The influence of whole sonicated *Pseudomonas aeruginosa* antigens on experimental *p. aeruginosa* arthritis in rabbits

Ahmed Q. Al-Awadi[@] and Mohammed J. Alwan
Department of Pathology, College of Veterinary Medicine, University of Baghdad, Iraq
E- mail: aqh_1974@yahoo.com
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Summary

To study the influence of whole sonicated *Pseudomonas aeruginosa* antigens (WSPAgs) on experimental arthritis induced by this bacteria, 15 rabbits were divided into 3 equal groups. The 1st group was inoculated intraarticular with 0.2 ml of *p. aeruginosa* suspension (2×10^8 cfu/ml), the 2nd group was immunized with WSP Ags, and inoculated intraarticular as in the 1st group. The 3rd group was served as negative control group. At 30 day post inoculation the immunized (2nd) group showed increase in the cellular (DTH and IFN-γ) and humeral (IgG) immunity and moderate bacterial isolation from joints, blood and internal organs comparing with other groups. The 1st group showed sever symptoms and inflammatory reaction as well as very obvious gross and microscopical lesions in their joints including supportive reaction, pyogranulomatous lesions, necrosis, pannus reaction and destruction of the articular cartilage and the lesion extended to the subchondral bone leading to osteomyelitis, the 2nd group (immunized group) expressed mild to moderate inflammatory reaction and the microscopic examination indicate that the lesion was confined in the articular capsule. In conclusion the whole *Pseudononas aeruginosa* sonicated Ags (WSPAgs) protect the joint from the experimental infection by *P. aeruginosa* in a rabbit model.

Keywords: *P. aeruginosa*, Arthritis, Rabbits.

Introduction

Bacterial septic arthritis (SA) is a severe infection of a native joint that can cause destruction and loss of function. Bacteria gain access to the joint space by hematogenous spread, trauma, direct inoculation (e.g. intraarticular injection), or contiguous spread from a nearby, localized infection within soft tissue or bone (1).

P. aeruginosa an important pathogen in intravenous drug users and has a particular affinity for fibrocartiloginous articular structures. Most cases of SA caused by *P. aeruginosa* occurred in high-risk patients with underlying medical conditions such as diabetes mellitus, chronic cardiac diseases, blood stream or urinary tract infections and malignancies, but recently SA caused by *P. aeruginosa* reported in a patient after intra-articular ozone injection into the knee joint in an immunocompetent patient with no medical history of intravenous drug abuse, recent hospitalization or chronic diseases like rheumatoid arthritis (2). The host response to acute bacterial SA produces much of the destruction to the joint space. The influx of inflammatory cells causes a purulent effusion, possibly leading to cartilage destruction and increased joint pressure (1).

The development of vaccines for *p. aeruginosa* infections has become hindered due to complexity of the organisms pathogenesis elaboration of wide array of virulence factors with a propensity to infect many different tissues which have made difficulty to determine which host immune effectors and which microbial factors needed to be target for effective immune response (3), however *P. aeruginosa* vaccine candidate includes outer membrane proteins (4 and 5), cytosolic protein (6), extracellular proteins, such as those of flagella (7) and pili (8), and extracellular polysaccharides, such as alginate (9) and LPS (10). For these reasons this study aimed to determine the role of *P. aeruginosa* in inducing SA and to determine the protective effects of immunization with whole sonicated *P. aeruginosa* antigens (WSPAgs).

Material and Methods

P. aeruginosa obtained from the department of Biology/ College of Science/University of Baghdad. This strain was isolated from a patient suffering from burns and the biochemical features of this strain were done

by the Central Public Health Laboratories/Ministry of Health.

Whole sonicated *pseudomonas aeruginosa* antigens (WSPAgs) used in the immunization and soluble sonicated *P. aeruginosa* antigen (SSPAgs) used in the skin test were prepared according to (11), the total protein concentrations of WSPAgs (25 mg/ml) and SSPAgs (0.5 mg/ml) were measured according to (12)

The bacterial suspension was determination of the challenge at dose (2×108 cfu/ml) prepared according to (13).

Immunological examination was determined by delayed type hypersensitivity test (DTH) for cellular immunity was performed at day 28 post immunization (11) using 0.1 ml of SSPAgs and the thickness of skin was measured after 24 hr and 48 hr posttest.

Serum collected at day 30 post immunization for interferon gamma (IFN-γ) assay using IFN-γ ELISA kit, provided by Cusabio Biotech. CO., LTD. China, and immunoglobulin G (IgG) assay using rabbit IgG ELISA kit, (Immunoperoxidase assay for determination of IgG in rabbit sera) provided by Immunology Consultants laboratory, Inc. Portland, USA.

At day 30 post immunization blood samples (4-5 ml) were collected from the hearts of rabbits directly using a 5 ml disposable syringes (23-gage needle), draw in sterile plain tubes and left in a standing position for about 20-30 minutes till clotting, then centrifuged at 3000 rpm for 10 minutes, serum collected immediately, aliquoted using Eppendorf tubes and stored at -20˚C. The sera were used latter to measure the level of both IFN-γ and IgG concentrations.

Fifteen male local breed rabbits, weight range 1.125-1.575 kg were randomly divided into 3 equal groups, The 1st group (n=5) was inoculated with 0.2 ml (2×108 cfu/ml) of *P. aeruginosa* suspension in the right ankle joint.

The 2nd group was (n=5) immunized subcutaneously with WSPAgs, 0.5 ml (protein concentration 25 mg/ml), two doses with 14 day intervals. At day 30 post immunization, the rabbits were infected intraarticularly in the right ankle joint as in the 1st group. The 3rd group (n=5) was served as negative control group.

All animals were sacrificed after 30 day post inoculation, bacterial isolation and histopathology were preformed from the infected joint and internal organs (liver, spleen, lung and kidney). Organs were fixed in 10% buffer formaldehyde solution for 72 hr, then used the routine tissue section preparation, except bone specimens which decalcified using formic acid before routine processing (14).

Data were analyzed by using one way analysis of variance (ANOVA) for more than two groups and differences of means were tested by unpaired t-test, where as paired t-test was used to test the differences between paired groups. The analysis was conducted using SAS program (2000).

Results and Discussion

The skin test showed that the mean values of the skin thickness in the immunized group 24 hr post examination was 2.25±0.25, the value was declined at 48 hr post test 2.14±0.23 ($P<0.001$).

The results showed that the mean values of IFN-γ were high in the rabbits of the 2nd group (456.15±37.61) compared with the 3rd group (70.94±8.63) with LSD=246.93. IgG were high in the rabbits of the 2nd group (23.87±1.41) compared with the 3rd group (15.52 ± 1.94) with LSD (2.75).

The results of DTH in the present study may indicate that the WSPAgs elicited a cell mediated immune (CMI) response in the immunized rabbits, since DTH is the principle pattern of CMI, and it is mediated by CD4+ T cell and CD8+ T cell cytokines production (15), also Th1 cells is the inducer of a DTH response since they secretes IFN-γ which is a potent stimulator and recruitment of macrophages chemotaxis to local site (16), and the activated macrophages produced high level of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-8, these cytokines stimulated the degranulaiton of mast cells and produced chemical mediators such as histamine that caused increased vascular permeability which facilitates the extravasations of fluids, inflammatory cells, edema and congestion of

blood vessels in the dermis and subcutaneous tissue and induced the indurations and swelling of the inoculated site during 24-48 hrs post antigen exposure, these evidences were supported by (17) who explained that the development of highly activated macrophages, depended on lymphokines secreted by Th1 subset, were correlated with the onset of DTH. The induction of DTH reaction in rabbits immunized with WSPAgs in the present study may be due to that the protein nature of extracellular secretion of *P. aeruginosa* considered a good stimulator of CMI responses (18), this supported the current results in which there is significant increase in IFN- γ production in immunized group.

In addition, the results of this study also showed that the vaccine enhanced the humoral immunity in the immunized rabbits. The WSPAgs can stimulate humoral immunity because it contains large amounts of proteins and bacterial O-polysaccharide taken in consideration that carbohydrates are a good stimulator for the humoral immunity (19). Proteins stimulate the B cell through T-dependent antibody response (CD4) while, polysaccharides and lipids do not need participation of antigen-specific helper T cell, thus these antigens are said to be T-independent (20).

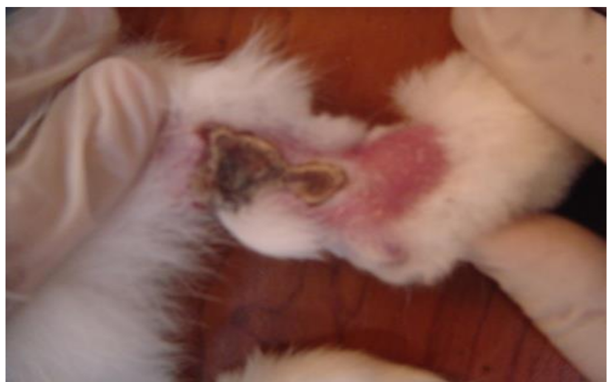
B cells that are in direct contact with activated T cells are exposed to high concentration of cytokines secreted by the activated helper T cell (IL-2, IL4 and IL-5) and acts on B cell to induce proliferation and activate transcription of immunoglobulin genes. Some of the B cells that have proliferated differentiate into effector cells that actively secrete antibodies that have same specificity to the surface Ig receptor that captured the antigen. Many of the antibody secreting B cells change into plasma cells which are characterized by abundant antibody production (20).

Also Th17 cells not only trigger B-cell proliferation but also promote the formation of germinal centers (GCs) together with isotype switching to IgG1, IgG2a, IgG2b, and IgG3. Interestingly, IL-17 on its own drove class switch recombination to IgG2a and IgG3, whereas IL-21 in addition promoted the switch to IgG2b and IgG1(21).

These observations are in consistance with (22) who showed enhancement of DTH and Abs titer in mice immunized with WSPAgs and infected intraperitonealy with *P. aeruginosa*.

All rabbits (n=5) in the 1st group (non immunized) showed lameness, swelling and stiffness of ankle joint due to inflammatory reaction, inflammation of the soft tissue surrounded the joint and 4 of 5 rabbits showed fistula formation which drain white or green pus and blood (Fig. 1). The rabbits lose function in their ankle joints, and suffer from obvious pain, the rabbits have difficulty in standing from laying position and move more slowly than normal (appear stiff when moving), and inability to use the hind limb and severe pain, also this group showed bacterial isolation from the joint (5:5), blood (3:5), liver (4:5), spleen (3:5), lung (1:5) and kindey (0:5) (Table-1).

The immunized group showed mild inflammation and swelling in the joints (2 of 5 rabbits). No fistula formation, and no lesions or clear signs had been seen. The bacteria was isolated from the joint (3:5) while there is no bacterial growth from examined internal organs except one rabbit which has shown bacterial growth from blood, liver and spleen(Table-2).



Fig, 1: The ankle join in the non-immunized rabbit, 30 day post inoculation showed: swelling, redness and a sinus which drained pus.

The heavy isolation of bacteria from the joint and internal organs in the non immunized rabbits indicated that the bacteria overcome

the natural defense mechanisms and proliferate in the joint tissues causing severe damage and disseminated to internal organs. The ability of *p. aeruginosa* to produce pyoverdin was probably more important for growth and dissemination *in vivo* (23) also after colonization, *P. aeruginosa* produced several extracellular virulence factors (alkaline protease and staphylolytic protease, elastase, protease IV, heat-labile and heat-stable hemolysins, phospholipases C and exotoxins A, S, T, U, Y), responsible for extensive tissue damage, bloodstream invasion, and dissemination (24).

Table,1: Bacteria:l isolation from the blood, joint and internal organs of the 1st (non immunized) group.

NO.	Bacterial isolation					
	Joint	Blood	Liver	Spleen	Lung	Kidney
1	+	+	+	+	-	-
2	+	+	+	+	-	-
3	+	-	-	-	-	-
4	+	-	+	-	+	-
5	+	+	+	+	-	-

(–) no growth ; bacteria growth.

Table,2: Bacterial isolation from the blood, joint and internal organs of the 2st (immunized) group.

NO.	Bacterial isolation					
	Joint	Blood	Liver	Spleen	Lung	Kidney
1	+	-	-	-	-	-
2	-	-	-	-	-	-
3	+	-	-	-	-	-
4	-	-	-	-	-	-
5	+	+	+	+	-	-

The immunized rabbits showed the ability of the vaccine to stimulate the immune system, but the isolation of bacteria from the joint of some immunized rabbit indicated that activation of quorum sensing signaling cascade promotes biofilm formation and persistence in the joint (25). Biofilm allows *P. aeruginosa* to evade the immune system and increases antibiotic resistance, making it extremely difficult to eradicate the infection (26).

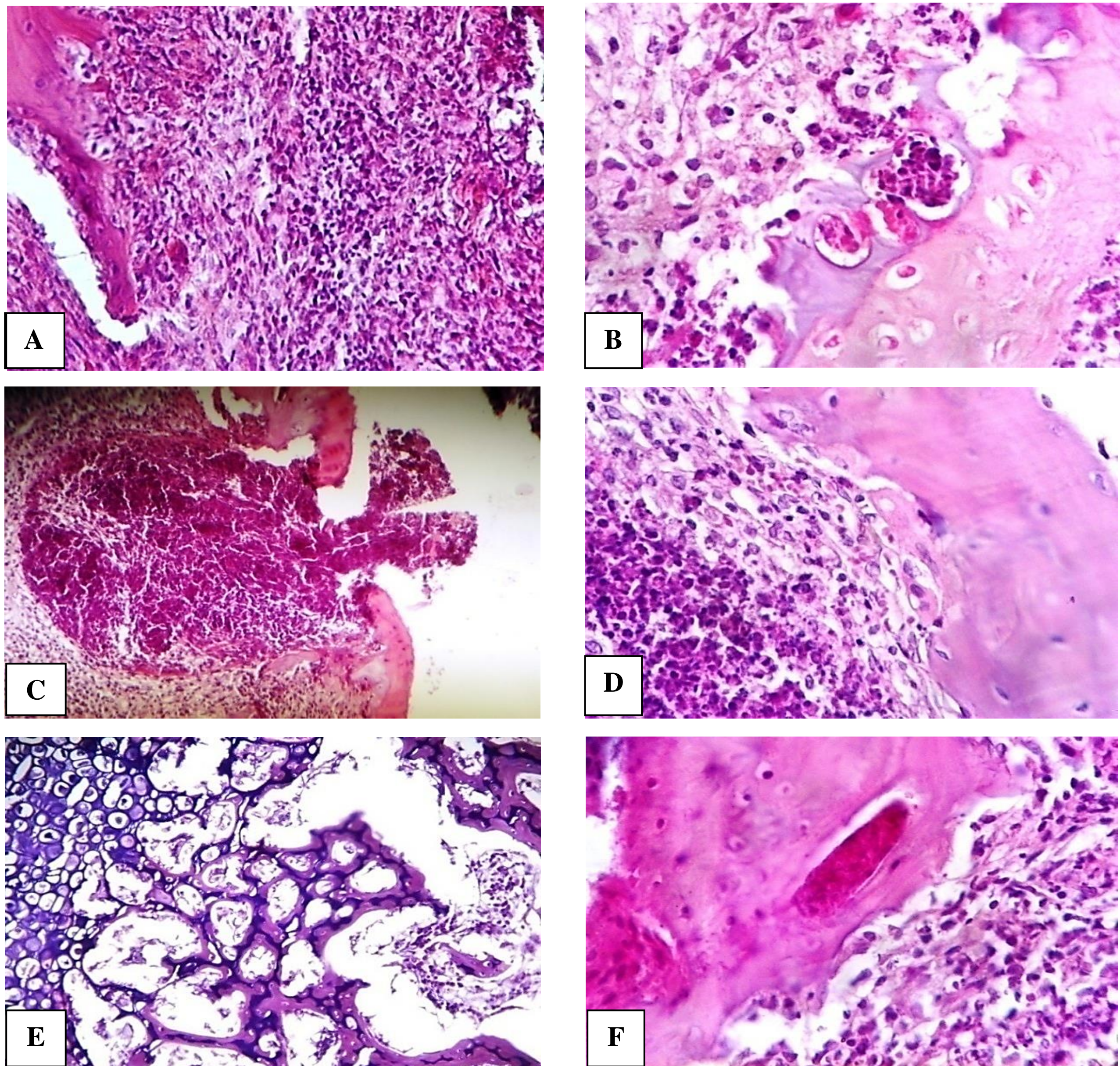
All sacrificed rabbits in the 1st group showed severe arthritis characterized by swelling, edema, redness and heat, also abscess formation which drained through a sinus tract and there is sever destruction to the joint. The articular cartilage which covered the tibia head loss it smoothness and shiny color, also one of the rabbits joint showed sever consolidation.

The histopathological examination of ankle joint in the 1st group expressed severe inflammatory cells infiltration mainly neutrophils in the synovial membrane and articular capsule (Fig.2A), also in other section large abscess consisting from aggregation of neutrophils and surrounded by pyogenic F.C.T. was reported in the synovial membrane. Necrosis, erosion and fragmentation of articular cartilage with sever neutrophils invaded the necrotic articular cartilage (Fig.2B). pannus reaction consisting from fibrovascular tissue, neutrophils, lymphocytes, and macrophages extended from the synovial membrane through necrotic articular cartilage to subnchondral bone (Fig.2C).

Microscopic section also revealed bone marrow sclerosis with inflammatory cells infiltration particularly neutrophils and macrophages in the medullary cavity of subchondral bones (Fig.2D). Pyogranulomatous lesion consisting from aggregation of neutrophils in the center surrounded by macrophages was seen in medullary cavity of subchondral bone. Fragment bone sequestration lining with active osteoblasts and surrounded with fibrous connective tissues, inflammatory cells. Other section expressed necrotic fragment pieces of subchondral cartilages were surrounded with severe inflammatory reaction, also incomplete endochondral ossification, primary spongeosa and unopened hypertrophic chondrocytes, in addition fragment dead bone surrounded by vascular fibrous connective tissue and inflammatory cells (Fig.2E).

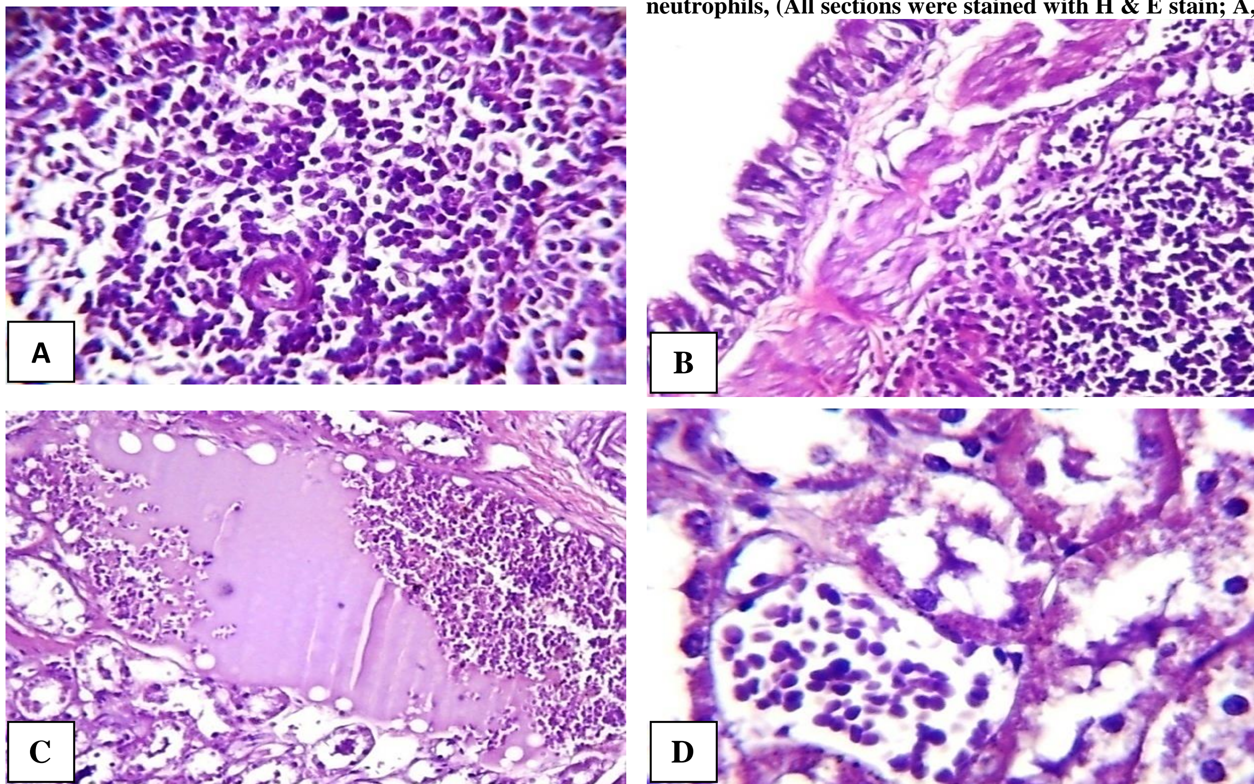
There is necrosis in the cortical part of subchondral bone characterized by pyknotic or disappear of osteocytes and dilated lacuna space and the Haversian canals were compacted with neutrophils (Fig.2F). Also the inflammatory reaction mainly neutrophils and MNCs extended to the medullary cavity with proliferation of fibrous connective tissue leading to erosion of endosteum and

destruction of Haversian system. The microscopic examination of the spleen showed depletion of white pulp of the spleen (Fig.3A), while the liver revealed coagulative necrosis of the hepatocytes with inflammatory cells mainly neutrophils and macrophages in the necrotic area. The lung showed inflammatory cells particularly mononuclear cells and neutrophils in the alveolar space and in the interalveolar septa, in another section, vacuolation of the epithelial layer of the bronchi, and inflammatory cells invasion of these layer, in addition to hyperplasia of lymphoid tissue in the wall of bronchi (Fig.3B). The main lesion in the kidney consisted of thrombus in the blood vessels (Fig.3C) with inflammatory cells mainly neutrophils appeared in the lumen of the blood vessels with severe cellular degeneration characterized by vacuolation, necrosis and sloughing of the epithelial lining cells of the renal tubules with cellular debris in their lumen (Fig.3D).



Fig, 2: Histological sections in the ankle joint of the non-immunized animals (30 day post inoculaiton) showed, A: neutrophils infiltration in the synovial membrane and articular cartilage. B: necrosis and destruction of the articular cartilage which invaded by neutrophils. C: pannus reaction. D: inflammatory cells neutrophils and macrophages in the medullary cavity of subchondral bone. E: incomplete endochondral ossification and and fragment of dead bone surrounded by surrounded by

vascular fibrous connective tissue. F: necrosis in the cortical part of subchondral bone and Haversian canals compacted with neutrophils, (All sections were stained with H & E stain; A, C and E 200X; B, D and F 400X).



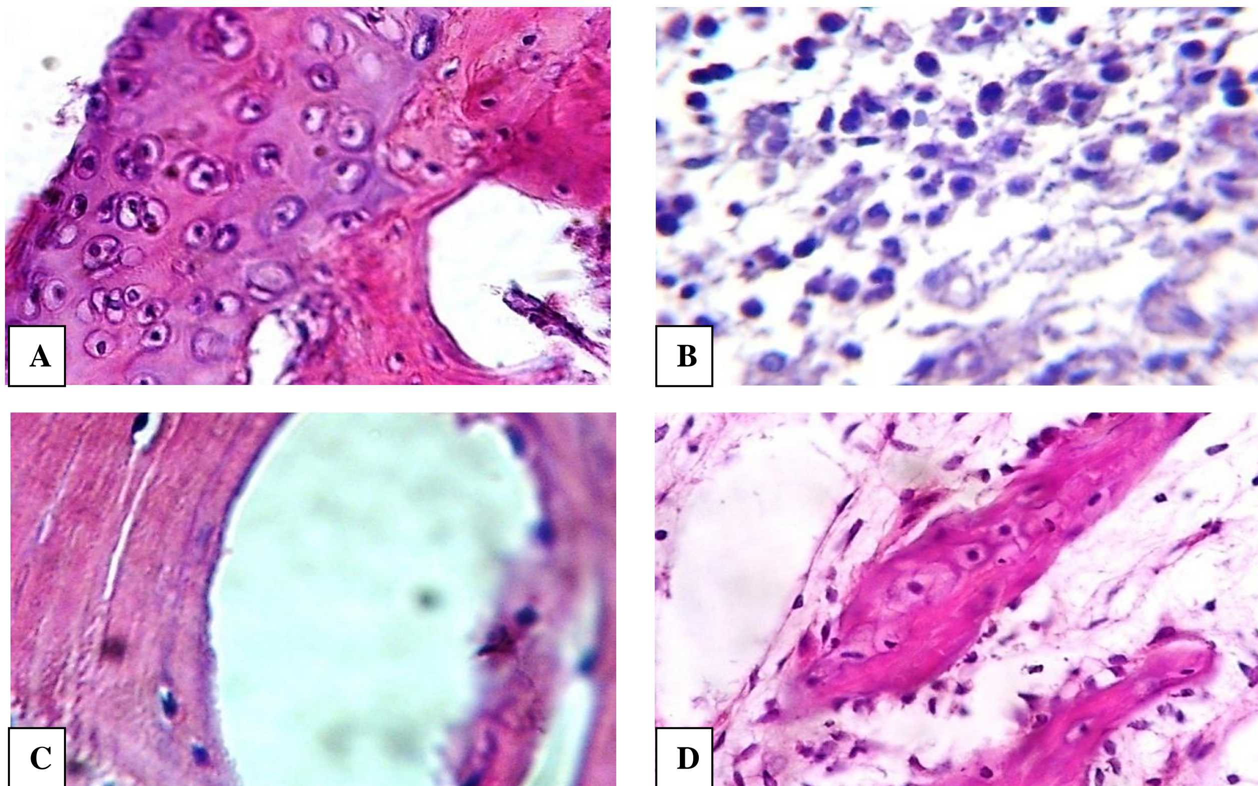
Fig, 3: Histological sections in the internal organs of the non-immunized group at 30 day post inoculation showed, A: Spleen: depletion of white pulp. B: Lung: hyperplasia of lymphoid tissue in the wall of bronchi. C: Kidney: thrombus in the blood vessels with acute cellular degeneration of epithelial lining cells of renal tubules. D: kidney: congestion of the blood vessels with inflammatory cells in their lumen, vacuolation and sloughing of epithelial lining cells of renel tubules, (All sections were stained with H & E stain; A and B 400X; C 200X; D 600X).

The histopathological examination of the ankle joint of the immunized infected animals (group 2) showed inflammation in the synovial membrane and the articular capsule characterized by infiltration of neutrophils and MNCs (Fig. 4A), also there is no clear lesions in the articular cartilage (Fig. 4B), cortical part of the subchondral bone (Fig. 4C), bone trabeculae and the bone marrow (Fig. 4D).

The histopathological section in the spleen showed hyperplasia of the white pulp (Fig. 5A), while the lesion in the liver characterize by proliferation of kupffer cells and dilation of sinusoids. In the lung there was congestion of blood vessels and infiltration of inflammatory cells in the wall and lumen of the alveoli (Fig. 5B). The kidney showed aggregation of MNCs around the blood vessels (Fig. 5C) and acute cellular degeneration in the renal tubules.

The heavy infiltration of neutrophils in the synovial membrane and articular capsule seen in the current experiment may be due the fact that the neutrophils that are considered the major cells type responsible for the clearance of *p. aeruginosa* (27). This is demonstrated by the high susceptibility of neutropenic patients to *p. aeruginosa* infections and the use of neutropenic mouse models to study *p. aeruginosa* infections (28). The extensive accumulation of neutrophils lead to abscess formation which may indicat that *p. aeruginosa* can resist the neutrophils even when it phagocytosed it by their protective LPS and this may protect and hide the bacteria from the immune system and may aid in the dissemination of the organism, also *p. aeruginosa* synthesizes Fe- or Mn- containing superoxide dismutase (SOD) enzymes, which catalyze the very reactive O- to H2O2 and O2.

It also detoxifies H_2O_2 to O_2 and H_2O by using catalase (29).



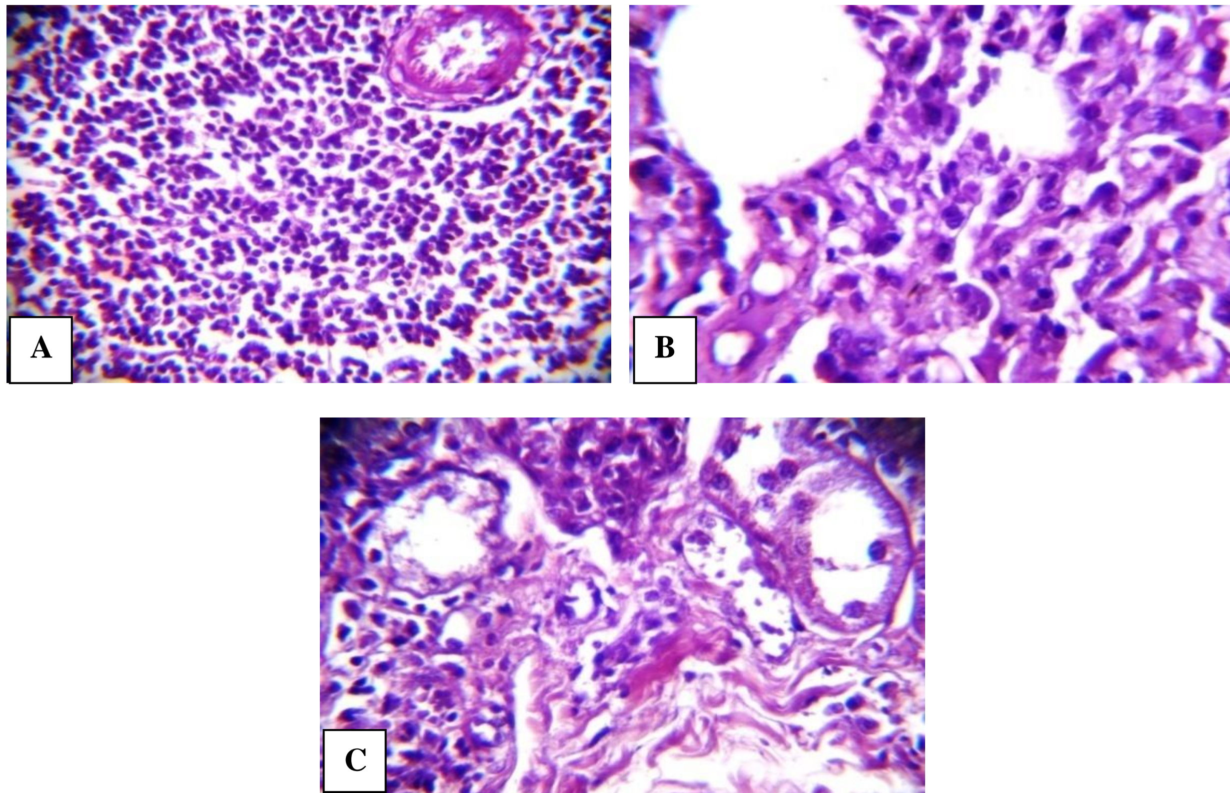
Fig, 4: Histological sections in the ankle joint of the immunized animals (30 day post inoculaiton) showed, A: few neutrophils and MNCs in the articular capsule. B: no clear lesion in the articular cartilage. C: no clear lesion in the cortical part of the subchondral bone. D: no clear inflammatory reaction in the bone trabeculae and the bone marrow with active osteoblasts lining the trabecular bone, (All sections were stained with H & E stain; A, B, C and D 400X).

The progression of inflammatory reaction may be due to the interaction of bacterial flagellins (flagellin A and B) with TLR5 which induced the secretion of proinflammatory cytokines TNF-α and IL-6. Also large amount of TNF- α were released in response to LPS and other bacterial product, TNF-α with IL-6 and IL-1 stimulating the acute phase response, leading to increase in C-reactive protein and a number of other mediators, also TNF-α is a potent chemoattractant for neutrophils, and promotes the expression of adhesion molecules on endothelial cells, helping neutrophils migrate, which explain the large number of neutrophils seen in the 1st group, also TNF-α stimulates macrophages to induce phagocytosis, and production of the inflammatory lipid prostaglandin E2 (PGE2) (30).

The chronisity, the massive destruction of the joint and the extension of the lesion to the subchondral bone may be due to the particular tropism of *P. aeruginosa* for fibrocartilagenous tissue (31). In addition to this fact, the current results suggested that the host's immune response to infection can damage the joint through several common cytokines which have osteolytic properties, and phagocytes produce toxic oxygen radicals and proteolytic enzymes that can harm host cells and increase the damage. For these reasons the increase of pressure caused by the abscesses in the joint lead to extension of infection from the joint to the articular cartilage and the subchondral bone.

Also the results suggested that the severe damage and necrosis in the articular cartilage is due to the ability of the *P. aeruginosa* to evade the immune system using their

virulence factor such as alkaline protease and LasB which reduce the phagocytic activity against *P. aeruginosa* and the ability of these virulence factors (elastase and alkaline protease) to degraded IL-2 which impair lymphocyte function (32), and the ability of rhamnolipid which produced cellular distortion of macrophages and inhibited their ability to bind and /or ingest preopsonized *p. aeruginosa*. Also rhamnolipids play a role in the degeneration of lipids and lecithin, which may contribute to tissue invasion and necrosis (33). Also Phospholipase C hydr-lyzes phosphatidylcholine and sphingomyclin, and cause tissue necrosis and cell death *in vivo* (34). ExoS and ExoT can cause rearrangement of the actin cytoskeleton and inhibition of phagocytosis (35).



Fig, 5: Histological sections in the internal organs of the non-immunized group at 30 day post inoculation showed, A: spleen hyperplasia of white pulp. B: Lung inflammatory cells in the wall and lumen of the alveoli. C: Kidney aggregation of inflammatory cell MNCs around the blood vessels, (All sections were stained with H & E stain; A and C 200X; B 400X).

The necrosis seen in the internal organs probably due to the phospolipase C which is toxic to animals at microgram amounts and cause vascular permeability, hepato-necrosis, renal tubular necrosis, organ damage and death at high doses (36). Also exotoxin A has necrotizing activity at the site of bacterial colonization and is thereby thought to contribute to the colonization process (31). The thrombus formation in the blood vessels is due to the action of protease IV which degraded plasminogen (37) since plasminogen deficiency lead to thrombosis and the clots are not degraded adequately.

While in the immunized group the lymphoid hyperplasia of the white pulp in the spleen may be due to the late events in helper T cell dependent antibody response, including affinity maturation and generation of memory B cells occur in the germinal centers of lymphoid follicles and after antigen exposure, some of the activated B cells migrate deep into the follicle and begin to proliferate rapidly, forming the germinal center (38).

In conclusion *Pseudomonas aeruginosa* could cause septic arthritis in rabbit model and the main lesion in the joint is suppurative reaction and the infection may extend to the

bone causing osteomyelitis. The WSPAgs vaccine used against this infection can enhance both cellular (DTH and IFN-γ) and humoral (IgG) immunity.

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دراسة تأثير المستضد الكلي المتكسر لبكتيريا *Pseudomonas aeruginosa* على اصابات المفصل التجريبية في الارانب

احمد قاسم العوادي و محمد جويد علوان
فرع الامراض - كلية الطب البيطري - جامعة بغداد - العراق

الخلاصة

لدراسة تأثير المستضد الكلي المتكسر لبكتريا *P. aeruginosa* على الاصابة التجريبية للمفصل بهذه البكتريا، استعمل خمسة عشر ارنب في هذه التجربة وقسمت الى 3 مجاميع متساوية. المجموعة الاولى حقنت في مفصل الكاحل ب 0.2 مل من عالق بكتريا *P. aeruginosa* (108×2 cfu/ml)، اما المجموعة الثانية فقد تم تمنيعها بالمستضد الكلي المتكسر لهذه البكتريا ثم حقنت في مفصل الكاحل ب 0.2 مل من عالق بكتريا *P. aeruginosa* (108×2 cfu/ml). اما المجموعة الثالثة اعتبرت كمجموعة سيطرة سالبة. في اليوم الثلاثين بعد الحقن اظهرت المجموعة الممنعة زيادة في مستويات المناعة الخلوية (DTH و IFN-γ) والخلطية (IgG) وعزل بكتيري محدود من المفصل والدم والاعضاء الداخلية مقارنة بالمجاميع الاخرى. اظهرت المجموعة الاولى اعراض شديدة وتفاعل التهابي شديد وتغيرات عيانية ونسيجية واضحة في المفصل شملت استجابة قيحية وقيحية حبيبية، تنكس وتحطم في غضروف المفصل وامتداد الاصابة الى النسيج العظمي تحت الغضروف مسببة التهاب العظم. اما المجموعة الثانية (الممنعة) فقد كانت التغيرات المرضية محدودة وقد اظهر الفحص النسيجي ان التغيرات كانت محددة بغشاء المفصل. نستنتج من هذا ان المستضد المتكسر الكلي لبكتريا *P. aeruginosa* قد وفر الحماية للمفاصل من الاصابة التجريبية بالـ *P. aeruginosa* في الارانب.

الكلمات المفتاحية: بكتريا ، التهاب المفصل، الارانب.