Effect of Physical Defenses of the Respiratory Tract on the Development of Pneumonia in Pigs Inoculated Endobronchially with Actinobacillus pleuropneumoniae

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ABSTRACT. Seven pigs inoculated endobronchially with Actinobacillus pleuropneumoniae (App) serotype 1 developed acute and subacute necrotizing pleuropneumonia. When treated with high doses of atropine (0.25 mg per kg) and/or xylocaine spray using a bronchoscope, which suppressed mucus secretion and ciliary activity, the pigs showed severe pleuropneumonia and 2 treated pigs died within 36 hr after inoculating 320 colony forming units (CFU) 2 ml of App serotype 1. Histopathologically, their lungs had alveolar and interlobular edema and intravascular fibrinous thrombosis. In the surviving pigs, the lymph nodes had App antigens in the germinal centers corresponding closely with activated follicular dendritic cells and increased in the number of IgG- and IgM-containing cells. The bacterial antigens were also observed as small sized granules in the cytoplasm of bronchoalveolar macrophages. These findings suggest that the attachment of App to the mucosal surface may be crucial in the development of pneumonic lesions. —KEY WORDS: Actinobacillus pleuropneumoniae serotype 1, pneumonia (swine).


Pleuropneumonia in pigs caused by Actinobacillus pleuropneumoniae (App) continues to be a serious threat to the pig industry throughout the world. The acute and subacute states of the disease are characterized by fibrinohemorrhagic pleuropneumonia and coagulative necrosis [15–18]. The severity of the disease is thought to be related to the number of bacteria reaching the lower respiratory tract [2, 21].

Defense mechanisms of the lung against pathogenic bacteria may be their physical removal of bacteria from the respiratory tract and destruction of the viability of the organisms [3]. Mucociliary clearance and bactericidal activity can be compromised by virus infections. Most previous studies have demonstrated the effect of combined infections with viruses and bacteria in the establishment of respiratory tract diseases [2, 4, 5, 7, 8, 12, 14, 19, 23], but there is little information on the interaction between respiratory physical defenses and A. pleuropneumoniae infection in pigs.

The purpose of the present study was to evaluate the effect of respiratory mucociliary clearance on the induction of pneumonia in pigs inoculated endobronchially (EB) with App.

MATERIALS AND METHODS

Animals: Eight specific pathogen-free pigs, 14–15 kg body weight, were used. The animals had no serum antibodies against App serotype 1 or Aujeszky’s disease virus. The pigs were divided into 6 groups for experiment and housed separately in different blocks to prevent cross-infection. The air from each block was exhausted through high efficiency sterilizing filters.

Bacteria: App serotype 1 strain 4074 was provided by Dr. Nicolet, University of Bern, Switzerland. The strain 4074 was cultured on PPLO agar (Difco) containing 5% horse serum, 1% glucose and 1% fresh yeast extract. An inoculated plate culture, incubated overnight at 37°C in 5% CO2, was suspended in ‘Mist. desiccans’ up to 106 CFU/ml and kept at -80°C. Bacterial inocula were prepared from 105 to 107 dilutions of the stock cultures with Brain Heart Infusion Broth (Difco) immediately prior to inoculation.

Experimental procedures: Before inoculation of App and/or growth culture medium, all pigs were sedated with azaperon (5 mg per kg body weight, intramuscularly (IM)) and atropine (0.05 mg per kg body weight, IM), and then anesthetized with pentobarbitral (10 mg per kg body weight, intravenously (IV)). Especially, 2 pigs (nos. 1 and 2) were injected with 5 times the above-mentioned dose (0.25 mg per kg of body weight) of atropine to suppress airway secretions. Two pigs (nos. 3 and 4), after anesthetization, were sprayed with xylocaine using a bronchoscope for 5 sec transiently to prevent ciliary activity of bronchial epithelial cells in the right lung lobus. One pig (no. 5) received both treatments (5 times the above-mentioned dose of atropine and 5 sec of xylocaine spray). Then, 6 pigs (nos. 1 to 6) were inoculated EB using a bronchoscope with 2 ml of growth culture medium containing 320 CFU of App. One pig (no. 7) was inoculated EB with 2 ml containing 6000 CFU of App. One pig (no. 8) received EB with 2 ml of growth culture medium and served as control. All pigs were clinically observed for 10 days and then sacrificed (Table 1).

Cell collection from broncho-alveolar lavage (BAL) fluid: After removal of the lung at necropsy, a cannula (4 mm diameter) was inserted into the right main bronchus, and 30 ml of sterile phosphate-buffered saline (PBS) was introduced and recovered by suction. BAL fluid was placed
in a sterile bottle and kept on ice, and the BAL fluid was centrifuged by autosmear. Air-dried smears of sediment were fixed in acetone for 5 min and subjected to the demonstration of App antigen by immunoperoxidase staining.

Pathological examination: Pigs were euthanized by IV administration of barbiturate. The pigs that died before the end of the experiment were immediately submitted to necropsy. All of the pigs were examined for gross morphological changes, and pulmonary parts of the lungs were dissected. Tissues were fixed in buffered 10% formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (HE), and phosphotungstic acid hematoxylin (PTAH) for light microscopy.

App antigen was demonstrated in respiratory tissues, associated lymph nodes and cells in BAL fluid by the avidin-biotin-complex immunoperoxidase (ABC) method using the Vectastain ABC kit (Vectastain, Vector Laboratories, Burlingame, Calif). Anti-App serotype 1 rabbit serum was provided by Dr. T. Yamamoto, National Institute of Animal Health, Japan, and used at a dilution of 1:16384. Anti-porcine IgG, and IgM rabbit sera (Miles Laboratories, Naperville, Ill) and anti-S100a mice serum (Japan Immunoresearch Laboratories, Takasaki, Japan) were used at dilutions of 1:2,560, 1:160 and 1:200, respectively. Sections were counter-stained with methyl green. Tissue sections from pig no. 6, and serum from a non-immunized rabbit were used as controls.

Isolation of bacteria: Lung samples from the right and left caudal lobes were cultured using Tryptic Soy Agar (TSA; Difco) containing 5% horse blood and 1% fresh yeast extract. Suspected colonies of App were identified biochemically and serologically. Quantitative analyses of viable organisms of App from lung samples were performed using the Miles and Misra technique [9].

RESULTS

Clinical observation: Five pigs (nos. 1 to 5) treated with atropine and/or xylcaine spray, and one pig (no. 7) inoculated with 6000 CFU of App showed pyrexia (up to 41°C), which persisted for 8 days after infection, and severe polynepa, dyspnea and anorexia. Three of the infected pigs (nos. 1, 4 and 5) showed vomiting. The 2 pigs (nos. 2 and 3) treated with atropine or xylcaine spray showed a marked fall in rectal temperature and epistaxis and died within 36 hr after infection. One pig (no. 6) that was non-medicated and inoculated with 320 CFU of App, showed only a slight pyrexia between 3 and 4 days after inoculation.

The non-infected control pig (no. 8) was free of clinical abnormalities.

Detection of App antigen in BAL fluid: App antigen was detected in BAL fluid from all the infected pigs. In 2 pigs (nos. 2 and 3) that died within 36 hr after inoculation, the rod-shaped bacterial antigens correlated closely with the amount of organism present and were distributed in BAL fluid (Fig. 1). A positive reaction in pigs killed 10 days after inoculation was detected in the cytoplasm of bronchoalveolar macrophages as small sized granules (Fig. 2).

One infected pig (no. 6) and non-infected control pig (no. 8) revealed a negative reaction.

Isolation of App: App was isolated from the right or left caudal lobes in all infected pigs, as shown in Table 1. The highest titer was 1.7 x 10⁶ CFU/g in left caudal lobe and 3.0 x 10⁹ CFU/g in right caudal lobe.

No App was isolated from tissues of the non-infected control pig.

Pathological examination: Gross lesions were usually confined to the thoracic cavity. The lung lesions were predominantly unilateral (Fig. 3). The severity of the pneumatic lesions in pigs treated with atropine and/or xylcaine spray and received 320 CFU of App was almost the same as that in pig inoculated with 6000 CFU of App, in spite of the small amount of inoculum (Table 1).

Pneumatic lesions in the acute cases (pig nos. 2 and 3), which died within 36 hr after inoculation, were characterized by lobar pleuropneumonia and extensive congestion, haemorrhage and fibrinous exudate. Interlobular edema was

Fig. 1. Large numbers of rod-shaped App antigens in BAL fluid in a dead pig (no. 2). Immunoperoxidase (IP) stain, ×1,000.

Fig. 2. App antigen as small sized granules in the cytoplasm of bronchoalveolar macrophages in a pig (no. 7). IP stain, ×1,000.
Table 1. Distribution of pulmonic lesions and isolation of *A. pleuropneumoniae* serotype 1 in pigs

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Treatment</th>
<th>PID</th>
<th>Distribution of pulmonic lesion</th>
<th>Isolation of <em>A. pp</em> (CFU/g)</th>
<th>Detection of App antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Atropine</td>
<td>10</td>
<td><img src="diagram1.png" alt="Diagram" /></td>
<td>$1.4 \times 10^7$</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>Atropine</td>
<td>1.5d</td>
<td><img src="diagram2.png" alt="Diagram" /></td>
<td>$2.0 \times 10^7$</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>Xylocaine</td>
<td>1.5d</td>
<td><img src="diagram3.png" alt="Diagram" /></td>
<td>$1.7 \times 10^6$</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>Xylocaine</td>
<td>10</td>
<td><img src="diagram4.png" alt="Diagram" /></td>
<td>$6.0 \times 10^6$</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>Atropine</td>
<td>10</td>
<td><img src="diagram5.png" alt="Diagram" /></td>
<td>$5.0 \times 10^2$</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Xylocaine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(320 CFU)</td>
<td>10</td>
<td><img src="diagram6.png" alt="Diagram" /></td>
<td>$3.0 \times 10^6$</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(6000 CFU)</td>
<td>10</td>
<td><img src="diagram7.png" alt="Diagram" /></td>
<td>$5.0 \times 10^6$</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>(non-infected)</td>
<td></td>
<td><img src="diagram8.png" alt="Diagram" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

prominent. On the cut surface, firm, haemorrhagic, irregularly shaped areas, often with centrally located necrotic foci, were seen. In the subacute cases (pig nos. 1, 4, 5, and 7), killed 10 days after inoculation, abscess-like nodules of various sizes were detected in middle and caudal parts of the caudal lobes. The nodules contained a thick capsule of connective tissue and were marked by extensive adhesive pleuritis. The infected, non-medicated pig (no. 6) had only a small abscess-like nodule in the bottom of the right caudal lobe. In all infected pigs, the bronchial and mediastinal lymph nodes were enlarged and edematous.

The characteristic features of App lesions in stained sections were seen with the naked eye or under a very low magnification (Fig. 4). Areas of coagulative necrosis of varying sizes were surrounded by darkly stained bands comprised of packed alveolar macrophages and lymphocytes. The acute cases (pig nos. 2 and 3) revealed marked congestion of alveolar capillaries and larger blood vessels with haemorrhage, exudation of fluid and fibrin into the pulmonary parenchyma. The interlobular septa and their lymphatic vessels were distended and fibrin thrombi were present in many vessels throughout the lungs. In the subacute cases (pig nos. 1, 4, 5 and 7), the necrotic lesions became fused and appeared as a large abscess with fibroblastic proliferation and connective tissue surrounding it.

No gross and histologic lesions were observed in the non-infected control pig.

**Immunohistological examination:** Diffuse distribution of App antigens was detected in the areas of coagulative necrosis in the lungs. They were also found in the dense zone of necrotic tissue and in the cytoplasm of macrophages around the coagulative necrosis, in some inflammatory cells in the lymphatics and bronchioles associated with necrotic lesion, and in germinai centers of the bronchial lymph node (Fig. 5). In the cases that died, the antigen appeared prominently in the interalveolar and interlobular septa, the dilated lymphatic vessels and lymphatic sinuses of the bronchial lymph nodes. In the bronchial lymph nodes, the number of follicular dendritic cells in the germinai center and IgG- and IgM-containing cells in the para-follicular areas (Fig. 6) in pigs killed 10 days after inoculation increased more significantly than those in 2 pigs that died and the non-infected control pig.

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**Fig. 3.** Localization of subacute pleuropneumonic lesion after endobronchial inoculation of App in a pig (no. 5).

**Fig. 4.** Irregular areas of coagulative necrosis demarcated by dense bands in a pig (no. 1). HE stain.

**Fig. 5.** App antigens detected as a meshed structure in the germinai center of the lymph node in a pig (no. 4). IP stain, ×200.

**Fig. 6.** Proliferation of IgG-containing cells in the para-follicular areas of the lymph node in a pig (no. 5). IP stain, ×125.
DISCUSSION

Experimental studies indicate that App is transmissible by the respiratory route. Inoculation with pure cultures of App in an aerosol or via the intratracheal or intranasal routes commonly produced pneumonia lesions in the caudal lung lobe [18, 22]. The severity of the pneumonia lesions correlated closely with the dose of App. In the present experiment, the pigs inoculated endobronchially with App revealed the presence of necrotizing pleuropneumonia. These characteristic pneumonia lesions were closely associated with the sites of deposition of the inoculum. This technique induced pneumonia readily and appears to be a useful means for the practical examination.

It is well known that mild or severe pleuropneumonia can be caused by different amounts of App that actually reach to the lung. The irregular coagulative necrosis caused by App in the acute form has been compared with that caused by endotoxin shock [13]. In the present study, the pigs that died within 36 hr after inoculation showed alveolar and interlobular edema in combination with dilation of lymph vessels, congestion, haemorrhage and intravascular fibrinous thrombosis. Thus, these findings might be attributed to the action of toxins, which agreed with the results reported in previous studies [1, 6, 20, 2].

Immunohistologically, App serotype 1 antigens were demonstrated in the areas of coagulative necrosis, in the lymphatic vessels at the acute stage of infection, as well as in the dense zone of necrotic tissue, in the cytoplasm of macrophages around necrotic nodules and in the germinal centers of the lymph nodes at the subacute stage of infection. Location of follicular dendritic cells corresponded closely with the presence of App antigens. Moreover, there was a great increase in the number of IgG- and IgM-containing cells in the para-follicular areas. Thus, these findings suggested that App is disseminated via the lymphatic vessels and subsequently stimulates the immune response in affected organs.

The mucociliary escalator plays a major role in the lung’s physical defense against the challenge of inhaled microorganisms [3]. In some viral respiratory diseases, including swine influenza and Aujeszky’s disease, ciliary activity in the respiratory mucosa is disrupted and mucociliary clearance is significantly reduced [2, 10, 11]. Subsequently, pneumonic lesions develop following bacterial infection [2, 4, 5, 7, 8, 12, 14, 19, 23]. In the present study, the pigs treated with a high dose of atropine and/or xylocaine spray using a bronchoscope, which suppressed mucus secretion and ciliary activity, developed severe pleuropneumonic lesions. The 2 treated pigs died within 36 hr after infection, despite they have received a small amount of App serotype 1 inoculum (320 CFU/2 ml). These results suggested that bacterial attachment to the mucosal surface might influence the severity of pleuropneumonic lesions in App infection.

Phagocytosis by alveolar macrophages is the principal means by which pathogenic organisms are cleared from the lungs. App, however, produces toxins that are cytotoxic to alveolar macrophages[1, 6, 20, 21]. In the present study, a large number of App serotype 1 organisms were detected in BAL fluid at the acute stage of infection. After that, the phagocytized bacterial antigens were detected as small sized granules in the cytoplasm of macrophages. These results strongly suggest that at the initial stage of infection, alveolar macrophages failed to digest engulfed App. Subsequently, infection with App might stimulate the development of a local immune response and then enhances phagocytic activity of macrophages.

Our findings suggest that suppression of the mucociliary escalator might promote the susceptibility of pigs to App infection. Further studies are needed to investigate the immunological response in the BAL fluid following App infection.

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