Identification of Interleukin-1, Tumor Necrosis Factor- α , and Interleukin-6 Expression in Lungs from Pigs Naturally Infected with *Mycoplasma hyopneumoniae* by *In situ* Hybridization

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ABSTRACT. The detection and distribution of interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α) and IL-6 were studied, by *in situ* hybridization with a non-radioactive digoxigenin-labeled probe, in formalin-fixed, paraffin-embedded lung tissue from 10 pigs naturally infected with *Mycoplasma hyopneumoniae*. The morphology of host cells was preserved despite the relatively high temperature required during the incubation procedure. Examination of three serial sections from each of the 10 lung samples showed that the three cytokines closely resembled each other in respect of cellular distribution. Three inflammatory cytokines are expressed in response to *M. hyopneumoniae* infection, with IL-6 localized primarily to peribronchiolar lymphoid hyperplastic tissues, and both IL-1 and TNF- α expressed in alveolar macrophages. Although statistically non-significant, IL-1 ($r=0.5744$, $p=0.0883$) showed potentially important correlation with histopathological lesions. No other potentially clinically important correlations ($r>0.30$) were observed between any of the other cytokines (TNF- α ; $r=0.2045$, $p=0.5603$ and IL-6; $r=-0.06607$, $p=0.8651$) and histopathological lesion score. The results suggest that inflammatory cytokines are associated with the development of pneumonia in *M. hyopneumoniae* infection and may contribute to disease severity.

KEY WORDS: Interleukin-1, interleukin-6, *Mycoplasma hyopneumoniae*, swine, tumor necrosis factor- α .

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Mycoplasma hyopneumoniae is a causative agent of mycoplasmal pneumonia of swine. It is widespread and causes economic losses in the swine industry through stunted growth caused by poor feeding efficiency and susceptibility of swine to infection by other pathogenic organisms [18]. Adherence of *M. hyopneumoniae* to the mucosa of the distal portion of the respiratory tract of swine is an important initial event in the development of mycoplasma pneumonia [14, 22]. During *in vivo* infection, *M. hyopneumoniae* is intimately associated with the cilia and causes an extensive loss of these structures [4, 14].

Histopathological lesions in the acute stage of disease are characterized by loss of respiratory cilia, exfoliation of ciliated cells, and small accumulation of neutrophils in lumina and around airway. In chronic stages, there is lymphoid hyperplasia or cuffing and thickening of the interalveolar septa in these areas. In convalescent stages, collapsed alveoli, alveolar emphysema, and rather extensive hyperplastic lymphoid nodules can be seen [4, 8]. *M. hyopneumoniae* was detected mainly in the luminal surface of bronchial and bronchiolar lining epithelial cells [11].

There are several reports of the gene expression of cytokines

in the organs which were infected with mycoplasmas [9, 17]. Induction of tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1) and IL-6 in *M. pulmonis* infection may play an important role in the pathogenesis of disease [9, 17]. Although the levels of bioactive IL-1, TNF- α and IL-6 in bronchoalveolar lavage fluids increased significantly following *M. hyopneumoniae* infection [1, 2], little is known about the cellular production sites of cytokines and their involvement in the pathogenesis of respiratory *M. hyopneumoniae* infection. *In situ* hybridization can identify host cell types undergoing secretion of cytokines with high specificity and sensitivity [5, 7, 21]. The objective of this study was to detect by *in situ* hybridization the expression of inflammatory cytokines and to identify their cellular production sites in the lungs of pigs naturally infected with *M. hyopneumoniae*.

MATERIALS AND METHODS

Animals: Ten pigs (Nos. 1–10) approximately 58 days of age, from ten different herds were selected on the basis of clinical signs such as severe coughing, characteristic pathological lesions, and positive *in situ* hybridization for *M. hyopneumoniae* infections [11]. Ten pigs were negative for *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, and porcine reproductive and respiratory syndrome virus by culture and *in situ* hybridization [6, 16]. Negative control tissues were collected from a 1-day-old colostrum-deprived piglet. Lung tissues from pigs naturally infected with *A.*

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pleuropneumoniae were used as positive control of IL-1, TNF- α , and IL-6 as previously described [7].

Tissue processing: Samples of lung, liver, kidney, tonsil, lymph node, and large and small intestine specimens were collected from infected and noninfected animals, fixed in 10% (w/v) neutral buffered formalin for 24–48 hr, and embedded in paraffin wax according to standard laboratory procedures. Sections were cut 4 μ m thick, floated on a water bath containing diethylpyrocarbonate-treated (DEPC) water and mounted on Superfrost/plus slides (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Probe: The 417 base pair (bp), 291 bp and 532 bp cDNA fragment from a genomic library were used as a probe for IL-1, TNF- α and IL-6, respectively. The reverse transcription polymerase chain reaction (PCR) was carried out as previously described [20]. The forward and reverse primers for IL-1 were 5'-TCAGGCAGATGGTGTCTGTGC-3' and 5'-GGTCTATATCCTCCAGCTGC-3', respectively. The forward and reverse primers for TNF- α were 5'-GCTGTACCTCATCTACTCCC-3' and 5'-TAGACCTGCCCA-GATTCAGC-3', respectively. The forward and reverse primers for IL-6 were 5'-AACGCCTGGAAGAAGATGCC-3' and 5'-CTCAGGCTGAACTGCAGGAA-3', respectively. The PCR products were purified with Wizard PCR Preps (Promega Biotech, Madison, WI, U.S.A.) and labeled by random priming with digoxigenin-dUTP with a commercial kit (Boehringer Mannheim, Indianapolis, IN, U.S.A.) following the manufacturer's instruction.

In situ hybridization: Tissues from each pig were collected in 10% neutral buffered formalin, and after 1 or 2 days' fixation were dehydrated through graded alcohols and a xylene step and embedded in paraffin wax. Sections were then cut at 4 μ m, placed on Superfrost/plus slides (Fisher Scientific), and stored at room temperature. Just before use, sections were dewaxed in xylene, rehydrated in phosphate-buffered saline (PBS; pH 7.4, 0.01 M) for 5 min. De-proteinization was carried out in 0.2 N HCl for 20 min at room temperature. Tissues were then digested at 37°C for 20 min in proteinase K (Gibco BRL, Grand Island, NY, U.S.A.) 150 μ g/ml in PBS. Serial sections of each tissue section examined were treated with RNase A (Boehringer Mannheim) at 100 μ g/ml in 10 mM Tris-HCl (pH 7.4) for 30 min at 37°C to remove target RNA as a specificity control. After digestion, tissues were fixed in 4% paraformaldehyde in PBS for 10 min. After rinsing with PBS twice, the slides were acetylated in 300 ml of 0.1 mM triethanolamine-HCl buffer (pH 8.0) to which 0.75 ml of acetic anhydride (0.25%) had been added. After 5 min, a further 0.75 ml of acetic anhydride was added, and 5 min later the slides were rinsed in 2 $\times$ saline sodium citrate (SSC) (1 $\times$ SSC contains 50 mM NaCl and 15 mM sodium citrate pH 7.0).

Hybridization was carried out overnight at 43°C for IL-1, and at 45°C for TNF- α and IL-6. The digoxigenin-labeled probe (1 ng/ μ l) was diluted in 50 μ l of the standard hybridization buffer that consisted of 2 $\times$ SSC containing deionized formamide 50%, salmon sperm DNA (Oncor, Gaithersburg, MD, U.S.A.) 10 mg, sodium dodecyl sulphate

(SDS) 0.02%, Denhart's solution 1%, dextran sulphate solution (50% concentration) 50%; it was then heated for 10 min in a 95°C heating block, and quenched on ice. Approximately, 75 ng of digoxigenin-labelled probe were added to standard hybridization buffer (70 μ l), which was then layered over the section. Fluid was held in place by a coverslip (the edges of which were sealed with rubber cement). After overnight hybridization, sections were thoroughly washed, twice in 4 $\times$ SSC for 10 min at room temperature, twice in 2 $\times$ SSC for 10 min at 45°C, twice in 2 $\times$ SSC for 10 min at room temperature, twice in 0.2 $\times$ SSC for 10 min, once in maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5) for 5 min and once in 1 $\times$ blocking reagent (Boehringer Mannheim) for 40 min at room temperature.

For detection of hybridization, sections were incubated with anti-digoxigenin conjugated with alkaline phosphatase (Boehringer Mannheim) diluted 1 in 500 in 1 $\times$ blocking reagent (Boehringer Mannheim). After three washes in buffer, substrate consisting of nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolylphosphate (BCIP) was layered over the sections. Color was allowed to develop for 1–4 hr in the dark, and development was stopped by dipping slides briefly in tri-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Sections were counterstained with methyl green 0.5%, and the slides were then washed with distilled water for 1 min and dried completely.

Immunohistochemistry: Tissues were processed by routine methods, sectioned (4 μ m), and placed on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA, U.S.A.). The sections were dewaxed in xylene, rehydrated through graded alcohols and air-dried. Endogenous alkaline phosphatase was quenched with glacial acetic acid 20% for 2 min at 4°C. All slides were then treated with proteinase K (Gibco BRL, Grand Island, NY, U.S.A.) 100 μ g/ml in phosphate-buffered saline (PBS; 0.1M, pH 7.4) for 20 min at 36°C. All slides were incubated with normal goat serum (Sigma Chemical Company, St Louis, MO, U.S.A.) in PBS (0.1 M, pH 7.4) for 30 min at room temperature to saturate nonspecific protein-binding sites. SWC3a monoclonal antibody, anti-swine macrophage marker [22], was diluted 1 in 50 in PBS (0.01 M, pH 7.4) containing Tween 20 0.1%. The slides were incubated with antibody overnight at 4°C in a humid chamber.

After three washes with Tween 20 0.1% in PBS (0.01 M, pH 7.4), sections were flooded and incubated for 1 hr at 36°C with alkaline phosphatase-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) diluted 1 in 100 in PBS (0.01 M, pH 7.4) containing Tween 20 0.1%. The slides were washed in Tween 20 0.1%. Then, sections were equilibrated with Tris-buffer (pH 9.5) for 5 min at room temperature. The final reaction was produced by immersing the sections in a solution of red substrate (Vector Laboratories, Burlingame, CA, U.S.A.) for 20 min at room temperature. The sections were lightly counterstained with Mayer's haematoxylin, dehydrated through graded concentrations of ethanol and xylene, and mounted.

Morphometric analysis: For morphometric analysis of histopathological changes, three lung sections were examined blind and given an estimated severity score for the parameters of pulmonary pathology (i. e., atelectasia, airway plugging, leukocyte infiltration, peribronchiolar lymphoid hyperplasia, interstitial change). Each individual parameter was given a score ranging from 0 (no lesion visible) to 3 (severe lesions). These scores were added to obtain an overall score for each section. For morphometric analysis of *in situ* hybridization, three sections were cut from each of three blocks of tissue from the middle pulmonary lobe of each pig. To obtain quantitative data, the morphometric analysis of *in situ* hybridization slides was performed with the NIH Image J Program (National Institutes of Health, Bethesda, MA, U.S.A.). In each case, 10 fields were randomly selected, the number of positive cells per unit area (0.95 mm²) being counted.

Statistical analysis: Spearman correlation coefficients were used to assess the relationship between expression of inflammatory cytokine and histopathological lesion. A *P* value less than 0.05 was considered to indicate a statistically significant.

RESULTS

***In situ* hybridization:** *In situ* hybridization results for the 10 infected pigs are presented in Table 1. By *in situ* hybridization, a distinct positive signal for IL-1, TNF- α and IL-6 was detected in all 10 pigs. The morphology of host cells was preserved despite the relatively high temperature required during the incubation procedure. Examination of three serial sections from each of the 10 lung samples showed that the three cytokines closely resembled each other in respect of cellular distribution. The signal intensity varied within and between histological structures in any one section, and between pigs. Positive cells typically exhibited dark-brown to black reaction product without background staining.

IL-1 (Fig. 1) and TNF- α (Fig. 2) nucleic acids were detected in the cells of alveolar spaces and interstitium. Identification of the cell types expressing inflammatory cytokines (IL-1 and TNF- α) was difficult in the *in situ* hybridization preparations; however, the positive cells generally had large oval nuclei and abundant cytoplasm. Comparison with adjacent sections stained with immunohistochemistry indicated that most of the IL-1 or TNF- α positive cells (Fig. 3) were positive for SWC3a, which is a macrophage marker. In addition, examination of adjacent sections stained with HE confirmed that positive cells resembled macrophage-like cells, which had large oval nuclei and abundant cytoplasm. A strong hybridization signal of IL-6 was detected in lymphocytes in the peribronchiolar lymphoid hyperplastic tissues (Fig. 4) whereas no hybridization signal of IL-1 and TNF- α was detected in lymphocytes in these areas. Expression of inflammatory cytokines was minimal in non-lesional lung of *M. hyopneu-*

Table 1. *In situ* hybridization for interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α) and IL-6 in 10 pigs naturally infected with *Mycoplasma hyopneumoniae*

Pig No.	Age (days)	Morphometric analysis ^{a)}			Histopathological lesion score ^{b)}
		IL-1	TNF- α	IL-6	
1	24	20	15	16	12
2	30	33	42	17	10
3	35	17	28	32	7
4	40	24	26	33	7
5	45	37	17	29	10
6	50	40	47	52	13
7	65	24	27	21	7
8	75	15	18	31	6
9	95	40	42	33	8
10	120	34	33	19	7

a) Number of signal positive cells/0.95 mm².

b) From 0 (no lesion) to 3 (severe lesion). Total scores of 3 sections.

Fig. 1. A section of lung from a naturally infected pig No. 5, showing interleukin-1 nucleic acid in the cells of alveolar spaces. *In situ* hybridization, methyl green counterstain. $\times 200$.

moniae-infected pigs and in normal lung from control pigs.

No hybridization signals were consistently seen in consecutive tissue sections with a solution of RNase A prior to the performance of *in situ* hybridization. Sections from positive control showed hybridization signal for IL-1, TNF- α and IL-6, and hybridization signal was not detected in lung sections from negative control pigs.

Statistical analysis: Although statistically non-significant, a number of potentially important correlations were observed between IL-1 and histopathological lesion score. IL-1 ($r=0.5744$, $p=0.0883$) showed potentially important correlation with histopathological lesions. No other potentially clinically important correlations ($r>0.30$) were observed between any of the other cytokines (TNF- α ; $r=0.2045$, $p=0.5603$ and IL-6; $r=-0.06607$, $p=0.8651$) and histopathological lesion score.

Fig. 2. A section of lung from a naturally infected pig No. 6, showing tumor necrosis factor- α nucleic acid in the macrophages. *In situ* hybridization, methyl green counterstain. $\times 400$.

Fig. 3. A section of lung from a naturally infected pig No. 9, showing SWC3a positive signals in the macrophages. Immunohistochemistry. $\times 400$.

DISCUSSION

The technique of non-radioactive *in situ* hybridization with digoxigenin-labeled oligonucleotide probes was successfully applied to *M. hyopneumoniae*-infected lung tissue to detect inflammatory cytokines and to identify the sites at which they were expressed. Intense and consistent expression of IL-1, TNF- α and IL-6 was demonstrated in lesions of pneumonia caused by *M. hyopneumoniae*. In contrast, expression of inflammatory cytokines was minimal in non-lesional lung of infected pigs and in normal lung from control pigs. The results strongly suggest that cytokines are important mediators in the pathophysiology of pneumonia.

The results of this study demonstrated potentially relationships between IL-1 expression and histopathological lesion score in pneumonia from pigs naturally infected with

Fig. 4. A section of lung from a naturally infected pig No. 10, showing interleukin-6 nucleic acid in the peribronchial lymphoid tissues. *In situ* hybridization, methyl green counterstain. $\times 200$.

M. hyopneumoniae. Although intense and consistent expression of IL-1, TNF- α and IL-6 was detected in lesions of pneumonia caused by *M. hyopneumoniae*, it is not possible to explain that no other potentially clinically important correlations were observed between any of the other cytokines and histopathological lesion score. Because of natural infection of *M. hyopneumoniae*, it could be due to different strains of *M. hyopneumoniae* infection or to a difference in the duration of infection. Further studies on expression of cytokines are needed.

IL-6 has been recognized for its effect on differentiation of B cells [19]. In this study, expression of IL-6 was detected mainly in the peribronchiolar hyperplastic lymphoid tissues. The B and T lymphocytes infiltrating in the hyperplastic tissues represent a significant source of IL-6 in response to *M. hyopneumoniae* infection. Since hybridization signals for *M. hyopneumoniae* were not seen in the peribronchiolar lymphoid hyperplastic tissues [11], *M. hyopneumoniae* probably induce the secretion of IL-6 from lymphocytes in peribronchiolar lymphoid hyperplastic tissues indirectly. Increased IL-6 levels were detected in bronchoalveolar lavage fluids of *M. hyopneumoniae* infected pigs [2]. Numbers of IgA and IgG producing cells were also increased in the lungs of *M. hyopneumoniae* infected pigs [15]. IL-6 might play a role in the *M. hyopneumoniae* specific antibody responses via promoting the terminal differentiation of B cells into Ig-secreting plasma cells.

While our study have shown that *M. hyopneumoniae* can induce TNF- α release, the identification of a specific inducer remains elusive. Identification of the masses of the molecules responsible for TNF- α induction should help in ultimately identifying and characterizing the molecules responsible. *M. hyopneumoniae* is an important pathogen in swine, in which it retards the growth rate and causes enzootic pneumonia [13]. The retardation of growth may be due to *M. hyopneumoniae*'s inducing high levels of TNF- α ,

which is known to cause cachexia or wasting syndrome [3]. A 48 kd protein from the membrane of *M. hyorhinis* induces the secretion of TNF- α from human monocytes [10]. Thus, by identifying the proteins responsible for the induction of TNF- α release, it is possible to test whether they are indeed responsible for the retardation of growth rate.

The technique of non-radioactive *in situ* hybridization with digoxigenin-labeled oligonucleotide probes was successfully applied to *M. hyopneumoniae*-infected lung tissue to detect inflammatory cytokines and to identify the sites at which they were expressed. All of the inflammatory cytokines are expressed in response to *M. hyopneumoniae* infection, with IL-6 localized primarily to peribronchiolar lymphoid hyperplastic tissues, and both IL-1 and TNF- α expressed in alveolar macrophages. Since the expression of these three cytokines is associated with areas of inflammatory lesion, *M. hyopneumoniae* induced the expression of the inflammatory cytokines and inflammatory lesions.

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