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Research article

Clinical Isolates of *Pseudomonas aeruginosa* Stimulate Interleukin 1 Beta and Tumour Necrosis Factor Alpha in Mice Lungs.

Majid Neemah Ali[1*], Zainab Hani Hatif [2]

ABSTRACT

Pseudomonas aeruginosa has the ability to stimulate the pro-inflammatory immune response in addition to causing infection. The current study aims to evaluate the effect of this bacteria on generating the pro-inflammatory immune response and whether this response can be short-term or long-term, as well as to identify the persistence of bacterial infection in the lungs of experimental mice. Here, *P. aeruginosa* (PAC) was isolated from sputum samples collected from patients suffering from acute respiratory tract infections. Experimental mice were given 10^8 c.f.u of *P. aeruginosa* intra-nasal (i.n.). The lungs were harvested at different time intervals (1, 2, 4, 24, 48h) to check the Interleukin (IL-)1 Beta (β) and tumor necrosis factor (TNF-) alpha (α) using Enzyme linkage immune sorbent assay (ELISA) and (4, 24, 48, 72h) for studying the bacterial burden in mice lung using plate count method. Significant increase in IL-1B with maximum level by 4 h post instillation. A similar finding was observed in studying the level of TNF-a. In both cytokines, a significant increase was observed up to 48 h post-instillation with *P. aeruginosa*. The study showed that the clinical isolate of *P. aeruginosa* could be persistent up to 72 h post-instillation with PAC. The current study confirms the ability of this bacteria to stimulate the pro-inflammatory cytokines for a long period, as well as its ability to remain in the lungs of animals for long periods, which confirms the ability of the bacteria to maintain its negative effect for long periods in experimental animal lungs.

Keywords: Clinical isolates, IL-1β, TNF-α, *Pseudomonas aeruginosa*.

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1. INTRODUCTION

Pseudomonas aeruginosa it is a Gram-negative bacterium, has long been recognized as a formidable pathogen, particularly within the context of respiratory tract infections. Its ability to colonize and thrive in various host environments, coupled with its inherent resistance to a multitude of antibiotics, poses a significant challenge in clinical settings [1]. It is involved in the stimulation of the innate immune system and inflammatory immune response of the mucosal layer of the respiratory tract system. It is a ubiquitously distributed opportunistic pathogen notorious for its ability to cause infections in diverse clinical settings [2]. It is perhaps best known for its impact on respiratory health, causing ailments ranging from acute pneumonia to chronic bronchiectasis in susceptible individuals. Its success as a pathogen can be attributed to a plethora of virulence factors and adaptability mechanisms [3]. The metabolic capabilities of this bacterial species allow it to grow in various environmental niches, including hospital water systems, soil, and the respiratory tracts of humans and animals. This adaptability is further

* Correspondence: **Majid Neemah Ali**. E. mail: sciencecity2015@gmail.com; https://orcid.org/0000-0001-7607-0839
Department of Biology, College Science, University of Baghdad, Baghdad, Iraq.
Full list of author information is available at the end of the article.

compounded by its ability to form resilient biofilms, which protect from both host immune responses and antimicrobial agents [4]. It invades the respiratory tract, the host immune system swiftly mobilizes to combat the threat. Neutrophils, macrophages, and other immune cells are recruited to the site of infection. However, this pathogen possesses an arsenal of virulence factors that enable it to evade immune detection and neutralization. The presence of *P. aeruginosa* in the respiratory tract stimulates the host immune response to produce pro-inflammatory cytokines, including Interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) [5]. These cytokines play pivotal roles in orchestrating the immune response, signaling danger, and promoting inflammation. Their secretion is often a double-edged sword - while they are essential for pathogen clearance, excessive or deregulated production can lead to tissue harm and exacerbate the inflammatory response [6].

IL-1β and TNF-α both are the central pro-inflammatory cytokines that emerge as key players in the host response to *P. aeruginosa* infection. These cytokines are produced by immune cells, particularly macrophages, in response to the presence of pathogenic invaders, including bacteria like *P. aeruginosa*. IL-1β, once secreted, exerts a multitude of effects. It enhances the recruitment of immune cells to the infection site and activates neutrophils and other phagocytic cells to engulf and destroy bacteria [7]. TNF-α, on the other hand, contributes to the recruitment and activation of immune cells and triggers various signaling cascades that intensify the immune response [8].

This bacterial species possesses several virulence factors that enable it to exploit the host immune response, leading to the deregulated secretion of IL-1β and TNF-α. These factors include lipopolysaccharides (LPS), flagellin, and various exotoxins. LPS, an integral component of the bacterial outer membrane, is a potent inducer of inflammation [9]. It activates toll-like receptors (TLRs) on immune cells, triggering the production of IL-1β and TNF-α. *P. aeruginosa* LPS is particularly adept at evading immune recognition, allowing it to persist within the host. Flagellin, the protein component of bacterial flagella, also activates TLRs and contributes to the deregulation of cytokine production. *P. aeruginosa* flagellin possesses unique structural features that enhance its ability to stimulate inflammatory responses [10]. The present study aims to highlight the role of stimulation of the respiratory tract of the animal model with *P. aeruginosa* (whole bacterial cells) in stimulating the pro-inflammatory cytokines including IL-1B and TNF-a as well as the persistence of this bacteria in the respiratory tract of animal model.

2. MATERIALS AND METHODS

2.1. Isolation and identification of bacteria

The specimens of 50 sputum samples (2-5 ml) were collected from indoor patients suffering from lower respiratory tract infections (Maysan Hospital, Maysan, Iraq). Five hundred microliter of sputum were placed in sterile tubes containing 9.5 ml of normal saline and transported directly to the technical diagnostic lab. The specimens were inoculated onto both MacConkey and blood agar, and incubated at 37°C for 18h. The non-lactose fermenting colonies were re-cultured onto cetramide agar. The suspected *Pseudomonas* isolates that were grown onto Cetrimide *agar* were sub-cultured onto nutrient agar to check for purity. Pure colonies were used for the biochemical tests for the identification of *P. aeruginosa* [11] including Gram stain, growth characteristics, and other test methods [11]. The VITEK 2 DensiCheck instrument, fluorescence system (bioMe´rieux) (ID-GNB card) was used for confirming the species of *P. aeruginosa* isolates [12]. The bacteria isolates were inoculated onto nutrient agar slant and incubated for 18 h at 37ºC and then stored at 4ºC for 2-4 weeks. For long-period storage, all clinical isolates of *P. aeruginosa* were maintained at -20ºC in trypticase soy broth (TSB) (Hi media, Mumbai) containing 20% glycerol.

2.3. Preparation of Bacterial Suspension (BS)

Clinical isolates of *P. aeruginosa* (PAC) were grown overnight in Luria Broth (LB) (Hi-media, India) at 37ºC (18 h). The bacterial cells were washed three times with sterile phosphate buffer saline (PBS, 0.1 M, pH 7.2). The bacterial pellets were re-suspended by sterile PBS (0.1M, pH, 7.2), and the numbers of bacteria were adjusted to 10^8 colony form unite (c.f.u.)/milliliter (ml).

2.4. ANIMALS

BALB/c mice 6-8 weeks old, weighing 20-25 gm were procured from Central Animal House, AL-Nahrain University, Baghdad, Iraq. Animals were kept in clean polypropylene cages and fed on a standard antibiotic-free diet

2.5. Experiment

To determine the ability of bacterial cells of clinical isolates of *P. aeruginosa* to generate the pro-inflammatory immune response in the lungs of normal mice, the following mice groups were established. In the test group, 15 mice were given 50 µl PBS (0.1 M, pH 7.2) containing $5x10^6$ c.f.u/ml clinical isolate of *P. aeruginosa* intranasally. In the control group A, 15 mice were given 50 µl sterile LB broth. Three animals were sacrificed at each time point: 4 hours, 1 day, 2 days, and 3 days post-bacterial administration. In the control group B, 15 mice were given 50 µl of PBS (0.1 M, pH 7.2). Three animals were sacrificed at each time point: 4 hours, 1 day, 2 days, and 3 days post-bacterial administration. The lungs from respective groups were sampled to determine the viable bacterial count and pro-inflammatory cytokines.

2.6. Quantification of bacteria in lungs

The Lungs were sectioned into several pieces. One piece of lung was placed in separate sterile tubes and weighted. Lung tissue was homogenized in PBS (0.1 M, pH 7.2) in a corning glass homogenizer. Serial dilutions of homogenized lung tissue in PBS (0.1 M, pH 7.2) were made and plated on LB agar plates. The plates were incubated at 37ºC overnight and quantitative bacterial counts were determined [13].

2.7. Levels of IL-1β and TNF-α

The mice's lungs were weighted and homogenized in 2 ml of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, and 1 mM MgCl (pH 7.4), using a tissue homogenizer. The homogenates were centrifuged (3000 ×g, for 10 min). Two hundred microliters of the supernatants of homogenizes of mice lungs of different groups were collected at different intervals of time (1, 2, 4, 24, and 48 h) after being stimulated with bacterial cells (PAC). The enzyme linkage immunosorbent assay (ELISA) was used to measure the mice's pro-inflammatory cytokines. The mouse TNF-α ELISA kit and mouse IL-1β ELISA kit (KOMA BIOTECH INC) in a wavelength of 450 nm were used. The manufacturer's instructions of companies were followed [13].

2.8. Statistical analysis

The Origin 8 software was used to do all operations of statistical analysis. The data were expressed as means ± SE. The differences were evaluated using a student t-test and one-way ANOVA. The P values less than 0.05 were considered to be statistically significant.

3. RESULT

3.1. Isolation and Identification of *P. aeruginosa*

Fifty sputum samples were collected from patients suffering from respiratory tract infections. From fifty sputum samples, only eight (16 %) samples were identified as suspected *P. aeruginosa*. These suspected isolates were run in VITEK 2 DensiCheck instrument (bioMe´rieux) the results demonstrated five *P. aeruginosa* (10 %). In the current study, the VITEK 2 was used to identify *P. aeruginosa* as this method is a highly accurate and very fast method as compared with the classical method (Biochemical method). The *P. aeruginosa* that resists the highest number of antibiotics was used for further study.

3.2. Levels of IL-1β in Mice Lung

ELISA was used to measure the levels of IL-1β in lung homogenates of three groups (test and control groups) at different time intervals (1, 2, 4, 24, 48 h) post-instillation intranasally (i.n.) with a standard inoculum of bacterial suspension. The significant elevation of IL-1β was observed as early as 1 h post-instillation in the test group (as compared with control groups A and B) and the levels of IL-1β increased dramatically with time. The maximum levels of IL-1β were observed at 4 h post-instillation. After this time point, the levels of IL-1β decreased dramatically with time and the decrease was slow. At time point 48h, the significant elevation of IL-1β was still observed as compared with control groups (A and B). The current study proved that a clinical isolate of *P. aeruginosa* stimulates the production of IL-1β at a high level (**Fig. 1**).

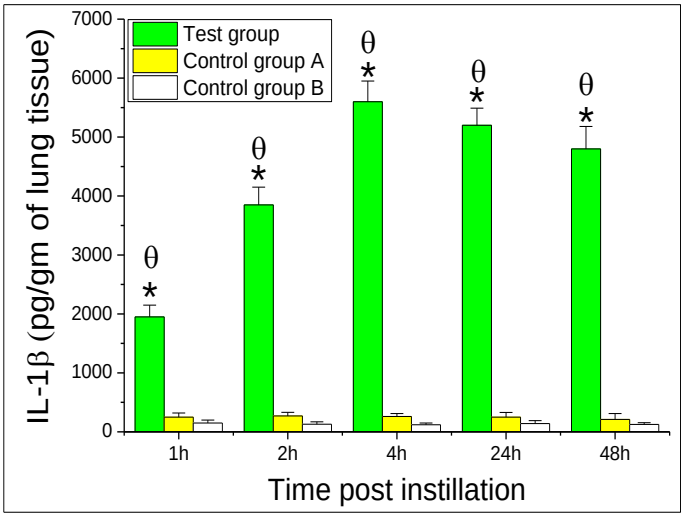

Fig. 1. Time-course of changes in IL-1β levels of homogenized lungs obtained from different groups of mice [test group, mice instilled (i.n.) with clinical isolate *P. aeruginosa* (PAC); control group A, mice instilled (i.n.) with sterile LB broth; control group B, mice instilled (i.n.) with sterile PBS (0.1 M, pH7.2)]. θ, p < 0.05 vs control group A, *, P < 0.05 vs control group B.

3.3. Levels of TNF-α in Mice Lung

The levels of TNF-α were estimated in lung homogenized that collected from test and control groups. The ELISA technique was used to measure the level of TNF-α. The results are shown in **Fig. 2**. This figure revealed that the significant production of TNF-α was started as early as one hour post mice instillation. At this time point the significant (P<0.05) elevation of TNF-α was found in lungs collected from the test group as compared with the level of TNF-α in the mice lungs collected from the control group. The elevation of TNF-α was increased dramatically with the maximum production of TNF-α by 4 h post instillation (i.n.) and after this time a sharp decline in TNF-α levels was observed. At the time point 48 h the significant elevation of TNF-α was also observed in the lung homogenized test group as compared to control groups A and B). From the current study, it can be concluded that clinical isolate of bacterial cells of *P. aeruginosa* could stimulate the lungs to produce TNF-α at a high level.

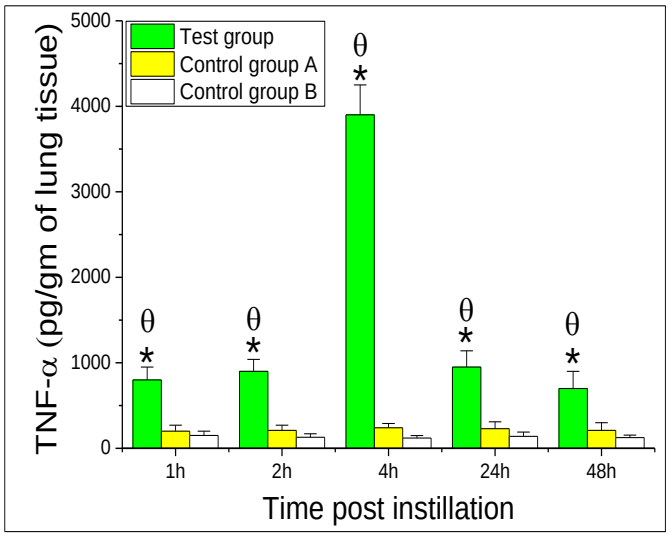

Fig. 2. Time-course of changes in TNF-α levels of homogenized lungs obtained from different groups of mice [test group, mice instilled (i.n.) with clinical isolate *P. aeruginosa* (PAC); control group A, mice instilled (i.n.) with sterile LB broth; control group B, mice instilled (i.n.) with sterile PBS (0.1 M, pH7.2)]. θ, p < 0.05 vs control group A, *, P < 0.05 vs control group B.

3.4. Lung Bacterial Load

In the present study, the standard inoculum of a clinical isolate of *P. aeruginosa* was installed (i.n.) to test groups. The bacterial count was checked at each time post-installation with bacteria. The results in Fig. 3 show the bacterial number (c.f.u./gm tissue lung) of a clinical isolate of *P. aeruginosa* (PAC) in lung homogenized was high at all-time points. The peak of the bacterial count of PAC was found at 48 h and the number of bacteria was decreased after this time point.

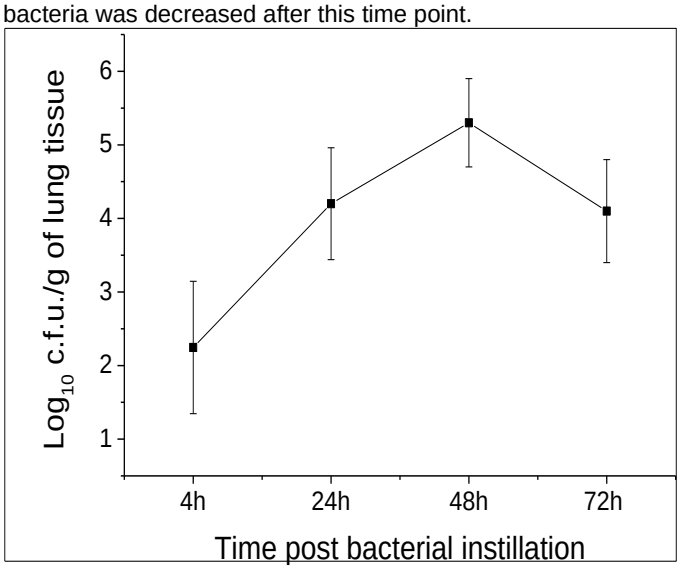

Fig 3. Lung bacterial burden in the test group (mice instilled (i.n.) with 50 µl (5 x 10^8 c.f.u./ml) of a clinical isolate of *P. aeruginosa* (PAC).

4. DISCUSSION

In the respiratory tract of mice, the innate immune response to *P.aeruginosa* is a multifaceted process. It begins with the recognition of *P. aeruginosa* by pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), triggering the recruitment of immune cells [14]. *P. aeruginosa* infection stimulates the production of pro-inflammatory cytokines like IL-1β and TNF-α. These cytokines enhance the immune response by promoting inflammation and the recruitment of additional immune cells. In the present study, the instillation of mice (animal model) with bacterial cell bodies (*P. aeruginosa*) intranasally (i.n.) stimulated the pro-inflammatory immune response, which was observed by evaluation of IL-1β and TNF-α in experimental mice lungs. The present study showed that the elevation of both cytokines was high up to 48 h post-instillation with bacterial cells. That was concomitant with the persistence of viable bacterial cells in the lungs of mice up to 72 h post-installation. That remark the infection of this bacterial (clinical isolates) along with persistence in the pro-inflammatory cytokines and bacterial count may affect negatively the lung tissue. The previous study proved that the persistence of proinflammatory cytokine in the tissue may lead to tissue damage [15]. Moreover, that elevation in both kinds of proinflammatory cytokine did not help the mice's lungs to clear up the bacterial cells from the respiratory tract system and that can be proved by the presence of viable bacteria of *P. aeruginosa* up to 72h. That is why, the instillation with infection dose of *P. aeruginosa* affects negatively on the host by promoting the pathogenicity of bacteria instead of stimulating the innate immune system.

5. CONCLUSION

It can concluded that the instillation of the respiratory tract system with clinical isolates of *P. aeruginosa* induces the pathogenicity effect of this bacteria in terms of increasing the level of pro-inflammatory cytokines for a long time and that may damage the lung tissue. Moreover, the instillation with clinical isolates of *P. aeruginosa* that are isolated from patients' sputum induces the infection status instead of an innate immune response.

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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 Ethical Committee of the University of Baghdad, Baghdad, Iraq (No 149, 2020).

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Author affiliation

1. *Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.*
2. *Ministry of science and technology, Baghdad, Iraq.*