoplasmal lipopeptides, all signaling via TLR2 (28-30), induced cytokine production in synergy with LPS (31, 32). Moreover, intravenously injected PepG synergised with LPS to cause systemic inflammation and organ injury in rats in vivo (33). Hence, the data of the present study support the concept that LTA and PepG act in synergy to cause pulmonary inflammatory in the early phase of pneumonia caused by S. aureus. This synergy may either function as a safety mechanism for the host by triggering an adequate innate immune response (34), or on the other hand may cause lung injury and dysfunction as observed during fulminant pneumonia.

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REFERENCES


