Effects of Cytokines and Minocycline on Subacute Lung Injuries Induced by Repeated Injection of Lipopolysaccharide

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Summary

Pathological changes were seen in the lungs of ddY mice one week after repeated intraperitoneal injections of lipopolysaccharide (LPS) of *Klebsiella pneumoniae*. The infiltration of polymorphonuclear cells (PMN), mainly neutrophils, and lymphocytes into the alveolar septum, the infiltration of PMN into perivascular areas and microthrombi were recognized in this murine model. The blood levels of TNFα and IL-1α did not rise at this time, suggesting that the most important cytokine promoting inflammation one week after LPS stimulation was neither TNFα nor IL-1α. In the lungs of mice administered minocycline together with LPS, lymphocyte infiltration of alveoli and perivascular areas as well as microthrombi were suppressed. The blood levels of TNFα, IL-1α, IL-4 and IL-6 were elevated in these groups, suggesting the suppression of pathological changes to be associated with the anti-inflammatory effect of IL-6 and/or persistent elevation of TNFα and/or IL-1α levels. In conclusion, subacute pathological changes in the lung were induced by repeated intraperitoneal injections of LPS to mice. These pathological changes were suppressed by minocycline, suggesting the anti-inflammatory effects of this antibiotic to be the result of stimulating certain cytokines.

Introduction

Septic shock induced by gram-negative bacterial infections causes multiple organ failure which is associated with decreased responsiveness of hypotension to vasoconstrictive drugs, possibly leading to disseminated intravascular syndrome, and even death. Lipopolysaccharide (LPS), a constituent of the bacterial membrane, is considered to cause shock due to infections with gram-negative bacteria. When LPS is administered to experimental animals such as mice, blood levels of cytokines, tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1), increase, and septic shock may ensue. When animals are given the inflammatory cytokine TNFα, changes similar to those caused by septic shock are induced.

When these animals are given a single dose of LPS (i.v. or i.p.), rapid rises in TNFα, IL-1, and IL-6 are triggered. While the level of TNFα peaks in 2 to 4 h and normalizes within several hours, the levels of IL-1 and IL-6 are slower to peak than that of TNFα and normalize within 24 h. It has been shown that acute injuries caused by LPS are due mainly to TNFα and IL-1 (in particular, TNFα).

The purpose of the present study is to examine subacute pathological changes (1 week post-
treatment) and associated changes in cytokines induced by repeated injection of mice with LPS, and the effect of minocycline, which is a potent stimulator of cytokine production by lymphocytes\(^\text{[46]}\).

**Materials and Methods**

**Animal:** male ddY mice, aged 12 weeks and weighing approximately 40 g, were used.

**Test drugs:** Lipopolysaccharide (LPS), extracted and purified from *Klebsiella pneumoniae*, Kasuya strain, by the Department of Microbiology, Nagoya University School of Medicine, was used\(^\text{[11,12]}\). Minocycline (MINO) was generously provided by Lederle Japan, Co., Ltd. (Tokyo).

**Method:** The mice were divided into the following five groups. LPS group: LPS 3 mg/kg was administered i.p. daily from days 1 to 3. LPS + MINO (low dose) group: LPS 3 mg/kg was administered i.p. daily from days 1 to 3 and MINO 1 mg/kg was administered i.p. daily from days 1 to 7. LPS + MINO (high dose) group: LPS 3 mg/kg was administered i.p. daily from days 1 to 3 and MINO 4 mg/kg was administered i.p. daily from days 1 to 7. MINO group: MINO 4 mg/kg was administered i.p. daily from days 1 to 7. Control: Saline solution 0.1 ml was administered i.p. daily from days 1 to 7. At the completion of treatment, blood samples were taken from the hearts of animals in all five groups on day 8, and the serum was stored frozen at \(-80^\circ\text{C}\). The animals were sacrificed by cervical dislocation, and the lungs, liver, and spleen were removed and fixed in 10% formalin.

**Pathological studies:** Preparations stained with Hematoxylin and eosin and elastica van Gieson stains were evaluated by microscopy. Lesions were divided into the alveolar septal, alveolar, broncho-bronchiolar, perivascular, and subpleural regions. Lesions were scored on a scale of 0–3, as follows. 0: no pathological change, 1: mild pathological change, 2: diffuse or severe pathological change, 3: diffuse and severe pathological change. Individual scores of all mice were determined, and the mean score of each group was calculated.

**Measurement of cytokines:** TNF\(\alpha\), IL-1\(\alpha\), IL-1\(\beta\), IL-4, and IL-6 levels were determined using a BIOTRAK ELIZA system for mice (Amersham International plc, Buckinghamshire, England).

**Statistical analysis:** The mean and standard deviation of cytokine levels in each group were calculated. Significant differences were examined by the unpaired Student’s t-test and differences were considered to be statistically significant when \(p<0.05\).

**Results**

**Pathological studies:** The incidence of pathological changes and scored observations are shown in Tables 1 and 2, and Figures 1 and 2. Alveolar septal lesions: No clear fibrosis was observed. Edematous thickening, infiltration of lymphocytes and PMN, and formation of microthrombi were observed in the LPS group (Figure 3, 4). These pathological changes were less severe in the groups coadministered MINO and no microthrombi were observed in the high dose MINO group. Alveolar lesions: Fibrin deposition was observed in approximately half of the animals in the LPS group, whereas little fibrin deposition was observed in the groups coadministered MINO (low and high doses). Microemphysema suggesting post-injury restructuring of alveoli was evident in the LPS group but was minimal in the groups coadministered MINO. No pathological changes were observed in the MINO or control groups. Broncho-bronchiolar lesions: Mild infiltration of lymphocytes was observed in approximately half of the animals in the LPS group, whereas little fibrin deposition was observed in the groups coadministered LPS alone or coadministered NINO. Polymorphonuclear cell infiltration was negligible in all of the groups. Perivascular lesions: Mild lymphocytic infiltration was observed in nearly all animals in the LPS group (Figure 5) and in approximately half of the animals in the groups coadministered MINO. Subpleural lesions: Mild lymphocytic infiltration was observed in the groups administered LPS alone or coadministered
Table 1 Incidence of Pathological Changes in Lungs of ddY Mice Induced by Repeated Injections of Lipopolysaccharide of Klebsiella pneumoniae (1)

<table>
<thead>
<tr>
<th>Alveolar lesion</th>
<th>macrophages</th>
<th>fibrin exudation</th>
<th>microemphysema</th>
<th>lymphocytes</th>
<th>polymorphonuclear cells</th>
<th>microthrombi</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>0/6</td>
<td>3/6</td>
<td>5/6</td>
<td>6/6</td>
<td>6/6</td>
<td>5/6</td>
</tr>
<tr>
<td>LPS+MINO(L)</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
<td>2/6</td>
<td>3/6</td>
<td>2/6</td>
</tr>
<tr>
<td>LPS+MINO(H)</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>MINO</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>control</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

The incidence of pathological changes of alveoli of each group are shown. In mice repeatedly injected with lipopolysaccharide (LPS), many changes are recognized. LPS: Group of mice administered LPS intraperitonealy, LPS+MINO(L): Group of mice administered LPS plus minocycline (MINO) (1mg/g), LPS+MINO(H): Group of mice administered LPS plus MINO (4mg/kg), MINO: Group of mice administered MINO (4mg/kg), control: Group of mice administered saline.

Table 2 Incidence of Pathological Changes in Lungs of ddY Mice Induced by Repeated Injections of Lipopolysaccharide of Klebsiella pneumoniae (2)

<table>
<thead>
<tr>
<th>Broncho-bronchiolar lesion</th>
<th>Perivascular lesion</th>
<th>Subpleural lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>lymphocytes</td>
<td>polymorphonuclear cells</td>
<td>lymphocytes</td>
</tr>
<tr>
<td>LPS</td>
<td>3/6</td>
<td>0/6</td>
</tr>
<tr>
<td>LPS+MINO(L)</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>LPS+MINO(H)</td>
<td>2/6</td>
<td>0/6</td>
</tr>
<tr>
<td>MINO</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>control</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

The incidence of pathological changes in broncho-bronchiolar, perivascular and subpleural area of each group are shown.

MINO. Surprisingly, mesothelial cell hyperplasia was observed in some animals in the low dose MINO group and in approximately half of those in the high dose MINO group (Figure 6).

Cytokines: The TNFα level in the LPS group was 50 ± 63 pg/ml (mean ± S.D.) whereas the corresponding levels in the groups coadministered low and high dose MINO were raised to 167 ± 118 and 228 ± 44 pg/ml, respectively (Figure 7). The level of IL-1α in the LPS group was 18 ± 12 pg/ml while the corresponding levels in the groups coadministered low and high dose MINO were 20 ± 13 and 57 ± 44 pg/ml, respectively (Figure 8). Thus, IL-1α was significantly elevated in the group coadministered a high dose of MINO. No changes in the level of IL-1β were observed in any of the groups. The level of IL-4 in the LPS group was 71 ± 36 pg/ml while the corresponding levels in the groups coadministered low and high dose MINO were 200 ± 77 and 68 ± 22 pg/ml, respectively (Figure 9). Thus, IL-4 was significantly elevated in the group coadministered a low dose of MINO. The level of IL-6 in the LPS group was 97 ± 41 pg/ml whereas the corresponding levels in the groups coadministered low and high dose MINO were 271 ± 215 and 58 ± 18 pg/ml, respectively. Thus, IL-6 was significantly elevated in the group coadministered a low dose of MINO (Figure 10).

Discussion

LPS is used to obtain a model of acute respiratory distress syndrome (ARDS). LPS-induced pathological changes include acute edema of the alveolar septum and infiltration of neutrophils, and leakage of protein and increased neutrophils are also observed in BAL6.9. It is assumed that these changes occur rapidly and, in surviving animals, are rapidly reversed.

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Figure 1. The degrees of alveolar lesions in experimental mice.

The degree of pathological change was evaluated in each mouse, as follows: 0: no pathological change, 1: mild pathological change, 2: diffuse or severe pathological change and 3: diffuse and severe pathological change. The mean scores of each group and items assessed are shown.

Figure 2. The degrees of broncho-bronchiolar, perivascular and subpleural lesions in experimental mice.

Subacute inflammatory pathological changes were seen one week after repeated injections of LPS into mice in the present study. The characteristic pathological changes in the lung in this model included infiltrating PMN, mainly neutrophils, and lymphocytes in the alveolar septum, perivascular infiltration of lymphocytes, and the formation of microthrombi. The changes were relatively mild and...
no interstitial fibrosis was observed.

As the levels of TNFα and IL-1α rise rapidly after a single dose of LPS and reach a steady state within 24 h, it has been proposed that the elevations of TNFα and IL-1α trigger the acute pulmonary injury associated with LPS. However, since inflammatory pathological changes in the lung are observed one week after repeated injections of LPS, at which time levels of TNFα and IL-1α have reached a steady state, this observation suggests that subacute inflammation is caused by cytokines other than TNFα and IL-1α. Thus, secondary factors induced by elevated TNFα and IL-1α may be involved in the inflammation. It should be noted that, in the limited number of mice examined, the levels of IL-1β, IL-4, and IL-6 were similar to those in the control group.

In mice coadministered MINO, an improvement was seen in three pathological changes: infiltration of PMN, mainly neutrophils, into the alveolar septum, infiltration of PMN into perivascular areas, and the formation of microthrombi. Amelioration of the pathological changes induced by LPS has not been reported using other antibiotics. Blood levels of TNFα and IL-1α were increased by the administration of MINO. In particular, levels were significantly elevated in the group coadministered a high dose of MINO in which pathological changes were significantly suppressed. Interestingly, inflammation was inhibited, a finding apparently incongruous with increased TNFα and IL-1α. We
speculate that inhibiting the normalization of TNFα and IL-1α levels may be associated with the suppression of pathological changes. This would suggest that persistently elevated TNFα and/or IL-1α levels suppress acute and/or chronic inflammation. Levels of IL-4 and IL-6, on the other hand, were elevated in the group coadministered a low dose of MINO. As it has been reported that IL-6 has an anti-inflammatory effect on lesions in pulmonary inflammation models, production of IL-6 in animals administered MINO apparently suppresses pathological changes induced by LPS. Kloppenburg et al. reported that MINO exerts anti-inflammatory effects via IL-6 production by T lymphocytes and monocytes. It is also possible that elevated IL-4, which is linked to increased IL-1α and IL-6, is expressed as an anti-inflammatory effect. However, further study is needed to explain why IL-6 and IL-4 levels in the present study were not elevated in animals coadministered a high dose of MINO.

As mesothelial cell hyperplasia in the pleura was observed in half of the animals in the group coadministered high dose MINO, MINO was assumed to stimulate the growth of mesothelial cells. Dryzer et al. reported that desquamation of mesothelial cells was observed with injection of MINO.
into the thoracic cavity\textsuperscript{16}). Based on the observation that mesothelial cell hyperplasia occurred in one animal from the MINO group, it was concluded that even when administered intraperitoneally MINO has a stimulating effect on mesothelial cells and, combined with the effect of LPS, results in more vigorous growth of mesothelial cells.

In addition to the anti-bacterial effects of MINO, reduced production of chemotactic factors by a direct action on neutrophils\textsuperscript{17,18)}, suppressed production of hydroxy radicals from neutrophils\textsuperscript{19)}, suppressed growth of peripheral lymphocytes\textsuperscript{20)}, and decreased phospholipase A\textsubscript{2} activity in synovial membrane derived from chronic rheumatoid arthritis patients\textsuperscript{21)} have been reported as anti-inflammatory effects. Further studies on the anti-inflammatory, rather than the anti-bacterial, effects of MINO are warranted.

In summary, we generated a murine model with clear, although mild, lesions including infiltration of PMN, mainly neutrophils, and lymphocytes into the alveolar septum, infiltration of perivascular areas by lymphocytes, and microthrombus formation in the subacute phase were seen one week after repeated injections of LPS. In addition, it was found that these lesions were partially suppressed by the administration of MINO, suggesting that this antibiotic exerts anti-inflammatory effects by regulating cytokine production.

Acknowledgements

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References


Lipopolysaccharide 反復投与にによるマウスの亜急性肺傷害に
およばす cytokine と minocycline の影響

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要 旨
LPS をくりかえしマウスに投与し、亜急性期として1週間後に炎症性の病理学的変化を誘導することができた。本モデルにおける肺の病理学的変化の特徴は、間質への線維化が認められなかったが、胞巣への好中球を主とした多核球とリンパ球の浸潤、小血管周囲への多核球の浸潤、そして微小血栓の形成が認められた。この時の TNFα と IL-1α は高値ではなく平常値化していたことから、亜急性期において炎症を進行させる cytokine は TNFα と IL-1α ではなく、TNFα と IL-1α の上昇によって引き起こされた二次的な要因が炎症を進行していることが考えられた。MINO を投与したマウスで、胞巣への好中球を主とした多核球の浸潤、小血管周囲へのリンパ球の浸潤、そして微小血栓の形成の3つの病理学的変化は抑制がみられ、この時の TNFα, IL-1α, IL-4と IL-6は高値であったことから、病理学的変化が抑制されたことにより、IL-6が抗炎症作用をしたこと、または TNFα と IL-1α の値が高値を持続したこと、が考えられた。以上をまとめると、LPS を繰り返し投与することで1週間後の亜急性期に肺に病変をもたらすマウスのモデルをつくることができた。この病変は MINO を投与することにより一部抑制でき、その機序として、cytokine の産生を調整し抗炎症作用を示すことが示唆された。