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In situ Analysis of CD4, CD8 and Mast Cells in Lung of *Mycoplasma hyopneumoniae* Experimentally Infected Pigs

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Abstract: Mast cells (MC) and $CD4^+$ and $CD8^+$ cell subpopulations, from the apical border of the right cranial lobe of lungs of pigs experimentally infected with *Mycoplasma hyopneumoniae*, were histologically (MC) and immunohistochemically (CD4 and CD8) analyzed during the first 20 days post infection (PI). Twelve weaned, four-week-old healthy pigs were endotracheally inoculated with a lung homogenate infected with *M. hyopneumoniae*, strain 194 and 6 control animals were inoculated with Friis medium only. A control animal and two infected animals were euthanized at days 0, 4, 8, 12, 16 and 20 PI. *M. hyopneumoniae* induced an early increase of CD4 cells at day 4 PI ($p<0.001$) and at day 8 PI there was a significant and coincident increase in all three cell populations ($p<0.001$), which intermittently persisted until day 20 (CD8 $p<0.001$, MC $p<0.001$). The results of this work suggest that infection with *M. hyopneumoniae* induces an early response of CD4+ cells, followed by an infiltration of CD8+ cells and MCs, possibly related to the pathophysiological events of the disease, since, despite the evident immune infiltration, the infection continued its course.

Key words: *Mycoplasma hyopneumoniae*, Immune response, CD4, CD8, Mast cell, swine lung

INTRODUCTION

Mycoplasma hyopneumoniae is the primary etiologic agent of Enzootic Pneumonia (EP) in swine. EP is a disease with a high incidence in swine production, which causes lung tissue damage, growth decrease, a reduction in daily weight gain and higher mortality, increasing the days of age at slaughter (Thacker, 2001). In mycoplasma infection, a widespread lung inflammation occurs, as evidenced by an infiltrate of T and B lymphocytes, NK cells and macrophages (Cassell *et al*., 1974; Fernald, 1997; Romero-Rojas *et al*., 2001). Histological changes are characterized by the presence of an inflammatory exudate in the airways, thickening of the alveolar septa, lymphocyte accumulation around the bronchia, the bronchioles and blood vessels and bronchus-associated lymphoid tissue (BALT) hyperplasia, causing obliteration of the bronchiole lumen and alveolar atelectasis (Kwon *et al*., 2002). *M. hyopneumoniae* colonizes the epithelial cell surface of the bronchia and bronchioles without penetrating lung parenchyma and induces a decrease in ciliary activity, cilia destruction and micro-colony formation (Blanchard *et al*., 1992; Hsu and Minion, 1998). The changes induced in the respiratory airways are critical due to the establishment of secondary infections, particularly with Pasteurella multocida (Ciprián *et al*., 1988). Previous studies on cell immunity against *M. hyopneumoniae* have revealed the presence of lymphocyte stimulating and suppressing factors in its membrane, such as MSF (mitogen-supressing factor), which is potentially responsible for lymphocyte infiltration in pneumonic lesions (Kishima and Ross, 1985). On the other hand, the experimental suppression of T-cell response has reduced the severity of pneumonic lesions caused by *M. hyopneumoniae*, suggesting that cell immunity is involved in their development (Tajima *et al*., 1983). It is also known that bronchoalveolar lavage of swine experimentally infected

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with *M. hyopneumoniae* supresses the chemoluminescent response of pig neutrophils (Asai *et al*., 1993). Flow cytometry analysis of the cellular response induced by *M. hyopneumoniae* demonstrated a significant increase of $CD8^+$ and CD16+ cells (FCRγIII) in bronchial lymph nodes, which may be related to the pulmonary lesions found (Dayalu and Ross, 1990; Bhogal *et al*., 1992). A greater number of CD4 than CD8 cells has been reported in BALT of pigs naturally infected with *M. hyopneumoniae* (Sarredell *et al*., 2003), although in these reports the infection stage or the potential presence of secondary agents are not described. Furthermore, mast cells (MCs) are crucial immune effector cells in inflammatory response; they are capable of recruiting neutrophils and of promoting the proliferation of epithelial cells and mucus secretion from the mucosae and they stimulate angiogenesis and bronchial smooth muscle proliferation by IL-4 release, which also attracts T-helper lymphocytes. Therefore, Mcs have a remarkable ability to modulate the innate and adaptative response to infections (Abraham *et al*., 1997). *Mycoplasma pneumoniae* is known to act on MC, activating the granule release with â-hexosaminidase and IL-4 when the pathogen is adhered to a biological surface, but this only occurs within the first 10 h following infection (Hoeck *et al*., 2002). No studies have been reported to date on the kinetics of the appearance of T-lymphocytes and MC during lung infections with *M. hyopneumoniae* and its potential relationship with the pathogenesis of the disease. In this work, the presence of these cells in the lung during the first 20 days after the experimental infection with *M. hyopneumoniae* was quantified, resulting in an early increase of CD4 cells, followed by an increase in CD8 and MC. Such increases were simultaneous to the appearance of lesions and remained high throughout the study.

MATERIALS AND METHODS

Animals: Twenty one weaned Yorkshire pigs, between 4 and 6 weeks of age, serologically negative for *Mycoplasma hyopneumoniae*, Actinobacillus pleuropneumoniae and Pasteurella multocida from a farm free of respiratory diseases were used. Three pigs were used to obtain the mycoplasma inoculum by means of a lung homogenate, six were used as non-infected controls and the other 12 in experimental infection.

Preparation of the *M. hyopneumoniae* inoculum: *M. hyopneumoniae* 194 strain (provided by Dr. Richard Ross, Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University, Ames, Iowa) was cultured in 10 mL of Friis medium for 72 h at 37°C. Then, it was inoculated into 250 mL of Friis medium and afterwards into 1000 mL of medium, incubating it at 37°C with constant stirring (70 rpm) during 1 h. The culture was subsequently titrated by serial dilutions in Friis liquid medium, from 10° to 10^{10}; tubes were incubated at 37°C for 5 days and the last tube showing a change in color to acidity (yellow) was taken as the culture titer, expressed as CCU (Color Changing Units), which in this case was 10^4 CCU. The culture was stored in sterile vials at -20°C until use.

Preparation of lung homogenate: To elaborate the lung homogenate, 10 mL of the *M. hyopneumoniae* 194 strain inoculum (10^4 CCU mL^{-1}) were administered by endotracheal route with a nasopharyngeal probe, to a pig sedated with azaperone (2 mg kg^{-1} weight, by intramuscular route) and anesthetized with methomidate hydrochloride (1.5 mg kg^{-1} of weight, by intravenous route) (Hannan *et al*., 1984). The animal was observed daily and when cough and disnea signs occurred, the animal was euthanized (23 days PI). Lungs were obtained aseptically and samples from pneumonic lesions were taken for isolation and immunofluorescence test. Lung fragments were embedded in Tissue Tek® and snap frozen in order to obtain frozen sections and subsequently apply the immunofluorescence conjugate. Another fragment of tissue was used for isolation in Friis medium according to the techniques described by Armstrong (1994). From this first passage in pigs, approximately 1% lung lesion was obtained, as well as a positive reaction to the immunofluorescence test and isolation. A new 20% (weight/volume) lung homogenate was prepared from pneumonic areas in Friis medium. Ten milliliter of this homogenate were endotracheally administered to 2 pigs as described before; the pigs were euthanized at 20 days PI. In this second phase, the pigs were positive to immunofluorescence test and bacterial isolation and both showed a 4% lung lesion. Another lung homogenate (with a 10^4 CCU mL^{-1} titer) was prepared from this second passage, to inoculate the experimental animals.

Experimental infection: Twelve pigs were previously sedated and anesthetized and were inoculated with 10 mL of the homogenate by endotracheal route; 6 control pigs were inoculated with 10 mL of Friis medium only. The day of inoculation was designated as day zero. The animals were observed daily in order to determine body temperature and assess clinical respiratory signs, such as cough and disnea.

Necropsy procedures and sample collection: Every 4 days (from day 0-20 day), 2 inoculated pigs and a control pig were randomly euthanized by exsanguination, after being sedated and anesthetized. Lungs were obtained aseptically from the euthanized animals; the extent of lesions was evaluated by the planimetry method (Ciprián *et al.*, 1988) and fragments (aprox. 1 cm^3) with pneumonic lesions from all pigs were fixed (1:10 V/V) in paraformaldehyde-lysine-periodate (PLP) (Mc Lean and Nakane, 1974) for 24 h at 4°C for the detection of mast cells by histochenistry and CD4 and CD8 cells by immunohistochemistry. Other fragments were fixed in 10% buffered formalin for histopatology. Sections were taken from the cranial lobes with pneumonic lesions, which were embedded in Tissue Tek® and snap frozen at -20°C in order to subsequently obtain frozen sections for the immunofluorescence test.

Histopatology: Histological sections from lung fragments kept in buffered formalin were obtained and subjected to hematoxylin and eosin staining to look for microscopic changes in lung microscopic structure.

Microbiology: For mycoplasma isolation, 1 cm^3 portions from the lung lobes were taken and homogenized with liquid Friis medium and sample dilutions (10^0-10^{-5}) were incubated at 37°C (Armstrong, 1994). Then, subcultures were carried out in solid Friis medium and were incubated at 37°C with a 5% CO_2 atmosphere until colony isolation was achieved. *Mycoplasma hyopneumoniae* was identified by the growth inhibition test (Armstrong, 1994; Kobish, 1982).

Immunofluorescence: A conjugate against *M. hyopneumoniae*, made in rabbits in our laboratory, was applied at a 1:16 dilution to frozen sections. These were subsequently observed in a Carl Zeiss WL fluorescence microscope, furnished with a dark field condenser and a HB22 W/4 mercury-vapor lamp. The intensity of positive reactions was considered from 1+ to 4+ according to Amanfu *et al.* (1984).

Inmunohistochemistry for CD4 and CD8 cells: For the determination of CD4 and CD8 cells, fixed samples of lungs were stained using the commercial kit Extra Avidin Stock No. EXTRA-2 (Immunochemicals-Sigma), with the use of monocolonal antibodies against CD4 (4-12-4, Pescovitz *et al.*, 1984) and CD8 (76-2-11, Pescovitz *et al.*, 1994), both at a 1: 50 dilution; diamino benzidine (Immunochemicals-Sigma) was used as developer and Harris' hematoxylin was used as counterstaining, following the protocol by Vega-Lopez *et al.* (1993) and the recommendations from Chainini *et al.* (2001).

Detection of mast cells: For the determination of MC, histologic sections were stained with 1% toluidine blue (Sigma) in distilled water for 20 min, the excess dye was eliminated with absolute methyl alcohol. Slides were clarified and mounted on resin. They were observed under the microscope at 40X magnification; cells with metachromatic granules yielded an intense pink color. The metachromasia of bronchial cartilage's fundamental substance by chondroitin sulfate was considered as the positive control of staining (Joseph *et al.*, 2003; Sheenan and Hrapchak, 1980).

Cell count and statistical analysis: Positive cells were counted in at least 10 randomly selected fields of each lung sample, using the Image Pro-Express program (version 4.01 Media Cybernetics) at 400 X and the mean and standard error were obtained from each animal. The results were analyzed by ANOVA and Tukey's multiple comparison test with the Graph Pad Prism software, version 3. The results for control pigs were similar for all cells throughout the experiment, therefore, they were grouped to form a single control group (n = 6).

RESULTS AND DISCUSSION

The study of the respiratory immunity in swine and the kinetics of cellular response during lung infection, are topics of great interest. In this research, the kinetics of arrival of CD4, CD8 and mast cells in pig lungs "*in situ*" was evaluated after *M. hyopneumoniae* experimental infection.

Control animals did not show lung lesions, they were negative to specific fluorescence against *M. hyopneumoniae* was detected and the bacterial isolate was always negative. In infected animals, characteristic lesions began to be observed in the lungs from day 4 PI onwards. Mean lesion percentages in consolidated lung surface at day 0 were 0%; day 4 , 2.4%; day 8, 7.4%; day 12, 8.1%; day 16, 13.3% and day 20, 17.2% (Table 1). In infected pigs at day 0, a normal lung histological morphology was observed and from day 4 onwards, peribronchial and perivascular lymphoid infiltration was seen; starting from day 12 PI, perivascular and peribronchial lymphoid hyperplasia was observed. All the inoculated pigs showed positive fluorescence in bronchioles, with readings from 1+ to 3+. From day 8 onwards, the upper respiratory tract cilia aggregation phenomenon was observed. Starting from day 4, mycoplasma was isolated in all inoculated pigs in dilutions ranging from 10^{-3} to 10^{-4} (Table 1).

CD4 + cells were preferably located around the bronchia and bronchioles in control and infected animals, as determined by immunohistochemistry. In control

Table 1: Post mortem evaluation of control and experimentally infected pigs with *Mycoplasma hyopneumoniae* from day 0 to day 20 PI

Day of euthanization	0		4		8		12		16		20	
Animals	C	I	C	I	C	I	C	I	C	I	C	I
Number	1	2	1	2	1	2	1	2	1	2	1	2
Percentage of pneumonic lesion*	0	0	0	2.4	0	7.4	0	8.1	0	13.3	0	17.2
Microscopic lesion•	-	-	-	+	-	+	-	+	-	+	-	+
Culture°	-	-	-	10^{-3}	-	10^{-3}	-	10^{-3}	-	10^{-4}	-	10^{-4}
Immunofluorescence against Mh♦	-	-	-	+	-	+	-	++	-	+++	-	++

Control pigs (C) were inoculated with 10 ml of Friis medium and were negative to all tests. Infected pigs (I) were endotracheally inoculated with 10 mL of lung homogenate with *M. hyopneumoniae* (10^4 CCU mL^{-1}). * Average of two infected animals. •Microscopic lesion with peribronchial lymphocytic infiltration, characteristic of proliferative pneumonia. °Dilution of the *M. hyopneumoniae* isolate from pneumonic lesions found in cranial lobes. ♦Degree of fluorescence detected

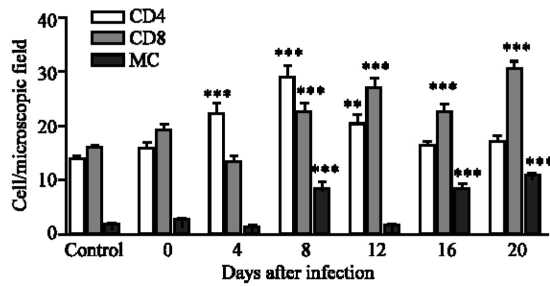

Fig. 1: Number of CD4$^+$, CD8$^+$ and mast cells (MC) for microscopic field in the lung tissue of control (n = 6) and *Mycoplasma hyopneumoniae* experimentally infected pigs (n = 2/day). The results for control pigs were similar on each day of euthanization, therefore, they are exemplified only once. Bars represent the mean and standard error of positive cells in a minimum of 10 microscopic fields per animal, CD4$^+$ (■) CD8$^+$ (□) and mast cells MC (▩). Statistically significant differences between control animals and infected animals: **(p<0.01), ***(p<0.001)

animals, mean counts were between 13 and 15 cells/field during all days of the experiment. The infected pigs had a significant rise on days 4 (22.20±1.93 cells/field p<0.001), 8 (28.95 cells/field p <0.001) and 12 (20.45±1.51 cells/field p<0.01), returning to normal values after this day (Fig. 1). CD8 cells were distributed in the whole parenchyma of control animals, with an average number ranging between 13 and 15 cells/field. In infected animals, they were found in the whole tissue, around blood vessels and had significant elevations at days 8 (22.5±1.7 cells/field p<0.001), 12 (27±1.7 cells/field p<0.001), 16 (22.80±1.2 cells/field p<0.001) and 20 PI (30.35±1.4 cells/field p<0.001) (Fig. 1).

Regarding MC, few cells were observed in the bronchial tree and the submucosa in control animals, with an average number ranging between 1 and 3 cells/field throughout the experiment. In infected animals, a statistically significant elevation was seen at days 8 (8.4±1.4, p<0.001), 16 (8.2±1.1, p<0.001) and 20 PI (10.6±0.5, p<0.001) (Fig. 1). At day 8, the increase occurred mainly in small diameter bronchioles (<80 μm). Also, at the boundary between the external connective tissue and the muscular layer, some cells were observed in the bronchiolar mucosa, the pleura and the interlobar septa, with an apparent association to lymphatic vessels in some cases. These cells were found to be associated to BALT, but not within BALT nor in large diameter blood vessels and were evident in the venules and septa in infected animals.

So far, there are no reports on the kinetics of arrival of lymphocytes and MC into the lung during *M. hyopneumoniae* infection in swine. By immunohistochemistry, Bernd and Muller (1995) studied the distribution of CD4+ and CD8+ cells, in an intratracheal infection with type A Pasteurella multocida in SPF swine lungs, showing an early accumulation in lung parenchyma and the perivascular and peribronchial zones after 8 h. However, the increase vanishes by day 16 PI. In our experiment, an early increase in the number of CD4+ cells, starting from day 4 IP, was observed in infected animals, reaching a maximum at day 8 PI, where BALT infiltration was seen, but such increase disappeared after day 12 PI, suggesting that these cells may be rapidly activated, releasing cytokines that would recruit CD8 cells and mast cells. In contrast, the late increase of CD8+ cells coincides with the appearance of microscopic and macroscopic lesions in the lungs, similarly to lesions observed in pulmonary infection of poultry with Mycoplasma gallisepticum, where an increase in CD8 and NK cells was also reported (Gaunson *et al*., 2006). The CD8 marker is present in swine T cells, but other cells such as CD4+ 8^{low} cells, NK cells (Ig-CD2+3-4-8^{low}), macrophages and dendritic cells also express this marker in a small degree (Zuckerman *et al*., 1998). In this reseaech, in order to prevent an overstimation of CD4 and CD8 cells it was verified that the counted cells were mononuclear, small and round cells and with peripheral positive staining.

On the other hand, in the present work, a significant increase of mast cells was seen at days 8, 16 and 20 PI in lung parenchyma, in agreement with the increase of CD8 cells at day 8. This coincidence may be explained by the model proposed by Abraham *et al.* (1997) in infections with respiratory pathogens, such as Klebsiella pneumonie and Bordetella pertusis in humans, suggesting three ways in which these cells are implicated. Firstly, by increasing mucosal secretions and epithelial cell proliferation and causing bronchoconstriction. Secondly, by taking part in phagocytosis and neutrophil recruitment by releasing TNF α and thirdly, by stimulating Th1 and Th2 responses and the development of lung pathophysiology. On the other hand, the apparent reduction of MC at day 12 may be due to the intense degranulation of these cells, which would make their detection difficult by metachromatic staining methods, such as the one used here. However, these degranulated cells are active and generate inflammatory factors, such as prostaglandins and leukotrienes, releasing potent chemotactic factors (Abraham *et al.*, 1997); therefore, it would be necessary to use different methods for the detection of these cells.

Finally, in this preliminary work, the extent of the macroscopic lesions, histopathology, fluorescence test and *M. hyopneumoniae* isolation were analyzed in the swine lung, as has been done by Kristensen *et al.* (1981) and Thacker *et al.* (1998) in vaccinated animals and by Strasser *et al.* (1992) in animals infected with *M. hyopneumoniae* In our work, besides these analysis, immunohistochemistry showed (Table 1) that the increase in CD4, CD8 and mast cells in the tissue did not mean a reduction in the number of bacteria, since at day 16 PI, the number of mycoplasma increased from a dilution of 10^{-3} to 10^{-4} CCU and there was an increase in fluorescence, suggesting infection progression, probably because the microorganism is externally located in the bronchia and bronchioles' lumen, where the immune reaction cannot effectively reach it, allowing it to continue releasing bacterial products that keep stimulating the immune response (Cruz *et al.*, 2003) and cytokine production (Rodriguez *et al.*, 2004; Choi *et al.*, 2006). This may explain in part the appearance of lesions induced by the ongoing inflammatory process.

CONCLUSION

The experimental infection with *Mycoplasma hyopneumoniae* in swine induced an early and transient increase of CD4 cells in lung parenchyma, followed by a persistent increase of CD8 and mast cells. This cell infiltrate may trigger the organ's pathology, since it coincides with the appearance of lesions and the increase in the number of bacteria in the tissue, suggesting that the developed response does not stop infection.

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