THE PATHOLOGICAL EFFECT OF PEPTIDOGLYCAN ON RATS’ LUNGS PART ONE: PATHOGENIC BACTERIA

STREPTOCOCCUS PNEUMONIAE

*May T. Fleih, *Harith J.F. Al-Mathkhury and **Zahraa S. Mahmod

*University of Baghdad, College of Science, Department of Biology.
**University of Al-Mustansiriyah, College of Science, Department of Biology.

Abstract

Twenty isolates of Streptococcus were obtained from fifty sputum specimens collected from patients presented with respiratory tract infections. Two of these isolates were identified as S. pneumoniae by conventional biochemical tests. For extraction of peptidoglycan from S. pneumoniae, mechanical disintegration by glass beads and vortex plus enzymatic digestion by DNase, RNase and pronase were applied. The partial purified peptidoglycan showed four protein bands compared with crude peptidoglycan which showed six bands when performed in polyacrylamide gel electrophoresis under undenaturing conditions.

The rats injected with germ suspension or the peptidoglycan showed similar histopathological changes included disruption of alveoli walls, haemorrhage, infiltration of inflammatory cells, sloughing of the epithelial cells lining the bronchioles, and edema. The effects of histopathological changes of the peptidoglycan of S. pneumoniae were more severe than those of cell suspension.

Introduction

Streptococcus pneumoniae is a leading cause of pneumonia in all ages often after "damage" to the upper respiratory tract (e.g. following viral infection). It also causes middle ear infections (otitis media). The organism often spreads causing bacteremia and meningitis. S. pneumoniae is α hemolytic and there is no group antigen. Direct Gram staining or detection of capsular antigen in sputum can be diagnostic. The organism grows well on sheep blood agar (1,2).

Although the structure varies in gram positive and gram negative cells, the rigidity of bacterial cell walls is due to a layer of peptidoglycan, a macromolecule found only in bacteria. The basic structure of peptidoglycan is an alternating series of two major subunits, N-acetylmuramic (NAM) acid and N-acetylglucosamine (NAG). These subunits, are covalently joined together to form a glycan chain which serves as the backbone of the peptidoglycan molecule. Attached to each of the NAM molecules a string of four amino acids; a tetrapeptide chain. Cross-linkage can form between tetrapeptide chains, thus joining adjacent glycan chains to form a single, very large three dimensional molecule (2).

In S. pneumoniae the peptidoglycan consists of the basic structure, the backbone attached to a tetrapeptide consists of L-alanine, D-isoglutamic acid, L-lysine and D-alanine (3,4).

Inflammation and septic shock are considered as the characteristic of infection caused by gram positive and gram negative bacteria (5). Recently a great attention was paid to the role of gram positive bacteria in the pathogenicity especially septic shock (6,7).

It was shown that the peptidoglycan, teichoic acid and lipoteichoic acid are immunostimulators as they stimulate the release of tumor necrosis factor (TNF) , IL-1β and IL-6 from Peripheral Blood Mononuclear Cells (PBMCs) (8). However the synergistic effect of peptidoglycan and lipoteichoic acid induced the production of nitric oxide a vasodilator can lead to circulatory failure, hypotension, and vascular hyporeactivity (9).

Injection of peptidoglycan in rats (10) and rabbits (11) was found to cause multiorgan dysfunction due to increase in level of aspartate aminotransferase, alanine aminotransferase and bilirubin which means a hepatic injury has occurred also in level of urea and creatinine and indication of renal dysfunction.

Majcherczyk and his coworkers (12) pointed out to the role of oligomeric stem peptides of S. pneumoniae peptidoglycan, but not the monopeptides or dipeptides, in
inflammation and release the TNF from PBMCs.

The present study aimed to study the pathological effects that may result from *S. pneumoniae* peptidoglycan injection in rats’ lungs.

**Materials and Methods**

**Isolation**

Fifty sputum specimens were collected from respiratory tract infection patients with different age visiting Medical city and central children hospital from November, 1\textsuperscript{st} 2004 to March, 1\textsuperscript{st} 2005 in order to isolate *S. pneumoniae*.

The specimens were cultured on blood agar and heated blood agar plates at 37° C for 24 h, thereafter, the discrete colonies that produce α heamolysis were selected for further conventional biochemical tests (13,14).

**Antibiotic sensitivity**

Susceptibility to the antibiotics listed in Table 1 is tested using the disk diffusion method described by (15).

**Table (1)**

**Antibiotics used in study.**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Disc code</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>Ax</td>
<td>10</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>AM</td>
<td>10</td>
</tr>
<tr>
<td>Augmentin</td>
<td>AC</td>
<td>20</td>
</tr>
<tr>
<td>Ceftzidime</td>
<td>CA</td>
<td>30</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>CK</td>
<td>30</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>TC</td>
<td>75</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>TM</td>
<td>10</td>
</tr>
</tbody>
</table>

**Peptidoglycan extraction**

The peptidoglycan of *S. pneumoniae* was extracted according to Majcherczyk et al. (12).

Two liters of brain heart infusion were inoculated with *S. pneumoniae* at 37 °C for 18 h. The bacterial culture was quickly chilled in an ice / ethanol bath at 0 - 4° C. and the cells were harvested by centrifugation at 8000 xg for 20 min at 4 °C. and washed three times by sterile distilled water. Seven milliliters 4% sodium dodecyl sulfate (SDS) were added to the pellets and incubated in boiling water bath at 100 °C for 15 min. Once again the cells were washed 10 times by sterile distilled water. The precipitate was suspended in 15 ml of DW. Protein (16), carbohydrate (17) and nucleic acids (18) were assayed. Protein electrophoresis was carried out to testify the purity of the extract by the procedure suggested by Piljac and his colleagues (19).

Glass beads of 0.2 mm were added to the suspension and mixed by vortex for 10 min. The suspension was aspirated by aid of Pasteur pipette and centrifuged at 8000 xg for 20 min at 4 °C. The residual was resuspended in 2 ml of 0.1 M Tris–HCl buffer pH 7.5, then treated with DNase (50 µg/ml) and RNase (50 µg/ml) for 2 h. and trypsin (100 µg/ml) for 18 h. The suspension was centrifuged at 3000 xg for 20 min at 20° C. The precipitate was mixed with 3 ml of 1% SDS and incubated in boiling water bath at 100 °C for 15 min. Thereafter, it was washed twice with sterile distilled water. Five milliliters of 8 M Lithium chloride were added to the precipitate of last wash and incubated at 37 °C for 15 min. subsequently; five milliliters of 100 mM EDTA were added. The suspension was centrifuged at 3000 xg for 15 min at 4 °C. and the residual was washed with DW. Ten milliliters of acetone were added to the precipitate and submitted to dryness at room temperature. Dray weight was estimated in addition to protein (16), carbohydrate (17) and nucleic acids (18). Protein electrophoresis was carried out to testify the purity of the extract (19).

**In vivo study**

**Animals**

White female rats, aged 6–7 weeks, weighing 260 to 330 g were obtained from the animal house of University of Baghdad, College of Science, Department of biology. The animals were divided into three groups (A, B and C) as three animals per group.

**Injection protocol**

Group A was injected intranasally using sterile catheter (0.6 mm in diameter) with the *S. pneumoniae* peptidoglycan (37×10⁴ µg/ml). Group B was injected with 1 × 10⁹ cfu / ml of bacterial suspension while group C was injected with normal saline following the same manner achieved with group A.
Two days later, the animals were killed and the lungs were preserved in 10 % formalin as they prepared for histopathological study (20).

**Results and Discussion**

Out of 20 *Streptococcus* isolates, 2 (10 %) isolates were identified as *S. pneumoniae*. A result is considered very low in comparison to other studies. Soepandi and his colleagues (21) isolated 47 bacterial isolates from 34 sputum specimens collected from patients presented with acute pulmonary infections, *S. pneumoniae* isolation percentage reached 10.63 %. And in another study it reached 25.8 % (22), while Alqaddo (23) and Ibrahim (24) have isolated the pneumococci separately in percentage reached 24 % and 34.8 % respectively.

The two isolates were susceptible to tobramycin, augmentin and ampicillin.

Table (2) demonstrated the result of protein, carbohydrate and nucleic acids estimation. The protein concentration of the peptidoglycan extract dropped from 150 μg / ml, at the first step of extraction after treatment with SDS, to 21.25 μg / ml at the end of extraction process, an indication of the efficiency of the procedure of extraction. However, carbohydrate concentration increased from 24 to 91 μg / ml. Nucleic acids estimation was accomplished as the RNA and DNA were dropped from 1.2 and 1.0, respectively, to 0.0 μg / ml. These results agreed with Umeda *et al.* (25) and Al-heety (11).

![Fig. (1) : Electrophoresis of *S. pneumoniae* peptidoglycan after treatment with SDS (right) and at the end of extraction procedure (left).](image)

The results of the histopathological study of the rats’ lung injected with *S. pneumoniae* showed several pathological changes in comparison to control Fig.(2) represented by disruption in the alveoli walls, haemorrhage, as a body response to these foreign bodies, sloughing of epithelial cells inside the bronchiole and hydropic degeneration of cells lining the bronchiole Fig.(3) disruption in the vessel wall and infiltration of inflammatory cells Fig.(4).
Edema have been seen inside the lung tissue due to imbalance of hydrodynamic forces through pulmonary capillaries in addition to increase the permeability of capillary endothelial layer which result in leaking of fluids more than the adjacent tissue, plus the vacuolation inside the blood tissue due to the cellular transport hyperactivity of the endothelial cells Fig.(5) (26).

The peptidoglycan caused many pathological changes demonstrated by disruption of alveoli walls, edema inside the blood vessel and hydropic degeneration of cells lining the bronchioles, in addition to aggregation of leukocytes as it illustrated in Figs. (7) and aggregation of erythrocytes (congestion) Fig.(8).
It is so noticeable that the peptidoglycan effects were more severe than bacterial suspension since the amount of sloughing of the epithelium made by peptidoglycan was more than by bacterial suspension in addition the extent of damage that occurred in the blood vessel represented by amount of infiltration of inflammatory cells, hydorpic degeneration and congestion.

Our results indicate that Peptidoglycan of S. pneumoniae play an important pathogenic role in inflammatory lung disease.

References


الملاحظة

تم الحصول على عشرين عينة من مرضى مصابين بخمج المجاري التنفسية واثبت عزلتين من س. بوساطة الطرق الكيميائية التقليدية. S. pneumoniae

ولعرض استخلاص الببتيدوكلاياكان من هذه البكتيريا تم اعتماد التكسير الميكانيكي ووساطة الكرات الزجاجية والوارج مع الهضم الانزيمي بايزي مات الدناز والرنزين والبروناز. وعند إجراء الرحلات الكهربائي عند ظرف غير ماسخة، أظهر الببتيدوكلاياكان المنقى جزئياً أربع حزم بروتينية مقارنة مع الببتيدوكلاياكان الخام الذي أظهر ست حزم.

ظهرت الجرذان المحفون بعالم خلايا S. pneumoniae أو بالببتيدوكلاياكان تغييرات نسيجية مرضية مشابهة شملت تمرق جذرات الخوصلة الرئوية والقرح و ارتشاح الخلايا الانتهائية وبسلاخ الخلايا الطالانية المبطنة للفصيات وتكوين الودمة. في حين كانت التغييرات النسيجية المرضية الناتجة عن الببتيدوكلاياكان أشد من تلك الظاهرة عن عامل الخلايا.