THE PATHOLOGICAL EFFECT OF PEPTIDOGLYCAN ON RATS' LUNGS PART TWO: NON PATHOGENIC BACTERIA *BACILLUS SUBTILIS*

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Abstract

Fourteen isolates of Bacillus were obtained from twenty samples isolated from soil. Three of which were identified as *B. subtilis* by biochemical tests. For extraction of peptidoglycan from *B. subtilis*, 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 several bands when it performed in polyacrylamide gel electrophoresis under undenaturing conditions.

The histopathological changes for the injected group of rats with the germ suspension of *B. subtilis* were increased number of alveolar macrophages, thickening of alveoli walls in addition to accumulation of fluids (edema) inside the lung tissue. Whereas the peptidoglycan injected group revealed heamorrhage, edema formation, vacuolation, increased number of lymphocytes, precipitation of collagen fibers, and rupture of alveoli walls and sloughing of epithelial cells inside bronchioles lumen.

The effects of histopathological changes caused by peptidoglycan were more severe than those of cell suspension, which suggesting the important role of this polymer in the pathogensis of this bacteria.

Introduction

In 1872, Ferdinand Cohn, a contemporary of Robert Koch, recognized and named the bacterium *Bacillus subtilis.* The organism was made to represent a large and diverse genus of Bacteria, *Bacillus***,** and was placed in the family *Bacillaceae***.** The family's distinguishing feature is the production of endospores, which are highly refractile resting structures formed within the bacterial cells. Since this time, members of the genus *Bacillus* are characterized as Gram-positive, rod-shaped, aerobic or facultative, endospore-forming bacteria (1).

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).

Inflammation and septic shock are considered as the characteristic of infection caused by gram positive and gram negative bacteria (3) recently a great attention was paid to the role of gram positive bacteria in the pathogenicity especially septic shock (4,5).

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) (6). 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 (7).

Injection of peptidoglycan in rats (8) and rabbits (9) 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.

The basic structure of peptidoglycan is considerably similar in structure among gram positive bacteria (10). In a previous study we proved that the peptidoglycan of *Streptococcus pneumoniae* has the ability to cause damage to rat lungs, hence the present study aimed to

investigate the pathological role of a peptidoglycan that extracted from non pathogenic bacteria such as *B. subtilis* in pathogenicity.

Materials and Methods

Isolation

Twenty samples were collected form soil in order to isolate *B. subtilis*, the specimens were cultured on blood agar plates at 37 °C for 24 h (11), thereafter, the discrete colonies were selected for further conventional biochemical tests (12, 13).

Peptidoglycan extraction

The peptidoglycan of *B. subtilis* was extracted in same manner followed by Atrih *et al*. (14).

One liter of brain hearth infusion was inoculated with *B. subtilis at* 37 °C for 18 h. The bacterial culture was incubated in boiling water bath for 7 minutes in order to avoid peptidoglycan hydrolysis. Thereafter the cells were harvested by centrifugation at 8000 xg for 15 min at 4° C. Twelve milliliters of 5 % sodium dodecyl sulfate (SDS) were added and centrifuged at 20 °C for 15 min. Once again Twelve milliliters of 4 % SDS were added, heated at 100° C for 15 min and the SDS was washed by centrifugation (3000 xg) at 4 °C for 15 min. The precipitate was suspended in 15 ml of deionized distilled water (DDW). Protein (15), carbohydrate (16) and nucleic acids (17) were assayed. Protein electrophoresis was carried out as well, by following the procedure suggested by Piljac and his colleagues (18).

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 pronase (2 mg/ml) for one hour. The suspension was centrifuged at 3000 xg for 15 min at 4 °C. The precipitate was mixed with 2 ml of sterile DDW, 400 µl of 48 % hydrofluoric acid were added and incubated 2 °C for 24 hours. The suspension was centrifuged (3000 xg) at 4 °C for 15 min. subsequently; the residual was washed with 50 mM Tris–HCl buffer pH 7, washed again with cold DDW five times. Dry weight was estimated in addition to protein (15), carbohydrate (16) and nucleic acids (17). Protein electrophoresis was carried out to testify the purity of the extract (18). The extract was kept at -20 °C.

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.

Group A was injected intranasally using sterile catheter (0.6 mm in diameter) with the *B. subtilis* peptidoglycan (37×10^4 µg/ml), group B was injected with 1×10^9 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 % formaline as they prepared for histopathological study (19).

Results and discussion

Out of 14 *Bacillus* isolates, 3 (21 %) isolates were identified as *B. subtilis*. A result is considered high in comparison to the study of (20) since he was isolated one isolate, however, the present study agreed with Aslim *et al*. (11) as they achieved 20 % of *B. subtilis* out of 40 bacterial isolates.

Table (1) demonstrated the results of protein, carbohydrate and nucleic acids estimation; the protein concentration of the peptidoglycan extract dropped from 287 µg / ml, at the first step of extraction after treatment with SDS, to 117.5 µg / ml at the end of extraction process, an indication of the efficiency of the procedure of extraction. However, carbohydrate concentration has increased from 17 - 88 µg / ml. nucleic acids estimation was accomplished as the RNA and DNA were dropped from 1.5 and 0.3, respectively, to 0.0 µg / ml. an indication to the efficiency of hydrolytic enzymes those were employed in this study. These results

have agreed with Umeda *et al.* (21) and Al-heety (9).

Fig.(1) illustrated the result of electrophoresis to indicate the purity of peptidoglycan extract, as we can see the specimen has developed several bands after treatment with SDS while they were reduced to 4 bands at the end of extraction procedure.

Table (1)
Protein, carbohydrate and nucleic acids estimation of the peptidoglycan extracted from B.

Extract-ion step	Protein concentration µg/ml	Carbohy-drate concentration µg/ml	Nucleic acids concentration µg/ml	
			DNA	RNA
After treatment with 4% SDS	287	17	0.3	1.5
Last step of the extraction	117.5	88	0.0	0.0

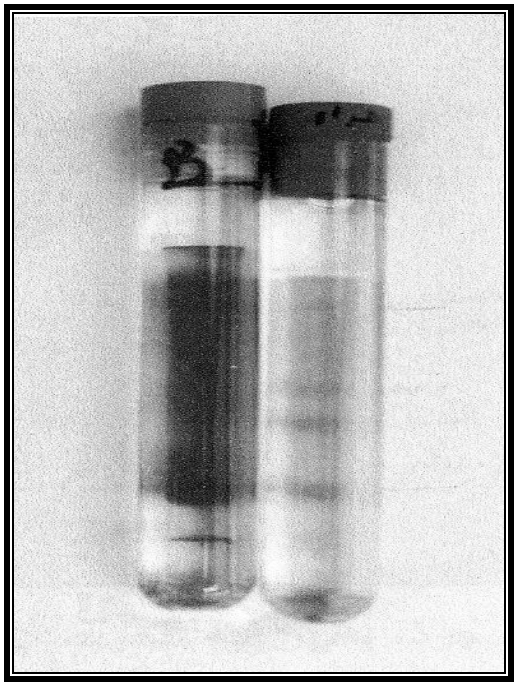

Fig.(1) : Electrophoresis of *B. subtilis* peptidoglycan; after treatment with SDS (left) and at the end of extraction procedure (right).

The results of the histopathological study of the rats' lung injected with *B. subtilis* showed several pathological changes in comparison to control Fig.(2) represented by increased number of alveolar macrophages, thickening of alveoli walls in addition to accumulation of fluids (edema) inside the lung tissue Fig. (3).

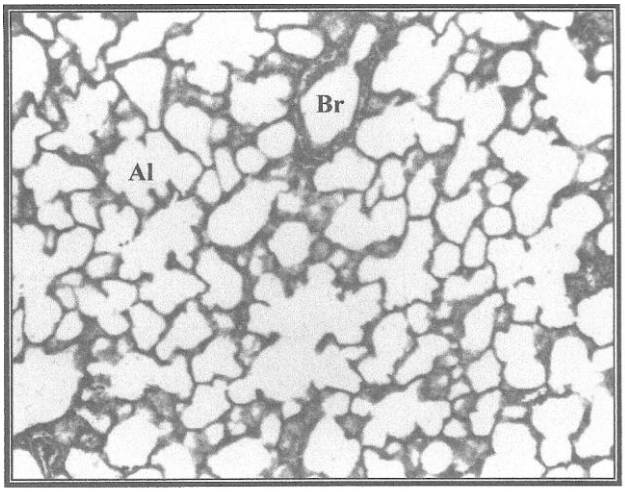

Fig.(2) : Cross section in rat lung showed the alveoli (A1) and the bronchiole (Br). X400 H&E.

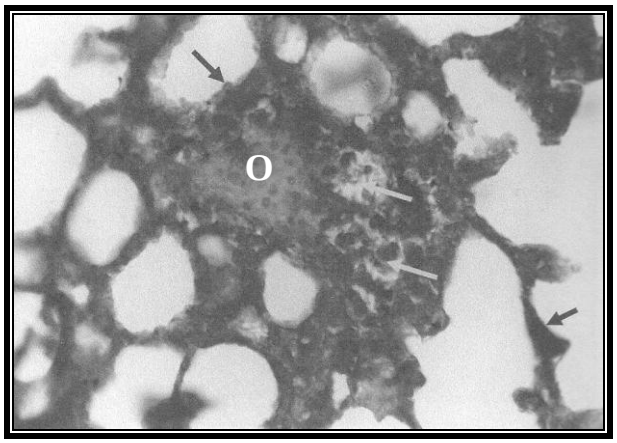

Fig.(3) : Cross section in rat lung injected with 1×10^9 cfu / ml of *B. subtilis* showed the alveolar macrophages (white arrows), edema (O), and thickening of alveoli walls (black arrows). X400 H&E.

Hisopathological changes in rats' lungs injected with 37×10^4 µg/ml *B. subtilis* peptidoglycan characterized by heamorrhage, edema formation, vacculation Fig.(4), increased number of lymphocytes and precipitation of collagen fibers Fig.(4) and (5), rupture of alveoli walls and sloughing of epithelial cells inside bronchioles lumen Fig.(6).

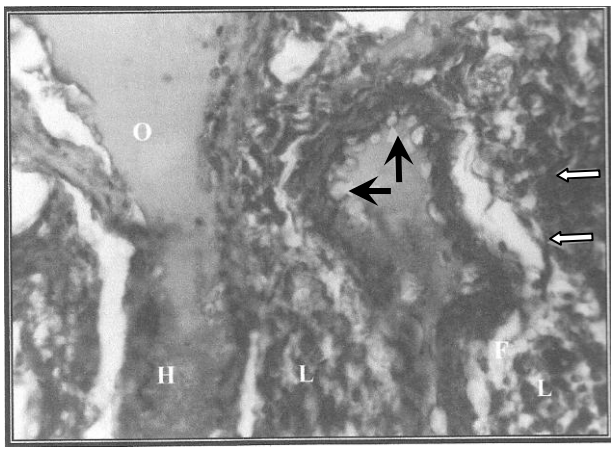

Fig.(4) : Cross section in rat lung injected with 37×10^4 µg/ml *B. subtilis* peptidoglycan showed heamorrhage (H), edema (O), vacuolation (black arrows), lymphocytes (L) and collagen fibers (white arrows). X400 H&E.

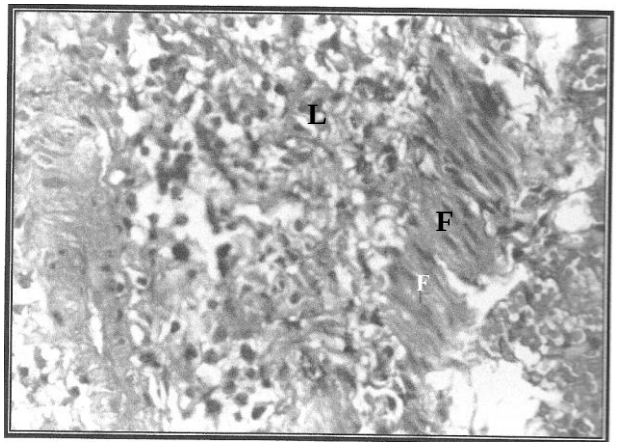

Fig.(5) : Cross section in rat lung injected with 37×10^4 µg/ml *B. subtilis* peptidoglycan showed lymphocytes (L) and collagen fibers (F). X400 H&E.

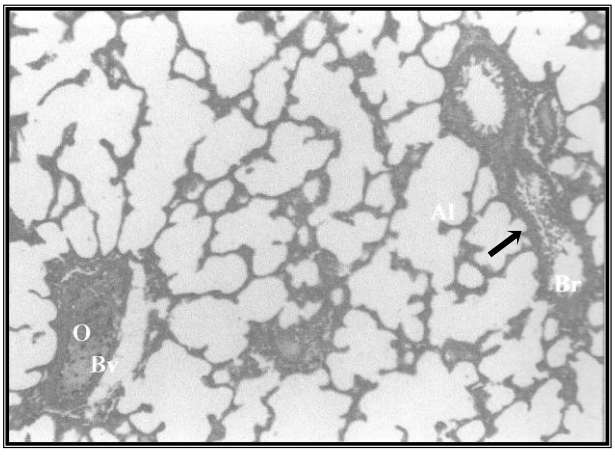

Fig.(6) : Cross section in rat lung injected with 37×10^4 µg/ml *B. subtilis* peptidoglycan showed edema (O) in blood vessel (Bv), vacuolation (O), rupture of alveoli walls (A1) and sloughing of epithelial cells (➞) inside bronchioles lumen (Br). X400 H&E.

It is so obvious that the peptidoglycan has caused damage more than the bacterial suspension due to the presence of haemorrhage, the edema formation was bigger than in lungs injected with bacterial suspension, number of inflammatory cells in peptidoglycan injected lung was higher than bacterial suspension injected lung in addition to the rupture of alveoli walls and the fibrosis as well. These changes in both animal models (peptidoglycan injected animals and bacterial suspension injected animals) could be attributed to the peptidoglycan since there is no evidence point to pathogenicity of this "non pathogenic" bacteria. Also the severe changes were noticed in lung injected with peptidoglycan could be attributed to concentration of the peptidoglycan (since its concentration would be, necessarily, more than in bacterial suspension).

These results highly recommended studying the pathogenicity of this polymer both immunologically and biochemically.

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الخلاصة

جمع 14 عزلة *Bacillus* من 20 عينة تربة ثلاثة منها شخصت على انها *B. subtilis* بوساطة الاختبارات الكيماحيوية. ولغرض استخلاص الببتيدوكلايكان من هذه البكتريا تم اعتماد التكسير الميكانيكي بوساطة الكرات الزجاجية والمازج مع الهضم الانزيمي بانزيمات الدناز والرناز والبروناز . وعند اجراء الرحلان الكهربائي عند ظرف غير ماسخة، اظهر الببتيدوكلايكان المنقى جزئيا اربع حزم بروتينة مقارنة مع الببتيدوكلايكان الخام الذي اظهر حزم عديدة.

اظهرت مجموعة الجرذان المحقونة بعالق خلايا *B. subtilis* تغييرات نسيجية مرضية شملت زيادة اعداد البلعم الكبير في الاسناخ و تثخن جدران الاسناخ الرئوية فضلا عن تجمع السوائل (وذمة) في انسجة الرئة . في حين اظهرت المجموعة المحقونة بمستخلص الببتيدوكلايكان تغييرات نسيجية مرضية تضمنت شملت النزف و تكوين الوذمة والتفجي وزيادة اعداد الخلايا اللمفاوي ة وترسب الياف الكولاجينية و تمزق جدران الحويصلات الرئوية وانسلاخ الخلايا الطلائية المبطنة للقصيبات.

لقد كانت التغييرات النسيجية المرضية الناتجة عن الببتيدوكلايكان اشد من تلك الناتجة عن عالق الخلايا . الامر الذي يشير الى اهمية الدور الذي يلعبه الببتيوكلايكا ن في امراضية هذه البكتريا.