Studies on Total Immune Response in Airway of Pigs Infected with Mycoplasma hyopneumoniae

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(豚マイコプラズマ肺炎における気道の局所免疫応答に関する研究)

Tetsuo Asai

CONTENTS

Chapter 3

ABBREVIATIONS

AM: alveolar macrophages
ATCC: American Type Culture Collection
BALF: bronchoalveolar lavage fluid
BHL medium: Brucella Hank's Lactoalbumin medium
CCU: color changing units
CL: chemiluminescence
EDTA: ethylendiaminetetraacetic acid
ELISA: enzyme-linked immuno-sorbent assay
FBS: fetal bovine serum
GL: gross lesions
HBSS: Hank' s balanced salt solution
HLT: hyperplasia of lymphoid tissue
Ig: immunogloblin
IL: interleukin
LPS: lipopolysaccharide
2-ME: 2-mercaptethanol
MPS: mycoplasmal pneumonia of swine
MTT: 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
OPZ: opsonized zymozan
PBS: phosphate-buffered saline
PGE2: prostaglandin E2
PI: postinoculation
PMN: polymorphonuclear neutrophils
r-: recombinant
rh-: recombinant human
RPMI: Roswell Park Memorial Institute
SPF: specific pathogen free
TNF-α: tumor necrosis factor alpha

PREFACE

Mycoplasma (M.) hyopneumoniae is the causative agent of mycoplasmal pneumonia of swine (MPS) and is widely spread throughout the world (55). Pulmonary lesions caused by *M. hyopneumoniae* are characterized by the infiltration of inflammatory cells into the alveoli and bronchi, lymphocytes accumulation in perivascular and peribronchiolar areas and hyperplasia of lymphoid tissue (HLT) around airways (5, 55). In the pathological processes of mycoplasmal pneumonia, the immunological response of the host against *M. hyopneumoniae* infection would play an important role (1, 55).

Cell-mediated immune responses have been demonstrated on mycoplasma infection in man and various animals (1, 55, 56). Previous reports have shown the systemic activation of lymphocytes and the skin reaction by the delayed-type hypersensitivity in pigs infected with *M. hyopneumoniae* (1, 41). Suppression of T cell function by thymectomy or treatment with anti-tymocyte sera decreased the extent of mycoplasamal pneumonic lesions such as lymphocytes accumulation around airways and blood vessels in mice (17), hamster (62) and swine (61). However, suppression of T cell function induced severe pathogenicity of mycoplasmas against various animals. Mycoplasmas were recovered from blood, brain, spleen and liver in immune suppressive mice with higher titer than controls

(17). Mice treated with cyclophosphamide, which is the drug that affects the T cell functions, increased the mortality in the *M. pulmonis* infection (58).

Earlier investigations have demonstrated that certain mycoplasmas modulate the induction of cytokines such as tumor necrosis factor alpha (TNF-α) (3, 7, 23, 38, 60) and interleukin-1 (IL-1) (6, 16) from macrophages and lymphoid cells. Mühlradt and co-workers (45, 53) reported that products of *M. fermentans* induced interleukin-6 (IL-6) and cytotoxic T cells. TNF-α and IL-1 are major cytokines produced by mononuclear phagocytes (18, 21, 49). Though these cytokines are biochemically and immunologically distinct proteins, their biological activities were similar to each other (18, 37). They play a critical role in the induction of inflammatory and immune responses causing activation of T cells, B cells and fibroblasts (18, 49). IL-6 is one of the B cell stimulatory factors, induces the final differentiation of B cells into antibody-producing cells (32). In addition to IL-6, the growth and differentiation of B cells require the other cytokines ; IL-4 for the activation of resting B cells and IL-5 for the growth of the activated B cells. IL-6 also has an important role in T cell activation and induction of acute phase protein to inflammation or tissue injury (32). Prostaglandin E2 (PGE2) also plays a role in the regulation of immune responses (25, 34). PGE2 suppresses the various cell function and acts as inhibitor of inflammatory response such as T cell proliferation and cytokine production (25, 34, 36). TNF-α and IL-1 stimulate the production of IL-6 (18, 21, 49) and PGE2 (11, 14, 15) from several kinds of cells. On the other hand, IL-6 and PGE2

suppress the production of TNF-α and IL-1 by negative feed-back (19, 34). Thus the production and functions of cytokines are regulated by each other.

On the other hand, most of the field cases of MPS occur as a mixed infection with *Pasteurella multocida*, *Haemophilus parasuis* or *Actinobacillus pleuropneumoniae* (27, 66). Earlier investigations have shown that experimental infection by *P. multocida* or *A. pleuropneumoniae* is exacerbated if animals are also infected with *M. hyopneumoniae* (55). It is well known that phagocytic cells play an important role in the host defense system against bacterial and fungal infection (40). Macrophages or polymorphonuclear neutrophils (PMN) infiltrate tissues invaded by pathogens and try to kill the organisms by phagocytosis and subsequent production of active oxygen-derived metabolites (40). Thomas et al. (63) reported that the chemiluminescence (CL) response of bovine neutrophils was suppressed by adhesion of *M. bovis* to the cell surface. Almeida et al. (2) have shown that the capsule of *M. dispar* had an inhibitory effect on the phagocytic activity of bovine alveolar macrophages. Caruso and Ross (12) have reported that phagocytosis by alveolar macrophages was suppressed by *M. hyopneumoniae* infection. These reports show that these phenomena might be related to the functional suppression of macrophages or PMN by mycoplasma.

Cytokines and PGE2 have been suggested as mediators of pathophysiologic event in various inflammatory disorders (18, 21, 25, 49). In the experimental pneumonia by *Chlamydia trachomatis,* the increased

activity of TNF-α, IL-1 and IL-6 were observed in lung tissues of mice (39, 64). In *Legionella pneumophila*-infected mice, TNF-α was detected in the lung lavage fluids and not in the sera (10). In the patients of the adult respiratory distress syndrome (13, 30) and sarcoidosis (29, 46), increased levels of TNF-α and IL-1 have been shown in airways. The increased activity of the cells producing PGE2 has observed in *Mycobacterium avium-intracellulare*-infected human (52). Thus, these inflammatory mediator have increased in pulmonary disorders, but their production appeared to be confined to the site of disorder in the adult respiratory distress syndrome (30) and sarcoidosis (46) as well as *Legionella pneumophila* infection (10). Local humoral immune responses in the respiratory tracts were demonstrated in various animals with mycoplasma infection (22, 41). Messier et al. (41) have reported the local activation of lymphocytes in bronchial lymph node at an early stage of *M. hyopneumoniae* infection. The increased number and percentage of B cells in bronchi and alveoli and the increased number of immunogloblin (Ig) G- and IgA- producing cells in perivascular and peribronchiolar areas have also been reported in *M. hyopneumoniae* infection (41). This study was carried out to demonstrate the relationship between cytokine levels in the bronchoalveolar lavage fluids (BALF) and the development of pulmonary lesions in mycoplasmal pneumonia as described in the Chapter 1. Then, as described in the Chapter 2, the kinetics of cytokine production in BALF were conducted after infection with *M. hyopneumoniae*. Furthermore, the author shows that the

BALF from *M. hyopneumoniae*-infected pigs suppressed the function of PMN and this suppression was correlated to the PGE2 levels in the BALF in the Chapter 3.

Chapter 1

Detection of inflammatory cytokines in bronchoalveolar lavage fluids from pigs infected with *Mycoplasma hyopneumoniae*

Introduction

MPS is a chronic inflammatory disease characterized by accumulations of neutrophils around airways and in alveoli, infiltration of lymphocytes around airways and blood vessels and HLT around airways (5, 55). Various cytokines appear to play an important role in the pathological processes of inflammatory lesions in animals (10, 64) and human beings (29, 30, 65). Earlier *in vitro* investigations have shown that some mycoplasmas modulate the induction of cytokines such as TNF-α (3, 7, 60), IL-1 (6, 16) and IL-6 (45, 53) from macrophages and lymphoid cells. The immunological response of the host against *M. hyopneumoniae* infection has been investigated mainly in terms of antibody response (41-43, 59) and lymphocytes activation (1, 41, 61). However, little is known regarding the roles of the cytokines induced by mycoplasma infection *in vivo*.

The author examined the levels of TNF-α, IL-1 and IL-6 in the BALF of *M. hyopneumoniae*-infected pigs after 4 weeks postinoculation (PI) and the relationship between cytokine levels in the BALF and the development of pulmonary lesions in mycoplasmal pneumonia.

Materials and Methods

M. hyopneumoniae

M. hyopneumoniae strain E-1 (42), passaged in pigs four times and grown in Brucella Hank's Lactoalbumin (BHL) medium (66) at 37℃ for 3 days, was used to bring about experimental infection. The BHL medium consisted of basic broth [5.8 g of Brucella broth (Gibco, Grand Island, N. Y.), 2.0 g of Lactoalbumin hydrolysate, 500 ml of Hank's solusion and 250 ml of distilled water], containing 200 ml of heat inactivated specific pathogen free (SPF) pig serum, 50 ml of 25% extract of bakers' yeast and 1.0 g of aminobenzilpenicillin.

Infection of pigs

Nineteen, 8 week-old, primary SPF pigs, kept in a barrier-maintained room, were inoculated intranasally with 1×10^7 color changing units (CCU) of *M. hyopneumoniae* strain E-1 and kept in the same room for 4 weeks PI. After necropsy, 20 ml of phosphate-buffered saline (PBS) were infused into the bronchus and BALF was collected. BALFs were stored at -20 ℃ until use.

Pathological examination

To determine the extent of gross pneumonia lesions, the abdominal and dorsal sides of lung were photographed, and the ratio of gross lesions

(GL) was calculated using a image processor (Nexus, 600, Tokyo, Japan). Individual pigs with 3% or more gross lung lesions were considered positive. Histopathological examination was performed according to routine procedures. In brief, the lungs were fixed in 10% neutral phosphate-buffered formalin. Tissue slices were dehydrated and embedded in paraffin wax, sectioned at 4 μm and stained with hematoxilin-eosin.

Preparation of culture supernatant

Alveolar macrophages (AM) were obtained from lungs of 2 SPF pigs by bronchoalveolar lavage as above-mentioned. Lavage fruids were filtrated through sterile gauze and AM was collected by centrifugation at 250 × g for 5 min. After three times washing with PBS, the AM were resuspended at 2×10^6/ml in Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% heat-inactivated fetal bovine serum (FBS) supplemented with 1 mM L-glutamine, 24 mM HEPES, 50 μM 2-mercaptethanol (2-ME) and antibiotics. After incubation at 37℃ in a humidified, 5% CO_2 atmosphere for 24 h, AM were cultured in culture medium with 10 μg of lipopolysaccharide (LPS) from *Escherichia coli* serotype O55, B5 (Sigma Chemical Co. USA). After incubation for 24 h, the culture fluids were harvested and centrifuged at 300 × g for 10 min. The supernatants were stored at -20℃ until assayed for cytokine activities.

Bioassay for TNF-α

TNF-α activity was determined with a cytotoxic assay using WEHI-164 (American Type Culture Collection (ATCC)) clone 28-4 cells, mouse fibrosarcoma cells, in the presence of actinomycin D (45). In brief, WEHI-164 cells were cultured in RPMI 1640 medium containing 10% FBS, supplemented with 1 mM L-glutamine, 24 mM HEPES, 1 mM sodium pyruvate and antibiotics at 37℃ in a 5% CO_2 incubator. The WEHI-164 cells (3×10^4) were added to the wells of 96-well microtiter plates containing serially two-fold-diluted BALF samples in the presence of 1 μg/ml actinomycin D. After incubation at 37℃ for 18 h, surviving cells were determined using the tetrazolium salt (MTT) method (26, 44). Twenty-five μl of 5 mg/ml MTT in PBS were added to each well and the plate was incubated at 37℃ for 2 h. After the addition of 100 μl of extraction buffer (12.5% SDS, 50% dimethylfolmamide and pH 4.7), the plate was incubated for overnight at 37℃. Then the color development was measured at 570 nm using enzyme-linked immuno-sorbent assay (ELISA) reader. When 50% or more cells were lysed, cytolitic activity was deemed positive and TNF-α concentrations were determined from the standard curve with recombinant human (rh-) TNF-α (Bachem, Torrance, CA, USA). Identification of TNF-α in tested samples was determined by the neutralization test with rabbit anti human TNF-α serum (Genzyme, Cambridge, MA, USA), because human TNF-α has 85% of protein homology to porcine TNF-α (50). Serial two-fold-dilutions of BALF samples were prepared and equal volume of fifty-fold-

diluted rabbit anti human TNF-α serum was added to each well. After reaction for 30 min at room temperature, TNF assay was performed.

Bioassays for IL-1 activity

IL-1 activity was determined in terms of ablity to stimulate proliferation of D10.G4.1 cells (ATCC), mouse helper T cell clone, in the presence of 2.5 μg/ml concanavalin A (Con A), according to the ATCC procedure. D10.G4.1 cells were cultured in RPMI 1640 medium containing 10% FBS, 10 % rat growth factor, 1 mM L-glutamine, 24 mM HEPES, 50 μM 2-ME and antibiotics. Serial two-fold dilutions of BALF samples were prepared in RPMI 1640 containing 10% FBS, 1 mM L-glutamine, 24 mM HEPES, 50 μM 2-ME and antibiotics. D10.G4.1 cells (2×10^4) were mixed with serially diluted samples in wells of the microtiter plates. After incubation for 72 h at 37℃, cell proliferation was determined by MTT assay using rh-IL-1 α (Genzyme) as standards.

Bioassays for IL-6 activity

IL-6 assay was performed in 96-well tissue culture plates using the 7TD1 (ATCC) hybridoma proliferation assay (67). 7TD1 cells were cultured in RPMI 1640 medium containing 10% FBS and 10 IU/ml rh-IL-6 (Genzyme), 1 mM L-glutamine, 24 mM HEPES, 50 μM 2-ME and antibiotics. Serial two-fold dilutions of BALF samples were prepared in RPMI 1640 containing 10% FBS 1 mM L-glutamine, 24 mM HEPES, 50 μM 2-ME and

antibiotics. 7TD1 cells (1×10^4) were added to each well and incubated for 72 h at 37℃ in a 5% CO_2 incubator. After incubation, MTT assays were performed. The concentrations of IL-6 were calculated from a standard curve, which was constructed by the use of rh-IL-6.

Statistical analysis

Statistical analysis was conducted according to the Student's *t*-test.

Results

The 30 to 50% of infused PBS were recovered by the bronchoalveolar lavage, and cytokine activity in BALFs from nineteen infected pigs was assayed. Various pneumonic lesions were recognized in the pigs. The infiltration of lymphocytes in perivascular and peribronchiolar areas by *M. hyopneumoniae* infection was commonly recognized in all infected pigs. Thirteen individual pigs with GL were positive for the infiltration of inflammatory cells, such as macrophages and polymorphonuclear cells, into alveoli and bronchi. HLT was found in thirteen of nineteen pigs. The uninoculated control pigs had no pneumonic lesion. The author classified the pneumonic lesions into two types and evaluated the relationship between cytokine levels and the development of pneumonic lesions.

In a preliminary experiment, the author confirmed the cytotoxic activity of rh-TNF-α and culture supernatant of porcine AM stimulated by LPS on WEHI-164 cells (Figures 1 and 2). The cytotoxic activity in culture supernatant was inhibited by anti human TNF-α serum (Figure 2). The proliferative responses of D10. G4. 1 cells to rh-IL-1 and the culture supernatant were determined (Figures 3 and 4). The proliferative responses of 7TD1 cells to r-IL-6 and the culture supernatant were shown in Figures 5 and 6. These results indicate that these cell lines can be used for bioassay of porcine cytokines. The concentrations of porcine cytokines were calculated from the standard curves.

TNF-α activity was detected in ten pigs at a concentration range from 3 to 48 pg/ml. Thirteen pigs with GL had higher geometric mean levels of TNF-α (7.4 pg/ml) in BALF than six pigs without GL and uninfected pigs (<3 pg/ml)(Table 1). Mean TNF-α levels (8.0 pg/ml) in the pigs with HLT in peribronchiolar areas were higher than those (<3 pg/ml) without HLT and controls ($p<0.05$).

IL-1 activity was detected in ten pigs at a concentration range from 16 to 1,024 pg/ml (Table 2). IL-1 (29.5 pg/ml) was significantly increased in the group of pigs with GL compared to those without GL ($p<0.01$) and uninfected pigs($p<0.05$). IL-1 in BALF was significantly ($p<0.05$) elevated in pigs with HLT (25.9 pg/ml).

IL-6 activity was detected on eleven pigs at a concentration range from 200 to 6,400 pg/ml (Table 3). Increased geometric mean levels of IL-6 (1,055.6 pg/ml) were observed in the pigs with GL compared to those without GL (200.0 pg/ml) and uninfected pigs (<100 pg/ml)($P<0.05$). Increased geometric mean levels of IL-6 (1,345.4 pg/ml) were observed in the pigs with HLT compared to those without HLT (123.1 pg/ml)($p<0.01$) and to uninfected pigs (<100 pg/ml)($P<0.01$).

Discussion

The presence of cytokines in microbial pulmonary infection has been reported (10, 39, 64, 65). IL-1 levels in BALF from children with bacterial pulmonary infection are higher than in those from children without infection (65). TNF-α, IL-1 and IL-6 in the lung have been reported in mice infected with *Chlamydia trachomatis* (39, 64). The presence of TNF-α, IL-1 and IL-6 was demonstrated in BALF from pigs experimentally infected with *M. hyopneumoniae*. The author demonstrates that, for the first time, TNF-α, IL-1 and IL-6 levels in BALF are increased in mycoplasmal pneumonia in pigs.

BALF is a complex mixture of cellular products, soluble receptors, small molecules and other components. Small molecules such as prostaglandins interfere with the measurement of the proliferation of responder cells such as D10.G4.1. Thus, additional treatment may be required to remove small contaminating molecules. In this study, the specificity of porcine TNF-α was identified using anti human TNF-α serum, since a previous report has revealed that anti human TNF-α serum is cross reactive with porcine TNF-α (4). Though the proliferation of D10. G4. 1 cells the cells was dependent on IL-1 and IL-2 (31), D10.G4.1 cells appear to express IL-2 receptors low to negative on the cell surfaces when co-cultured with mitomycin C inactivated syngenic mouse spleen cells and conalbumin (ATCC information). D10. G4. 1 cells with low expression of IL-2 receptors

were prepared for the IL-1 bioassay in this study. Porcine IL-6 was detected using 7TD1 cells. 7TD1 cells have been used to determine IL-6 activity in pig serum from experimental pneumococcal septicemia (67). Since recombinant porcine TNF-α, IL-1 and IL-6 were not available, rh-TNF-α, IL-1 and IL-6 were purchased and used in the quantitative assay for cytokines as standards.

TNF-α, IL-1 and IL-6 in BALF were elevated when GL were present and HLT was demonstrated histopathologically. The mean levels of TNF-α, IL-1 and IL-6 in BALF of pigs having pneumonitis with HLT were significantly elevated ($P<0.05$) (Tables 1-3). The mean IL-1 level in BALF of the pigs with GL was significantly higher than those in pigs without lesions ($P<0.01$). The presence of TNF-α, IL-1 and IL-6 were correlated significantly with the development of pneumonic lesions. Tajima et al. (61) have reported that the extent of pneumonic lesions in *M. hyopneumoniae*-infection can be decreased by thymectomy and treatment with antithymocyte serum. Messier et al. (41) have reported that numbers of IgA- and IgG-producing cells increased in the lungs of *M. hyopneumoniae*-infected pigs. TNF-α and IL-1 are multifunctional cytokines and known to be activators of T lymphocytes (37, 49). IL-6 is known to promote T cell activation and B cell differentiation (32). In the present study, significantly increased levels of TNF-α, IL-1 and IL-6 in BALF were observed in the *M. hyopneumoniae*-infected pigs with GL and/or HLT. The level of these cytokines might be

related to the development of pneumonic lesions in *M. hyopneumoniae* infection.

Summary

The author examined the levels of TNF-α, IL-1 and IL-6 in BALF from pigs experimentally infected with *Mycoplasma hyopneumoniae* using WEHI-164 cells, D10.G4.1 cells and 7TD1 cells, respectively. Increased levels of TNF-α, IL-1 and IL-6 in BALF were found in infected pigs with gross and/or microscopic lesions. The presence of these cytokines in BALF would thus appear to be associated with the development of pneumonic lesions in *M. hyopneumoniae* infected pigs.

Legends to Figures

Figure 1. Cytotoxic activity of recombinant human TNF-α on WEHI164 cells. Values represent the mean from duplicate wells.

Figure 2. Cytotoxic activity of porcine TNF-α on WEHI164 cells and neutralization of cytotoxic activity with anti-human TNF serum. Symbols: Cytotoxic activity of No.1 with (◆) or without (◇) anti-human TNF serum, and No.2 with (●) or without (○) anti-human TNF serum. Values represent the mean from duplicate wells.

Figure 3. Proliferative response of D10.G4.1 cells to recombinant human IL-1. Values represent the mean from duplicate wells.

Figure 4. Proliferative response of D10.G4.1 to porcine IL-1. Symbols: Proliferative activity of No.1 (◆) and No.2 (●). Values represent the mean from duplicate wells.

Figure 5. Proliferative response of 7TD1 cells to human recombinant IL-6. Values represent the mean from duplicate wells.

Figure 6. Proliferative response of 7TD1 cells to porcine IL-6. Symbols are as described in the legend to Figure 4. Values represent the mean from duplicate wells.

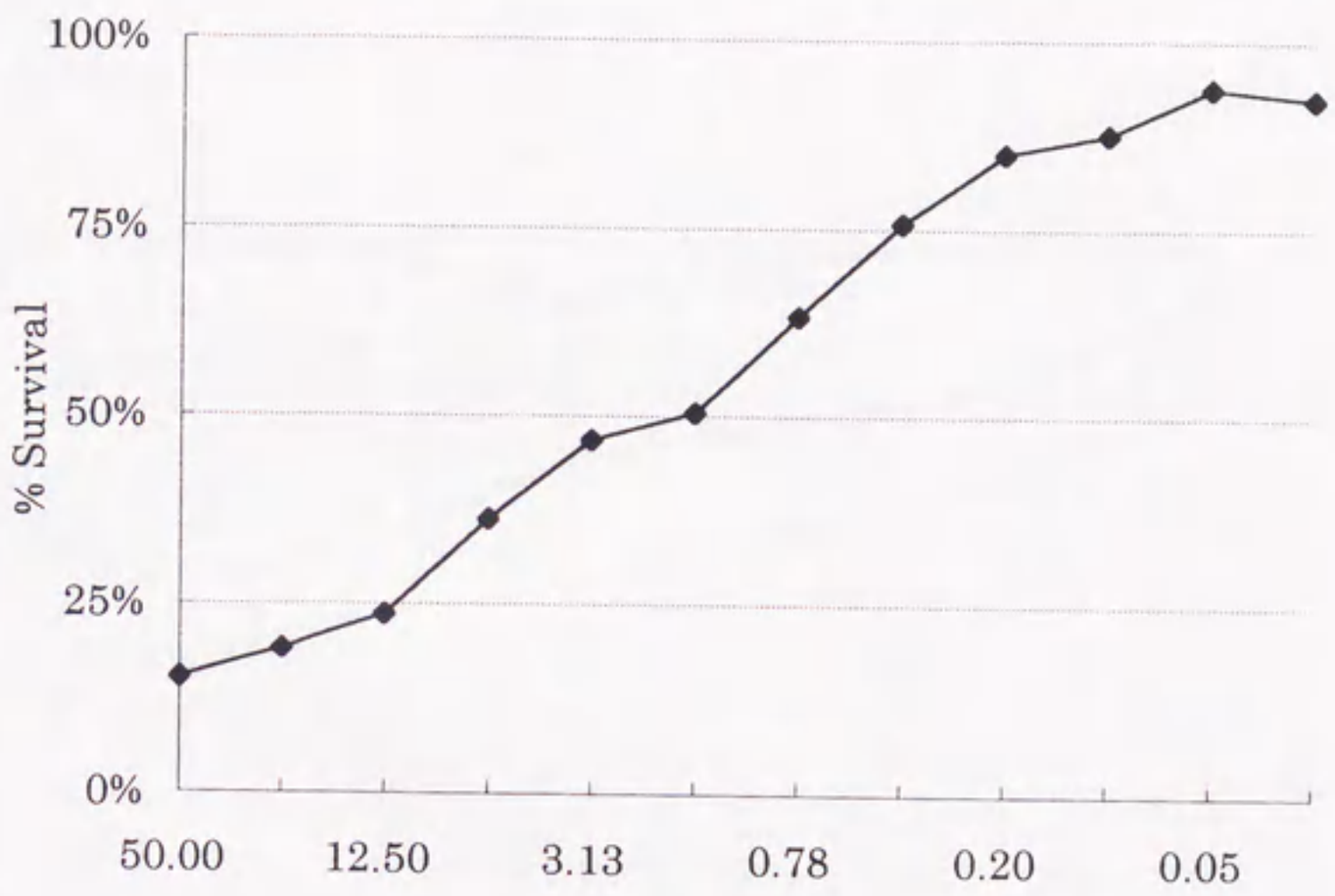

100%
75%
50%
25%
0%
% Survival
50.00
12.50
3.13
0.78
0.20
0.05
Recombinant human TNF (pg/ml)

Figure 1

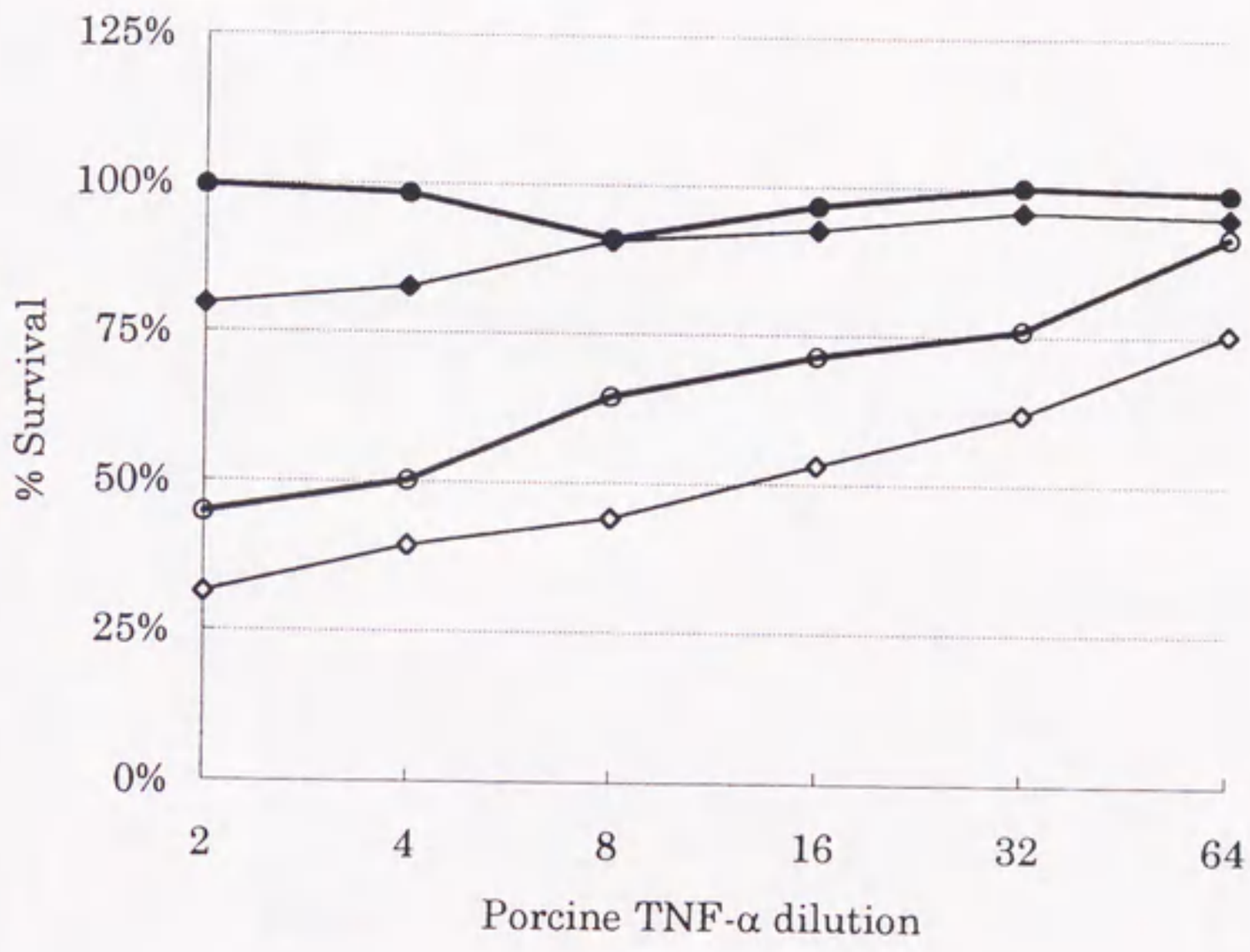


Figure 2

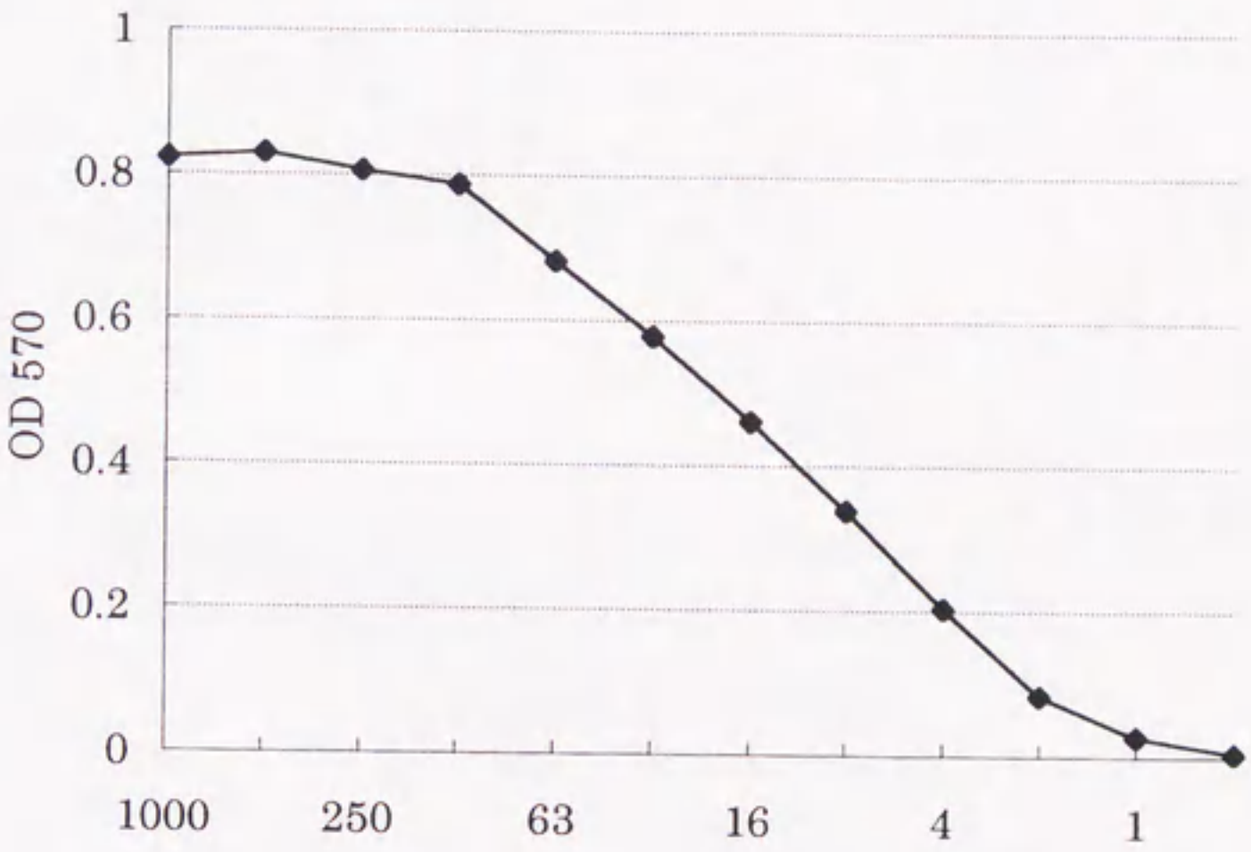

1
0.8
0.6
0.4
0.2
0
OD 570
1000
250
63
16
4
1
Recombinant human IL-1(pg/ml)

Figure 3

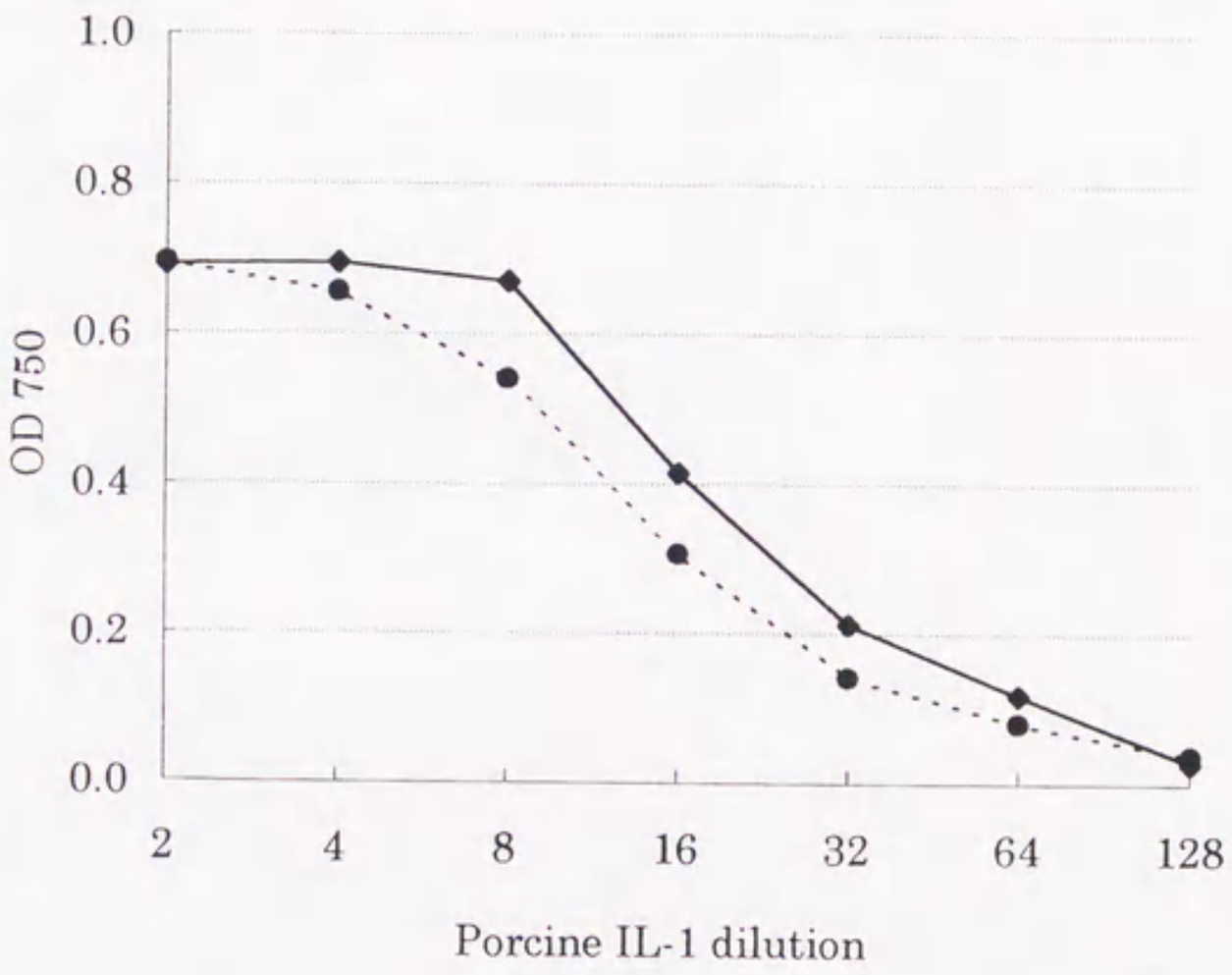


Figure 4

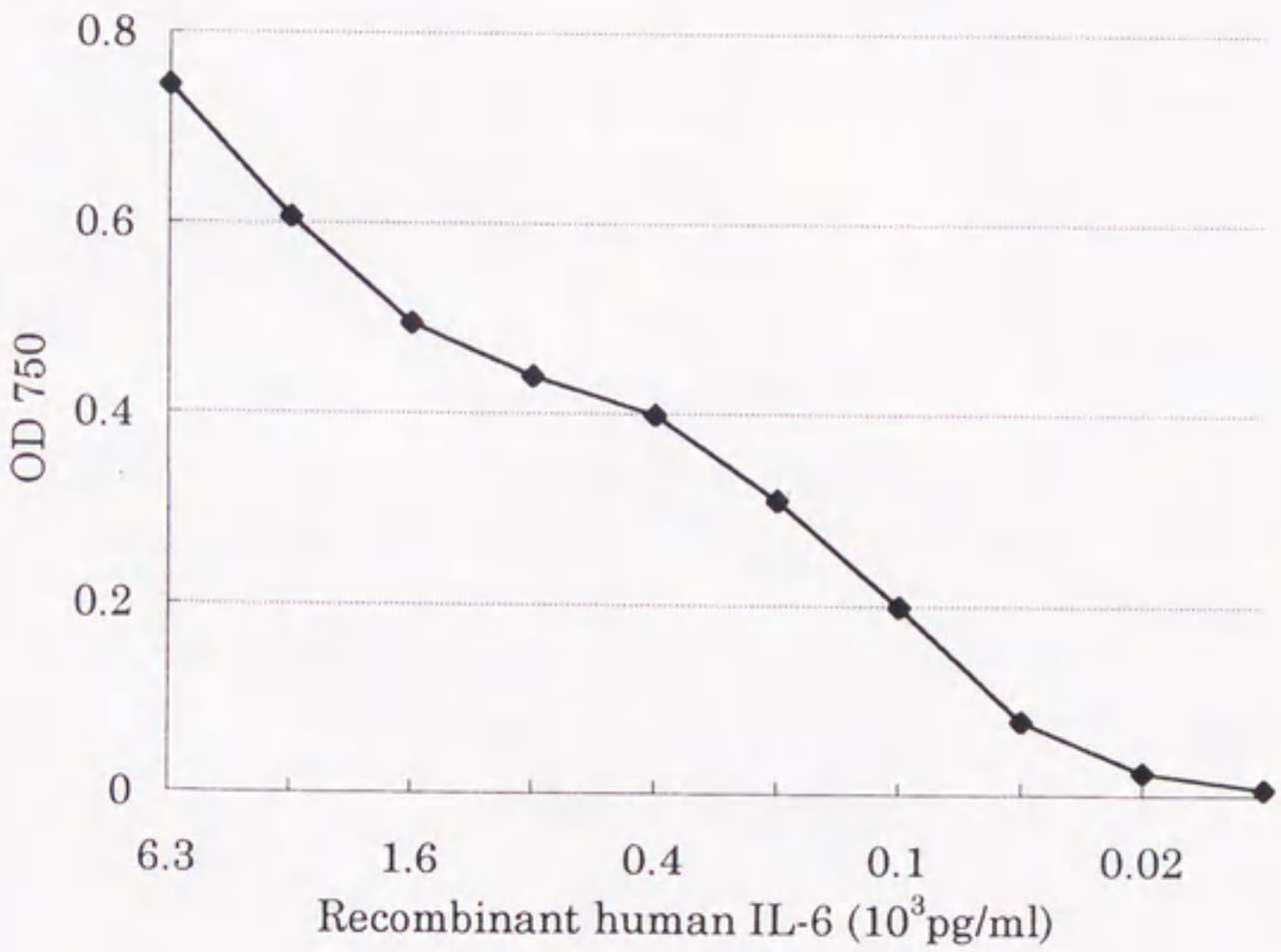

0.8
0.6
0.4
0.2
0
OD 750
6.3
1.6
0.4
0.1
0.02
Recombinant human IL-6 (10^3pg/ml)

Figure 5

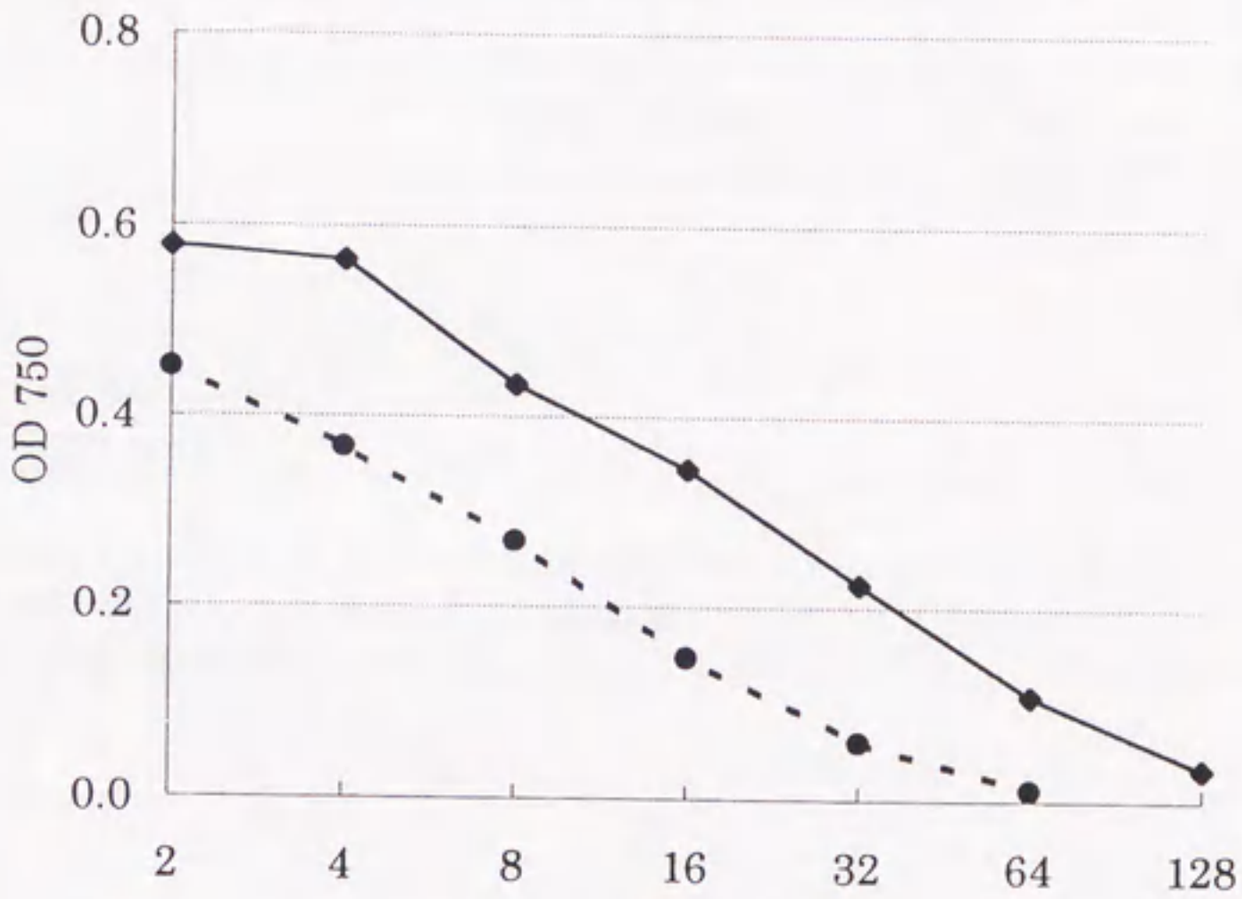


Figure 6

Table 1. TNF-α in BALF from *M. hyopheumoniae*-infected pigs with and without lung lesions at 4 weeks postinoculation

Lung lesion	Number of pigs with bioactivity						Geometric mean
	<3[1]	3	6	12	24	48	
Controls (n=4)	4						<3[1]
Gross lesion negative[2] (n=6)	5		1				<3
Gross lesion positive (n=13)	4	2	2		3	2	7.4
Not observed HLT[3] (n=6)	6						<3
Observed HLT (n=13)	3	2	3		3	2	8.0*[4]

1 pg/ml.
2 Individual pigs with 3% or more gross lesions were deemed positive.
3 Lymphocyte infiltration with hyperplasia of lymphoid tissue.
4 *: $p<0.05$ compared with control group and not observed HLT group.

Table 2. IL-1 activity in BALF from *M. hyopheumoniae*-infected pigs with and without lung lesions at 4 weeks postinoculation

Lung lesion	Number of pigs with bioactivity										Geometric
	<4[1]	4	8	16	32	64	128	256	512	1,024	mean
Controls (n=4)	4										<4[1]
Gross lesion negative (n=6)	6										<4
Gross lesion positive (n=13)	3			3	2	2	1	1		1	29.5*[2]
Not observed HLT (n=6)	5			1							<4
Observed HLT (n=13)	4			2	2	2	1	1		1	25.9**

1 pg/ml.

2 *: $p \leq 0.05$ compared with control group and $p < 0.01$ with gross lesion negative group.
**: $p < 0.05$ compared with control group and not observed HLT group.

ble 3. IL-6 activity in BALF from *M. hyopheumoniae*-infected pigs with and without lung lesions at 4 weeks postinoculation

Lung lesion	Number of pigs with bioactivity							Geometric
	<100[1]	100	200	400	800	1,600	3,200	6,400	mean
Controls (n=4)	4								<100[1]
Gross lesion negative (n=5)	2		1	1	1				200.0
Gross lesion positive (n=10)	2			2	1		2	3	1055.6*[2]
Not observed HLT (n=5)	3		1	1					123.1
Observed HLT (n=10)	1			2	2		2	3	1345.4**

pg/ml.

*: $p<0.05$ compared with control group.

**: $p<0.01$ compared with control group and not observed HLT group.

Chapter 2

Time course study of inflammatory cytokines in bronchoalveolar lavage fluids from pigs infected with *Mycoplasma hyopneumoniae*

Introduction

Inflammation is the local or systemic response of host against a various kind of invasion (40). Infiltration of neutrophils is the first pathological response in the site of inflammation and various cytokines participate in activation of the neutrophils (18, 40). It has been shown that TNF-α, IL-1 and IL-6 were detected in BALF from pigs infected with *M. hyopneumoniae* as defined in the Chapter 1. The gene expression of TNF-α and interferon-γ in lung tissues have been observed in mice experimentally infected with *M. pulmonis* (47). These reports also indicate the relationship between these cytokines released from lung tissues and the pathological process of pulmonary lesions. Thus, cytokines are probably essential mediators in the pathogenesis of mycoplasmal pneumonia.

The production and functions of cytokines are regulated by each other. TNF-α and IL-1 stimulate the production of IL-6 by several kinds of cells (18, 49). On the other hand, IL-6 suppress the production of TNF-α and IL-1 by negative feed-back (19, 34). In the Chapter 1, the author showed that the levels of TNF-α, IL-1 and IL-6 in the BALF were increased in mycoplasmal pneumonia in pigs. However, little is known about the kinetics of these cytokine production in BALF in *M. hyopneumoniae*-infected pigs. In this chapter, the author examined the kinetics of TNF-α, IL-1 and IL-6 production in BALF of pigs infected with *M. hyopneumoniae*, and the

relationship between these cytokines levels in BALF and the infiltration of the cells into the airways.

Materials and Methods

Infection of pigs

Experimental infection of pigs with strain E-1 of *M. hyopneumoniae* (42) were performed as described in the Chapter 1. Five of nine 8-week-old primary SPF pigs were inoculated with *M. hyopneumoniae* and the remaining four SPF pigs served as the uninoculated controls. These two groups of pigs were housed separately in barrier-maintained rooms for 4 weeks after inoculation.

Preparation of BALF

BALF was collected from each of five infected pigs at weekly intervals and from control pigs at two week intervals using fiber-optic bronchoscopes (BF type P20, Olympus, Tokyo, Japan). In brief, each pig was anesthetized with an intravenous injection of 30 mg/kg pentobarbital after being preanesthetized by an intramuscular injection of 4 mg/kg azaperone and 0.03 mg/kg atropine. The bronchoscope was inserted into the bronchus. With the tip of bronchoscope wedged in the bronchus of right frontal pulmonary lobe, 30 ml of PBS were infused and BALF was collected. After centrifugation at 250 × g for 5 min, the cell pellet was used for cytological examination. The supernatant was filtrated with a 0.20 μm filter and the filtrates were stored at -20℃ until used for TNF-α, IL-1 and IL-6 assays. At

4 weeks post-inoculation (PI), the pigs were killed for pathological evaluation and collection of BALFs.

Bioassay for TNF-α, IL-1 and IL-6.

Bioassay for these cytokines were performed as described in the Chapter 1.

Cytological evaluation of BALF.

Cell differentials were performed by observing cytocentrifuged slide prepared at 100 × g for 5 min (Cytospine 2, Shandon, England) and stained with modified Wright-Giemsa-stained (Diff-quiq, Baxter Healthacre Corp., Chicago, IL). Two hundreds cells or more from a representative area of preparation were examined. The results were expressed as percentage of the total number of cells.

Statistical analysis

Statistical analysis was conducted according to the Student's *t*-test.

Results

The 65 to 80% of infused PBS were recovered by using fiber-optic bronchoscopes. All five inoculated pigs had GL on 4 weeks PI. The results of a time-course study of cytological changes in BALFs from five infected pigs are shown in Table 4. The percentage of macrophages in BALF was 97.4 and 96.8% before inoculation and on one week PI, respectively. The decreased percentage of macrophages and increased percentages of neutrophils and lymphocytes were observed at 2, 3 and 4 weeks PI.

TNF-α, IL-1 and IL-6 were not detected at any time examined in BALFs from the four control pigs and in those from inoculated pigs collected within 1 week PI. TNF-α, IL-1 and IL-6 were found in pigs inoculated with *M. hyopneumoniae* on 2, 3 and 4 weeks PI. Geometric mean levels of TNF-α on 2, 3 and 4 weeks PI were 3.4, 7.9 and 3.2 pg/ml, respectively (Figure 7). The significantly increased level of TNF-α in BALF was observed on 3 weeks PI ($p<0.05$) compared to that before inoculation. Elevated level of IL-1 were 14.9, 55.7 and 64.0 pg/ml at 2, 3 and 4 weeks PI, respectively (Figure 8). The level of IL-1 in BALF on 3 and 4 weeks PI was significantly ($p<0.01$) higher than that before inoculation. The geometric mean levels of IL-6 in BALF of the infected pigs were significantly increased at 2 (428.7 pg/ml), 3 (400.0 pg/ml) and 4 (746.4 pg/ml) weeks PI compared to those before inoculation ($p<0.05$) (Figure 9).

Discussion

The author showed that the mean levels of TNF-α, IL-1 and IL-6 in BALFs were elevated following *M. hyopneumoniae* infection and all infected pigs had gross pneumonia lesions. In this study, TNF-α, IL-1 and IL-6 were detected by 2 weeks PI. The levels of TNF-α were maximal at 3 weeks PI and reduced at 4 weeks PI. High levels of IL-1 were detected at 3 and 4 weeks PI. The significantly increased levels of IL-6 were observed at 2 to 4 weeks PI. It is known that IL-6 production is induced by TNF-α and IL-1 and then suppresses the production of TNF-α and IL-1 (18, 32, 49). Hence, in the present study, it is suggested that IL-6 in BALF suppresses TNF-α production, though the regulation of IL-1 production by IL-6 is still obscure. Further study is needed to clarify the regulation of these cytokines produced by lung tissues in MPS.

The cells in BALF collected before inoculation and one week PI were almost all alveolar macrophages, while those at 2, 3 and 4 weeks PI were composed of macrophages, neutrophils and lymphocytes. These results indicate that the inflammatory responses in the lungs of pigs infected with *M. hyopneumoniae* occurred by 2 weeks PI. The appearance of the PMN and lymphocytes in the airways were correlated to the presence of inflammatory cytokines in the BALF. TNF-α and IL-1 are thought to be inducers of IL-8. IL-8 is a chemotaxis factor produced by macrophages and also directly affects the migration of polymorphonuclear cells (18, 49). The increased

levels of the cytokines in BALF of *M. hyopneumoniae*-infected pigs might affect the accumulation of the inflammatory cells in the airways.

TNF-α, IL-1 and IL-6 were persistently released into the airways in pigs with *M. hyopneumoniae* infection. Presence of these cytokines in the airways would induce the activation of infiltrated lymphocytes and PMN, and B cell activation resulting in differentiation to plasma cells (18, 32, 49). Nishimoto et al. (47) have reported that the mRNA expression of TNF-α in the lungs of *M. pulmonis*-infected mice was demonstrated at 24 h PI and continued for 35 d PI (throughout the observation). In mice experimentally infected with *Legionella pneumophilla*, TNF-α was detected from 3 to 48 h PI (10). In the model of mouse pneumonia caused by *Chlamydia trachomatis*, the mRNA expressions of IL-1 and IL-6 were detected by 24 h PI, with a peak at 48 h PI and reduced levels at 72 h PI (39). Thus, compared with these pneumonia, the cytokines produced in infectious foci seem to release for a long time into the airways in mycoplasmal pneumonia of pigs and mice. The persistent presence of cytokines in the airways of the infected pigs may be involved in the pathogenesis of chronic pneumonia. Moreover, the cytokines induced by mycoplasma infection appear to play an essential role not only for the development of pneumonic lesions as described in the Chapter 1, but also for the maintenance of the inflammation resulting in persistent activation of immune systems.

Summary

A time-course study suggested that TNF-α, IL-1 and IL-6 to be persistently produced in BALF from pigs experimentally infected with *M. hyopneumoniae*. TNF-α , IL-1 and IL-6 were detected at 2, 3 and 4 weeks PI. The cytological examination suggested that the presence of these cytokines may be associated with the increased number of neutrophils and lymphocytes in the airways. The cytokines released persistently into the airways of infected pigs may play a role not only for development of pneumonic lesion, but also for the maintenance of the inflammation.

Legends to Figures

Figure 7. Time-course study of TNF-α levels in BALF from *M. hyopneumoniae* infected pigs. Assay was performed in duplicate and values represent the mean ± standard deviation of TNF-α levels in BALF from five infected pig. ＊: Significant difference compared with BALF samples before inoculation at $P<0.05$.

Figure 8. Time-course study of IL-1 levels in BALF from *M. hyopneumoniae* infected pigs. Assay was performed in duplicate and values represent the mean ± standard deviation of IL-1 levels in BALF from five infected pig. ＊＊: Significant difference compared with BALF samples before inoculation at $P<0.01$.

Figure 9. Time-course study of IL-6 levels in BALF from *M. hyopneumoniae* infected pigs. Assay was performed in duplicate and values represent the mean ± standard deviation of IL-6 levels in BALF from five infected pig. ＊: Significant difference compared with BALF samples before inoculation at $P<0.05$.

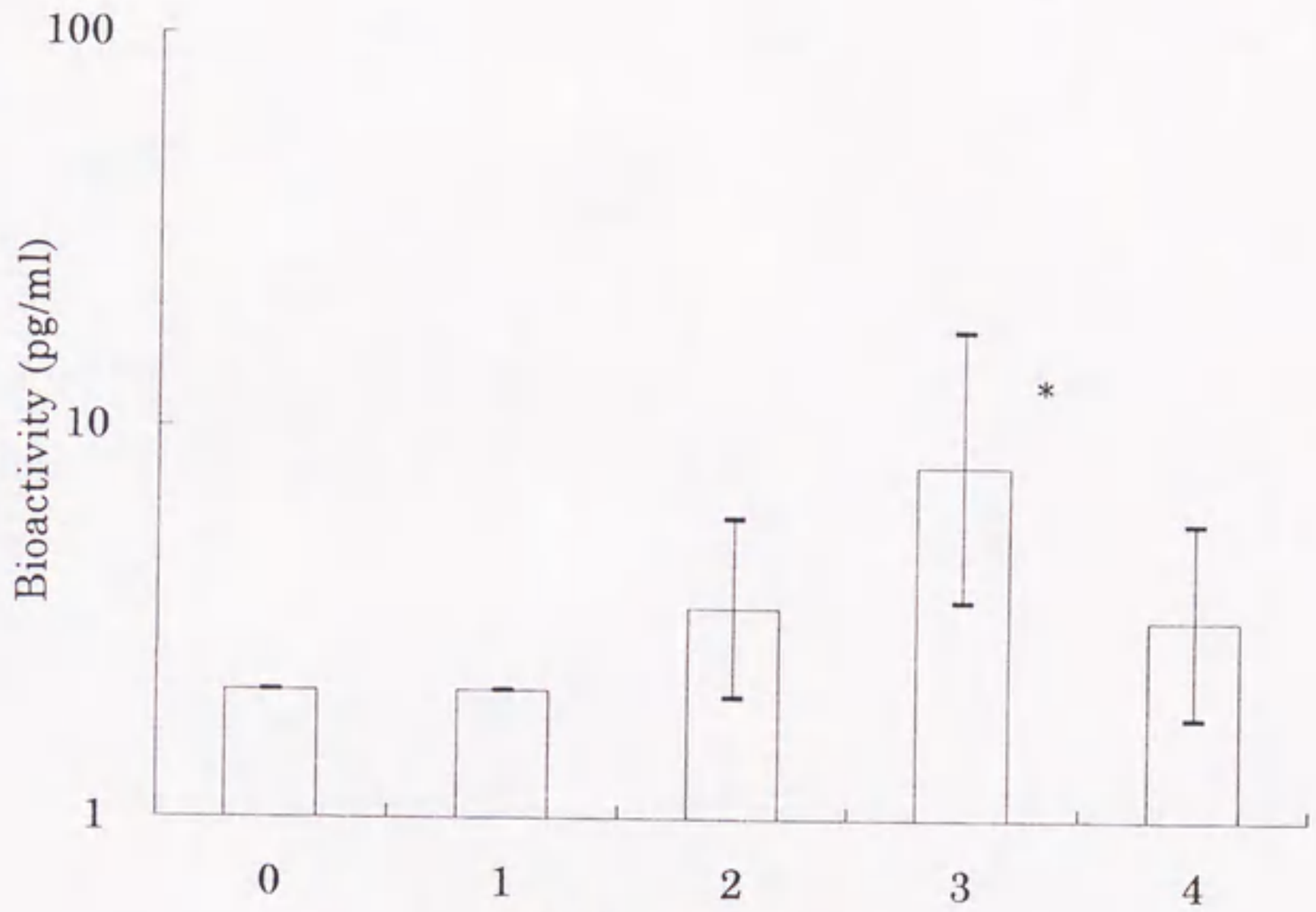


Figure 7

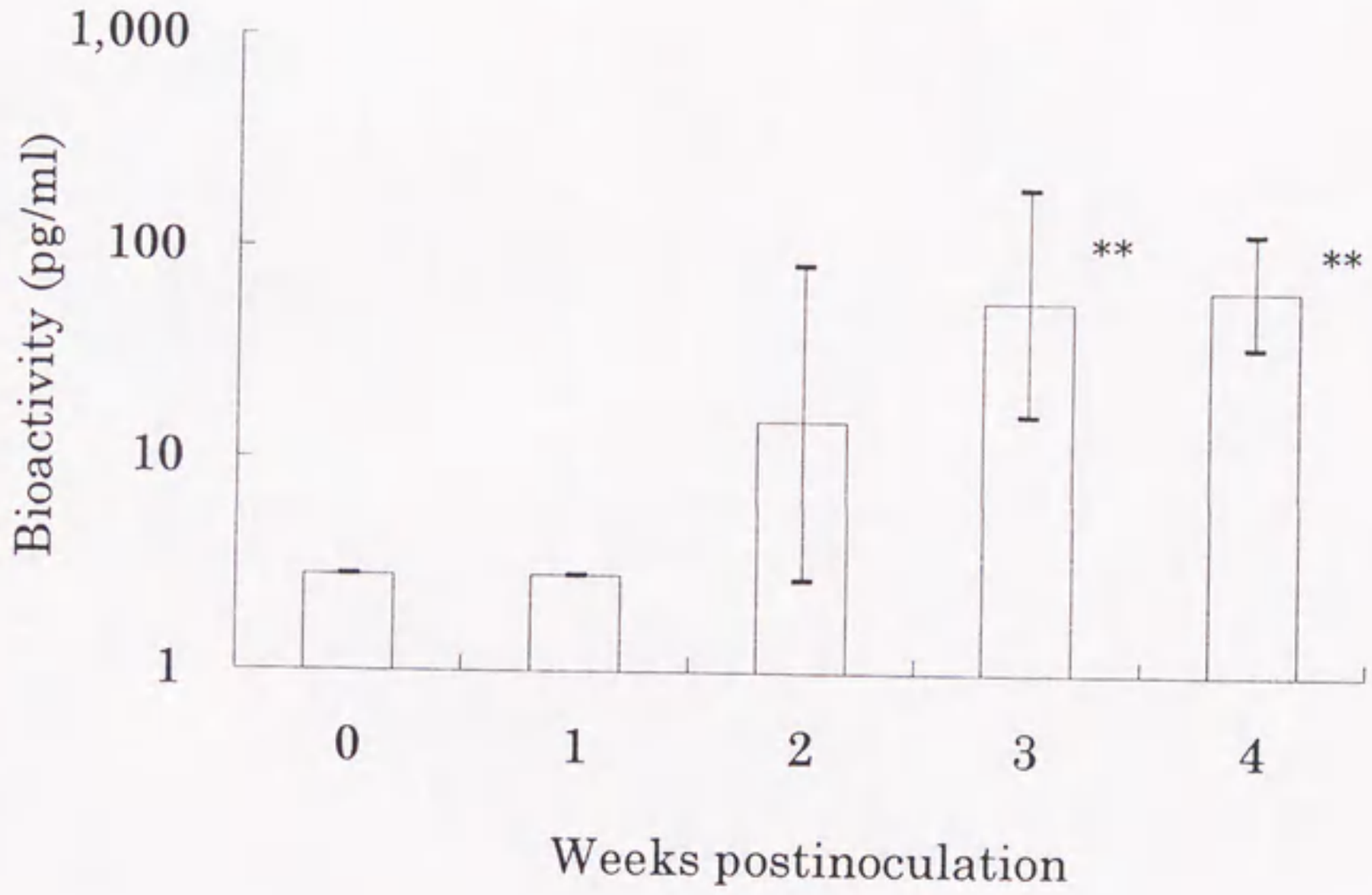
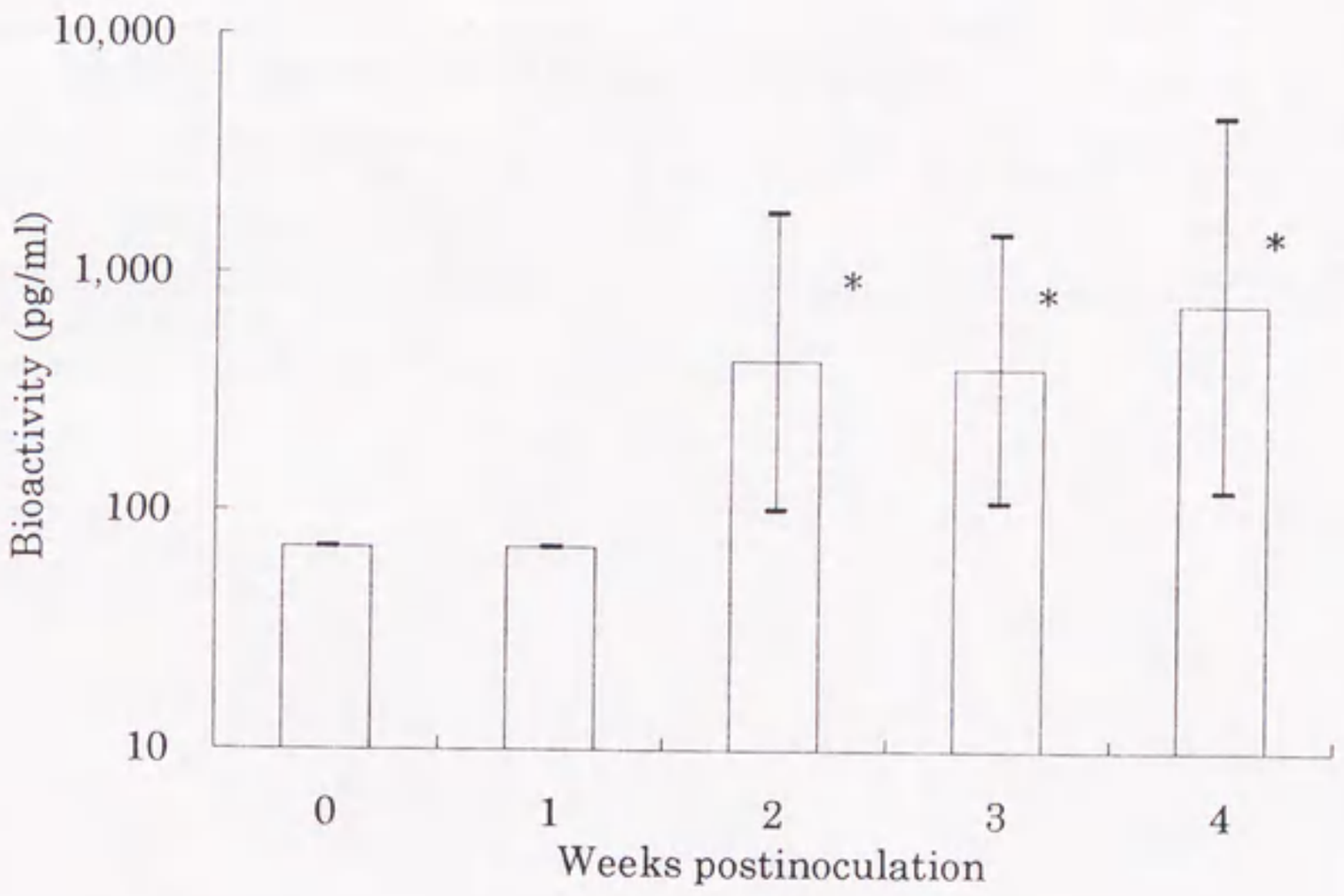

1,000
100
10
1
Bioactivity (pg/ml)
**
**
0
1
2
3
4
Weeks postinoculation

Figure 8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Bioactivity (pg/ml)
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Weeks postinoculation

Figure 9

Table 4. Time-couse study of cytological changes in BALF from infected pigs

	Mean of cellular composition in BAL cells (%)				
	0[1]	1	2	3	4
Macrophages	97.4±1.9[2]	96.8±2.4	51.6±26.2	42.2±22.2	32.6±14.8
Neutrophils	0.6±0.6	2.1±2.5	35.7±23.2	41.7±25.7	36.5±24.7
Lymphocytes	1.8±1.8	1.0±1.2	10.6± 6.0	15.7± 5.3	30.7±25.6

1 Weeks postinoculation.
2 Values represent mean ± the standard deviation.

Chapter 3

Suppressive effect of bronchoalveolar lavage fluid from pigs infected with *Mycoplasma hyopneumoniae* on chemiluminescence of porcine peripheral neutrophils

Introduction

It is well known that pigs affected with mycoplasmal pneumonia are more susceptible to other pulmonary pathogens (55). The field cases of MPS are often mixed infections with pulmonary pathogens (27, 66), and the results of experimental infection reveal an exacerbation of the pneumonia by secondary infection (55). As reported by several workers, this might be related to the functional suppression of macrophages or PMN by some *Mycoplasma* species (2, 12, 63). Phagocytic cells such as macrophages and PMN play an important role in the host defense system against bacterial infection (40). The phagocytic cells try to kill the invaded pathogens by several mechanisms such as chemotaxis, phagocytosis and the microbicidal metabolites by the oxidative burst (40). The author has shown the increased levels of TNF-α and IL-1 in BALF from pigs experimentally infected with *M. hyopneumoniae* as described in the Chapter 1 and 2. In the proportion of cells in BALF, the percentages of neutrophils were increased in *M. hyopneumoniae*-infected pigs as defined in the Chapter 2. It has been reported that TNF-α and IL-1 prime neutrophils and enhance their oxidative response to subsequent stimuli (8, 33). Although it has been reported that phagocytosis by alveolar macrophages was suppressed by *M. hyopneumoniae* infection (12), little information is available concerning the effect of *M. hyopneumoniae* infection on PMN function. In this chapter, the author shows that the BALF from *M. hyopneumoniae*-infected pigs

suppressed the CL response of PMN stimulated subsequently with opsonized zymozan (OPZ), and the suppressive effect was correlated to the concentration of PGE2 in the BALF.

Materials and Methods

Infection of pigs with *M. hyopneumoniae*

Five, 8-week-old, primary SPF pigs were employed for experimental infection with *M. hyopneumoniae* strain E1 (42) as previously described in the Chapter 1. Three SPF pigs served as uninoculated controls.

Preparation of bronchoalveolar lavage fluids (BALF)

The bronchoalveolar lavage of pigs was performed using fiberoptic bronchoscopes (BF type P20, Olympus, Tokyo, Japan) as previously described in the Chapter 2. BALFs were collected from infected pigs at weekly intervals after inoculation and from control pigs at two week intervals. The BALFs were centrifuged at 20,000 × g for 10 min, filtrated through a 0.2 μm pore-sized filter and stored at -20℃ until use.

Prostaglandin E2 assay

Assays of PGE2 in BALF were performed by using a competitive enzyme immunoassay kit for determination of PGE2 (Advanced Magnetics, Inc., Cambridge, MA.). PGE2 assays were performed according to the manufacturer's directions.

Isolation of porcine peripheral polymorphonuclear neutrophils (PMNs)

Three healthy pigs from 3 to 5 month-old were maintained as blood donors. All pigs were negative for *M. hyopneumoniae* infection as determined by the complement fixation test (43). Blood was collected using 0.2% ethylendiaminetetraacetic acid (EDTA) as an anticoagulant. PMNs were isolated by density gradient centrifugation according to the method of EI-Away and Hahn (20) with slight modification. Briefly, 10 ml of blood was mixed with 5 ml of 6% dextran sulfate in PBS. After incubation for 45 min at 37℃, the mixture was centrifuged, the cells were washed twice with PBS and resuspended in 3 ml of PBS. The cells were carefully overlaid on a discontinuous gradient made of 80 and 70% percoll (Pharmacia LKB Biotechnology, Uppsala, Sweden). After centrifugation at 400 × g for 30 min, the 80% fraction was collected and erythrocytes were removed by hypotonic lysis. The remaining cells were resuspended in Hank's balanced salt solution (HBSS) and adjusted to 2×10^6 cells/ml. The percentage of neutrophils in this suspension was higher than 93% with more than 98% viability as determined by nigrosin exclusion.

Preparation of opsonized zymozan

Zymozan A (Sigma Chemical Co. , St. Louis, MO, USA) suspended in water was boiled for 20 min, and washed twice with PBS and resuspended at a concentration of 20 mg/ml in PBS. The zymozan was opsonized with an

equal volume of fresh pig serum for 45 min at 37℃. After two washings with HBSS, the opsonized zymozan (OPZ) was suspended at 20 mg/ml in HBSS.

Incubation of porcine neutrophils with BALF or PGE2

One hundred μl of the PMN suspension was mixed with an equal volume of BALF in round-bottom tubes. After addition of luminol (Wako Chemical, Tokyo, Japan) to a final concentration of 0.2 μg/ml, the tubes were incubated at 37℃ for 30 min. Purified human PGE2 (Advanced Magnetics, Inc., Cambridge, MA, USA) was diluted with PBS (5,000 pg/ml to 8 pg/ml) and mixed with porcine PMN and luminol solution as described above to help quantitate the PGE2 in BALF samples. PBS was employed for unstimulated controls.

Measurement of chemiluminescence

CL was measured using Biolumat LB 9505 (Berthold Co., Germany). After incubation of PMN with BALF or PGE2, 50 μl of OPZ suspension was added to each tube, and the light emission was measured at 37℃ for 60 min. Results were expressed as the ratio calculated as follows;

CL index (%) = (The peak response of PMN incubated with the BALF or PGE2) ÷(The peak response of PMN incubated with PBS) × 100

Statistic analysis

Statistical difference of CL index was calculated by Student's t test.

The correlation between CL index and PGE2 concentration were tested by Pearson's correlation coefficient.

Results

The CL indexes of PMN incubated with BALF from control pigs were 104.9 ± 10.5, 117.8 ± 21.8 and 111.6 ± 14.3% at 0, 2 and 4 weeks PI, respectively. PMN's incubated with BALF from pigs before inoculation was 114.4 ± 13.7%. In contrast, PMN's incubated with BALF obtained at 1, 2, 3 and 4 weeks PI were 65.8 ± 35.6, 82.8 ± 21.2, 68.2 ± 16.7 and 52.3± 25.2%, respectively. Thus, the CL index of PMN was decreased by addition of BALF from infected pigs. This suppressive effect of BALF obtained from pigs at 4 weeks PI was significantly different compared with those of uninoculated control pigs that were similarly treated (Figure 10).

The geometric means of PGE2 level in the same BALF samples were 23.4, 85.9, 233.4, 130.6 and 6,884.0 pg/ml at 0, 1, 2, 3 and 4 weeks PI, respectively. Significantly increased geometric mean levels of PGE2 were observed at 4 weeks PI ($p<0.001$) (Figure 11).

The effect of PGE2 on the CL response of PMN was examined using purified human PGE2. As shown in Figure 12, PGE2 suppressed the CL response of PMN in a dose dependent manner and there was a significant correlation between the CL index and the PGE2 concentration ($r=-0.989$, $p<0.01$). In addition, the relationship between the CL and the PGE2 concentration in the BALF is shown in Figure 13. The CL index of PMN incubated with BALF was significantly correlated with the PGE2 concentration in the BALF from infected pigs ($r=-0.676$, $p<0.001$).

The relationship between pulmonary lesion and PGE2 was examined (Table 5). The levels of PGE2 in BALF from all the infected pigs increased significantly compared to controls ($p<0.01$). Increased geometric means of PGE2 levels (2,449.2 pg/ml) were observed in the pigs with HLT compared to those without them (794.3 pg/ml)($p<0.05$). There is no significant difference between the pigs with and without GL.

Discussion

In the present study, the author demonstrated that the CL response of PMN was suppressed by BALF from infected pigs with *M. hyopneumoniae*. Adhesion of *M. bovis* to the bovine neutrophils was considered to be responsible for suppression of the CL response (63). However, in our study, *M. hyopneumoniae* was not detected in the BALFs, because they were centrifuged at 20,000 × g and filtered through 0.2 μm pore-sized membrane filter. Thus, it is considered that the CL suppression of porcine neutrophils caused by the BALF is not due to the direct interaction of *M. hyopneumoniae* with neutrophils. These results suggested that soluble mediators may be involved in modulating PMN function.

Prostaglandins are important mediators in the inflammatory reaction (25) and exert suppressive effects on immunological responses in vitro (19, 34, 36, 51). Phillips et al. (51) showed that PGE2 suppressed the CL response of bovine PMN. The suppressive effect of PGE2 on porcine PMN was also demonstrated in the present study. The suppressive effect of the BALF from infected pigs was significantly correlated with the concentration of PGE2 in the BALF. However, it is not known whether PGE2 is the only one causative factor of this suppression, because BALF contains many different types of immunomodulators (35, 48). Studies have shown that several different cytokines, including TNF-α, IL-I and gamma-interferon (47) were produced in the airway during the mycoplasmal infection. It is

known that these cytokines modulate PMN function and enhance the oxidative response of PMN (8, 9, 33). Thus cytokine may also play a role in these events. Recently, Sample and Czuprynski (57) have reported that human IL-1 receptor antagonist (IL-1ra) inhibits CL of bovine PMN. Although the author can not exclude the involvement of various mediators, the present study has indicated that PGE2 is the predominant factor contributing to the suppression of the CL response of PMN by BALF from *M. hyopneumoniae*-infected pigs.

Productions of PGE2 and IL-6 are performed by induction of TNF-α and IL-1 (15, 18, 49). Significantly increased levels of PGE2 were observed at 4 weeks PI which was later than that for IL-6 as defined in chapter 2. In this regard, PGE2 production may be stimulated in a different manner to IL-6 production. PGE2 suppresses the production of TNF-α and IL-1 (19, 34, 36). In the acute arthritis by *Erysipelothrix rhusiopathine* in rats, though the peaks of TNF-α and IL-1 released from macrophages were observed on 2 and 3 d after infection, the increased levels of PGE2 were observed in decreased level of them (54). Furthermore the treatment of the infected rats with indomethacin (prostaglandin synthesis inhibitor) prolonged the release of TNF-α from macrophages (54). In the Chapter 2, the author showed that IL-6 might be involved in the suppressing TNF-α production in *M. hyopneumoniae*-infected pigs. PGE2 levels elevated at 4 weeks PI might also be involved in the reduction of TNF-α levels at the time.

PGE2 has biological activities which not only affect inflammations, but also to suppress various cell functions (24). In various pulmonary disorders, increased levels of PGE2 were detected in airways. In BALF from acute ozone-induced pneumonic patients, PGE2 levels, numbers of polymorphonuclear cells and elastase activity were elevated after exposure (35). Ogushi et al. (48) reported that increased levels of PGE2 in BALF from patients with eosinophilic pneumonia were related to an increased number of eosinophils and suggested that the PGE2 may affect the decreased number and percentage of lymphocytes. However, the levels of PGE2 in BALF from *M. hyopneumoniae*-infected pigs increased regardless of the presence of GL and/or HLT. In the present study, the author suggests that PGE2 released into the airway of pigs infected with *M. hyopneumoniae* may suppress the CL activity of infiltrating PMN with a concomitant decrease in microbicidal activity of the PMN (28). Thus, PGE2 produced in the airway during *M. hyopneumoniae* infection may be important not only as an immunological mediator, but also as a factor that causes the exacerbation of mycoplasmal pneumonia.

Summary

BALF from pigs experimentally infected with *M. hyopneumoniae* suppressed the CL response of porcine PMN. The suppressive effect was significantly correlated to the concentration of PGE2 in the BALF. Purified human PGE2 suppressed the CL response of porcine PMN. The increased levels of PGE2 were observed in all the infected pigs. The increased level of PGE2 following infection with *M. hyopneumoniae* may be responsible for the suppression of PMN function in the airway of infected pigs with *M. hyopneumoniae*. Furthermore, the decrease of PMN function may be responsible for the exacerbation of mycoplasmal pneumonia by secondary infection with pulmonary bacterial pathogens.

Legends to Figures

Figure 10. Effect of BALF from pigs infected with *M. hyopneumoniae* on the CL response of porcine PMN. Values represent the mean ± standard deviation of CL index from infected pigs (□) and control pigs (■). NT: not tested. ＊: Significant difference compared with BALF samples before inoculation at $P<0.05$.

Figure 11. Time-course study of PGE2 levels in BALF from pigs infected with *M. hyopneumoniae*. Assay was performed in duplicate and values represent the mean ± standard deviation of PGE2 from five infected pig. ＊＊: Significant difference compared with BALF samples before inoculation at $P<0.01$.

Figure 12. Effect of human PGE2 on the CL response of porcine PMN.
CL index versus PGE2, $r=-0.989$, $P<0.01$.

Figure 13. The relationship between the CL response of porcine PMN and the PGE2 concentration in the BALF from pigs infected with *M. hyopneumoniae*. CL index versus PGE2, $r=-0.676$, $P<0.001$.

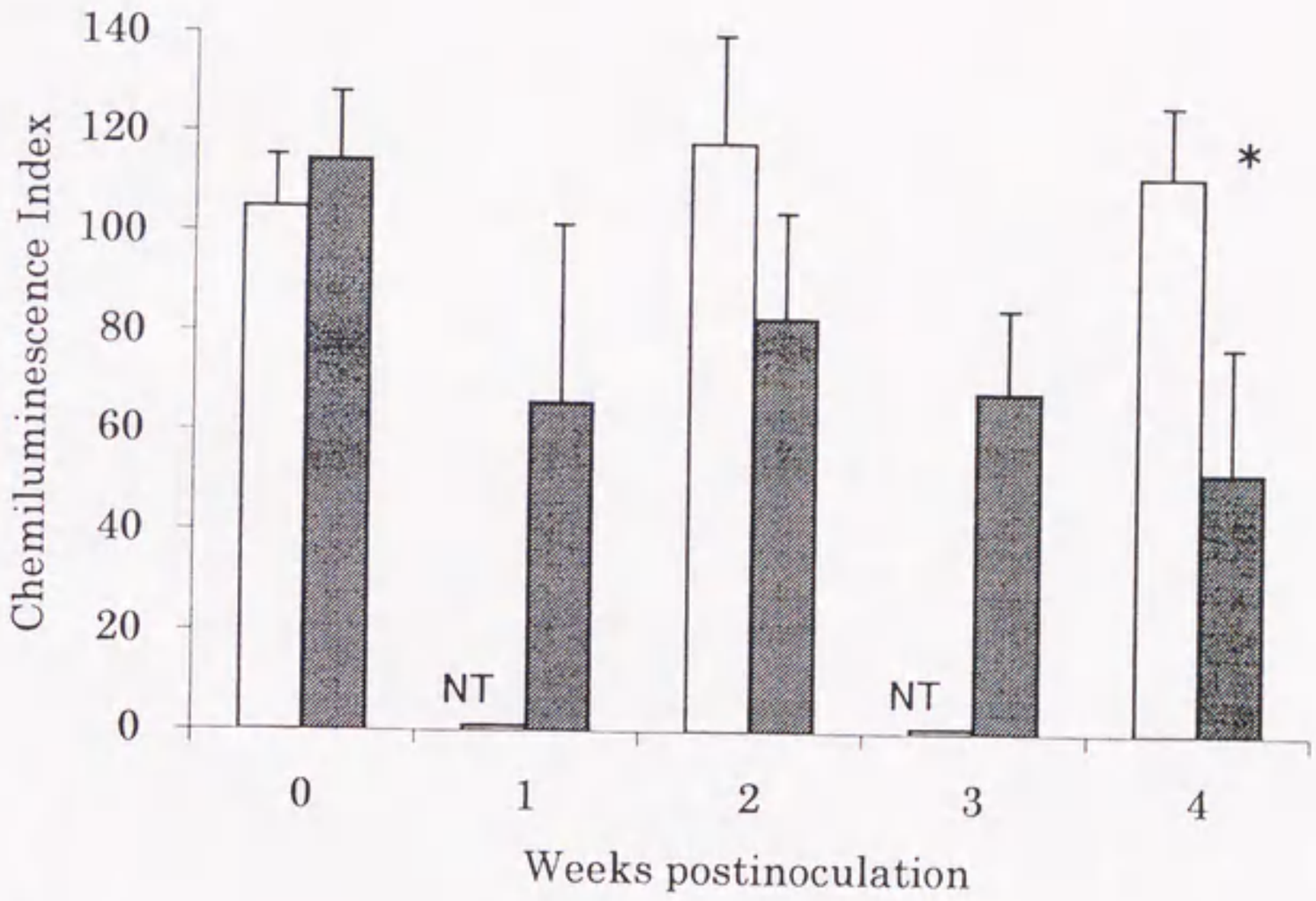
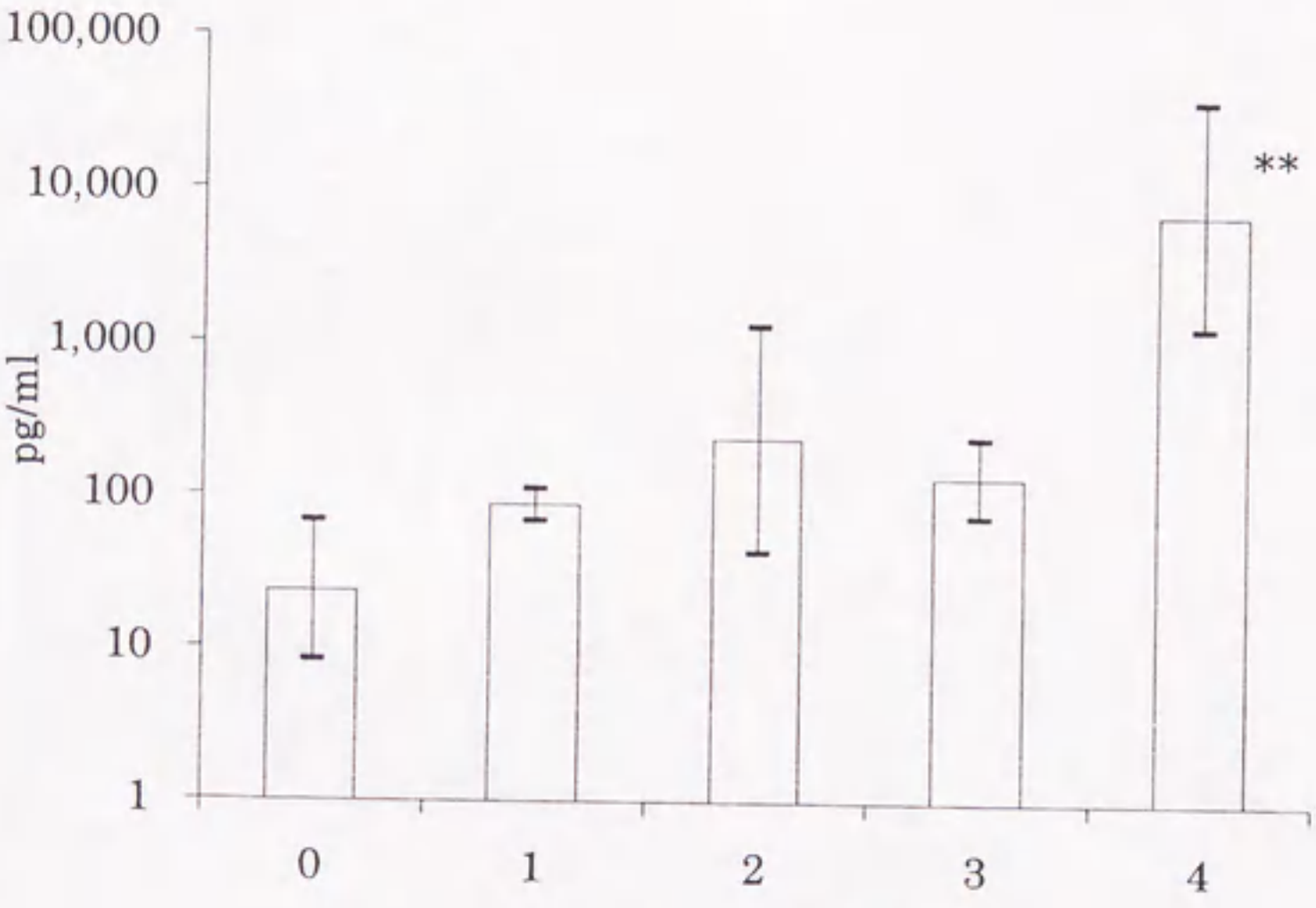

Chemiluminescence Index
140
120
100
80
60
40
20
0
NT
NT
*
0
1
2
3
4
Weeks postinoculation

Figure 10


100,000
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pg/ml
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Weeks after inoculation

Figure 11

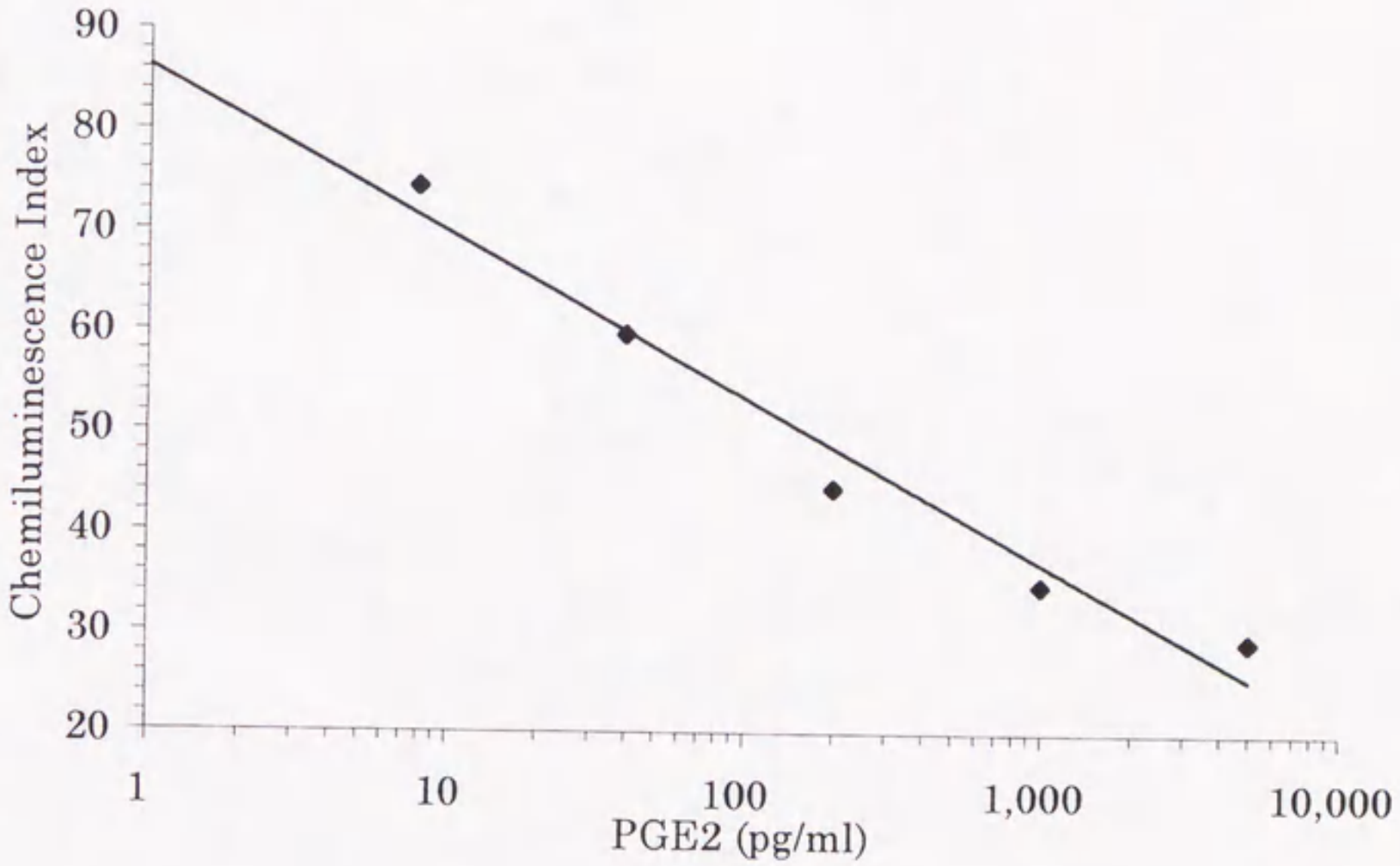
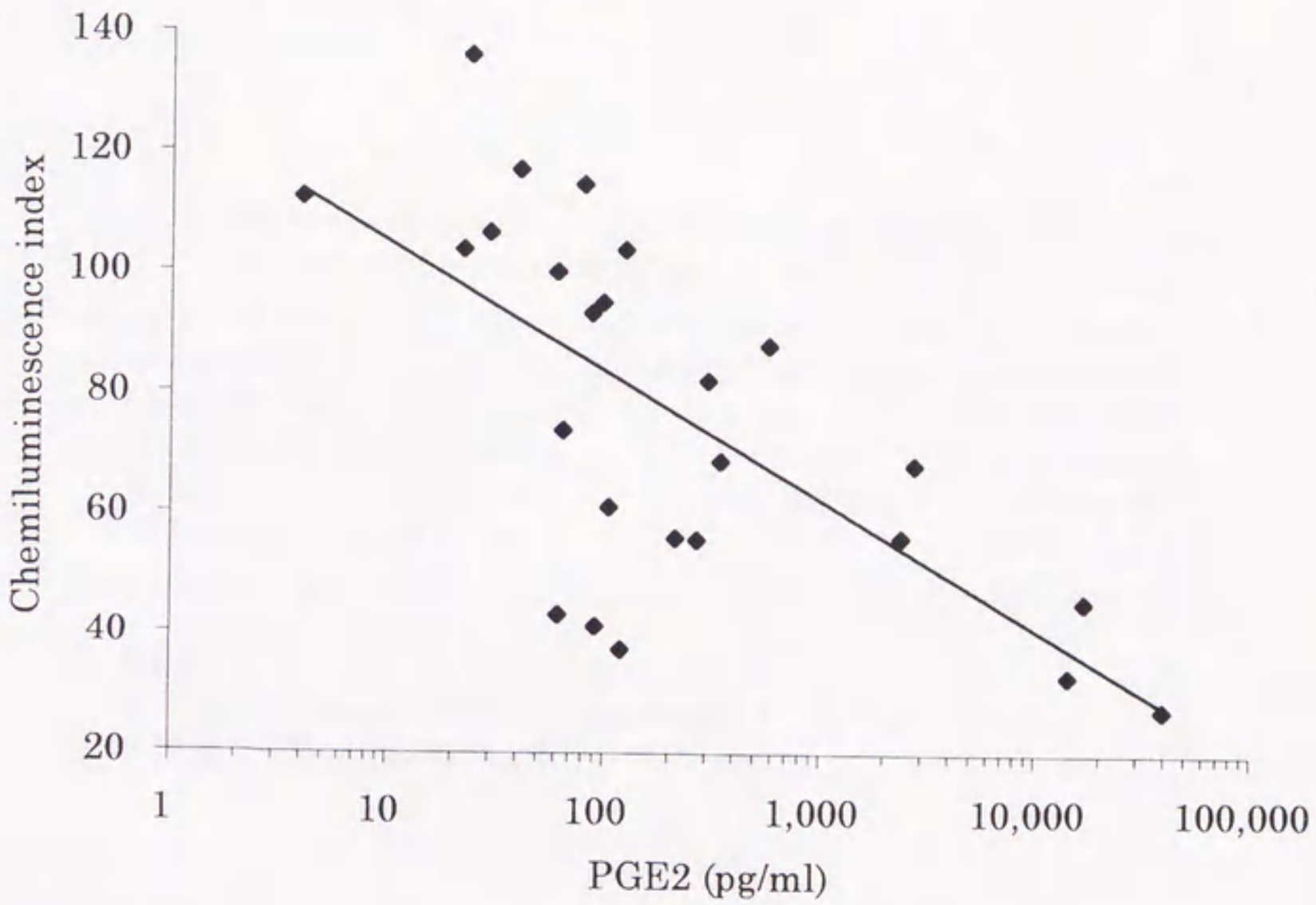

Chemiluminescence Index
90
80
70
60
50
40
30
20
1
10
100
1,000
10,000
PGE2 (pg/ml)

Figure 12


Chemiluminescence index
140
120
100
80
60
40
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1
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1,000
10,000
100,000
PGE2 (pg/ml)

Figure 13

Table 5. The levels of PGE2 in BALF from *M. hyopheumoniae*-infected pigs with and without lung lesions at 4 weeks postinoculation

Lung lesion	Geometric mean	Range of SD[1]
Controls (n=4)	40.2[2]	10.0- 51.6
Gross lesion negative (n=5)	972.8**[3]	397.3-2,382.0
Gross lesion positive (n=10)	2,213.3**	808.9-6,056.1
Not observed HLT (n=5)	794.3**	501.3-1,258.5
Observed HLT (n=10)	2,449.2*,**	876.4-6,845.1

1 Standard devation.
2 pg/ml.
3 *: $p<0.05$ compared with not observed HLT group.
**: $p<0.001$ compared with control group.

CONCLUSION

The purpose of this paper is to analyze the association of inflammatory cytokines in the airways of pigs infected with *Mycoplasma hyopneumoniae* with development of pneumonic lesions and kinetics of these cytokines in the airways. In addition, the relationship between the functional suppression of phagocytic cells and PGE2 released into the airways following infection with *M. hyopneumoniae* was studied. The results obtained are as follows:

1. TNF-α, IL-1 and IL-6 in BALF from pigs experimentally infected with *Mycoplasma hyopneumoniae* were detected using biological assays.
2. Increased levels of TNF-α in BALF were found in infected pigs with HLT compared with controls and infected pigs without HLT.
3. The levels of IL-1 in BALF were increased significantly in infected pigs with GL and HLT.
4. The levels of IL-6 in BALF from infected pigs with GL and HLT were significantly higher than that without pneumonic lesions.
5. TNF-α in BALF was detected on weeks 2, 3 and 4 PI and the significantly increased level of TNF-α in BALF was observed at 3 weeks PI.
6. Elevated levels of IL-1 were observed at 2, 3 and 4 weeks PI and the level of IL-1 in BALF on weeks 3 and 4 PI was significantly higher than that on before inoculation.

7. The levels of IL-6 in BALF of the infected pigs were significantly increased at 2 to 4 weeks PI compared to those before inoculation.
8. The increased number of neutrophils and lymphocytes in the airways were observed at 2, 3 and 4 weeks PI and the presence of the cytokines associated with the cytological change in BALF.
9. BALF from pigs experimentally infected with *M. hyopneumoniae* suppressed the CL response of porcine PMN.
10. The suppressive effect was significantly correlated to the concentration of PGE2 in the BALF.
11. The increased levels of PGE2 were observed in all the infected pigs.

The persistent presence of TNF-α, IL-1 and IL-6 in the airways of *M. hyopneumoniae*-infected pigs may be involved in the development of pneumonic lesions. And the PMN function suppressed by PGE2 released into the airway of infected pigs may be responsible for exacerbation of mycoplasmal pneumonia by secondary infection with pulmonary bacterial pathogens.

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