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Research article

Role of Lipopolysaccharide in Stimulating Rheumatoid Arthritis Leukocytes to Produce IL-1β *in vitro*

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ABSTRACT

The pro-inflammatory response plays an important role in the pathogenesis of rheumatoid arthritis (RA), which is considered one of the most common autoimmune diseases around the world. The role of lipopolysaccharide (LPS) in stimulating the pro-inflammatory response in patients with RA requires further clarification. The current study aims to determine the role of LPS in stimulating leukocytes in patients with RA to produce interleukin (IL-) 1 beta (β) in vitro. Here, LPS was extracted from *Pseudomonas aeruginosa*, and used in stimulating the cultures of leukocytes isolated from patients with RA and cultures of leukocytes isolated from healthy control (HC) people with LPS. Two control groups were employed, first, cultures of leukocytes isolated from patients with RA from HC people, and stimulated with phosphate buffer saline (PBS). The level of IL-1β was measured at the protein in supernatants of cell cultures and at the gene expression in leukocytes. The results showed the effect of LPS in stimulating leukocytes of patients with RA and HC people to produce IL-1β higher than the production of IL-1β in cultures of control groups (leukocytes of RA and HC exposed to PBS). The results also showed that leukocytes of patients with RA stimulated with LPS produced IL-1β (in terms of protein production and gene expression) higher than the production of IL-1β by leukocytes isolated from HC people and stimulated with LPS ($P<0.05$). It can be concluded that LPS stimulates leukocytes in RA patients to produce a high level of IL-1β and that plays a role in pro-inflammatory response in RA.

Keywords: Interleukin 1 beta, Leukocytes, Lipopolysaccharide, Rheumatoid arthritis.

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1. INTRODUCTION

Lipopolysaccharide (LPS) is a constituent of the Gram-negative bacterial cell wall which activates B cells, leading to the production of polyclonal antibodies. Different previous studies extracted this molecule from *Pseudomonas aeruginosa* which was isolated from clinical environments [1]. It is also a powerful substance that stimulates different somatic and immunological cells to secret different kinds of immune mediators, including pro-inflammatory cytokines such as Interleukin (IL)-1 beta (β) which is involved in pro-inflammatory immune response [2]. Previous studies reported that LPS plays a role in many diseases in which autoantibodies or self-antigen-specific T cells are involved [3]. Previous studies have shown that stimulating the inflammatory immune response for a long period will lead to damage to the tissues that are exposed to the mediators of this response [4].

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Rheumatoid arthritis (RA) is one of these autoimmune diseases, a chronic inflammatory disease that may lead to serious disability, especially in elder humans. It is systemic and often affects joints. The main symptoms of RA include joint pain or stiffness, weakness, and muscle aches. Joint deformity takes place in the final stage of disease progression. Extra-articular manifestations can also be present [5]. The pathogenesis of RA is complex and involves immune dysfunction, synovial inflammation and hyperplasia, cartilage degradation, and bone erosion [6]. Multiple studies have shown that the synovium in RA patients exhibits dysregulated cellular and molecular pathways, leading to the destruction of bone and cartilage. The effects of RA on the synovium and cartilage contribute to the clinical manifestations and progression of the disease, highlighting the need for targeted therapies for specific patient subsets [7].

The cause of RA is still a matter of speculation but may involve both genetic and environmental factors [8]. In a joint affected with RA, there is chronic pain and inflammation of the synovial tissue filling the joint capsule. Nevertheless, few studies have shown a role for LPS in the induction of autoimmune arthritis. The link between LPS and rheumatoid arthritis is so strong that they test medications for RA by testing their effect on LPS receptors. Therefore, this study aimed to observe the immunological aspects of patients with RA and the role of LPS isolated from *P. aeruginosa* in disease severity in terms of stimulating the proinflammatory cytokines (IL-1β) production for a bit a long time.

2. MATERIALS and METHODS

2.1. Isolation and identification of *P. aeruginosa*

The standard method of Subhi et al. (2016) was followed to isolate the bacterial strain. Briefly, the wound swabs were collected from indoor patients (Baghdad Teaching Hospital, Baghdad, Iraq). The swabs were transported directly to the laboratory and inoculated into MacConkey, and the lactose non-fermenting colonies were re-cultured onto Cetramide agar. Several biochemical tests were done to identify the bacterial isolates [1]. The VITEK 2 DensiCheck instrument, fluorescence system (bioMe´rieux) (ID-GNB card) includes 43 non-enterobacterial Gram-negative taxa. The instructions of the manufacturing company were followed. Data were analyzed using the VITEK 2 software version VT2- R03.1 [1, 9].

2.2. Partial purification of LPS

The hot EDTA method was followed to extract the LPS of *P. aeruginosa*. Briefly, 250 ml Brain Heart Infusion (BHI) broth (Himedia, India) was inoculated with 1 ml overnight bacterial growth of BHI broth and incubated for 18 h at 37ºC. The bacterial cells were harvested by washing three with phosphate buffer saline (PBS, pH, 7.2, 0.1 M) using a cooling centrifuge (10000 g for 15 min). The pellets were re-suspended by PBS (pH, 7.2, 0.1 M) containing 0.5% formalin and centrifuged (10000 g for 15 min). Bacterial cells were dried with acetone. Cell disruption using the enzymes Proteinase K, DNase, and RNase [10]. The extraction was done by adding EDTA-PBS solution, autoclaved (121 °C for 15 min at a pressure of 15 psi), and left to cool at room temperature. The mixture was centrifuged, and the supernatant was collected and placed in dialysis tubes. The supernatant was passed through a gel filtration column (Sephadex G-200, Sigma-Aldrich) for partial purification of LPS [10]. The sample was eluted and the absorbency was measured at 280 nm (protein), 490 nm (carbohydrates), 260 nm (nucleic acids), and 525 nm (lipids) [1]

2.3. Patients' samples

Peripheral blood was obtained from 20 RA patients and 18 age and sex-matched healthy controls. 2010 Rheumatoid arthritis classification criteria were followed by rheumatologists in Baghdad Teaching Hospital, Baghdad, Iraq [11]. All required laboratory tests were done and according to that the rheumatologist identified the patients with RA. The disease activity score 28 (DAS28) was 3.2 ± 1.2 (mean ± SD). All patients were under treatment with biological and chemical medicines. Erythrocyte sedimentation rate (ESR) was measured according to standard Westergren techniques (38.1 ± 21). The blood samples were collected in anticoagulant tubes (heparinized tubes).

2.4. Peripheral blood leukocyte isolation

Human peripheral blood leukocytes (PBLs) were isolated from buffy coats of patients suffering from RA (Central Public Health Laboratories, Baghdad, Iraq) by centrifugation (800g for 15 min). The blood samples collected in heparinized tubes were centrifuged at 2000 g for 10 min, and the white layer between the plasma layer and blood layer was collected by sterile pasture pipette. The collected PBLs were re-suspended in RPMI 1640 (Sigma) medium. The collected cells were washed extensively with RPMI 1640 medium (Sigma-Aldrich). The isolated PBLs were adjusted to 10^6 viable cells/ml. The number of viable cells was counted by Trypan blue method [12] and cultured under standard cell culture conditions (37 ºC, 5 % CO2) in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate (pH, 7.2), 4.5 g/l glucose and 10 mM HEPES (complete media), and supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Sigma).

2.5. Experiment

In this study, four groups of tissue cultures were prepared. 1st group (test group), tissue culture tubes containing 1 ml of complete tissue culture media [RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate (pH, 7.2), 4.5 g/l glucose and 10 mM HEPES (complete media), and supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Sigma)] containing 10^6 PBLs (95 % viable cells) collected from patients with RA and stimulated with 1 µg of purified LPS. 2nd group (test group), tissue culture tubes containing 1 ml of complete tissue culture media [RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate (pH, 7.2), 4.5 g/l glucose and 10 mM HEPES (complete media), and supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Sigma)] containing 10^6 PBLs (95 % viable cells) collected from healthy volunteers cohorts and stimulated with 1 µg of purified LPS. 3rd group (control group), tissue culture tubes containing 1 ml of complete tissue culture media [RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate (pH, 7.2), 4.5 g/l glucose and 10 mM HEPES (complete media) and supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Sigma)] containing 10^6 PBLs (95 % viable cells) collected from patients with RA and stimulated with 10 µl of sterile PBS (pH, 7.2, 0.1 M).4th group (control group), tissue culture tubes containing 1 ml of complete tissue culture media [RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate (pH, 7.2), 4.5 g/l glucose and 10 mM HEPES (complete media), and

supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Sigma)] containing 10^6 PBLs (95 % viable cells) collected from healthy volunteers cohorts and stimulated with 10 µl of PBS (pH, 7.2, 0.1 M). Five patients with RA and 5 healthy volunteers participated in the experiment. All tissue culture tubes were incubated at 37 ºC and 5 % CO_2.

2.6. IL-1β measurement

Two hundred fifty microliters were collected in microfuge tubes from each tissue culture at different time intervals (1 h, 24h, 48 h, and 72 h). The samples were centrifuged at 1000 g for 5 min to pellet cells. Supernatants were then collected and stored at -20 ºC until used for measuring. The IL-1β levels were measured using enzyme linkage immune sorbent assay (ELISA) [human IL-1β ELISA kit (BD Biosciences, USA)].

2.7. IL-1β mRNA expression in cultured PBLs

The pellets that remained in the microfuge tube after collecting the supernatants (in the step of IL-1β measurement**)** were mixed with 400 µl of Trizol reagent (Invitrogen, CA) using an Ultra Turrax homogenizer and stored at -80 ºC. The total RNA was isolated using an RNA isolation kit (Dongsheng Biotech, China). The IL-1β mRNA levels were measured using a real-time-polymerase chain reaction (RT-PCR). The purified total RNA was used as the template in the RT-PCR. Total RNA was reversely transcribed to complementary DNA (cDNA) using WizScript™ RT FDmix Kit. The PCR primers used were as described previously for IL-1β and G3PDH [13, 14] and the reactions were performed as described previously [14]. The relative expression of IL-10 with G3PDH as the reference gene was determined using the 2-T (Livak) method.

2.8. Statistical analysis

All values have been used to give a mean value and the standard deviation calculated. The differences were analyzed by using the Student's t-test and Chi-square test employing Origin version 8.0 software. A value of $P < 0.05$ was considered to be statistically significant.

3. RESULTS

3.1. IL-1β levels after stimulation with LPS

In the present study, the level of IL-1β was measured in LPB tissue culture post-adding (stimulating) with 1µ of LPS extracted from *P. aeruginosa* isolated from infected wounds at different time intervals (24, 48, 72h). Fig 1 showed the significant elevation of IL-1β in tissue culture of PBLs collected from patients with RA (146 pg/ ml, P<0.05) started as early as 24 h post-stimulation with 1µ of LPS, as compared with control groups (PBLs tissue culture that obtained from patients with RA and healthy control and stimulating with PBS). The level of IL-1β in the suspension of PBLs (collected from peripheral blood of RA) tissue culture that was stimulated with LPS increased with time, the highest level was seen by the hour 72 post-stimulation with LPS (160 pg/ ml, P<0.05). The significant level of IL-1β in the supernatants of PBLs (collected from healthy cohort) tissue culture started as early as 24 h post stimulating with 1µ of LPS extracted from *P. aeruginosa* isolated from infected wound (106 pg/ml, P<0.05). The levels of IL-1β in the last group of tissue culture decreased slightly with time but the levels of IL-1β still significantly higher than the levels of IL-1β in the supernatants of tissue cultures of control groups (PBLs tissue culture obtained from patients with RA and healthy control and stimulating with PBS). In the present study, it was seen significant increases (P<0.05) in the levels of IL-1β in the supernatants of tissue culture of PBLs collected from patients with RA and stimulated with LPS as compared with the levels of IL-1β in the supernatants of PBLs tissue culture that collected from healthy volunteers and stimulated with 1µ of LPS extracted from *P. aeruginosa* at all-time points (P<0.05).

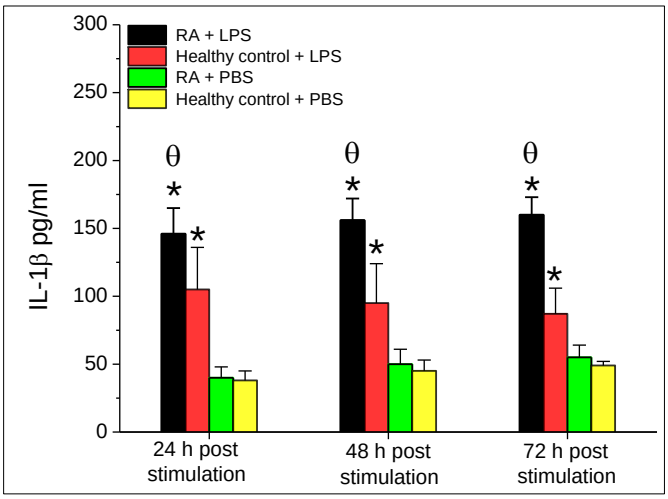

Fig 1. IL-1β levels in pg/ml in the suspension of peripheral blood leukocytes (PBL) tissue cultures stimulated with either LPS (1µg) or PBS (pH,7.2, 0.1 M) at different time intervals (24, 48, and 72h). Indicates of significant difference from control groups [PBL collected from RA patients and healthy control cohorts and exposed to PBS (pH 7.2, 0.1 M)]. Θ, indicates of significant difference from the healthy test group (PBL collected from healthy cohorts and stimulated with 1 µg of LPS). A P value of 0.05 is considered to be a significant difference from the control groups.

3.2. Effect of LPS on IL-1β mRNA expression

Fig. 2 shows the folds of IL-1β mRNA expression in PBLs that were cultured in vitro and exposed to 1µ of LPS extracted from *P. aeruginosa* isolated from infected wounds at different time intervals (1h and 24 h). The significant elevation (P<0.05) of IL-1β mRNA expression started as early as 1 h exposure to 1 µg/ml of bacterial LPS as compared to IL-1β mRNA expression of control groups [PBL collected from RA patients and healthy control cohorts and exposed to PBS (pH 7.2, 0.1 M)]. The IL-1β mRNA expression of this group increased at 24 h post exposing to LPS (fold of gene expression was 3.8). A similar finding was observed in the case of tissue cultures of PBLs that were collected from healthy control and exposed to 1µ of LPS extracted from *P. aeruginosa* but the elevation (P<0.05) in the last group was moderate as compared to the folds of IL-1β mRNA expression of control groups [PBL collected from RA patients and healthy control cohorts and exposed to PBS (pH 7.2, 0.1 M)]. The IL-1β mRNA expression in the PBLs collected from RA patients and stimulated with 1µ of LPS was higher significantly (P<0.05) than the IL-1β mRNA expression of PBLs collected from the healthy control group and stimulated with 1µ of LPS (at both time points).

4. DISCUSSION

The LPS generates a pro-inflammatory response in patients with RA through various mechanisms. One mechanism involves the activation of macrophages, which are key players in the inflammatory response. In RA patients, LPS stimulation leads to the production of pro-inflammatory cytokines, such as tumor

necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6), by mononuclear cells [15]. Another mechanism involves the dysbiosis of the gut microbiota, which can contribute to the pathogenesis of RA. Increased systemic bacterial exposure in RA patients leads to elevated levels of antimicrobial response factors (ARFs), including soluble CD14 (sCD14), lysozyme, and CXCL16. These ARFs are associated with disease severity and may contribute to the pro-inflammatory response [16].

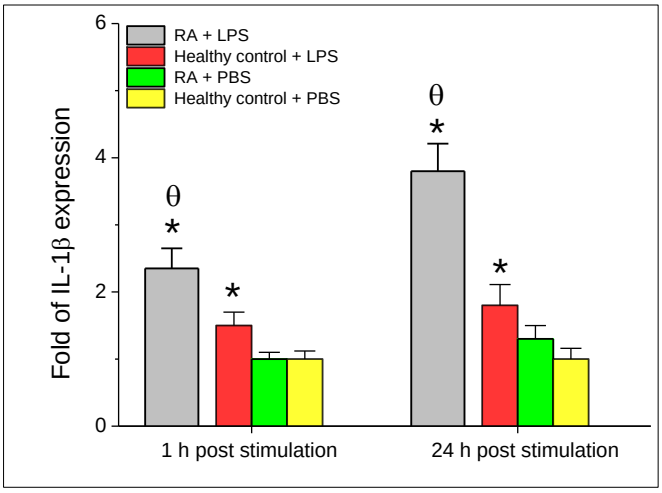

Fig 2. Gene expression of IL-1β post into PBLs that collected from patients with RA and healthy control and exposed to either 1 µg of LPS or PBS at two time points (1 h and 24 h) post-stimulation with LPS or PBS. *, indicates of significant difference from control groups [PBL collected from RA patients and healthy control cohorts and exposed to PBS (pH 7.2, 0.1 M)]. Θ, indicates of significant difference from the healthy test group (PBL collected from healthy cohorts and stimulated with 1 µg of LPS). A p-value less than 0.05 is considered a significant difference.

In the current study, LPS was extracted from *P. aeruginosa* isolated from infected wounds. Leukocytes were isolated from patients with RA and then stimulated with lipopolysaccharide *in vitro*. This stimulation was responsible for producing pro-inflammatory cytokines such as IL-1β. It was found that leukocytes of patients with RA have the ability, after stimulation, to produce IL-1β in an amount greater than leukocytes isolated from healthy people and stimulated with LPS, leukocytes isolated from RA patients, and healthy people not stimulated with LPS. That confirms the role of bacterial antigens, especially LPS, in stimulating the pro-inflammatory response in these patients, which plays an important role in the severity of RA. This confirms the role of bacterial infections in the possibility of increasing the pathogenicity of RA. These studies provide a mechanism for the role of LPS in increasing the pro-inflammatory response via IL-1β, which in turn plays an important role in the pathogenesis of RA.

Similar results were found by Wang et al. (2015), it was found that LPS can stimulate the cells to produce IL-1β higher than other stimulators at different time points. It is well known that LPS has specific receptors on leukocytes, toll-like receptor 4, and when the LPS binds the receptor that will stimulate the long cascades of intracellular cell signals resulting in to production of different pro-inflammatory mediators such as IL-1β, That is why we found a high level of IL-1β in the suspension of leukocyte cells that stimulated with LPS [17].

The persistence of pro-inflammatory immune response in the tissue area will produce tissue damage. LPS-induced tissue damage is mediated by several mechanisms. LPS promotes inflammation-induced organ dysfunction by activating the TLR4-MD2 receptor complex, leading to the production of pro-inflammatory mediators [18]. In the bovine endometrium, LPS induces the secretion of prostaglandin E2 (PGE2) and upregulates pro-inflammatory factors and damage-associated molecular patterns (DAMPs), resulting in tissue damage [19]. LPS also promotes macrophage necroptosis, but this is ameliorated by the release of high-mobility group box 1 (HMGB1) from damaged tissue, which decreases macrophage necroptosis [20]. Additionally, LPS can activate host degradative pathways, such as the matrix metalloproteinase pathway, leading to tissue destruction [21, 22]. These mechanisms collectively contribute to the tissue damage induced by LPS pro-inflammatory mediators.

5. CONCLUSION

The study showed that LPS stimulates the leukocytes of RA patients and the leukocytes of healthy control (HC) people to produce high levels of IL-1β *in vitro*. The study also showed that LPS stimulates the leukocytes of patients with RA to produce IL-1β greater than the leukocytes of HC people. The current study emphasizes the role of LPS in stimulating pro-inflammatory response mediators (IL-1β), which play an important role in the pathogenesis of RA. Therefore, it can be concluded that LPS may play a role in increasing the activity (severity) of RA, and this requires further in vivo studies.

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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 1205, 2022).

Author contributions

Israa M Subhi: Conceptualization; Data curation; Investigation; Methodology; Project administration; Roles/Writing - original draft; Supervision; Validation; Software; Visualization; Writing - review & editing.
Mohammed HM Al-Osami: Formal analysis; Clinical investigation; Methodology; Resources.

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