Veterinaria Italiana, **46** (4), 397-404

Effects of immune serum on macrophage cell cultures infected with *Mycoplasma mycoides* subsp. *mycoides* small colony: morphological analysis by scanning electron microscopy

Anna Rita D'Angelo, Flavio Sacchini, Tiziana Di Febo, Vincenzo Langella, Andrea Di Provvido, Gabriella Di Francesco, Rossella Lelli & Attilio Pini

Summary

Macrophages are pivotal cells of the immune system and play a key role in the host defence mechanism against pathogens. To date, the importance of macrophages and the role of humoral response in eliciting macrophage activity against *Mycoplasma mycoides* subsp. *mycoides* small colony (*Mmm*-SC), the causative agent of contagious bovine pleuropneumonia (CBPP), have only been marginally elucidated or are almost unknown. The present study was undertaken to investigate the changes in surface morphology of macrophages after *in vitro* infection with *Mmm*-SC in the presence of bovine immune serum. Morphological analysis was performed on macrophage cultures at 6 h post infection using the three-dimensional vision of scanning electron microscopy. Non-infected macrophages in the presence of negative or immune serum and macrophages infected with *Mmm*-SC in the absence of serum showed only minor cell surface changes. In contrast, clear surface modifications, broad veils, fine philopodia highlighting cell activation and small aggregates of mycoplasma closely attached to the macrophage membrane, were observed in infected macrophage cultures in the presence of immune serum. Our results suggest that specific humoral response to *Mmm*-SC may contribute and support phagocytic activity of macrophages.

Keywords

Contagious bovine pleuropneumonia, IgG, IgM, Immunoblotting, Macrophage, *Mycoplasma*, *Mycoplasma mycoides* subsp. *mycoides* small colony, Scanning electron microscopy.

Introduction

Mycoplasma mycoides subsp. *mycoides* small colony (*Mmm*-SC), the causative agent of contagious bovine pleuropneumonia (CBPP), is localised in the focal necrotic micro abscess of the lung, in the dense fibrotic pyogranulomatous area and in the infiltrations of macrophages around the bronchioles and perivascular cellular cuffs (5, 6, 8).

At the onset of mycoplasma infection, macrophages, derived from blood monocytes, are the major cellular component involved in the defence of the respiratory tract and they show a tendency to accumulate at the sites of mycoplasma localisation (5). The outcome of mycoplasma-macrophage interaction may determine the subsequent progression of disease (10). Macrophages not only perform effector functions, such as receptor-mediated phagocytosis, but they are also important accessory cells of the immune system and are involved in antigen presentation and cytokine production (1).

Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Via Campo Boario, 64100 Teramo, Italy
a.dangelo@izs.it

To date, pathogenic studies on *Mmm*-SC have marginally explained the role of macrophages and their phagocytic activity against mycoplasma. Moreover, the function of bovine humoral response during mycoplasma infections and the possible role of antibodies, as opsonins, able to elicit macrophage activity, have been partially investigated. Some studies on *Mycoplasma bovis* have demonstrated the importance of IgG in promoting mycoplasma phagocytosis and killing by bovine alveolar macrophages and neutrophils (9). In studies conducted on *Mmm*-SC, no correlation has been observed between IgM and IgG antibody levels and the clinical/pathological progression of the disease (2, 11). The current *in vitro* study was designed to observe the interaction between *Mmm*-SC and macrophage cultures in the presence or absence of specific immune serum. Our hypothesis is that antibodies to *Mmm*-SC may function as opsonins promoting macrophage activation, expressed as morphological changes.

The research was based on the ability of *Mmm*-SC and macrophages to adhere to glass surfaces; the use of the scanning electron microscopy (SEM) technique led to a better understanding of the morphological changes that occur with macrophage cells. With a relatively wide range of magnification, the use of SEM means that the area of interest of a specimen that had initially been scanned at a lower magnification can be focussed on with ease (13).

Materials and methods

Mycoplasma strains and growth condition

A Mmm-SC field strain isolated in the Caprivi region of Namibia in 2003 was cultured in contagious caprine pleuropneumonia (CCPP) medium (15) and incubated for 48 h at 37°C in an atmosphere of 5% CO_2, had a titre of 10^8 colony-forming units (cfu)/ml. The culture was centrifuged at 4 000 g for 15 min and the pellet washed and re-suspended in RPMI-1640 (Sigma, Munich) with 10% foetal bovine serum (Sigma) (R-10). *Mmm*-SC survival in R-10 medium was successfully assessed at intervals by titration on CCPP agar (15) as described previously (3).

Sera

The complement fixation test was performed in accordance with the *Manual of diagnostic tests and vaccines for terrestrial animals* of the World Organisation for Animal Health (*Office International des Épizooties*: OIE) (14). The *Mmm*-SC bovine immune serum had a titre of 1:1 280 (12). The negative serum that had a titre <1:10 was obtained from a healthy calf from a CBPP-free population. To evaluate the possible opsonising effects of immunoglobulins, all sera were heat inactivated at 56°C for 30 min before use.

In order to identify the dominant immunogenic antigens of *Mmm*-SC recognised by IgM and IgG antibody isotypes, the immunoblotting test was performed according to OIE procedures (14). To reduce background staining, negative control serum was diluted 1:3; whereas for IgG and IgM analysis, the immune serum was diluted at 1:80 and 1:10, respectively. An anti-bovine IgG horseradish peroxidase (HRP) conjugated (Sigma, A8917), diluted at 1:4 000 and an anti-bovine IgM HRP conjugated (Bethyl Laboratories, Montgomery, Texas), diluted at 1:2 000, were used as secondary antibodies.

Monocyte cell separation

Bovine monocytes were isolated from venous blood taken in ethylenediaminetetraacetic acid (EDTA) from a naive healthy calf from a CBPP-free population. Erythrocytes were lysed by adding 16 ml of whole blood lysis buffer into 4 ml of blood and the cell suspension was left standing at room temperature for 30 min (7). Cells were centrifuged at 400 g for 15 min, at 18°C and then washed twice with phosphate buffered saline (PBS). The pellet was re-suspended in R-10 medium. Cell count and cell viability were performed by trypan blue staining. Flow cytometric acquisition and analysis was performed using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Brea, California) with EXPO™ 32 software. The cell suspension was displayed by using forward and side-angle scatter properties, following which the percentage of monocytes

was calculated. The density of monocytes was adjusted to 3×10^6 monocytes/ml. One ml of cells suspension was cultured in tissue culture chamber slides (Nunc) with 0.5 ml of R-10 medium and incubated at 37°C in an atmosphere of 5% CO_2 for 2 h to allow the monocytes to attach to the glass surface. The supernatants were then discarded, cultures rinsed twice with fresh medium and incubated overnight at 37°C in an atmosphere of 5% CO_2. After discarding supernatants, monocyte cultures showing morphological characteristics of macrophages as observed with an optical microscope, were rinsed with PBS. Subsequently 0.5 ml of the mycoplasma culture (Table I: test 1) and 0.5 ml of the mycoplasma culture, to which an equal volume of immune or negative sera was added (Table I: tests 2 and 3), were dispensed into each of three chamber slides with macrophage monolayers and incubated for 6 h at 37°C in an atmosphere of 5% CO_2.

Table I
Tests for scanning electron microscopy analysis

Tests	Cells	*Mycoplasma*	Serum
Control 1	Macrophages		
Control 2		*Mmm*-SC	
Control 3	Macrophages		Negative
Control 4	Macrophages		Positive
Test 1	Macrophages	*Mmm*-SC	
Test 2	Macrophages	*Mmm*-SC	Negative
Test 3	Macrophages	*Mmm*-SC	Positive

Mmm-SC *Mycoplasma mycoides* subsp. *mycoides* small colony

Finally the chamber slides were washed twice in PBS to remove the non-adherent cells and mycoplasmas. A macrophage monolayer, a smear of *Mmm*-SC culture and a macrophage monolayer in the presence of immune and negative sera were used as controls (Table I: controls 1-4).

Scanning electron microscopy

The chamber slides and the *Mmm*-SC smear slide were placed in modified Karnovsky's fixative (Electron Microscopy Science, Hatfield, Pennsylvania) in 0.1 M cacodylate buffer, pH 7.2 at 4°C for 1 h and then washed twice with cold 0.1 M cacodylate buffer, pH 7.2 at 4°C for 1 h. Dehydration of fixed cells was performed in 25%, 50%, 75%, 95% and twice in 100% graded acetone solutions, at room temperature for 10 min each and were then air-dried immediately (3). Small sections (1 × 1 cm) of slides were then cut and glued onto polished aluminium stubs and coated uniformly *in vacuum* with a layer of approximately 20 nm thickness of gold. Coated samples were examined using a Zeiss DSM 940A SEM. Micrographs were recorded as electronic images and archived on an AxioVision system.

Results

Sera characterisation

Immunoblotting analysis, performed using whole *Mmm*-SC cells as antigen, revealed that the IgG contained in the immune serum identified a protein panel of 110, 98, 95, 85, 80, 72, 60-62, 48 and 39 kDa. No protein band was detected using negative serum (Fig. 1).

Repeated analysis investigating the *Mmm*-SC antigen profile identified by IgM showed that positive serum recognised the same protein antigens of IgG except for the fraction of 95 kDa. Results also demonstrated that IgM contained in the negative serum were able to identify proteins of 80, 60-62 and 48 kDa (Fig. 1).

Scanning electron microscopy

For SEM analysis, control tests 1 to 4 were observed before tests 1 to 3. In control tests, most of the monocytes observed under the SEM had developed into typical macrophages that adhered to the culture slides, showing distinct changes comparable to natural activated macrophages. The cells showed generally rounded shapes (Fig. 2) and had undergone certain morphological changes, such as the light spreading of cytoplasm and development of membrane ruffling and microprojections required for glass attachment (Fig. 3). The *Mmm*-SC culture smear showed rounded or pleomorphic micro-organisms on a non-specific background (Fig. 4). Most of the macrophages, after 6 h of incubation in the

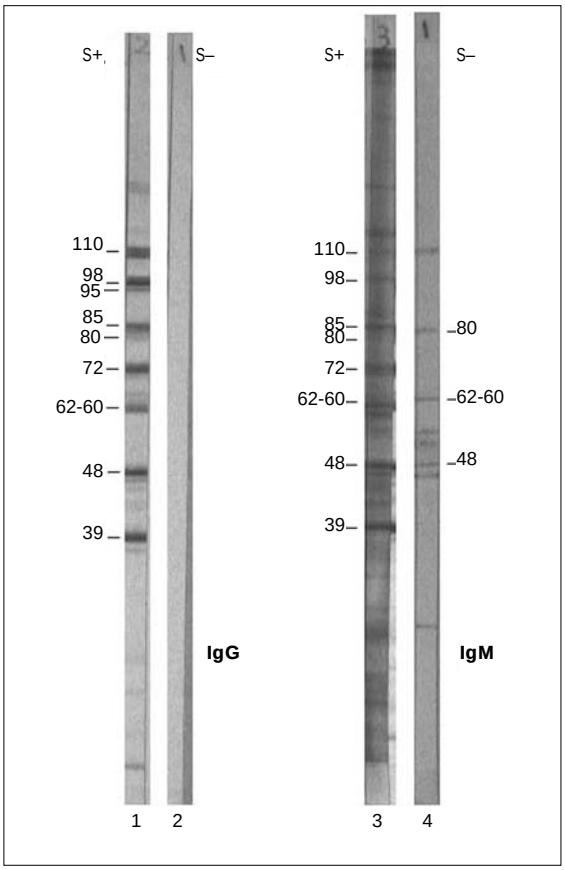

Figure 1
Immunoblots illustrate *Mycoplasma mycoides* subsp. *mycoides* small colony antigens identified by IgG (rows 1 and 2) and IgM (rows 3 and 4) of immune (S+) and negative (S–) sera
The molecular masses of the immunogenic protein fraction identified by IgG and IgM are indicated for each row

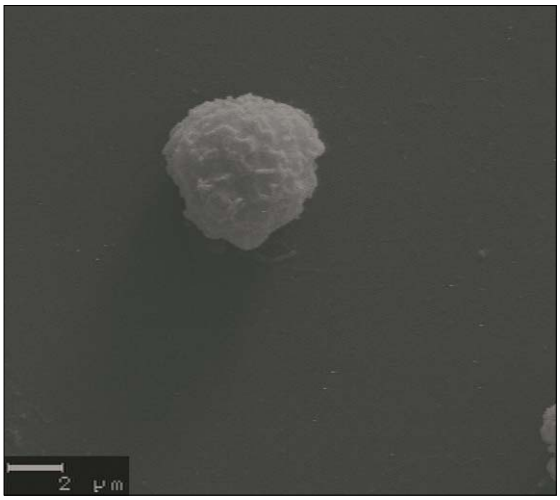

Figure 2
Control 1: a rounded shaped macrophage adheres to the culture slides
(Scanning electron microscope 5 000×)

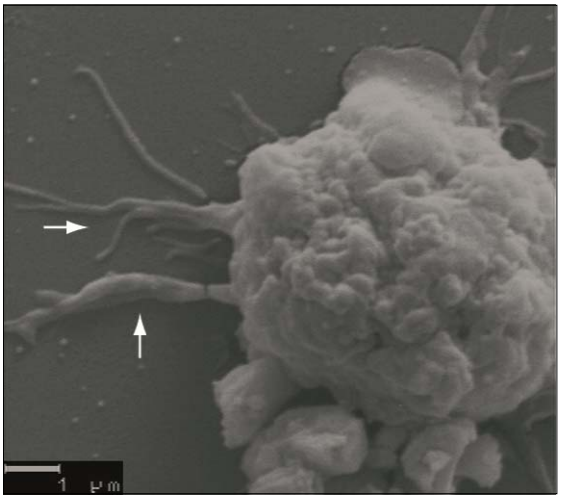

Figure 3
Control 1: a rounded shaped macrophage adheres to the culture slide
Note the light spreading of cytoplasm and development of microprojections (arrows)
(Scanning electron microscope 10 000×)

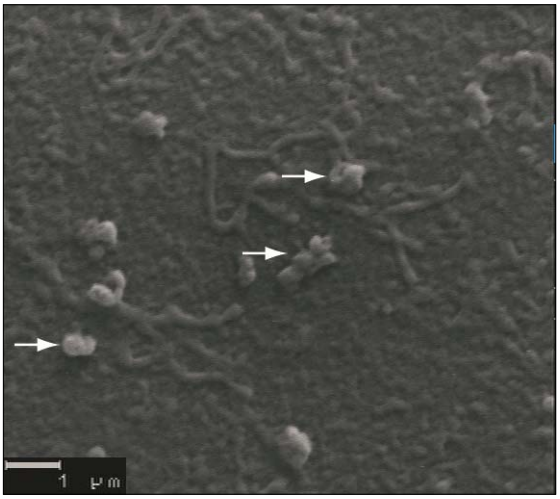

Figure 4
Control 2: *Mycoplasma mycoides* subsp. *mycoides* small colony culture smear
Note the variability in the shape of the organisms (arrows) on the non-specific background
(Scanning electron microscope 10 000×)

presence of bovine immune or negative sera, showed a more marked spreading of cytoplasm, large flange-like processes and development of philopodia, while maintaining a rounded shape (Figs 5 and 6).

An analysis was performed after 6 h of incubation with *Mmm*-SC. In test 1, macrophages showed rounded shapes, limited cell surface modifications and the presence of several plasmatic membrane microprojections;

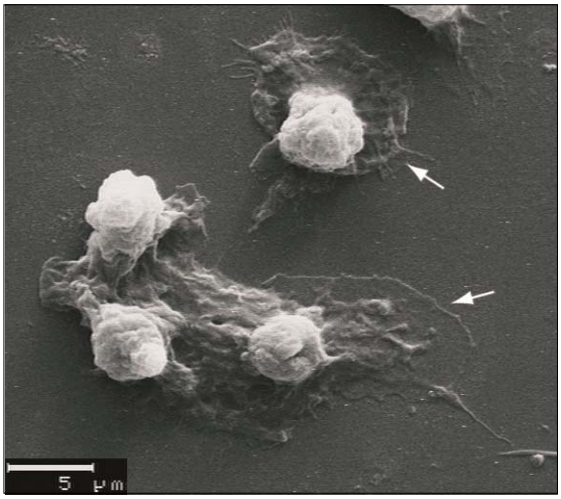

Figure 5
Control 3: macrophage culture following addition of negative serum
Note the marked spreading of the cytoplasm and large flange-like processes (arrows) while a rounded shape is maintained
(Scanning electron microscope 3 000×)

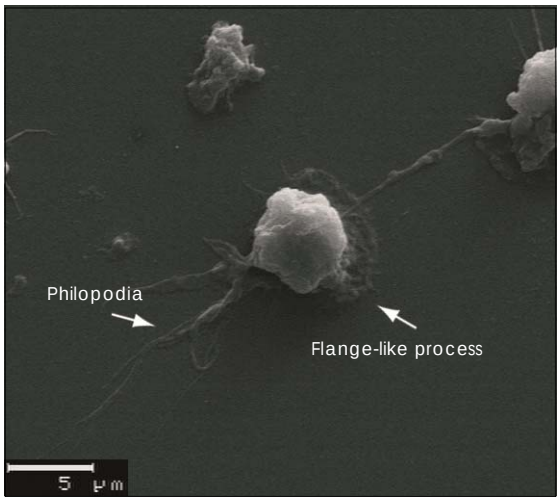

Figure 6
Control 4: macrophage culture following addition of immune serum
Note the marked spreading of the cytoplasm and large flange-like processes and philopodia while a rounded shape is maintained
(Scanning electron microscope 3 000×)

mycoplasmas formed aggregates that were separated from cells (Fig. 7). In test 2, macrophage cultures infected with *Mmm*-SC, in the presence of negative serum, showed similar modifications and free mycoplasma cells were observed around the cells (Fig. 8).

In test 3, following the addition of bovine immune serum, *Mmm*-SC-infected cultures showed significant morphological changes. Although they retained their structural individuality, a vast network of inter-connecting philopodia between cells, with the loss of their rounded shape, were seen (Fig. 9). They revealed the presence of wide veils, enveloping mycoplasma aggregates, fine philopodia (Fig. 10) and mycoplasmas that

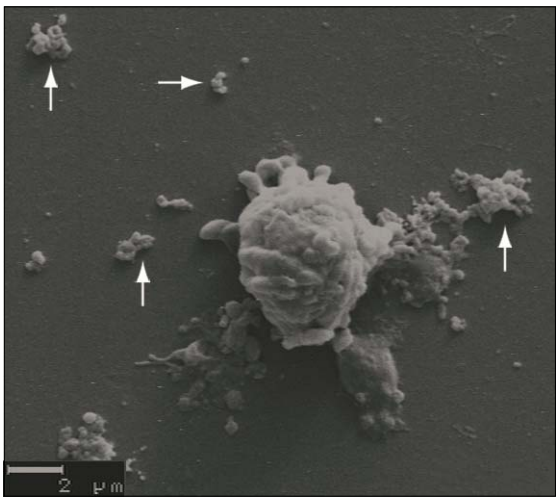

Figure 7
Test 1: macrophage culture infected with *Mycoplasma mycoides* subsp. *mycoides* small colony
Note the rounded shaped cell, limited surface modification and mycoplasma aggregates (arrows) separated from cell
(Scanning electron microscope 5 000×)

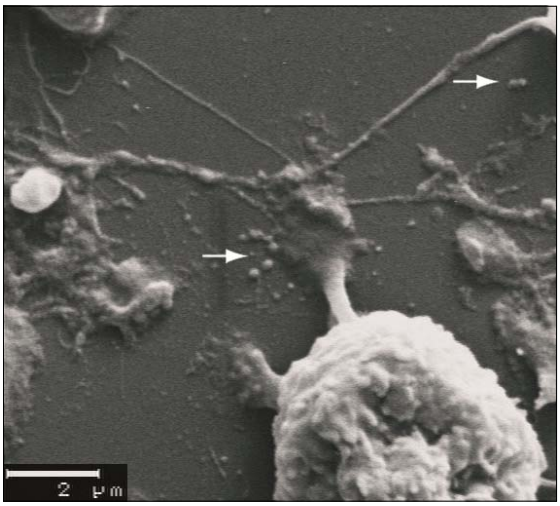

Figure 8
Test 2: macrophage culture infected with *Mycoplasma mycoides* subsp. *mycoides* small colony following addition of negative serum
Note the round shape of the macrophage and isolated mycoplasma organisms (arrows) separated from the cell
(Scanning electron microscope 8 000×)

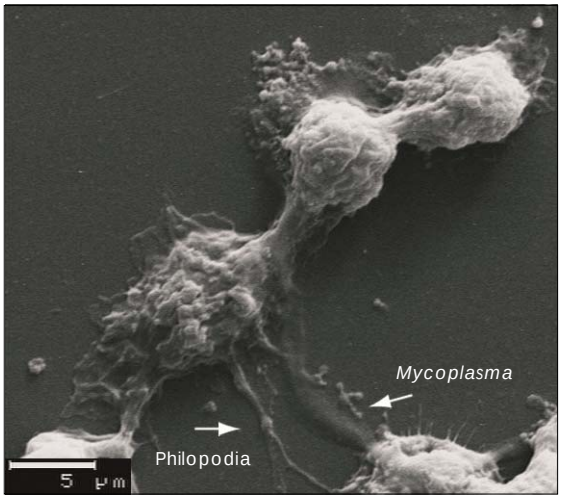

Figure 9
Test 3: macrophage culture infected with *Mycoplasma mycoides* subsp. *mycoides* small colony following addition of immune serum
Note the loss of the rounded shape of the cell and the vast interlocking network of philopodia between cells
(Scanning electron microscope 3 000×)

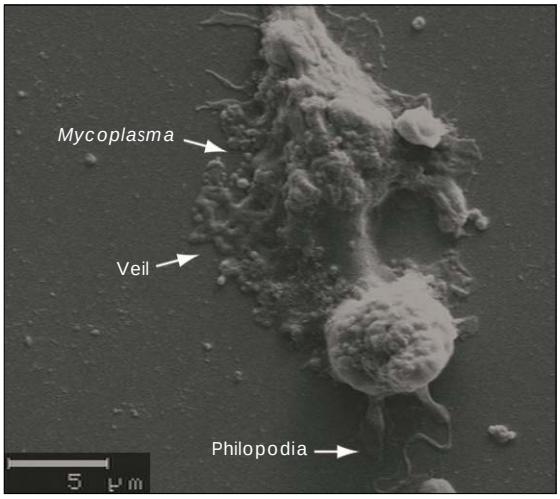

Figure 10
Test 3: macrophage culture infected with *Mycoplasma mycoides* subsp. *mycoides* small colony following addition of immune serum
Note the presence of a wide and transparent veil, enveloping mycoplasma aggregates and fine philopodia
(Scanning electron microscope 3 000×)

were closely attached to the membrane (Fig. 11).

Conclusions

CBPP is a disease that is transmitted by contact and the pathogen generally remains restricted to the lungs causing bronchiolitis and

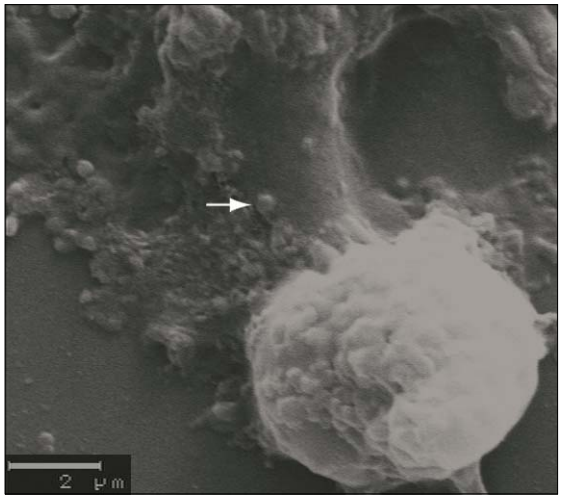

Figure 11
Test 3: macrophage culture infected with *Mycoplasma mycoides* subsp. *mycoides* small colony following addition of immune serum
Note the individual *mycoplasmas* (arrow) closely attached to the macrophage membrane
(Scanning electron microscope 8 000×)

pneumonia. The host immune mechanisms, following *Mmm*-SC invasion of the respiratory tract, are only partially known. A microscopic analysis of lung lesions revealed infiltration of neutrophils and macrophages in the alveoli during the early stages of inflammation, followed by recruitment of monocytes and plasmacells (5, 6, 8). Thus, alveolar macrophages and neutrophils represent the primary cells in the host defence mechanism. The initial mycoplasma-phagocyte interaction plays an important role in determining the progression of infection and the severity of disease (9).

Macrophages are important accessory cells of the immune system and are involved in antigen presentation and cytokine production. They act as secretory and regulatory cells, initiating and modulating the inflammatory processes. Furthermore, phagocytic activity may be enhanced by opsonising antibodies, produced as a result of exposure to mycoplasma antigens (1, 10).

Studies on the interactions of *Mycoplasma dispar* with bovine alveolar macrophages indicate that their ability to phagocyte mycoplasmas is strongly correlated with the production of opsonising antibodies that are mainly directed against capsular antigens (4).

The results of the *in vitro* study conducted here indicate that macrophage activation and morphological changes observed by SEM are induced by the presence of *Mmm*-SC immune serum. Macrophage activation results in the development of philopodia and micro-projections on the cell surface membrane and mycoplasma aggregation on the surface.

The experimental model suggests that humoral response, induced in cattle infected with *Mmm*-SC, may support macrophage activation.

Immunoblotting results demonstrated that IgM and IgG in immune serum mostly recognised the same protein fractions. The switching from a primary IgM to a more specific IgG response, however, may represent an important step of the immune system aimed to control *Mmm*-SC tissue spread; IgG may act as opsonins targeting mycoplasmas and favouring phagocytosis through the fragment crystallisable (Fc) γ receptor of macrophages.

The role of IgM in CBPP humoral response is not clear but immunoblotting analysis revealed that serum from naive cattle contained IgM that was able to detect certain *Mmm*-SC antigens. Probably the 80, 60-62 and 48 kDa fractions identified only by IgM contain epitopes similar to other micro-organisms against which the animal had been sensitised. IgM are characterised by a high avidity but low affinity that results in lesser specificity for the epitope and possible antigen cross-reactivity (1).

Additional studies are required to clarify the role of B-cell responses during CBPP infection and the mechanisms involved in Mmm-SC macrophage interaction.

References

1. Abbas A.K., Lichtman A.H. & Pillai S. 2007. Cells and tissues of the adaptive immune system. *In* Cellular and molecular immunology, 6th Ed. Saunders Elsevier, Philadelphia, 56 pp.
2. Abdo E.M., Nicolet J., Miserez R., Gonçalves R., Regalla J., Griot C., Bensaide A., Krampe M. & Frey J. 1998. Humoral and bronchial immune responses in cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* small colony type. *Vet Microbiol*, **59** (2-3), 109-122.
3. Al-Kaissi A. & Alley M.R. 1983. Electron microscopic studies of the interaction between ovine alveolar macrophages and *Mycoplasma ovipneumoniae in vitro*. *Vet Microbiol*, **8**, 571-584.
4. Almeida R.A., Wannemuehler M.J. & Rosenbusch R.F. 1992. Interaction of *Mycoplasma dispar* with bovine alveolar macrophages. *Infect Immun*, **60** (7), 2914-2919.
5. Bashiruddin J.B., Santini F.G., De Santis P., Visaggio M.C., Di Francesco G., D'Angelo A. & Nicholas R.A.J. 1999. Detection of *Mycoplasma mycoides* subspecies *mycoides* in tissue from outbreak of contagious bovine pleuropneumonia by culture, immunohistochemistry and polymerase chain reaction. *Vet Rec*, **145**, 271-274.
6. Coetzer J.A.W. & Tustin R.C. 2004. Contagious bovine pleuropneumonia. *In* Infectious diseases of livestock, 2nd Ed. Oxford University Press, Cape Town, Oxford and New York, Vol. 3, 2045-2059.
7. Faldyna M., Levá L., Knötigová P. & Toman M. 2001. Lymphocyte subsets in peripheral blood of dogs – a flow cytometric study. *Vet Immunol Immunopathol*, **82**, 23-37.
8. Food and Agriculture Organization (FAO) 2002. Preparation of contagious bovine pleuropneumonia contingency plans. *In* FAO Animal Health Manual. FAO, Rome, 14.
9. Howard C.J. & Taylor G. 1983. Interaction of mycoplasmas and phagocytes. *Yale J Biol Med*, **56**, 643-648.
10. Marshall A.J., Miles R.J. & Richards L. 1995. The phagocytosis of mycoplasma. *J Med Microbiol*, **43**, 239-250.
11. Niang M., Diallo M., Cissé O., Kone M., Doucoure M., Roth J.A., Balcer-Rodrigues V. & Dedieu L. 2006. Pulmonary and serum antibody responses elicited in zebu cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* SC by contact exposure. *Vet Res*, **37**, 733-744.
12. Pini A., De Santis P., Langella V., Ferri N., Scacchia M., Di Francesco G., Tittarelli M., Visaggio M. & Lelli R. 1999. Experimental infection of cattle with a strain of *Mycoplasma mycoides mycoides* small colony (*Mmm* SC). *In* COST Action 826 International Symposium: Mycoplasmas of ruminants, 2-4 June, Toulouse. European Commission, Brussels, 93.

13. Stadtländer H. 2007. Scanning electron microscopy and transmission electron microscopy of mollicutes: challenges and opportunities. *In* Modern research and educational topics in microscopy. Series 2 (A. Méndez-Vilas & J. Díaz, eds). Formetex, Badajoz, 122-131.
14. World Organisation for Animal Health (Office International des Épizooties: OIE) 2008. Contagious bovine pleuropneumonia. *In* Manual of diagnostic tests and vaccines for terrestrial animals, 6th Ed. OIE, Paris, 712-724.
15. World Organisation for Animal Health (Office International des Épizooties: OIE) 2008. Contagious bovine pleuropneumonia. *In* Manual of diagnostic tests and vaccines for terrestrial animals, 6th Ed. OIE, Paris, 1000-1003.

