**Immunohistochemical investigation of CD14 in experimental rabbit pneumonic pasteurellosis**

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**SUMMARY**

The present study investigated the immunolocalization of CD14 and the bacterial antigen of *Pasteurella multocida* serotype A:3 in normal and pneumonic rabbit lungs. To induce pneumonia, *P. multocida* serotype A:3 (4x10⁶ CFU/ml) was inoculated intra-tracheally to twelve New Zealand White rabbits (*Oryctolagus cuniculus*) at age of 8-10 weeks while four rabbits received physiologic saline with the same route. Nine infected rabbits died with clinical signs of the disease between the 3rd and 13th days post inoculation. On day 14, the remaining three infected and control rabbits were euthanized. The histopathological evaluation of the lungs samples collected from nine infected rabbits with clinical symptoms revealed severe cases of fibrinopurulent and necrotic pneumonia and fibrinopurulent pleuritis. The bacterial antigen was immunolocalized in macrophages and degenerating leukocytes around the necrotic tissue and in exudates occupying the lumens of bronchia, bronchioles, and alveoli. CD14 immunoreactivity was present in the alveolar macrophages, neutrophils, alveolar epithelial cell surfaces, and lumens of the capillaries of the pneumonic lungs. In three rabbits, inoculated but presenting no clinical symptoms and no histopathological lesions, the bacterial antigen was found in the alveolar wall; however, like in the controls, CD14 immunoreactivity was present only in the lumen of capillaries. Double labelling immunohistochemistry indicated that CD14 was closely associated with the bacterial antigen in inflamed regions of the lung. Results suggested that interaction of the bacterial antigens and CD14 may have a critical role in lung pathologies resulting from *P. multocida* serotype A:3 infection.

**Keyword : CD14 - Pasteurella multocida - experimental pasteurellosis - lung - rabbit - immunohistochemistry.**

**Introduction**

*Pasteurella multocida* causes an upper respiratory disease in rabbits, called snuffles, which is one of the major causes of morbidity and mortality. It may also cause an acute pneumonia that may kill rabbits few hours or in few days [5]. Conjunctivitis, rhinitis, oitis media, pleuritis, and abscess formation are among the current manifestations seen in *P. multocida* infections. Pasteurellosis among rabbit colonies causes a considerable economical loss especially in laboratory health departments, and, besides, interferes with the collection of reliable research data [6]. Basic and clinical research elucidated some aspects of *P. multocida* infections in rabbits [9, 14, 23]; however, some details especially in pathogenesis such as the role of CD14 in recognition of *P. multocida* antigens remains incomplete.

CD14 is a 55-kDa glycoprotein expressed by myeloid cells including macrophages and monocytes as a glycosphati-
Current literature raised a rational question if the lung manifestations in *P. multocida* infections in rabbits develop following a concomitant interaction between *P. multocida* and CD14 positive cells. Therefore, the aim of the present study was to illustrate the distribution of the bacterial antigen and its relationship with CD14 in the rabbit lung with pneumonia induced by *P. multocida* serotype A:3.

**Materials and methods**

**ANIMALS**

This study was conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Kirikkale University. Sixteen New Zealand White rabbits (*Oryctolagus cuniculus*) at age of 8-10 weeks from either sex were obtained from the Rabbit Breeding Unit of the Research and Development Branch of the Ministry of Agriculture, Ankara. Rabbits were randomly assigned to a treatment group (n=12) and a control group (n=4). Absence of *P. multocida* infection at beginning of the study was verified by the bacterial analysis of the nasal cultures collected from all rabbits.

**PREPARATION OF INOCULATE**

*Pasteurella multocida* serotype A:3, provided by Dr. Richard Rimler of National Animal Disease Center, Ames, Iowa, was grown from lyophilized stock cultures and inoculated intraperitoneally into mice. Upon re-isolation from blood collected from the heart of the infected mice, *P. multocida* serotype A:3 was propagated on 5 % sheep blood agar supplemented with 0.5 % yeast extract. The bacterial suspensions were adjusted to approximately 4x10^6 cfu/mL of inoculate prepared in phosphate buffered saline (PBS).

**EXPERIMENTAL INDUCTION OF PASTEURELLA MULTOCIDA INFECTION**

*P. multocida* serotype A:3 was inoculated intra-tracheally to rabbits in the treatment group (n=12). Physiological saline was administered to control rabbits (n=4) with the similar procedure. For this purpose, rabbits were anesthetized by intravenous injection of 0.5 ml of Ketamine hydrochloride (100 mg/ml) and intramuscular injection of 0.2 mL of Xylazine (20 mg/ml). Placing rabbits in a dorsal recumbency position, a midline incision was made to expose the anterior one third of the trachea. A 16 gauge 1” hypodermic needle was inserted between the cartilaginous rings of the trachea, and then a polyethylene catheter was pushed gently down to one third of the trachea. A 16 gauge 1” hypodermic needle was inserted between the cartilaginous rings of the trachea, and then a polyethylene catheter was pushed gently down to the bifurcation region of the trachea. Through the polyethylene catheter, 0.5 mL of *P. multocida A: 3 suspensions* (4x10^6 CFU/mL in PBS) were inoculated.

**BACTERIAL ISOLATION**

At necropsy, swabs from the nasal cavity and lung tissue samples were cultured from all rabbits either died or euthanatized. The swabs and tissue samples inoculated onto nutrient agar plates containing 5% defibrinated sheep blood with and without 2 µg/ml of clindamycin [12]. Plates were incubated aerobically at 37°C and examined after 24 and 48 hours. Isolates of *P. multocida* were identified using standard methods, including colonial morphology and biochemical reactions.

**NECROPSY AND PATHOLOGIC EXAMINATION**

All rabbits, either died or euthanatized at end of the clinical phase of the study, were subjected to necropsy. Euthanasia was accomplished with an intravenous injection of pentobarbital sodium 14 days post inoculation. Following a gross necropsy examination, tissue samples were collected from the lung (all lobes) for histological and immunohistochemical evaluations. The tissue samples were fixated in 10% neutral buffered formalin, embedded in paraffin wax, and sectioned at a thickness of 5µm. Sections were collected on glass slides and stained with haematoxylin-eosin and examined with an Olympus BX-50 microscope.

**PRODUCTION OF PRIMARY ANTISERA AGAINST P. MULTOCIDA**

*P. multocida* serotype A:3 strain was inoculated in Trypticase soy broth (Oxoid) and incubated at 37°C for 18 hours. 0.5 mL of the broth culture, inoculated with *P. multocida* serotype A:3 strain, was then inoculated onto Kolle flasks of Trypticase soy agar and incubated at 37°C. The growth was removed with 0.5% formalinized saline and washed four times with normal saline. Pairs of rabbits were injected intravenously on successive days with 0.1, 0.2, 0.4, 0.8, and 1.0 ml of inoculate containing *P. multocida* serotype A:3 strain. Ten days after the last injection, they were re-inoculated with 1 ml of inoculate. Seven days later, they were bled and serum samples were collected and prepared for immunohistochemistry.

**IMMUNOHISTOCHEMISTRY FOR CD14**

Following dehydration, tissue sections were boiled in citrate buffer (2.1 g sodium citrate/L, pH 6.0) in a microwave oven (600W) 4 times for 5 min. Following quenching of endogenous peroxidase activity with 1% H_2O_2 in methanol for 15 min, 10% normal goat serum (Dako, Glostrup Denmark) was applied onto sections to block non-specific binding of immunoglobulins. The sections were then incubated with a monoclonal mouse anti-rabbit CD14 antibody (generosity of Dr RJ Ulevitch of The Scripps Research Institute) at a dilution of 1:512 in PBS (pH 7.4) for 60 min. All steps were performed in a humidity chamber at room temperature. A universal LSAB2 horseradish peroxidase (HRP) kit (Dako) was used to demonstrate the CD14 antigen binding. A biotinylated polyvalent anti- mouse secondary antibody (Dako) and then streptavidin-peroxidase enzyme (Dako) were applied onto sections for 10 min each. For color reaction, 3, 3’-diaminobenzidine (DAB) chromogen (Dako) was applied onto sections for 10-15 min (controlled by visual observation with a microscope). Sections were rinsed with distilled water, counterstained with Mayer’s haema-
toxylin for 1-2 min, and mounted with aqueous mounting medium.

**IMMUNOHISTOCHEMISTRY FOR PASTEURELLA MULTOCIDA**

A similar procedure was followed; however, sections were labeled with rabbit anti-*P. multocida* at a dilution of 1: 256 in PBS (pH 7.4), which was further amplified by an anti-rabbit secondary antibody. 3-amino-9-ethylcarbazole (AEC) chromogen was used for color reaction.

**DOUBLE-LABELLING IMMUNOHISTOCHEMISTRY FOR CD14 AND PASTEURELLA MULTOCIDA**

The sections were first applied anti-*P. multocida* with same procedure described above. However, a universal LSAB2 alkaline phosphatase (AP) kit (Dako) and fuchsin for color reaction was used to visualize the immune reaction. Then, the monoclonal anti-rabbit CD14 was applied. Following application of the anti- mouse secondary antibody, sections were treated with streptavidin-peroxidase enzyme. Sections were then treated with DAB and counters- tained lightly with Mayer’s haematoxylin.

To validate the specificity of immunohistochemical procedure, primary antibodies and secondary antibodies were omitted in separate sections.

**Results**

**CLINICAL FINDINGS**

Nine rabbits in the treatment group died between the 3rd and 13th day post inoculation. They have exhibited high body temperature (up to 40°C), dyspnea, depression, sniffing, coughing, and anorexia for 24 hours or longer prior to death. The euthanatized rabbits, control and the three survival rabbits in the treatment group, did not show any clinical signs of the disease.

**BACTERIOLOGICAL FINDINGS**

At the beginning of the study, all rabbits were free of *P. multocida*. However, *P. multocida* serotype A: 3 was isolated from the nasal cavity swaps and the lung tissue collected during the necropsy of all 12 rabbits in the treatment group.

**NECROPSY FINDINGS**

Of the twelve rabbits infected with *P. multocida* serotype A: 3, nine rabbits had a marked consolidation of the whole of

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**Table I.**—Bacteriological, histopathological, immunohistochemical, and necropsy findings in rabbits with (treated) and without (control) experimental pneumonia induced by *Pasteurella multocida* serotype A: 3.

<table>
<thead>
<tr>
<th>Case No</th>
<th>Clinical outcome</th>
<th>Necropsy findings</th>
<th>Histopathological findings</th>
<th>Immuno-localization of bacterial antigen</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Died/ Euthanasia</td>
<td>Bacterial presence*</td>
<td>Consolidation</td>
<td>Pleuritis</td>
</tr>
<tr>
<td>1</td>
<td>Died</td>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Died</td>
<td>4</td>
<td>+</td>
<td>-</td>
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<td>4</td>
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<td>Died</td>
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<td>+</td>
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<td>Died</td>
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<td>Died</td>
<td>7</td>
<td>+</td>
<td>-</td>
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<tr>
<td>7</td>
<td>Died</td>
<td>8</td>
<td>+</td>
<td>-</td>
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<tr>
<td>8</td>
<td>Died</td>
<td>8</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Died</td>
<td>13</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Euthanasia</td>
<td>14</td>
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<tr>
<td>16</td>
<td>Euthanasia</td>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fib-prl= Fibrinopurulent  
* «Day post-inoculation» represents the day on which the incidence of death occurred or euthanasia was performed  
^ «Bacterial presence» stands for the isolation of *Pasteurella multocida* serotype A: 3 from the lung tissue and the nasal swaps collected during necropsy
A) In pneumonic rabbit lungs, the bacterial antigen was frequently observed (bacterial antigen immunoreactivity: arrows) in alveolar exudates.

B) The bacterial antigen (arrows) was also observed in the alveolar wall of the lung samples taken from rabbits with no clinical sign and lung pathology despite bacterial inoculation.

C) In normal rabbit lungs, CD14 expression is restricted to capillary lumens (arrows).

D) In pneumonic rabbit lungs, CD14 immunoreactive alveolar macrophages (arrows) were quite visible.

E) CD14 immunoreactivity was also observed in some bronchiolar epithelial cells and cells situated in exudates and in the lumen of the bronchioles (e).

F) CD14 immunoreactive macrophages (arrowheads; light brown staining) were in close contact with the bacterial antigens (arrows; red staining).

Figure 1.—Photomicrographs illustrating the immunolocalizations of the bacterial antigen and CD14 in the rabbit lung with and without pneumonia induced by Pasteurella multocida serotype A:3. Streptavidin-peroxidase staining in A, B, C, D, and E. In F, streptavidin-peroxidase for light brown and alkaline phosphatase for red. Bar = 20 μm in A, D, E and F, Bar = 30 μm in B, and Bar = 40 μm in C. al = alveolar lumen.
The bacterial antigen of *P. multocida* serotype A:3 was detected at the cell surface and cytoplasm of the bronchiolar epithelia and alveolar macrophages, at the edge of the bronchiolar exudates, in the necrotic alveolar walls, fibrin, serous exudates, and degenerating leukocytes in the lung samples collected from the infected rabbits. It was also detected in the dense zone of neutrophiles, intraluminal leukocytes in bronchioles, and inflammatory cells around the necrotic tissue (Fig. 1A). In the three rabbits inoculated with the bacteria but which had no significant lung pathologies, the bacterial antigen was also immunolocalized in the alveolar wall (Fig 1B). No antigen was observed in the lung samples collected from the control rabbits.

CD14 immunoreactivity was observed at the cell surface of alveolar macrophages, alveolar and bronchiolar epithelia in the lung samples collected from the nine infected rabbits died without anesthesia (Fig. 1D and 1E). Immunoreactivity was also present within the cytoplasm of alveolar macrophages (Fig. 1D) and neutrophiles (located in alveoli and alveolar wall) (Fig 1E). In the lung samples of control animals and of the three rabbits of the treatment group with no clinical signs and histopathological findings (Fig 1C), immunoreactivity was present only in the lumen of capillary vessels.

As observed in the double labeling immunohistochemistry, the CD14 was closely associated with the bacterial antigen in pulmonary inflammatory lesions (Fig 1F). Bacterial antigens were observed as they were in contact with the cell surface and engulfed within the cytoplasm of the C14 positive macrophages (Fig. 1F). The CD14 and the *P. multocida* antigen immunoreactivity on cells in the infected lung areas were summarized in Table II.

### Table II.—Immunolocalisation of *Pasteurella multocida* antigen and CD14 in cells of the infected lung areas.

<table>
<thead>
<tr>
<th>Macrophages</th>
<th>Cytoplasm</th>
<th>Cell membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neutrophiles</td>
<td>Cytoplasm</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cell membrane</td>
<td>+</td>
</tr>
<tr>
<td>Bronchiolar epithelial cells</td>
<td>Cytoplasm</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Cell membrane</td>
<td>-</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Cytoplasm</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cell membrane</td>
<td>-</td>
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</table>

### Discussion

*Pasteurella multocida* A:3 is among the most virulent strains of *P. multocida* that induces a severe case of pneumonia in rabbits [8, 23]. In the present study, pneumonic rabbits exhibited body temperature up 40°C, dyspnea, snuffling, coughing, and anorexia that subsequently led to death. Histopathologically, lesions were characterized focal to widespread extensive fibrinopurulent bronchopneumonia and fibrinopurulent pleuritis, which have been described previously [14, 23]. Nevertheless, the clinical response to *P. multocida* was variable among rabbits: clinical signs and the incidence of death occurred earlier in some rabbits, and three rabbits survived until the end of the clinical phase of the study (14 days) with no clinical symptoms. Although *P. multocida* serotype A:3 was isolated from the nasal swaps and the lung tissues of these three rabbits, no significant lung pathologies were present. We may think of the occurrence of a previous *P. multocida* infection in these three rabbits: indeed, this bacteria may reside in rabbits with no clinical symptoms until a stress factor exists in the surrounding [8].

An extensive invasion of the lung by *P. multocida* serotype A:3 was observed in the pneumonic lungs using immunoperoxidase staining method, a viable method to detect *Pasteurella* spp. in paraffin embedded tissue sections [17]. The bacterial antigen was detected at the cell surface and cytoplasm of the bronchiolar epithelia, alveolar macrophages, and degenerating leukocytes in the pneumonic rabbit lungs. Association of *P. multocida* serotype A:3 with these cells was also mentioned by AL-HADDAWI et al. [1].

Functioning as a receptor for LPS, CD14 is one of the key components of the innate immune response. It is expressed on myeloid cells, including monocytes and activated macrophages [2, 27, 31]. In the present study, CD14 immunoreactivity was observed at the cell surface and cytoplasm of alveolar macrophages and neutrophiles as well as alveolar and bronchiolar epithelial cell surfaces in pneumonic rabbits. Double labeling immunohistochemistry revealed that CD14 positive macrophages were in close contact with *P. multocida* serotype A:3 antigen. As observed in pneumonic lungs of the present study, activated macrophages express CD14 antigen, which is essential for the phagocytosis and killing of the bacteria. The CD14 and the bacterial antigen were present in close contact with each other, indicating a synergistic interaction that helps in the clearance of the bacteria from the lung tissue.
to that in controls). The results suggest that interaction of CD14 positive cells with P. multocida serotype A:3 may determine the outcome of the pneumonia.

Acknowledgements

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