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Research article

Histopathological Effect of Extracellular Products of Clinical Isolates of *Pseudomonas aeruginosa* on Mice Lungs.

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ABSTRACT

The bacterial secretions into the growth medium that are produced as a result of the dynamics of growth and death of bacteria such as proteins, nucleic acids, and polysaccharides stimulate inflammatory response. The effect of extracellular bacterial secretions that are released by bacteria during growth on inflammatory response as well as the body's tissues is not clear. The current study aims to highlight the effect of sterile bacterial secretion (IBS) of clinical isolate *Pseudomonas aeruginosa* on lung tissue. Here*, P. aeruginosa* was isolated from urine and grown on Luria-Bertani (LB) broth for a day, and IBSs were collected after filtration using a Millipore *filter*. These materials were administered to the experimental mice intranasally (i.n.). The tissue sections of the mouse lungs and the number of leukocytes in the bronchoalveolar lavage (BAL) were examined. The histological sections showed an increase in the leukocyte infiltration into the alveoli cavity and walls was seen as early as 4 h post-installation, and this continued until the second day. However, at 72 post instillation, it was found that the lung tissue had almost completely recovered and returned to its normal shape. The study also showed an increase in the number of Leukocytes number in BAL of mice that were given the IBS as early as 4 h post instillation this increase continued until 72 h. The results showed the effect of IBS in stimulating a safe inflammatory response in the lungs of mice, and this could help in the possibility of using these materials in the future to increase the immunostimulation of weak immunostimulatory antigens.

Keywords: Histopathology, leukocytes, Lungs, Mice, *Pseudomonas aeruginosa*.

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1. INTRODUCTION

The outbreak of *Pseudomonas aeruginosa* is well-known for its capacity to survive in a variety of settings, such as sewage water, freshwater, and moist surfaces. It is also a prominent human pathogen that takes advantage of opportunities and is linked to several diseases [1]. *P. aeruginosa* environmental isolates are useful in bioremediation. They are renowned for their capacity to decompose and disintegrate a wide range of organic substances, such as contaminants and hydrocarbons [2]. Their ability to adapt to different metabolic conditions makes them useful for decontaminating areas. *P. aeruginosa* is a member of the normal microbial population in the surroundings. It interacts with plants and other creatures and contributes to the cycling of nutrients [3]. The genetic characteristics present in clinical isolates may be influenced by this diversity. For example,

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genes resistant to antibiotics can be transferred between clinical and environmental bacteria. If environmental isolates are not well controlled, they may give rise to clinical infections [4]. The clinical isolates of *P. aeruginosa* are a major contributor to nosocomial infections, or infections acquired in hospitals, especially in immunocompromised patients and those with underlying illnesses and caused several infectious diseases i.e. sepsis, wound infections, urinary tract infections, and pneumonia [5]. They may efficiently colonize and infect host tissues because of their virulence factors, which include exotoxins, proteases, and the ability to build biofilms. Treatment for *P. aeruginosa* clinical isolates is difficult since these strains frequently show resistance to many medicines [6].

Bacterial products have been shown to affect lung tissue structure [7]. Tissue damage in the lungs can lead to the outgrowth of commensal bacterial species that promote fibrotic responses. In the context of chronic respiratory diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF), the microbiome of the lung has been found to play a role in maintaining lung health and in the pathogenesis of these diseases [8]. The presence and composition of bacteria in the respiratory tract can have significant effects on lung tissue structure and the pathogenesis of respiratory diseases [9]. Furthermore, studies suggest that the lung microbiome may contribute to local host inflammatory changes, including the Th17 response, which may have relevance to lung pathogen-esis. The presence and composition of bacterial appendages in the lungs can have significant implications for respiratory health and disease, including fibrosis, COPD, and lung cancer [8].

Bacterial secretions can affect inflammation in both specific and general ways. Gram-negative bacteria have specialized secretion systems that can both activate and inhibit inflammasome signaling, which is crucial for producing inflammatory cytokines [10]. Additionally, the composition of bacterial strains can dictate the degree of bacterially-driven intestinal inflammation, with specific combinations of bacteria having different inflammatory effects [11]. Furthermore, the expression of genes involved in inflammation regulation can be modulated by bacterial molec-ular patterns, leading to the suppression of inflammatory reacti-ons. These findings suggest that bacterial secretions can have both specific and general effects on inflammation, depending on the bacterial species and their interactions with the host immune system [12]. The scientific literature is devoid of studies that address the role of bacterial secretions in cultural media in regulating the inflammation immune response and tissue response, especially in the lungs, and the changes that can occur in the lungs. Therefore, the current study deals with the effect of bacterial secretions of strain isolated from pathological samples on tissue changes that occur in the lungs of mice.

2. MATERIALS AND METHODS

2.1. Bacterial Isolation and Identification

The urine of patients with urinary tract infection (UTI) was collected in a sterile container. The samples were examined in the laboratory for analysis. The non-lactose fermenting colonies were re-cultivated on cetramide agar, and oxidase and catalase tests were done. The pre-examined isolates of *P. aeruginosa* were identified using the VITEK 2 DensiCheck equipment and the bioMe´rieux fluorescence system, in accordance with the manufacturing company's recommendations [13].

2.2. Preparation of infiltrated bacterial secretion

Separate flasks of LB broth (HiMedia) were used to culture the clinical isolates of *P. aeruginosa* (PAC) for a whole night at 37 °C. By centrifuging the bacteria cultured in LB broth for 25 min at 4 °C, the supernatants of infiltrated bacterial secretions (IBSs) were obtained. A Millport filter (MF-Millipore Membrane Filter, 0.22 µm pore size) was used to filter the supernatants. Supernatants (IBSs) were cultured on LB agar plates to verify their sterility.

2.3. Experimental animals

Six to eight-week-old BALB/c mice weighing twenty to twenty-five grams were obtained from Al-Nahrain University's central animal house in Baghdad, Iraq. The animals were fed normal food devoid of antibiotics and housed in hygienic polypropylene cages. The University of Baghdad's College of Science's animal ethics committee gave its clearance before the study could be carried out.

2.4. Experiment

Three test groups of animals (mice) were employed in the current study. Test group (n: 12 mice), mice were given intranasal (i.n.) 50 µl IBS obtained from a PAC growth. In the present study, two control groups were used, each control group consisted of 3 mice instilled i.n. with 50 µl of sterile LB broth (1st control group) and PBS (pH, 7.2, 0.1 M) (2nd control group). At different intervals of time (1, 4, 24, 48, and 72 h), three animals were sacrificed post-IBS instillation intranasally.

2.5. Bronchoalveolar lavage (BAL) cell counts

A hemocytometer was used to count all the cells in bronchoalv-eolar lavage (BAL) specimens. Leishman stain-stained smears were examined to detect the differential count of cells [14].

2.6. Histopathological examinations of lungs

Formalin-fixed, paraffin-embedded lung sections were prepared using Zgair and Chhibber's procedure [14]. After the mice were killed, the lungs of the test and control groups were prepared. Hematoxylin and eosin were used to stain lung sections [15].

3. RESULT

3.1. Isolation and Identification of *P. aeruginosa*

While the environmental *P. aeruginosa* (PAE) was isolated from freshwater, the clinical isolate of *P. aeruginosa* (PAC) was obtained from urine. The isolates were identified using VITEK technology and biochemical techniques. From the above isolates, the IBSs were prepared.

3.2. Effect of IBS of PAC on lung tissue

In the present study, the histological study was performed also to find the pathological effect of the IBS of a clinical isolate of *P. aeruginosa* (PAC) on the lung tissue. Post instillation with IBS the histological changes were examined at different time intervals (4, 24, 48, and 72h) post instillation with IBS. The result of this experiment is demonstrated in Fig 1. Few infiltration of leukocytes was observed at 4 h post-instillation (Fig.1. a, and b). The infiltration of leukocytes was slightly increased with little changes in alveolar septa. These changes were observed at 24 h and 48 h post instillation (Fig. 1 c, d, and e).). After 72 h of the instillation with IBSs of a clinical isolate of *P. aeruginosa* (PAC) the lung tissue was completely cured and became normal (Fig. 1 f) whereas the thin alveolar wall with normal air space of alveoli

was observed clearly. To compare the changes that happened in the lungs of mice post-instillation with IBSs of PAC, the histological sections were made to control groups (A and B), mice installed (i.n.) with sterile LB broth and PBS, respectively. The results of the histological study for control groups are shown in Fig. 1. Only one-time point was shown in the figure because all time points gave the same results. The figure showed that no histological changes occurred in the structure of the lung of mice post-instillation with either sterile LB broth (Fig. 1 g) or PBS (Fig. 1 h). The normal structure of the alveoli was seen with a thin zigzag wall and empty alveolar space.

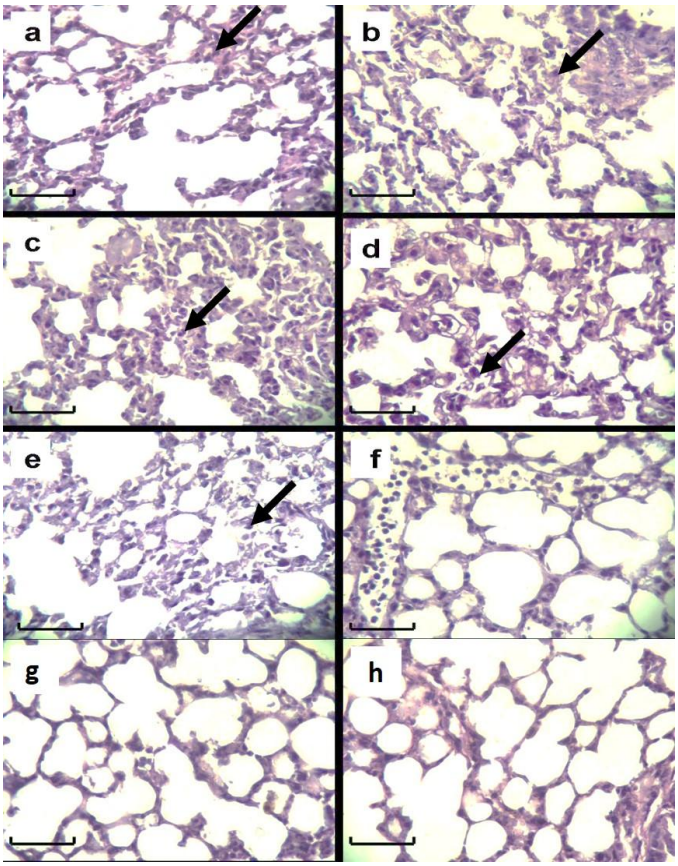

Fig 1. Histopathological photomicrographs depicting mice lung tissues obtained at different time intervals post-instillation intranasal (i.n.) with sterile bacterial secretions (IBSs) prepared by infiltrating the LB broth inoculated previously with clinical isolate *P. aeruginosa* (PAC). a, at 4 h; b, at 4 h; c, at 24 h; d, at 24 h; e, at 48 h; f, at 72 h post instillation with IBSs of PAC. g, mice instilled i.n. with sterile LB broth (1st control group); h, mice instilled i.n. with sterile PBS (2nd control group), bars, 50 µm. Arrows point at leukocytes (WBCs) infiltration.

3.3. Leukocyte infiltration in mice lungs (BALs)

The number of white blood cells (leukocytes) was estimated at different time intervals (4, 24, 46, 72 h) in bronchoalveolar lavages (BALs collected from three groups of mice [test group, 1st control group, and 2nd control group). Fig. 2 showed that a significant increase in leukocytes was observed as early as 4 h post-instillation (i.n.) with IBSs of PAC as compared with the 1st and 2nd control groups. The leukocyte infiltration in BALs of the test group of mice increased dramatically with time with the maximum number of leukocytes at 48 h post-instillation with IBSs of PAC. After 48 h the number of leukocytes was decreased but they were high significantly as compared with 1st and 2nd control groups. This finding proved that IBSs of a clinical isolate of *P. aeruginosa* (PAC) stimulate the WBCs infiltration in mice lungs and that proved the results of histopathological study.

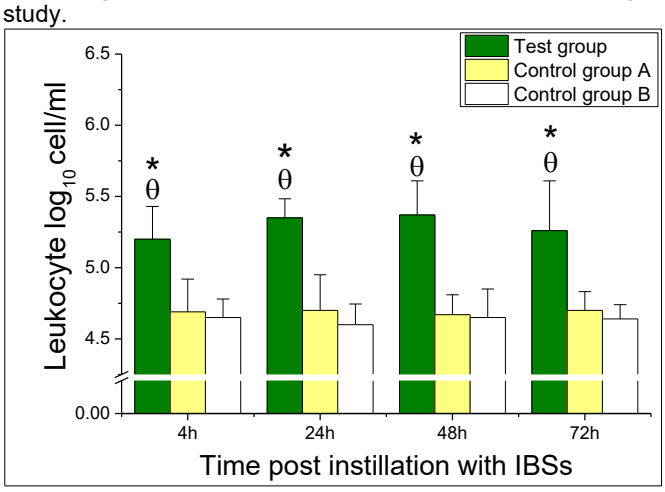

Fig 2. Time-course of changes in leukocytes in bronchoalveolar lavages (BALs) of lungs obtained from different groups of mice [test group, mice instilled (i.n.) with sterile bacterial secretion (IBSs) infiltrated growth medium (LB broth) post inoculated with *P. aeruginosa* (PAC); 1st control group, mice instilled (i.n.) with sterile LB broth; 2nd control group, mice instilled (i.n.) with sterile PBS. θ, $p < 0.05$ vs 1st control group; *, $P < 0.05$ vs 2nd control group.

4. DISCUSSION

Gram-negative bacteria possess specialized secretion systems, which can activate and evade innate immune signaling pathways, including caspase-1-activating inflammasomes [11]. Additionally, certain phyla of bacteria within the microbiota, through fermentation of fiber, produce short-chain fatty acids that can signal through the G-protein coupled receptor GPR43, leading to anti-inflammatory effects [11]. Bacterial products/components, such as lipopolysaccharide (LPS) and formylated peptides (fMLP), can synergistically induce inflammation by activating proinflammatory cytokine gene expression through the TLR4 and IKKβ-IκBα signaling pathways [16]. Bacteria also possess a diverse array of proinflammatory molecules, including soluble and membrane-associated inducers of cytokine release, inducers of host cell apoptosis, and immunostimulatory DNA [17]. Furthermore, commensal bacteria can protect against inflammation caused by enteric pathogens by modulating the innate immune system, potentially through the activation of alternatively activated macrophages [18]. The present study supports that the bacterial secretions of clinical isolates of *P. aeruginosa* stimulate the inflammation reaction in terms of leukocyte infiltration to lung tissue and to the airways of the respiratory tract and that was approved by the counting of the number of leukocytes in the BALs of the mice through instilled i.n. with serial bacterial secretions collected from the growth media of *P. aeruginosa* that were isolated from the urine of patients with UTI.

Previous studies by Vives-Flórez and Garnica, (2006) used the strains of *P. aeruginosa* were isolated from clinical specimens and studied the virulence factors of this isolate of bacteria [19]. Hoffmann et al. (2005) found that the lungs of both CF mice and BALB/c mice challenged with the mucoid *P. aeruginosa*, respectively, showed severe inflammation response including infiltration of inflammatory cells and edema [20]. As judged by lung scoring, significantly more severe changes in lung pathology were found in CF mice challenged with the mucoid

compared to the nonmucoid isolates. The histopathological effect of *P. aeruginosa* (ATCC 27853) on mice lungs was also investigated by Rodrigues et al. (2013). They reported large scattered areas of intense inflammation with mainly mononuclear inflammation infiltration. Previous studies showed a strong relation between the increase of pro-inflammatory cytokines production in the lung post-infection with *P. aeruginosa* and the increase in the level of leukocyte infiltration in the lung [21, 22]. The study focused on the role of bacterial secretion in the histological feature of the lungs is scanty in literature. A recent study by Ghafil and Zgair, (2022) reported that the effect of serial bacterial secretion in the growth media after inoculation with *P. aeruginosa t*hat was isolated from infected wounds (cut wound) stimulated the pro-inflammatory immune response and had a slight effect on the histological feature of an animal model [23], while in the present study, the *P. aeruginous* was isolated from urine.

The recruitment and accumulation of inflammatory cells is orchestrated by a large number of inflammatory mediators, defined as chemical messengers that act on blood vessels and/or cells to produce an inflammatory response [24]. The presence of inflammatory cells plays a central role in the inflammatory immune response [24]. That may help in the eradication of pathogenic bacteria by engulfing bacteria and killing them intracellularly. The previous studies (mentioned above) support the concept of the relationship between high levels of pro-inflammatory cytokines in the inflammatory area and the presence of leukocyte infiltration. This is completely going on with the results obtained in the present study.

5. CONCLUSION

The extracellular products generated in the growth media after *P. aeruginosa* inoculation (and incubation for 18 h) in our study demonstrate good activation of the cellular innate immune response and inflammation immune response by attracting the leukocytes to the lung alveoli and lung airways. This attraction generated was safe and from show time. No damage and negative effect on the mice's lung features.

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Conflict of interest

The authors declare that they have no conflict of interests.

Ethical Approval

This review was approved by the Ministry of Health, Baghdad, Iraq (No 312, 2023).

Author contributions

Majid N Ali. Conceptualization; Data curation; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; and Writing - review & editing

Mehdi Meskini Heydarlou. Formal analysis; Funding acquisition; Validation; Visualization; Roles/Writing - original draft; and Writing - review & editing

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