CYTOLOGICAL STUDIES

OF OVINE ALVEOLAR MACROPHAGES: INTERACTION WITH

MYCOPLASMA OVIPNEUMONIAE IN VITRO

This thesis is presented in partial fulfilment (30%) of the requirements for the degree of Master of Philosophy in Veterinary Pathology at Massey University.

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ABSTRACT

The attachment between *Mycoplasma ovipneumoniae* organisms and ovine alveolar macrophages was studied in culture for a 24 hour period and antibody-mediated phagocytosis of *M. ovipneumoniae* organisms was observed by both scanning and transmission electron microscopy. *Mycoplasma ovipneumoniae* organisms have the ability to attach to the alveolar macrophage membrane without inducing phagocytosis although they stimulated mitotic division in early cultured cells. The addition of specific antibody to the mycoplasma-macrophage cultures provoked phagocytosis of surface attached and surrounding *M. ovipneumoniae* organisms. Alveolar macrophages stimulated by specific antibody showed rapid and extensive spreading on the glass coverslip and prominent membrane ruffling and filopodia. Many exterior openings and fine cytoplasmic pits were also evident which may represent pinocytotic vesicle formation sites. With transmission electron microscopy *M. ovipneumoniae* organisms were observed surrounded by macrophage filopodia 2 hours after the addition of specific antibody and numerous micro-organisms were seen within phagocytic vacuoles. Some of the intracellular *M. ovipneumoniae* organisms appeared normal while others appeared partially or completely degraded. Twenty four hours after the addition of specific antibody, intracellular *M. ovipneumoniae* organisms had been digested.

A new procedure for collection of alveolar macrophages was developed. The procedure provides an alternative to other methods and may be particularly useful for collecting alveolar macrophages from the lungs of large animal species such as sheep and cattle. Acetone was used to dehydrate macrophages for SEM with excellent results.

In conclusion, it was found that the addition of specific antibody to an *M. ovipneumoniae* - macrophage culture stimulated phagocytosis of these micro-organisms. This suggests that if sheep gain high titres to *M. ovipneumoniae*, their alveolar macrophages will be able to destroy inhaled *M. ovipneumoniae* organisms quickly and effectively; a possibility which should be tested further *in vivo*.

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INTRODUCTION

Sheep are of special economic importance to New Zealand, and it is generally recognised that chronic non-progressive pneumonia (CNP) is one of the most prevalent diseases encountered in this species both here and overseas (54) (24) (101) (34) (4). The aetiology of this disease has not been clearly established, but natural and experimental studies undertaken to date have demonstrated a close association between *Mycoplasma ovipneumoniae* micro-organisms and CNP (32) (104) (105) (2) (3).

Because of their capacity to clear inhaled particles from lungs and process antigenic material (44) alveolar macrophages are considered to be one of the most important components of the pulmonary immune system. No studies of the normal ultrastructure and surface membrane morphology of ovine alveolar macrophages have been reported up until the present time. These cells are invariably seen in early lesions of CNP and are often predominant in the alveolar exudate of advanced lesions (1). Macrophages, therefore, have an important role in the pathogenesis of this disease.

The interaction between certain species of mycoplasmas and alveolar or peritoneal macrophages has been studied in considerable detail (92) (55) (56) (57). Studies of both natural and experimental *Mycoplasma pneumoniae* indicate that thymic-dependent immunity is an important aspect of host response, so that alveolar macrophages play an essential role in human mycoplasma pneumonia through the pulmonary cell-mediated immune system (92). Since it has been shown that *M. ovipneumoniae* has a role in the development of chronic non-progressive pneumonia in sheep, the interaction between this micro-organism and ovine alveolar macrophages deserved investigation using *in vitro* methods. Studies of the changes in the macrophage plasma membrane during infection by a living surface-associated mycoplasma and the fate of the intracellular

organisms in the presence and in the absence of specific antibody were therefore undertaken.

When cells are prepared for scanning electron microscopy by critical point or freeze drying, their surface architecture is well preserved and a variety of different surface structures can be recognised (91). Few papers describing the three-dimensional surface membrane changes that transpire during mycoplasm-macrophage interaction have appeared (92) (93) (91) (55). This study was therefore, designed to utilize the three-dimensional qualities of scanning electron microscopy to examine this interaction *in vitro*, and follow the events over a 24 hour period. Transmission electron microscopy was also used to confirm these changes and study the intracellular events in detail.

A further aim of the present work was to add to current knowledge of the macrophage plasma membrane and the normal ultrastructure of ovine alveolar macrophages. In addition, the study provided an opportunity to acquire information on the surface morphology of *M. ovipneumoniae* micro-organisms.

CHAPTER ONE

GENERAL REVIEW OF LITERATURE
SECTION ONE

THE ALVEOLAR MACROPHAGE

1. INTRODUCTION

The lung occupies a unique position among the internal organs of the body in that it is more constantly in contact with the external environment than any other structure. It is exposed to a variety of dusts, fumes and micro-organisms, many of which are potentially harmful. The lung is protected against inhaled particles by a combination of phagocytosis and the action of mucociliary apparatus (30). In addition, the mucus lining the respiratory tract possesses antibacterial properties and is particularly rich in lysozyme. The mucus also provides a layer of insulation between trapped particles and the respiratory epithelial cells (94).

The most important mechanism for processing inhaled particles which reach the lower respiratory tract is the phagocytic action of alveolar macrophages (43) (44). The alveolar macrophage is a highly motile free cell, which commonly resides on the alveolar wall. It is specialized for processing inhaled particles that reach terminal airways and alveoli. It contains phagocytic vacuoles, micropinocytic vesicles and possesses numerous processes.

2. ORIGIN OF THE ALVEOLAR MACROPHAGE

Since the end of the last century, the origin of the alveolar macrophage has been one of the major preoccupations of pulmonary histologists and pathologists. Many investigators

have attempted to determine the origin of these cells and until recently, histologists have with few exceptions, maintained that the alveolar macrophage was epithelial in nature (7) (8). The concept of epithelial origin has led to a considerable confusion in the terminology used to describe these cells (Table 1).

Betalanffy (7) and Stuart (103) have reviewed some of the diverse sites of origin for these cells proposed by earlier workers. They may be summarized as follows:-

1. The endothelial cells of the pulmonary capillaries.
2. Leukocytes which had left the pulmonary capillaries by diapedesis.
3. Histiocytes which had emigrated from the pulmonary parenchyma into alveolar spaces.
4. Alveolar epithelial cells (type I and II).
5. Bronchial epithelial cells.
6. The haemopoietic system.

In 1964 Moore and Schoenberg investigated the cellular response to Freund's adjuvant in the lung of rabbits. Their electron microscopic observations suggested that the population of cells within the alveolar space was contributed to by monocytes from the circulation, mesenchymal cells of the alveolar walls and epithelial lining cells of the alveoli (82) (83).

It was Pinkett *et al* (90) who were the first to provide experimental evidence for the haemopoietic origin of alveolar macrophages. They used chromosome markers in x-irradiated CBA mice, repopulated with T6T6 bone marrow, and showed that two-thirds of the alveolar macrophages were blood-derived cells and one-third were of pulmonary origin. However some CBA mouse haemopoietic cells survived irradiation, so that their results were not conclusive. A short time later, Virolainen (111) applied T6T6 genetic markers in CBA radiation chimeras and concluded that all dividing free macrophages, regardless of their site were of haemopoietic origin. The work of

Table 1: SOME EARLY TERMS FOR THE ALVEOLAR MACROPHAGE

Term	Comment	Author
Septal cell*	Due to the predominant location of macrophages on the alveolar septa.	Lang, 1925
Niche cell*	Because macrophages were often encountered at points of junction.	Gross, 1927
Epicytes		Clara, 1936
Pneumocytes*		Businco & Guinti, 1930
Pneumonocytes*		Macklin, 1953
Alveolar cell* Alveolar epithelial cell*		Aschoff & others
Dust cell	Macrophages containing foreign material	Von Ins. 1876
Alveolar macrophage Pulmonary macrophage	Often used by pathologists	
Alveolar histiocyte Respiratory histiocyte Pulmonary histiocyte	Usually used by cytodiagnosticians	
Alveolar phagocyte		Briscoe (1908)
Dust-laden cell Heart failure cell	Alveolar macrophage containing blood or haemosiderin	
Foam cell	Vacuolated alveolar macrophages are foamy in appearance under the light microscope.	Mettencheimer (1866) and Macklin (1949)
Siderophages	Macrophages containing haemosiderin	Lendrum (1950)

* Terms which are confused with alveolar epithelial cells.

Brunstter *et al*, (17) supported this conclusion. Using a pre-albumin esterase marker in the donor mouse, they established that alveolar macrophages were primarily of bone marrow origin. This work was extended by Godleski (42) who utilized an antigenic marker to identify cells of haemopoietic origin, thus confirming that alveolar macrophages are entirely of haemopoietic origin. The origin and turnover of alveolar macrophages in experimental pneumonia was studied by Velo & Spector (110) who also concluded that macrophages in the pulmonary inflammatory reaction were of bone marrow origin.

3. DISTRIBUTION OF MACROPHAGES IN PULMONARY TISSUE

Macrophages are widely distributed in pulmonary tissue and may be found in the following sites (97):-

1. The interstitial connective tissue of the alveolar wall.
2. Fixed to the alveolar wall.
3. Free in the lumen of the alveolus.

Occasionally, alveolar macrophages may be found extending through the interstitium from one alveolar surface to the next and may also pass through the pores of Kohn (98). Free cells found in the alveolar spaces however are not all true macrophages (20). From their work with Freund's adjuvant in rabbits, Moore & Schoenberg (82) (83) postulated that monocytes migrate from the small blood vessels through the tight junctions of endothelial cells into the interstitial tissue, and then between the cell junctions of the alveolar epithelial cells into the air spaces.

Once macrophages reach the alveoli they may persist there for a long period or they may enter the terminal airways and eventually be carried by the mucociliary apparatus to the oropharynx. Alternatively, if an inflammatory process is present they may migrate into the interstitium and thence to draining lymph nodes where they ultimately reside (94). The

life span of an alveolar macrophage depends upon the nature of the particles which it ingests. For example; if ingested particles are chemically inert, the alveolar macrophages may survive throughout the life of the individual. If ingested particles are not inert but easily digested by lysozomal enzymes the macrophages life span will be only slightly shortened. Some materials, such as asbestos particles however, are highly toxic to alveolar macrophages and they are killed shortly after phagocytosis (106).

4. THE ALVEOLAR MACROPHAGE AS PART OF THE MONONUCLEAR PHAGOCYTE SYSTEM

Macrophages regardless of their situation in the lung are all considered to be part of the 'Mononuclear Phagocyte System'. The terms 'Histiocytic System', 'Mononuclear Cell System', 'Macrophage system' and 'Reticuloendothelial System' have all been used by earlier workers to classify mononuclear cells and to define the cell system they are considered to form (20) (35) (36) (38) (103). van Furth (35) proposed that the term reticulo-endothelial system referred to a heterogenous collection of cells situated throughout the body and that these cells have a different origin, morphology and function. With these considerations in mind macrophages in various sites (Table 2) have now been grouped together into one system, the 'Mononuclear Phagocyte System' (MPS) (38). At present cell types accepted as descendants of the promonocytes in the bone marrow are all considered to be members of the MPS (Table 3).

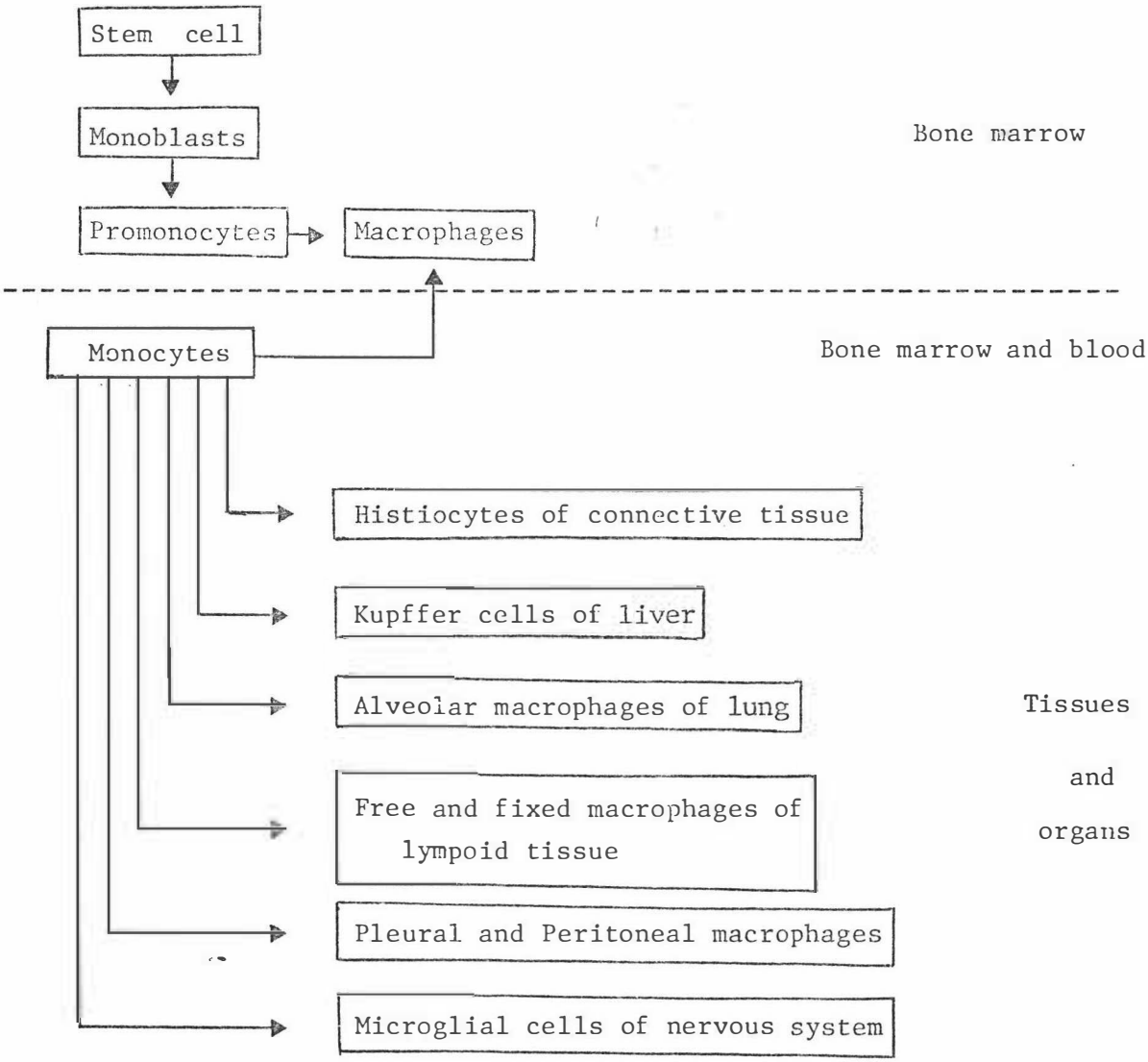
5. MORPHOLOGY OF THE ALVEOLAR MACROPHAGE

Although the structure of alveolar macrophages of the mammalian species studied to date is similar, it differs from that of macrophages from elsewhere in the body. This is because of the specialised structure, metabolism and blood supply of the

Table 2: THE DISTRIBUTION OF MACROPHAGES IN MAMMALIAN TISSUE (97)

Organ or tissue	Synonym	Location of macrophages
Liver	Kupffer cell	Lining hepatic sinusoids; connective tissue of portal tracts.
Spleen		Lining venous sinuses and enmeshed in Billroth cords of red pulp; scattered among lymphocytes of Malpigian follicles in white pule; in marginal zones.
Lymph nodes-Cortex	Dendritic macrophage	Scattered in lymphoid follicles of cortex
" " -Medulla		Lining subcapsular and medullary sinuses; scattered in medullary pulp.
Bone marrow	Monocyte and macrophage	Lining venous sinuses of red marrow; scattered in extra-sinusoidal tissues.
Thymus		Scattered throughout cortex and medulla.
Lung	Alveolar macrophage	Within interstitium of alveolar wall; fixed on alveolar wall; in alveolar spaces.
Central nervous system	Microglia; glial cell; neuroglia	Throughout central nervous tissue.
Pleural and peritoneal cavities	Peritoneal macrophage	Pleural and peritoneal fluid and milk spots.
Adrenal gland		Lining sinusoids of cortex; scattered in medulla.
Joints	Type A and M cells	Part of synovial wall; within synovial fluid.
Subcutaneous tissue Alimentary tract	Histiocyte)	Connective tissue.
Kidney	Mesangial cell	
Pituitary gland; testis; ovary; endometrium	)	Lining vascular channels
Blood	Monocyte; macrophage	Monocytes and some macrophages circulate in the blood stream.
All tissues and organs		Part of inflammatory exudate.

Table 3: THE MONONUCLEAR PHAGOCYTE SYSTEM (MPS)
Based on van Furth.et al.(38)



pulmonary tissue in which these cells reside and their high level of exposure to inhaled gases and foreign particles (89).

The alveolar macrophage in tissue section measures from 8 to 40 μm in diameter and may appear in the respiratory tissue in various morphological forms. Both 'granular' and 'vacuolated' types of these cells can frequently be distinguished. The cells have a variety of shape due to their ameboid properties. The nucleus has a spherical or oval shape and sometimes an irregular outline. It occupies one-third to two-thirds of the total cell, is eccentric in position and measures from 6 to 8 μm in diameter. The cytoplasm is acidophilic or slightly basophilic and may contain dense, dark, granules and vacuoles of various sizes. Under the light microscope, mitochondria can be seen in abundance evenly distributed throughout the cytoplasm by using the Altmann technique(7). A perinuclear Golgi zone, containing numerous small granules surrounding the nucleus is commonly seen in the cytoplasm using Golgi's silver method. Application of Perl's reaction and Best's technique usually reveals the presence of both iron and glycogen granules respectively in mature cells (7) (8).

The fine structure of alveolar macrophages has been described in detail by Karrer (60) (61) and Low (71). Comparatively, alveolar macrophages are larger than peritoneal macrophages of the same species (68). Their cytoplasm contains a moderate amount of smooth endoplasmic reticulum, relatively little rough endoplasmic reticulum, but more lysosomes than peritoneal macrophages. It contains a small number of mitochondria and a well developed Golgi apparatus. Many inclusion bodies or lysosomes are present, often containing myelin figures or ferritin. The cell outline is extremely irregular and shows multiple processes and invaginations. The nucleus appears oval with occasional indentations or clefts. The distribution of the chromatin varies somewhat but it usually appears as thin or scattered dark patches at the periphery of the nucleus. One or two nucleoli are

frequently present (41).

The morphology of alveolar macrophages from germ-free animals is essentially the same as that of conventionally reared animals (6). Leake & Heise (68), observed five major morphological differences between alveolar and peritoneal macrophages of germ-free rats (Table 4).

The morphology of free alveolar macrophages of the rat (12), mouse (21) (79) (50), monkey (59), rabbit (85) and cattle (33) have been studied in detail by light and electron microscopy.

Three morphological types of mononuclear cells have been distinguished in lavages of human lung by Cohn & Cline (25); these cells were termed types A, B and C and each type exhibited various morphological features as shown in Table 5 and Figure 1.

Williams (117) studied alveolar macrophages obtained from hysterectomy-derived pigs and classified them into three types, on the basis of size (Fig. 1). The proportion of each type is shown in Figure 1 and a wide variation from those of man is evident. Under the light microscope, however, the structure of hysterectomized pig alveolar macrophages is almost the same as that of man. From these studies it can be concluded that there are two major differences between alveolar macrophages of man and pigs: -

1. The size of the macrophages in each of the three categories is greater in man. This may be due to activation of the human alveolar macrophages by various inhaled aerosols during life, since hysterectomized pigs would have had only minimal exposure to antigens.

Table 4: THE MAJOR MORPHOLOGICAL DIFFERENCES BETWEEN ALVEOLAR AND PERITONEAL MACROPHAGES OF GERM-FREE RATS.*

Structure	Alveolar macrophage	Peritoneal macrophage
Nuclei	Round or slightly ovoid	Elongated, deeply-indented
Rough endoplasmic reticulum	Frequently seen	Rarely seen
Golgi apparatus	Extensive	Small
Cytoplasmic granules	Many and dense	Not present
Mitochondria	Large and elongated	Small and short

* Based on Bauer (6)

Table 5: THE STRUCTURAL DIFFERENCES BETWEEN THE TYPES OF ALVEOLAR MACROPHAGES OF MAN (GIEMSA STAIN).

Structure	Type A	Type B	Type C
Cytoplasm	Dark, contains many small, dark blue granules and occasional vacuoles	Contains few large green granules	Contains a large number of vacuoles
Nucleus	Oval to irregular in shape and dark blue in colour	Oval or irregular in shape and red to pink in colour	
Nucleus/ cytoplasm ratio	1:3	1:6 to 1:7	
Adherence to glass and plastic surfaces	Adhere	Not mentioned	Adhere

	Cell size (mean) μ		
	S	M	L
Man	25	30	40
Pig	10	16	30

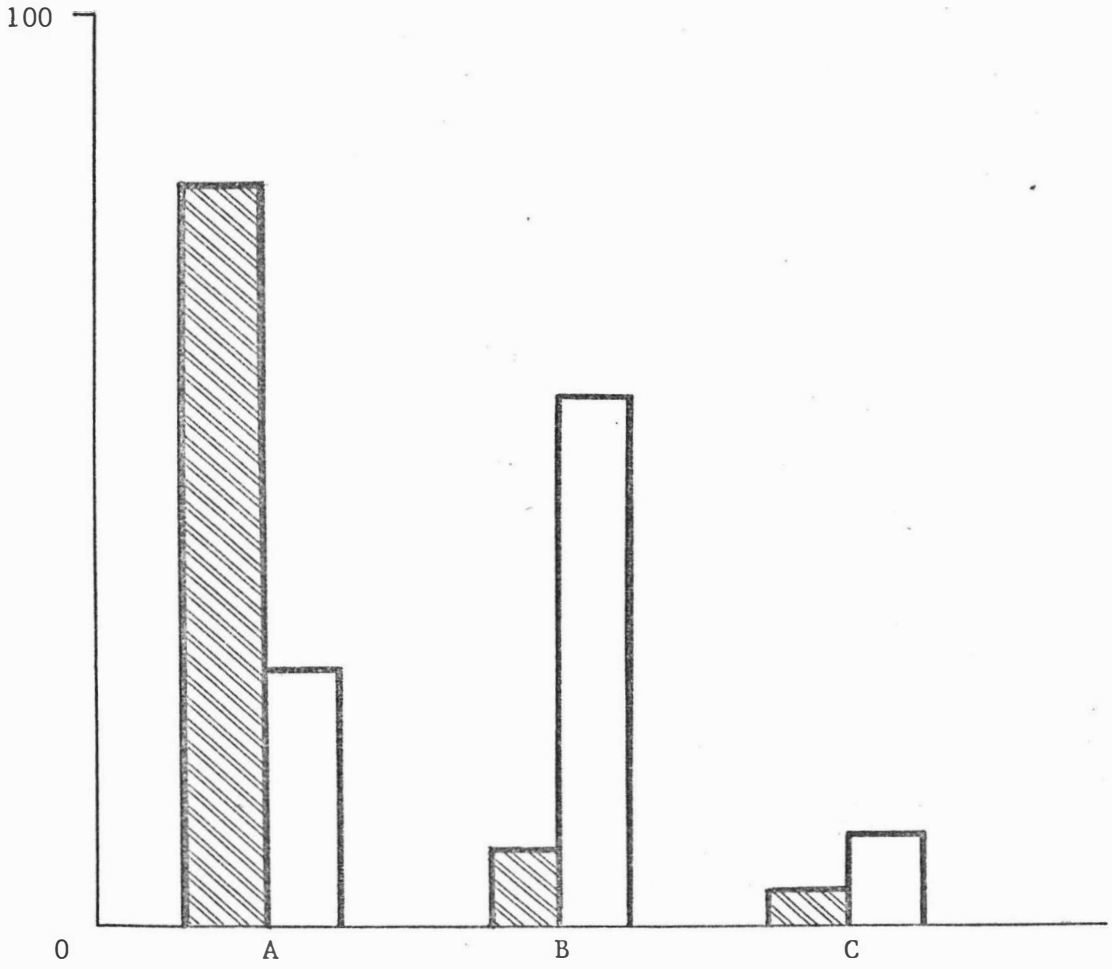


Fig. 1: THE PROPORTIONS AND SIZE OF EACH OF ALVEOLAR MACROPHAGE IN THE LAVAGE FLUID FROM MAN AND HYSTERECTOMIZED PIGS.

Key:

Man

Pig

2. The predominant type of macrophage in human lavage fluid is the small type, followed by the medium and then the large types. In pigs, however, the medium size predominates, followed by the smaller and then larger types.

SECTION TWO

MYCOPLASMA OVIPNEUMONIAE

1. INTRODUCTION

Mycoplasmas are widely distributed in nature and have been detected in man, animals and plants. They are of particular economic importance in veterinary medicine as they cause infection in the respiratory tract, mammary gland and genital tract in cattle, sheep, cats, mice, rats and birds (29).

Mycoplasma ovipneumoniae is one of the largest organisms in the family Mycoplasmaceae, but like other mycoplasmas it has no rigid cell wall and is a highly pleomorphic cell. It is bound by a trilaminar membrane bearing small projections (77). The organism has been associated with chronic respiratory diseases of sheep in Australia (102), New Zealand (24), Scotland (54) and Iraq (4).

When cultured on agar, *M. ovipneumoniae* forms slightly raised, granular, somewhat circular and centreless colonies. The organism has the ability to ferment glucose with acid production and shows marked haemolytic activity for ovine, bovine and equine erythrocytes. Serologically, it has been found to be distinct from other ovine and caprine mycoplasmas and because of this and its association with pneumonia in sheep the name *Mycoplasma ovipneumoniae* was proposed by Carmichael *et al.* (19).

2. DISTRIBUTION

A mycoplasma resembling *M. ovipneumoniae* was first isolated in Scotland (type A) by Mackay *et al.* (72) from cases of sheep pulmonary adenomatosis. Jones *et al.* (54), later showed that this strain of mycoplasma could be classified as *M. ovipneumoniae*

and was similar to the Australian strain.

In Australia an organism later shown to be *M. ovipneumoniae* was first isolated from the respiratory tract of pneumonic and apparently healthy sheep by St George *et al.* (102). Subsequently *M. ovipneumoniae* has been shown to be widely distributed throughout Australia and has been isolated from lambs in Tasmania, Victoria, New South Wales and Queensland. Strains from Victoria and Queensland have been compared and found identical (104) (105).

In New Zealand, Clarke *et al.* (24) were the first to isolate and identify mycoplasmas from the respiratory tract of sheep. Two groups of organisms were found in the nasal cavity and lungs of normal and pneumonic lambs and one of these was found to be morphologically and serologically similar if not identical to the Australian isolate of *M. ovipneumoniae*.

The organism has also been isolated and associated with macroscopic or microscopic lesions of chronic pneumonia in lambs from Hungary (101), Iceland (34) and recently in Iraq (4).

3. MORPHOLOGY

The morphology of mycoplasmas depends generally on the tonicity and nutritive properties of their growth medium. Of particular importance is the ratio of saturated to unsaturated fatty acids which influences the membrane structure of the organisms (29).

The exponential phase cell is roughly spherical and measures about 400 to 700 nm in diamter. The cell is limited by a plasma membrane, 8 to 10 nm thick, with two electron-dense layers separated by a translucent layer, bearing projections about 12 nm long and arranged vertically to the surface (77). The electron lucent part of the membrane represents lipids,

while the electron-dense part represents protein and carbohydrate.

The plasma membrane of this organism is the site of many metabolic reactions involving membrane-bound enzymes and transport mechanisms (29).

The cytoplasm does not contain an internal membrane (endoplasmic reticulum) or a defined mesosome as do bacteria. The cytoplasm is packed with ribosomes and there is electron-lucent, nuclear material in fibrillary form centrally placed or dispersed (77).

4. PATHOGENCITY

In Australia, St George *et al.* (102) induced pneumonic lesions in caesarian derived lambs experimentally, by the intratracheal inoculation of pneumonic lung tissue suspension which was obtained from naturally infected lambs. The gross and histopathological features, they observed in experimentally inoculated lambs were identical to naturally occurring cases and consisted of a proliferative interstitial pneumonia. Mycoplasma organisms were isolated from a suspension of ground lung tissue of the experimental lambs and incriminated as the primary aetiological agent (102).

Sullivan *et al.* (104) studied the pathogenesis of chronic pneumonia of sheep in Australia by sequential clinical, pathological and microbiological investigations of naturally affected lambs and recovered a mycoplasma similar to that described earlier (102), which they identified as *M. ovipneumoniae*. The infectivity and predilection of the organism for the lung has been studied experimentally by Sullivan *et al.* (105) who produced pulmonary lesions after intranasal and intravenous inoculation, but failed to recover the organism from the exposed lambs.

Following the isolation of the organism in New Zealand, Alley *et al.* (1) undertook a survey of pneumonic and normal lungs collected from abattior sources. They found a marked difference between both the proportion of positives and the titre of *M. ovipneumoniae* in normal and pneumonic sheep which suggested that the organism may have a role in the pathogenesis of chronic non-progressive pneumonia of lambs in New Zealand.

Although these studies have demonstrated a close association between *M. ovipneumoniae* and chronic pneumonia of sheep in Britain, Australia and New Zealand they have not succeeded in establishing whether the organism is a primary or secondary pathogen.

Since it was first discovered, Australian investigators have considered *M. ovipneumoniae* as a primary aetiological agent for chronic pneumonia in young lambs (19) (104) (105), but New Zealand investigators have differed in interpreting the role of this organism. The pneumonic lesions produced by Alley *et al.* (2), using an inoculum of pneumonic lung homogenate with a high titre of *M. ovipneumoniae* were identical to natural forms of sub-acute and chronic non-progressive pneumonia . Inoculation of a pure culture of *M. ovipneumoniae* however, failed to produce lesions that corresponded to those induced by homogenized pneumonic lung. These results were similar to those obtained by Foggie *et al.* (32) in Britain who produced mild macroscopic and microscopic lesions in only 50% of specific pathogen free (SPF) lambs inoculated endobronchially with a pure broth culture of *Mycoplasma ovipneumoniae*.

For these reasons the New Zealand investigators have suggested that *M. ovipneumoniae* is not the sole infective agent of chronic non-progressive pneumonia of young sheep in New Zealand and that a second factor or agent, is essential for the full development of lesions.

SECTION THREE

PHAGOCYTOSIS

1. INTRODUCTION

Phagocytosis is the term used to describe the process by which cells are able to engulf or interiorize a solid particle (97). The mechanism by which cells ingest extracellular soluble material, submicroscopic particles or fluid droplets is termed pinocytosis or microphagocytosis (89). The mechanisms of both processes have been shown to be identical and differ only in the content of the vesicle formed. For this reason the term endocytosis is now often used to encompass both of these processes (97).

The solid material or fluid droplets that are taken up by the cell are surrounded by a membrane formed by the interiorization of part of the plasma membrane. When the vesicle formed contains solid matter it is called a phagosome but if it contains fluid it is called pinocytotic vesicle or pinosome (97).

Although many kinds of epithelial, mesenchymal and tumour cells can display the ability to engulf particles under certain physiological and pathological conditions, macrophages and polymorphonuclear leukocytes have a special capacity to perform endocytosis. Only macrophages, however, have a highly developed ability to discriminate between various types of particle (98).

2. THE MECHANISM OF PHAGOCYTOSIS

Particles adhere to the surface of mononuclear phagocytes either by being trapped against a suitable surface or by specific attachment to receptors. The ability of macrophages to phagocytose virulent bacteria such as encapsulated pneumococci without the assistance of opsonin depends upon the physical

factor for promoting phagocytosis (120).

Regardless of how particles adhere to macrophages the mechanism of the phagocytic process is the same and may be arbitrarily divided into four stages, namely, chemotaxis; attachment; ingestion and destruction or digestion (86) (106).

A. Chemotaxis

Chemotaxis is the direct movement of cells under the influence of an external chemical stimulus (106). It is often regarded as being positive or negative according to whether the movement of the cells is towards or away from the substance acting as a chemotactic factor.

Any mononuclear phagocyte not firmly fixed in place may be responsive to a chemotactic stimulus. The fixed macrophages of the MPS which line the sinusoids of the liver, spleen, lymph nodes and bone marrow respond to foreign particles carried by the turbulent flow of blood and lymph but do not actively undergo chemotaxis (86) (106).

Many substances including polysaccharides, polypeptides and proteins have been found to exert a chemotactic activity for macrophages *in vitro* (Table 6). Macrophages are chemotactically attracted to many bacterial products, to factors liberated from damaged cells, to certain products generated as a result of antigen-antibody interaction and some lymphokines released by sensitised lymphocytes (106).

Chemotactic agents do not necessarily have a direct effect on cells. Boyden (11) showed that antigen-antibody complexes are not chemotactic *per se*, but exert their effect by inducing formation of chemotactic factors in fresh serum. Keller *et al.* (63) have shown that many biochemical and biological agents act indirectly in a similar way to antigen-antibody complexes. Other

Table 6: SOME AGENTS CHEMOTACTIC FOR MACROPHAGES *IN VITRO**

AGENT	CHEMOTACTIC EFFECT	AUTHOR
Plasma	-	Borel & Sorkin (1969)
Antigen/Antibody + plasma	+	ibid
Normal serum	+	Wilkinson *et al.* (1969)
Antigen/Antibody + serum	±	Sorkin *et al.* (1970)
Plasmin + serum	+	Ward (1968)
Granules from neutrophils + serum	-	Sorkin *et al.* (1970)
Granules from alveolar macrophages + serum	-	ibid
Post-granular supernate from neutrophils	-	ibid
Post-granular supernate from neutrophils + serum	+	ibid
Guanosine + serum	+	ibid
Sensitized lymphocytes + antigen	+	Ward (1969)
Pneumococcal chemotactic factor	+	Ward (1968)
Filtrates from *Escherichia coli* and *Staphylococcus albus*	-	Keller & Sorkin (1967b)
E. coli and *Staph. albus* bacteria + serum	-	ibid
Casein	+	Keller & Sorkin (1967a)

* based on Sorkin *et al.* (100)

substances were found to be chemotactic without serum, because they exert a direct effect on the cells. In general two types of chemotactic substances have been studied; those which exert a direct effect on cells and those materials which induce formation of chemotactic factors which act indirectly. Sorkin *et al.* (100) classified chemotaxis inducing substances according to their mode of action. Those with a direct effect on cells were termed cytotaxins and those inducing formation of cytotaxins were termed cytotaxigens.

B. Attachment

The second stage in the phagocytic process involves the firm attachment of the particle to a phagocytic cell. Nelson (86) defined attachment as an association between the plasma membrane of the phagocyte and the particle, sufficiently strong to resist mild mechanical trauma such as shaking or washing.

To date, two types of receptors have been recognised on the plasma membrane of macrophages. One for the Fc portion of immunoglobulin-G, termed Fc receptor and the other one for the cleaved third component of complement, termed C3b receptor (99). On murine macrophages there are at least two distinct classes of Fc receptor. These are:

1) trypsin sensitive Fc receptors that bind monomeric mouse immunoglobulins of subclass IgG2a, and

2) trypsin-resistant Fc receptors that will bind aggregated IgG from several other species (109).

The function of the trypsin-sensitive IgG2a receptor has not yet been resolved. However, the trypsin-resistant Fc receptor is involved in the phagocytosis of IgG-coated particles (9). Macrophages have no receptors for the Fc portions of pentameric IgM or for IgA, so that particles coated with these ligands (immunoglobulin molecules) will not bind to macrophage membranes.

Macrophages express receptors for the cleaved third component of complement, C3b. The function of C3b receptors varies with the physiological state of the macrophages. Thioglycollate-elicited macrophages plated on surfaces coated by complement show a marked reduction in binding and ingestion of complement-coated red cells. In contrast, normal macrophages exhibit no reduction in complement-receptor activity when plated on complement-coated surface (81).

Recently, Griffen *et al.* (45) and Michl *et al.* (81) found that although the Fc and C3b receptor systems are capable of mediating similar functions, they are not coupled to one another and that signals generated by ligands interacting with the C3b receptors are not transmitted to the Fc receptors, and *vice versa*.

C. Engulfment

Silverstein *et al.* (99), have taken advantage of the ease with which the macrophage plasma membrane receptors can be manipulated experimentally to study the ingestion phase of the phagocytic process. They demonstrated that the macrophage responds segmentally to a phagocytic stimulus. This segmental nature of the macrophage membrane response indicates that the molecules which couple receptors on the external surface of the cell, to the cytoskeletal system, do not diffuse readily within the cytoplasm. Instead, the coupling factors interact stoichiometrically with cytoplasmic proteins located at, or very near to, the site of receptor-ligand interaction.

The movement of the macrophage plasma membrane is governed by the sequential and circumferential interaction of receptors on the surface of the phagocyte with ligands distributed over the surface of the particle. Griffin *et al.* (45) have termed this the 'zipper mechanism' of phagocytosis (Fig. 2). Such a model predicts that ligands must be distributed uniformly over the surface of a particle for it to be completely phagocytosed (99).

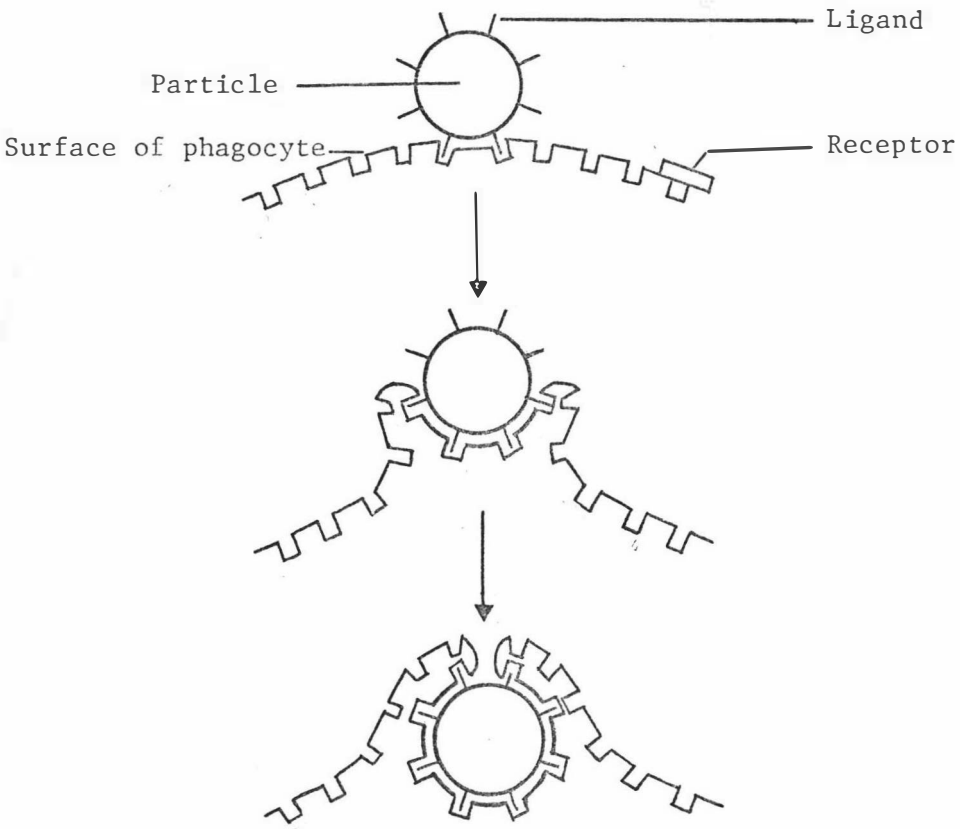


Fig. 2: THE "ZIPPER MECHANISM" OF PHAGOCYTOSIS. THE INTERACTION OF PARTICLE-BOUND LIGANDS WITH RECEPTORS ON THE SURFACE OF THE PHAGOCYTE DIRECTS THE MOVEMENT OF MEMBRANE OVER THE SURFACE OF THE PARTICLE (99).

Gribben *et al.* (46) noticed that the portion of macrophage cytoplasm directly adjacent to the particle undergoing digestion contains a thick meshwork of filaments from which virtually all cytoplasmic organelles are excluded. They have suggested that the initial interaction of ligand with receptor, generates a transmembrane signal that promotes the aggregation of contractile proteins in the adjacent cytopasm.

Hartwig *et al.* (48) have shown that actin-binding protein is released from the macrophage plasma membrane during phagocytosis. Actin-binding protein acts stoichiometrically and is a particularly attractive candidate for a molecule able to cause the assembly of contractile proteins within a restricted zone of cytoplasm. According to Silverstein *et al.* (99), receptor-ligand interaction stimulates the release of actin-binding protein. This in turn causes cross-linking of actin filaments and the assembly of an ordered array of contractile proteins. This structure promotes pseudopod extension, thereby bringing additional receptors into opposition with particle-bound ligands. The process continues until the particle is enclosed within a phagocytic vacuole.

D. Destruction and Digestion

A particle enclosed within the cytoplasm of a macrophage finds itself in a space known as a phagosome. The formation of the phagosome initiates the digestive phase of intracellular events. The fate of the ingested particle depends on its physical composition, the state of the macrophage and the nature of the opsonins initially stimulating its phagocytosis (86).

Destruction of the particle occurs when lyosomes containing hydrolytic enzymes fuse with the phagosome to form a vacuole known as a phagolysosome. The lysosomal enzymes of macrophages are a complex mixture (Table 7). This mixture of lysosomal enzymes is lethal to most micro-organisms (106).

Table 7: HYDROLYTIC ENZYMES OF MACROPHAGE LYSOSOMES (106)

HYDROLYTIC ENZYMES
Lysozyme
Acid phisphatase
Lipase
Cathesin
Acid ribonuclease
Acid deoxyribonuclease
Neuraminidase
β glucuronidase
Hyaluronidase
Aryl sulfatase
Nonspecific esterase
Phospholipases

The interaction between different types of micro-organisms and macrophages has been studied in considerable detail. In general, it can be said that some viruses are broken down in phagolysosomes and thus nucleic acid may be released to direct the synthesis of new virus at least in cases where the virus is capable of multiplying within the macrophages.

Many bacteria which are susceptible to lysozymes are rapidly destroyed by macrophages (e.g. *Eschericia coli, Bacillus subtilis, Mycobacterium lysodeikticus, Staphylococcus albus, Staphylococcus aureus,* strains of *Salmonella typhimurium* (26) *Pasteurella haemolytica* (78) and *Salmonella cholera-suis* (13). Other bacteria such as *Listeria monocytogenes* may survive and multiply in macrophages of non-immune animals because the organisms are highly resistant to the lethal effects of the lysosomal enzymes (87) (88). *Mycobacterium tuberculosis* also survives within macrophages after phagocytosis by blocking the fusion of lysosomes with the phagosome (106). The phagocytosis of mycoplasmas will be discussed separately in the next section.

Adenosine triphosphate (ATP) is thought to provide the chemical energy for phagocytosis. Support for this concept is derived from two types of experimental evidence:

1) Phagocytosis is accompanied by increases in O_2 consumption, glycolysis and hexose monophosphate shunt activity; processes that are associated with ATP generation (59).

2) Inhibitors of ATP biosynthesis such as sodium and iodoacetate also inhibit phagocytosis (58).

Recently, however, Silverstein *et al.* (99) examined the rate of ATP turnover during particle ingestion. They compared the incorporation of $^{32}PO_4$ into ATP, in phagocytosing and resting macrophages. The $^{32}PO_4$ was incorporated into the ATP of resting macrophages at a faster rate than into the ATP of phagocytosing macrophages. These findings led to a search for other possible

sources of energy for phagocytosis. Macrophages contain creatine phosphate in 2 to 5 fold molar excess of ATP, and Silverstein *et al*. (99) were able to show that creatine phosphate consumption increased during phagocytosis. They therefore suggested that the ATP content of phagocytosing macrophages is maintained at least in part , by rephosphorylation of ADP by the cell's creatine phosphate reserves.

SECTION FOUR

MYCOPLASMA-MACROPHAGE INTERACTION

The intereaction of some species of mycoplasma with macrophages has been studied in considerable detail (Table 8).

Jones (1971) (55) (53) examined the attachment and ingestion of *Mycoplasma pulmonis* by mouse macrophages with the electron microscope and typical membrane-bound mycoplasmas were seen associated with the macrophage membrane. The unit membrane of the mycoplasma is usually clearly separated from the plasma membrane of the cell by approximately 10 nm. Several factors which influence the kinetics of attachment of mycoplasmas to macrophages were defined by Jones (53). They are:

1) Mycoplasmas do not attach to macrophages in suspension. This was confirmed by a search for organisms using phase and electron microscopy and by assay of colony-forming units in a tumble suspension system.

2) The rate of attachment of mycoplasmas to macrophages spread on a glass surface is dependent on the length of time the macrophages remain in culture.

3) The rate of attachment of mycoplasmas is greater the more extensively spread the macrophages are over the glass surface.

4) The rate of attachment of mycoplasmas is greater if macrophages are in horizontal stationary cultures rather than vertical rotated cultures.

Attachment of living mycoplasmas to spread macrophages in culture may occur at any site on the exposed surface of the cell. After attachment, the mycoplasmas are moved centripetally by a wave-like motion of the macrophage membrane surface at a rate of 4 μm/min (27). The mechanism and functional significance of this

Table 8: STUDIES OF MYCOPLASMA-MACROPHAGE INTERACTION

Mycoplasma species	Source of macrophages	Method of study	Author
M. pulmonis	Mouse peritoneal macrophages	1) Phase-contrast microscopy 2) Time-lapse cinematography 3) T.E.M.* 4) Radio autography	Jones & Hirsch (1971)
"	"	T.E.M.	Jones (1975)
"	"	Biochemistry	Jones *et al.* (1977a)
"	"	S.E.M.†	Jones *et al.* (1977b)
	Mouse peritoneal macrophages	Radio-isotopes	Howard & Taylor (1979)
M. pneumoniae	Guinea pig alveolar macrophages	1) Phase-contrast microscopy 2) T.E.M. 3) Radio-isotopes	Powell *et al.* (1974)
"	Guinea pig peritoneal macrophages	Photo-microscopy	Bredt (1975)
"	Gunea pig alveolar macrophages	S.E.M.	Powell & Muse (1977)
"	"	Labelled-antibody	Erb & Bredt (1979)
M. dispar and *M. agalactiae sub sp. bovis*	Bovine alveolar macrophages	Immunochemistry	Howard *et al.* (1976)

* *Transmission electron microscopy*

† *Scanning electron microscopy*

movement are not understood (53).

Immunoglobulins have been shown to have a significant effect on the phagocytosis of some mycoplasmas. When mycoplasmas are cultivated on the surface of mouse peritoneal macrophages, as little as, 10 µg/ml of anti-mycoplasma antibody, induces ingestion of surface organisms. Ingestion of mycoplasma is preceded by an increase in macrophage membrane activity, the appearance of ruffles surrounding the mycoplasma and the disappearance from the macrophage surface, of the lawn of organisms within 10 minutes after the addition of antimycoplasma antibody (53).

Mycoplasmas may be seen intracellularly within phagocytic vacuoles under two conditions. Firstly, in the absence of antimycoplasma antibody, degenerate forms of organisms are seen infrequently by electron microscopy, within the macrophages. This could reflect a very slow rate of ingestion of normal mycoplasmas under these conditions, or it may reflect engulfment of damaged mycoplasmas. Secondly, in the presence of antimycoplasma antibody, surface organisms are readily phagocytized and then digested within lysosomes of macrophages (55).

Complement does not augment either the effect of anti-mycoplasma antibody on the ingestion phase of phagocytosis or increase the rate of ingestion induced by antibody (53). However, Bredt *et al.* (15) found that complement in guinea pig serum exerted a toxic effect on *M. pneumoniae* cells. In a second paper, Bredt (31) reported that macrophages alone are not able to efficiently phagocytize normal *M. pneumoniae* cells. Complement activated either *via* the classical or alternate pathway was found to induce phagocytosis and complement alone caused severe damage to *M. pneumoniae* cells.

CHAPTER TWO

MATERIALS AND METHODS

1. ANIMALS

Six, Romney or Romney-cross sheep, aged 6 to 12 months of both sexes were used as a source of lungs. The sheep were killed by intravenously administered pentobarbital sodium* and the lungs, trachea and larynx were removed immediately after death. Macrophage collection was undertaken at room temperature and commenced within 10 minutes of the time of death.

2. COLLECTION OF MACROPHAGES

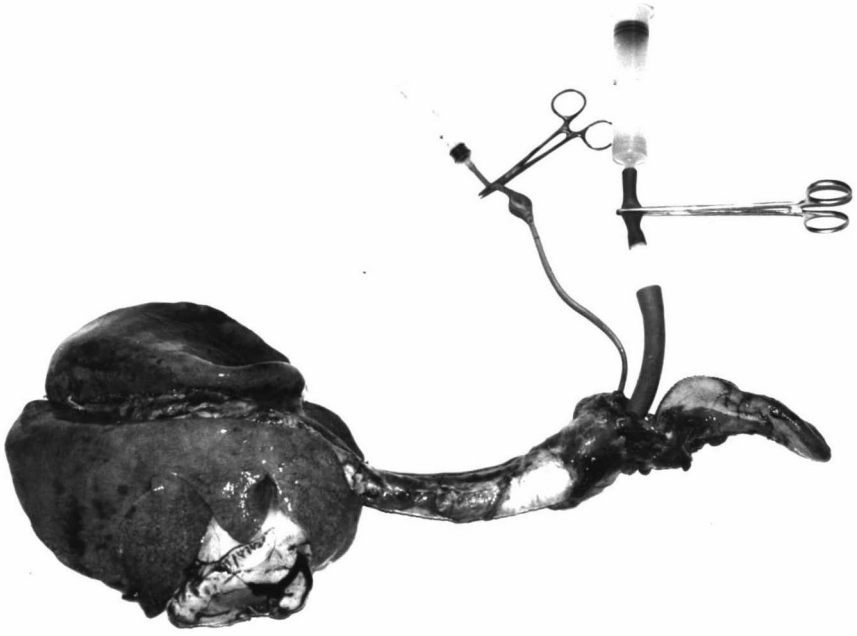
The macrophages were obtained aseptically by an intrapulmonary washing procedure. A sterile intratracheal tube, modified by the connection of a plastic adaptor to the cuff end, (Figure 3) was introduced into the trachea through the larynx and inserted as far as the bronchial bifurcation. The lungs were gradually and completely filled with sterile normal saline solution *via* the intratracheal tube using a 50 ml sterile plastic syringe. The lungs were then gently massaged and the washings were collected in a siliconized flask by suction with the syringe. Twelve to sixteen hundred millilitres of sterile normal saline solution were used and about two-thirds of this was usually recovered.

The washings were then centrifuged at 1000 g for 10 minutes within an hour of collection. The pellets obtained were washed with sterile normal saline solution. Some pellets were fixed immediately after washing and used for electron microscopic studies of normal ovine alveolar macrophages. Other pellets

* Euthesate (Willows Francis).

Figure 3

The intrapulmonary washing procedure. Note the intratracheal tube is modified by the connection of a plastic adaptor to its cuff end.



were resuspended, pooled and cultured in TC199 medium for various time intervals (see experimental design for details) before fixation for electron microscopy.

3. CULTURE MEDIA

The macrophages were cultured in TC199 medium to which antibiotics and foetal bovine serum were added (see appendix I). The cells were counted in a haemocytometer chamber and suspension was diluted to give 3 x 10^6 macrophages per ml.

4. *MYCOPLASMA OVIPNEUMONIAE*

The isolate of *Mycoplasma ovipneumoniae* used was strain 5 of a New Zealand isolate which was kindly supplied by Dr J.K. Clarke. It was originally isolated from the lungs of a lamb with chronic non-progressive pneumonia. The organism was propagated and grown to late exponential phase in modified FM4 broth, then subdivided into aliquots and stored at -70°C. The titre of culture used was approximately 10^9 CFU/ml

The modified FM4 medium in which the organism was cultured is detailed in the appendix II. The yeast autolysate was omitted from the medium and the swine serum and phytone were replaced to avoid cross-reactions with *M. ovipneumoniae* propagated in standard FM4 medium (76). Thallium acetate was excluded because of its toxicity to *M. ovipneumoniae* in cell culture (76).

5. PREPARATION OF ANTISERA

Anti-mycoplasma antibody was made by intramuscular inoculation of 0.3 ml of *M. ovipneumoniae* suspension with 10% adjuvant into rabbits. Two further inoculations of 0.3 ml of antigen suspension were given by the same route at monthly intervals. After which the rabbits were bled weekly and serum was collected.

6. EXPERIMENTAL DESIGN

A- PRELIMINARY EXPERIMENTS

Alveolar macrophages were obtained from 3 sheep and used in preliminary studies for both scanning and transmission electron microscopy (SEM and TEM). In these initial studies an estimate of the number of macrophages which it was possible to collect was obtained and the collection and processing techniques were refined.

B- *IN VITRO* STUDIES OF MYCOPLASMA-MACROPHAGE INTERACTION

The design of the experiment is shown in Table 9. Because of the difficulty in obtaining large enough numbers of macrophages for sequential studies of phagocytosis from one animal only and the problems of processing samples collected at close time intervals, it was necessary to divide this study into 2 parts; SEM and TEM. The source of macrophages in both cases were healthy, Romney sheep aged approximately one year.

For the study of the surface morphology of normal ovine alveolar macrophages, a 2 mls suspension of ovine alveolar macrophages in TC 199 medium was cultured on coverslips in 35 x 10 mm plastic tissue petrie dishes (Falcon) and incubated at 37°C (Table 9).

Observations were also made on infected alveolar macrophage cultures. Suspensions of ovine alveolar macrophages in TC 199 medium mixed with a suspension of *M. ovipneumoniae* were made. The mixed suspension was either cultured alone or diluted with rabbit antimycoplasma antibody and cultured on coverslips in 35 x 10 mm tissue culture dishes (Falcon) at 37°C for variable time intervals (Table 9).

TABLE 9: EXPERIMENTAL DESIGN OF *IN VITRO* ALVEOLAR MACROPHAGE - MYCOPLASMA INTERACTION STUDY

Time intervals of macrophage culture in hours	Normal macrophages		Macrophages infected with *M. ovipneumoniae*			
			with antibody		without antibody	
	SEM*	TEM†	SEM	TEM	SEM	TEM
0.5	X	X	X	X	X	X
1			X		X	
2			X		X	
6			X		X	
12	X	X	X		X	
24	X		X	X	X	X

*Scanning electron microscopy (SEM)

†Transmission electron microscopy (TEM)

For the study of the intracellular morphology of normal ovine alveolar macrophages a 5 mls suspension of ovine alveolar macrophages in TC199 medium was cultured in T-flasks and incubated at 37^{o}C for 12 hours.

Suspensions of ovine alveolar macrophages in TC199 were also mixed with a suspension of *M. ovipneumoniae* to give 3 x 10^{6} macrophages per ml. with and without the addition of rabbit antimycoplasma antibody (Table 9). There were also cultured in plastic T-flasks at 37^{o}C.

7. SCANNING ELECTRON MICROSCOPY

On each occasion when samples were taken, coverslips were removed from the culture and washed twice in sterile normal saline solution to remove the non-adherent macrophages

and mycoplasams. The coverslips were placed in modified Karnovsky's fixative (see appendix III) for one hour at 4°C and then washed twice with cold 0.1 M phosphate buffer, pH 7.2 for one hour at 4°C. Dehydration of the fixed coverslips was carried out in a graded acetone series at room temperature, for 10 minutes each in 25, 50, 75 and twice in 100%, after which they were immediately air dried. Small pieces of coverslip were then cut with a diamond pencil and glued, face up, to aluminium stubs with silver conducting paint. The preparations were coated with gold by routine methods and examined under a Cwikscan/100 field emission scanning electron microscope.

8. TRANSMISSION ELECTRON MICROSCOPY

The suspension of TC199 medium containing ovine alveolar macrophages and *M. ovipneumoniae* with or without rabbit antimycoplasma antibody was removed from plastic T-flasks. The flasks were washed with 0.25% trypsin (Difco 1:250) in Hank's basic salt solution (see appendix IV) at pH 7.6 to remove the macrophages and mycoplasmas adherent to the bottom surface of the flasks and these washings were added to the suspension. The suspension was placed in 10 mls conical tubes and centrifuged at 1000 g for 10 minutes. The supernatant was drawn off and the cell pellets were fixed for two hours, at 4°C, in modified Karnovsky's fixative (appendix III). The pellets were then resuspended in 0.3 mls of modified Karnovsky's solution and drawn into capillary tubes which were centrifuged by micro-capillary centrifuge,* for 10 minutes. The pellets were then removed from the capillary tubes, washed twice with phosphate buffer, fixed secondarily in 1% osmium tetraoxide, washed again twice, dehydrated for 10 minutes each in 25%, 50%, 75% and 95% alcohol and twice in 100% alcohol before infiltration and embedding in plastic capsules (see appendix V).

* Model MB, International

CHAPTER THREE

RESULTS

1. PRELIMINARY EXPERIMENTS

In these studies an estimate of the number of macrophages which could be obtained per wash was made. The number and size of the macrophages obtained varied with the age, size and health status of sheep used (Table 10). It was also found that the number of alveolar macrophages obtained when sterile Hank's balanced salt solution was used was much lower than that obtained by using normal saline solution. For this reason normal saline solution was used as a lavage fluid for the remaining experiments.

TABLE 10: PRELIMINARY STUDIES OF MACROPHAGE RECOVERY FROM OVINE LUNGS

Sheep No.	Amount of fluid* needed to fill the lungs	Amount of fluid recovered from the lungs	Number of macrophages per millilitre
1	1200 mls	830 mls	8.700.000
2+	1400 "	900 "	12.500.000
3	1350 "	850 "	9.200.000

* Normal saline solution

\+ The lungs were infected by *Dictyocaulus filaria* (lungworm)

These experiments also included preliminary scanning and transmission electron microscopy studies of the infective ability of *M. ovipneumoniae* organisms toward ovine alveolar macrophage cultures and the macrophage-mycoplasma interaction after 24 hours of incubation. The attachment between *M. ovipneumoniae* organisms and macrophage plasma membrane was

apparent at multiple points, particularly at the base of the cells and through short and long filopodia.

2. NORMAL OVINE ALVEOLAR MACROPHAGES

A. SURFACE MORPHOLOGY

After 12 hours incubation in TC199 medium, ovine alveolar macrophages were found as single cells or aggregates of cells grouped together and adherent to the underlying glass coverslip (Fig. 4). These cells were generally rounded in shape and measured 8 to 17 μm in diameter. The plasma membrane was characterized by the presence of numerous microprojections, ridge-like elevations and large flange-like processes (Figs. 5 and 6). Most of the cells had started to spread over the coverslip by means of transparent veils of cytoplasm which spread beneath the nuclear pole (Fig. 7). The nuclear pole was often dome-shaped and covered by a relatively large number of ridges and short ruffles (Figs. 5 and 6). The cytoplasmic veils had an undulating surface with scattered ridges. Elongated filopodia or pseudopodia were occasionally visible (Fig. 8) and finer pits 60 to 200 nm in diameter were seen in most cells (Figs. 5 and 6). Intracytoplasmic spherules 0.4 and 0.6 μm were also evident and may have represented lyosomes or lipid droplets (Fig. 7).

B. ULTRASTRUCTURE

Normal macrophages studied after 24 hours varied in shape and size and measured between 5.5 to 14 μm in diameter with a mean 9.6 μm. These cells possessed a well defined plasma membrane which displayed many surface microvilli and finger-like projections radiating in various directions (Fig. 9). Invaginations of the plasma membrane were also common and these extended appreciable distances into the cytoplasm and

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Figure 4

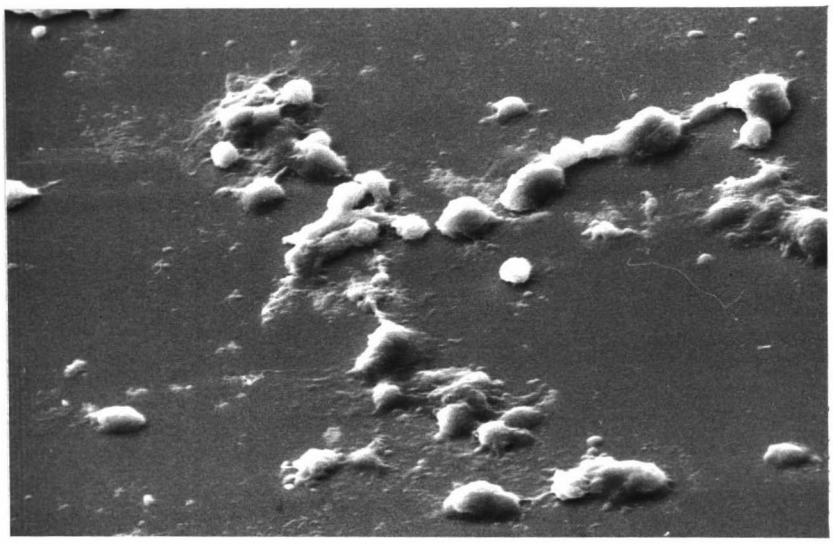
The distribution of normal ovine alveolar macrophages on a glass coverslip. The cells are distributed singly or form small groups of 2 to 6 cells. x 1000.

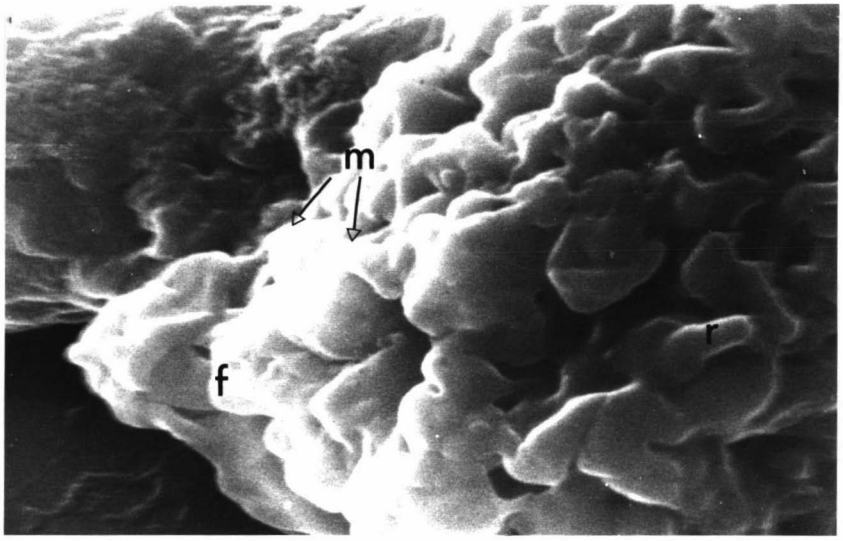
Figure 5

The plasma membrane of an ovine alveolar macrophage. It is characterised by microprojections (m), ridge-like elevations (r) and flange-like processes (f). x 12,000.

Figure 6

High magnification of the plasma membrane of a normal macrophage. Note the surface indentations (I), openings (L) and fine pits (P). x 16,000.

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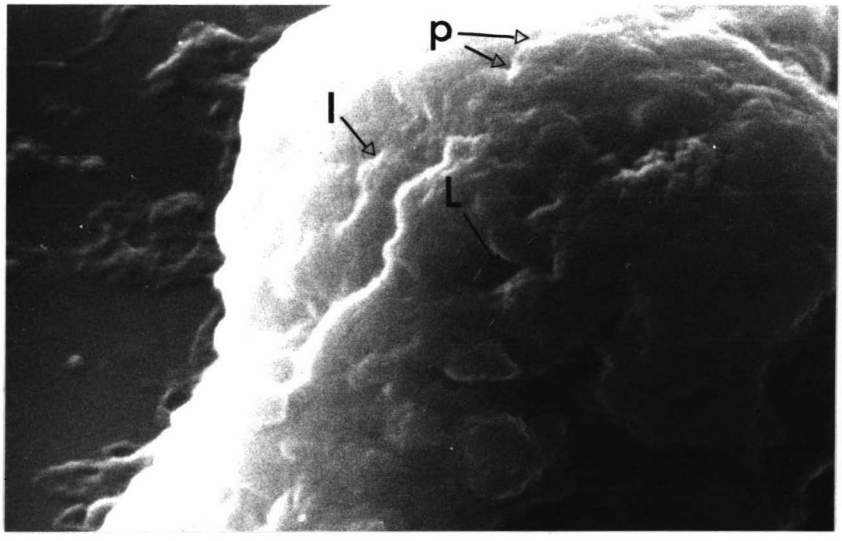
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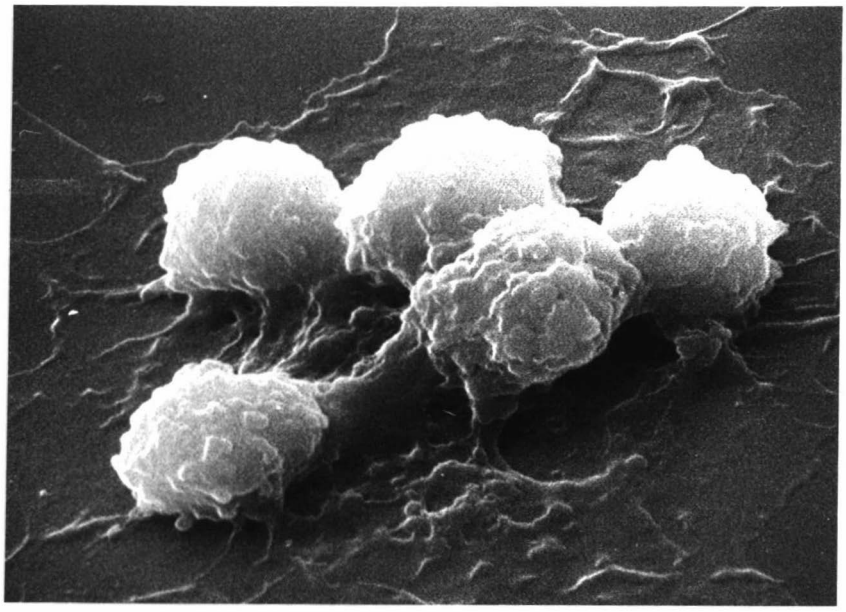
Figure 7

A small group of 5 normal alveolar macrophages. The cells are attached to the underlying coverslip by cytoplasmic veils which spread beneath a raised dome-shaped nuclear pole. Note the undulating surface of the cytoplasmic veils with scattered ridges and many spherules. x 6,000.

Figure 8

A group of 3 or 4 normal alveolar macrophages. The largest cell is beginning to form elongated filopodia. x 4,000.



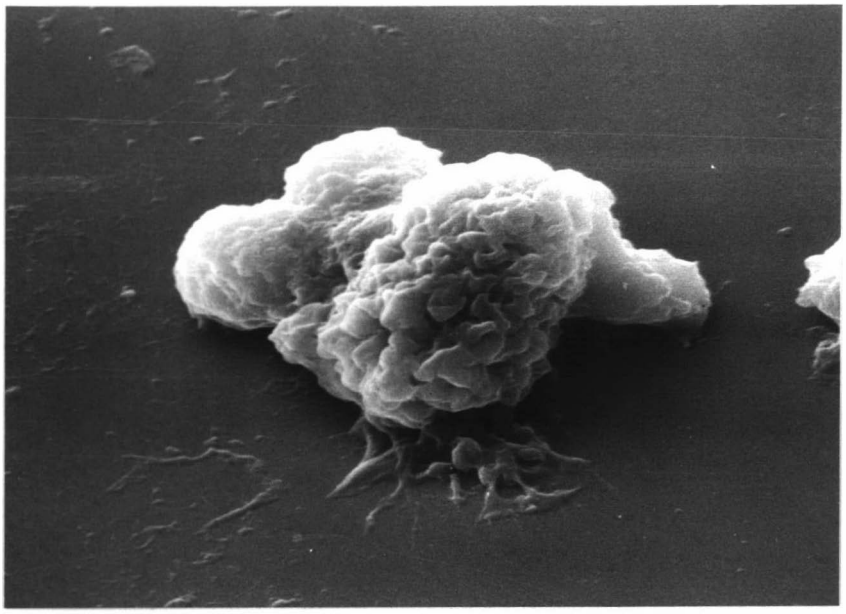


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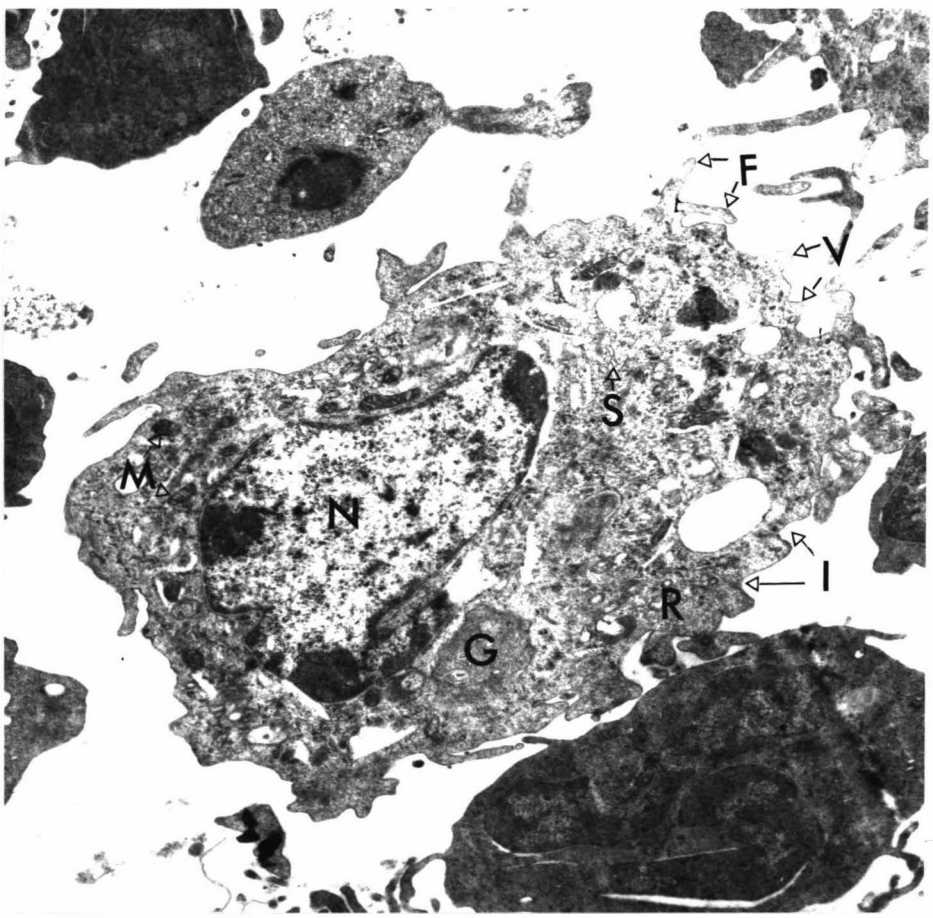
Figure 9

A normal ovine alveolar macrophage. This cell measured 14 μm in diameter and possessed a well-defined plasma membrane which displayed many surface microvilli (V), finger-like projections (F) and invaginations (I). The nucleus (N) is roughly oval in shape and contains a large amount of euchromatin. The cytoplasm is extensive and contains few mitochondria (M), rough (R) and smooth (S) endoplasmic reticulum and Golgi apparatus (G) x 8,100.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could therefore be confused with cytoplasmic vacuoles (Fig. 10).

The complexity of the cytoplasm varied between cells. They contained few or numerous mitochondia, strips of rough and smooth endoplasmic reticulum and granules and inclusions of various shapes and sizes. Although the peripheral cytoplasm contained elements of the endoplasmic reticulum, the cytoplasm of the projections or microvilli was devoid of membranes of the reticulum and consisted of fine granular material (Figs. 9 and 10). The internal cytoplasm contained a variety of structures, most of which had membranous components. The membranes of the reticulum were characteristically dotted with ribonucleoprotein (RNP) particles. These particles were not all attached to the membrane; most in fact were free within the cytoplasm. They were visible as dark staining granules embedded in an amorphous matrix (Figs. 9 and 13).

The mitochondria were cylindrical in form and measured about 0.3 to 0.49 μm in diameter and 0.6 to 1.1 μm in length. The number of mitochondria seen per cell was relatively small when compared to other cell types (Figs. 9 and 11).

The Golgi apparatus was very extensive and occupied a large part of the cytoplasm. It was composed of vesicles and moderately dense osmiophilic material which formed membranous structures (Fig. 9).

Vesicles enclosed by a unit membrane were very common and dark osmiophilic inclusions with variable densities were not uncommon (Fig. 11).

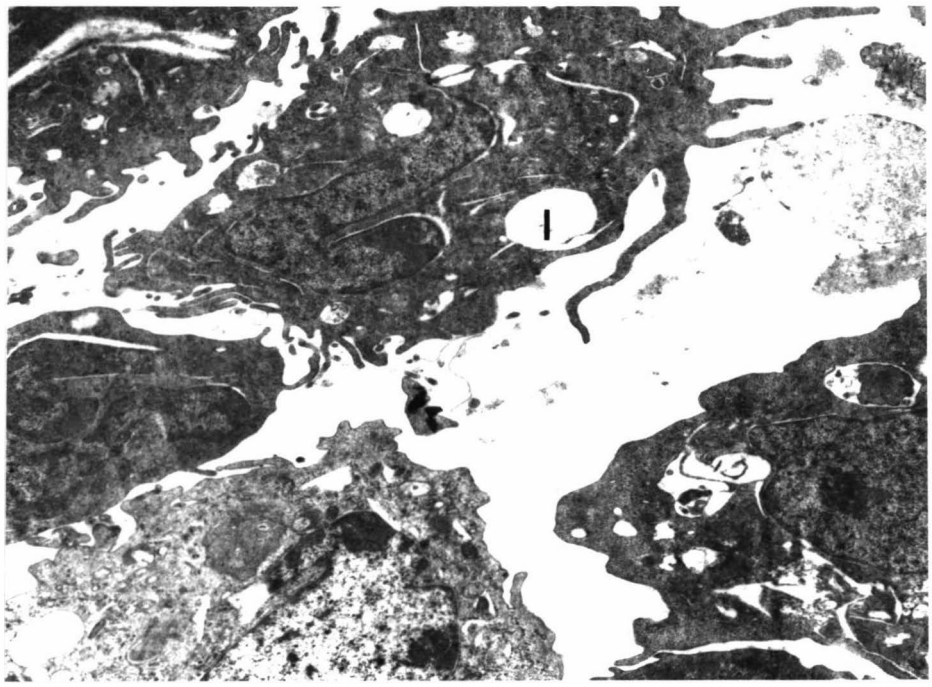
The nuclei were oval or irregular in shape with one or more indentations. Two unit membranes enclosed the nucleus and these were frequently interrupted to form pores. The external nuclear membrane invariably had RNP particles attached to it. Heterochromatin was distributed on the nuclear margins

Figure 10

A group of normal alveolar macrophages. The plasma membrane of these cells contains invaginations (I) which extend an appreciable distance into the cytoplasm. x 8,100.

Figure 11

High magnification of a normal ovine alveolar macrophage. The cytoplasm contains a large number of mitochrondria (M) together with strips of smooth (S) and rough (R) endoplasmic reticulum. Vesicles of variable size are common and dark osmiophilic inclusions (D) were occasionally seen. The nuclei (N) are irregular in shape with more than one invagination. Note that the two unit membrane enclosing the nucleus contains many nuclear pores (P). The external nuclear membrane has RNP particles attached to it. x 10,500.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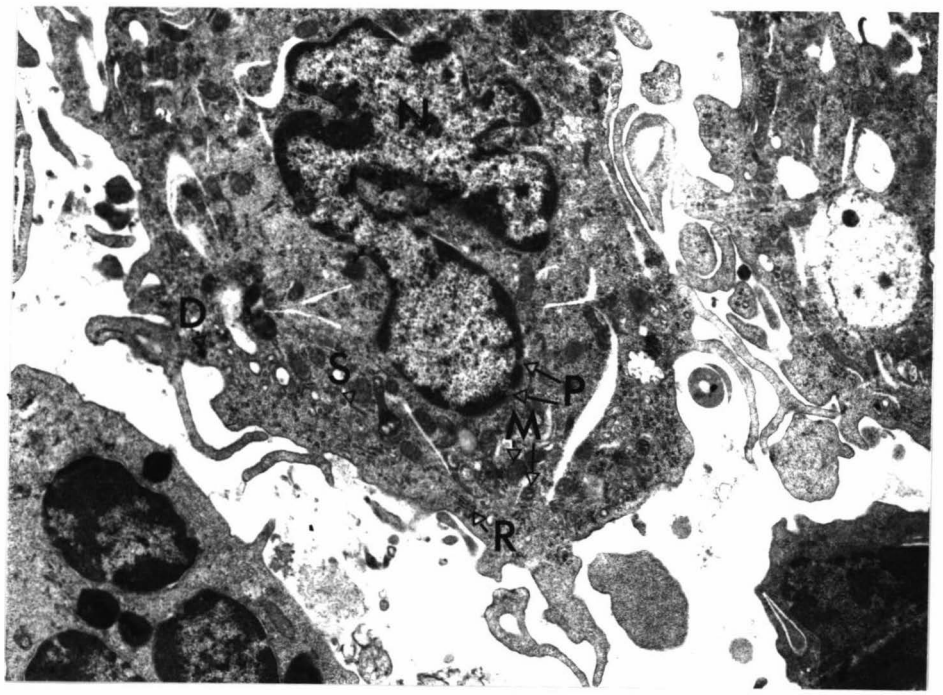
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and occasionally formed clumps elsewhere in the nucleus. The central portion of the nucleus contained a large amount of euchromatin or interchromatin and nucleoli were sometimes observed (Figs. 9, 10 and 11).

3. OBSERVATIONS ON MACROPHAGE-MYCOPLASMA INTERACTION

A. HALF HOUR POSTINOCULATION WITH *M. OVIPNEUMONIAE*

When examined by the scanning electron microscope, the macrophages were distributed mostly as single cells, adherent to the glass coverslip surface. The cells were roughly spherical and occasionally projected short filopodia (Figs. 12 and 13). Most of them measured between 8.4 to 12.13 μm. Macrophage surface membranes exhibited extensive ruffling and displayed ridge-like profiles. Occasional, single, randomly distributed *M. ovipneumoniae* organisms were seen around some macrophages. Cellular attachment between *M. ovipneumoniae* organisms and the plasma membrane of macrophages was not observed at this stage (Fig. 12).

Smooth cord-like extensions, measuring 1.75 to 4 μm in thickness, interconnecting two macrophages were relatively frequent findings (Fig. 13). Since these extensions appeared to be part of both cells it seemed probable that these macrophages were undergoing cellular division.

B. HALF HOUR POSTINOCULATION WITH *M. OVIPNEUMONIAE* AND ANTIBODY

After ½ hour of incubation with *M. ovipneumoniae* and rabbit antimycoplasma antibody, the macrophages measured between 8 to 11.75 μm in diameter and most cells had spread extensively over the glass coverslip and become flattened. They were attached to the coverslip by means of transparent veils of cytoplasm which spread beneath a raised dome-shaped nuclear pole (Fig. 14). The nuclear pole was relatively smooth and

Figure 12

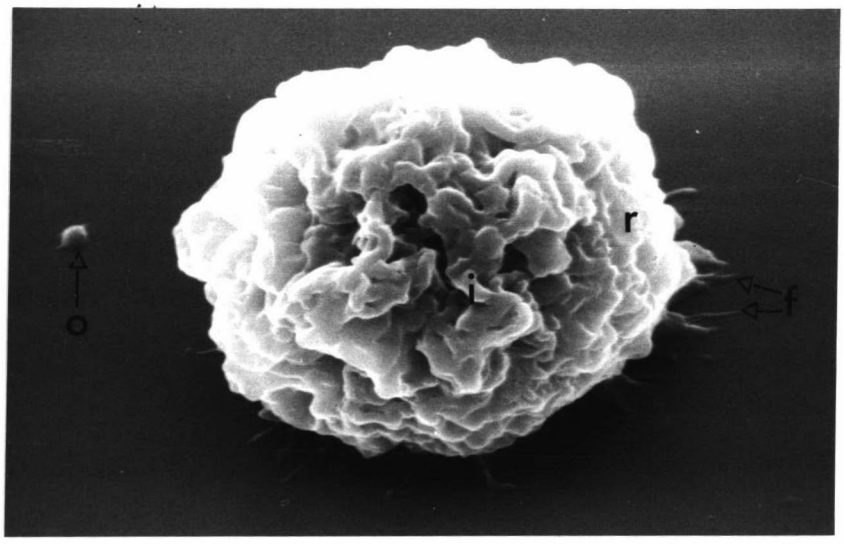
An alveolar macrophage cultured with *M. ovipneumoniae* for ½ an hour. It is roughly spherical in shape and attached to the substratum by short filopodia (f). The plasma membrane exhibits extensive ruffling (r) and displays ridge-like profiles (i). There is no interaction between the macrophage and the *M. ovipneumoniae* organism (o). x 3,600.

Figure 13

Mitotic division of an alveolar macrophage cultured with *M. ovipneumoniae* for ½ an hour. x 3,600.

Figure 14

Macrophage cultured for ½ an hour with *M. ovipneumoniae* and specific antibody. The formation of extensive cytoplasmic veils (c) was a characteristic feature of the cells in this culture. Intracytoplasmic spherules (s) can be seen within the veils. x 8,000.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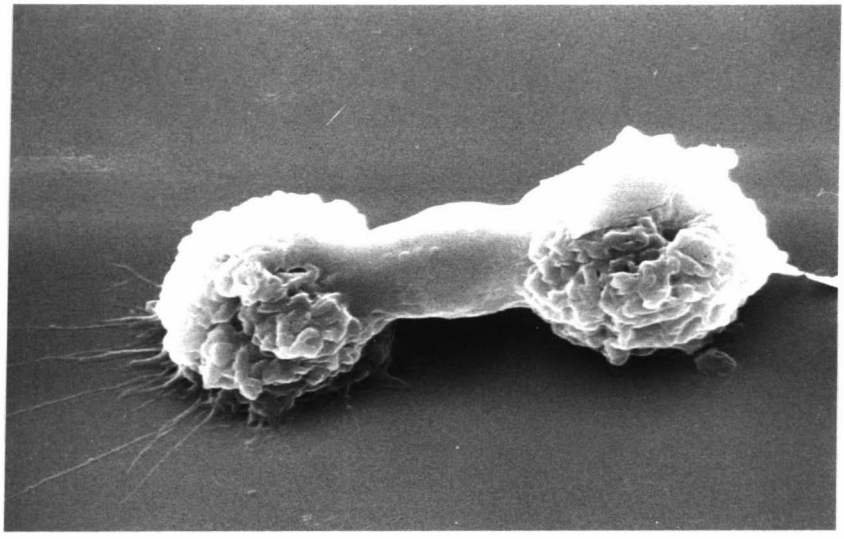
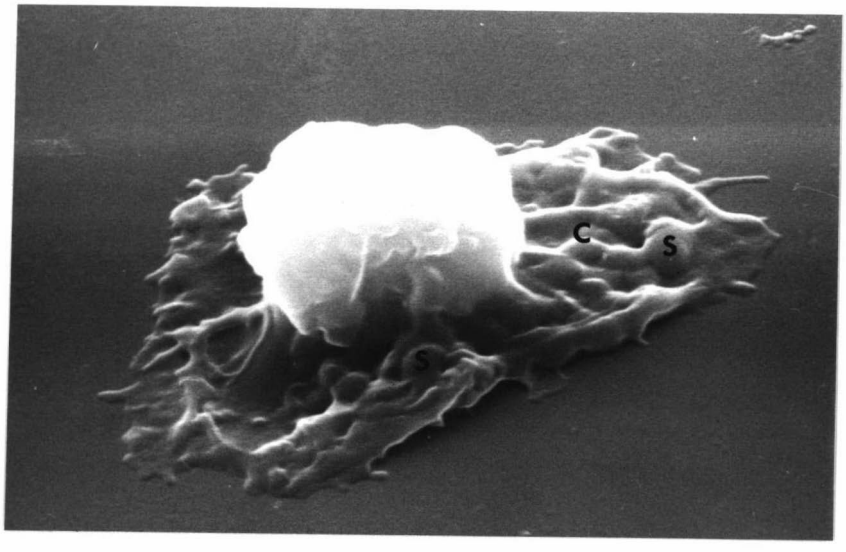



C
S
S

covered by fewer ridges than the specimens incubated without antimycoplasma antibody. The cytoplasmic veils were extensively expanded and had an undulating surface with scattered ridges. Intracytoplasmic spherules measuring between 0.4 to 1 μm in diamter were evident within the cytoplasmic veils and short filopodia sometimes extended from their margins (Fig. 14).

M. ovipneumoniae organisms were not seen attached to the macrophages, but the intracytoplasmic spherules seen beneath the cytoplasmic veils were compatible in size to these micro-organisms.

C. ONE HOUR POSTINOCULATION WITH *M. OVIPNEUMONIAE*

After one hour of incubation the macrophages were morphologically similar to cells from the ½ hour culture. However, although they were generally rounded and measured between 5.2 to 12.9 μm in diameter with a mean of 9 μm (Fig. 15). The cytoplasmic cord-like extensions between cells were longer, thinner and approximately 2.3 μm in thickness (Fig. 16). *Mycoplasma ovipneumoniae* organisms were distributed as single cells and occasional small aggregations of cells around adherent macrophages (Fig. 15).

D. ONE HOUR POSTINOCULATION WITH *M. OVIPNEUMONIAE* AND ANTIBODY

Most of the macrophages in this preparation measured between 12.3 to 20.5 μm in diameter but in contrast to the extensive expansion of cytoplasmic veils characterizing the ½ hour culture preparation, most cells were attached to the glass substratum by much smaller cytoplasmic veils. A large number of short and fine filopodia extended from the edges of the cell (Fig. 17). These findings gave the impression that the cells were in a contractile position rather than in expansion as seen in the ½ hour culture. The plasma

Figure 15

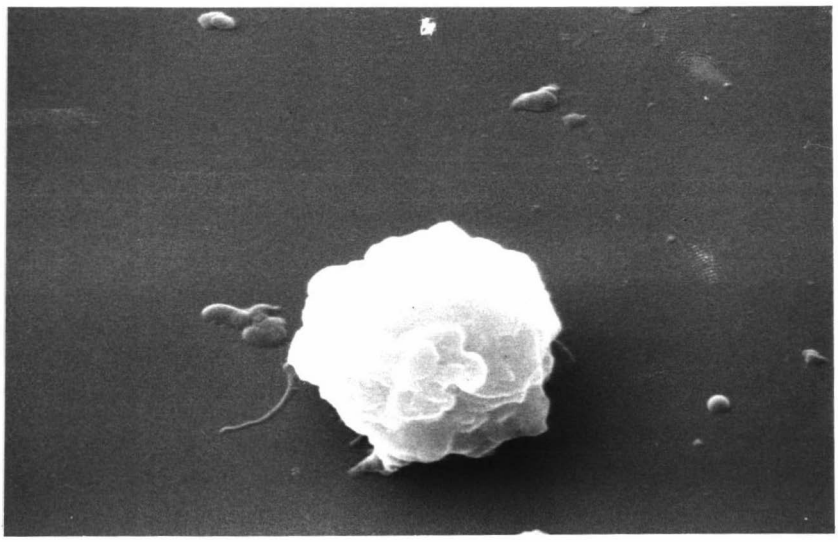
Macrophages cultured for 1 hour with *M. ovipneumoniae* were similar to cells from the ½ hour culture. Note the distribution of *M. ovipneumoniae* organisms surrounding the cell. x 7,000.

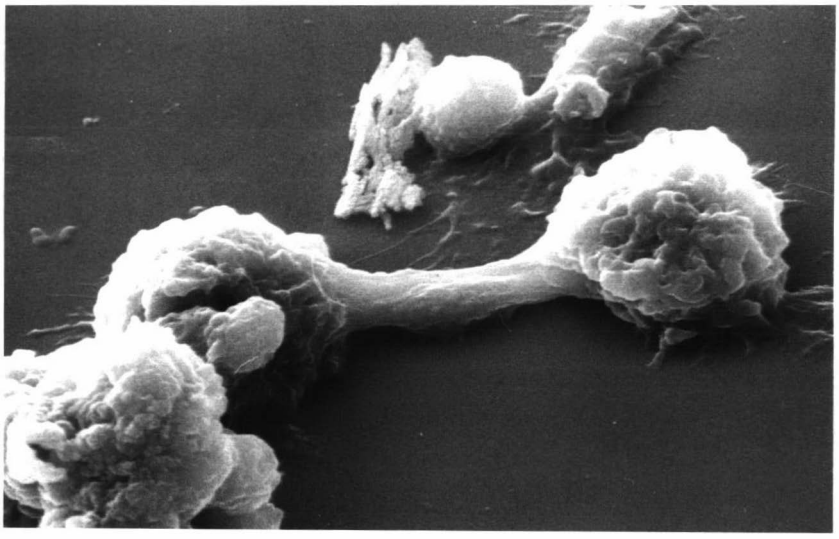
Figure 16

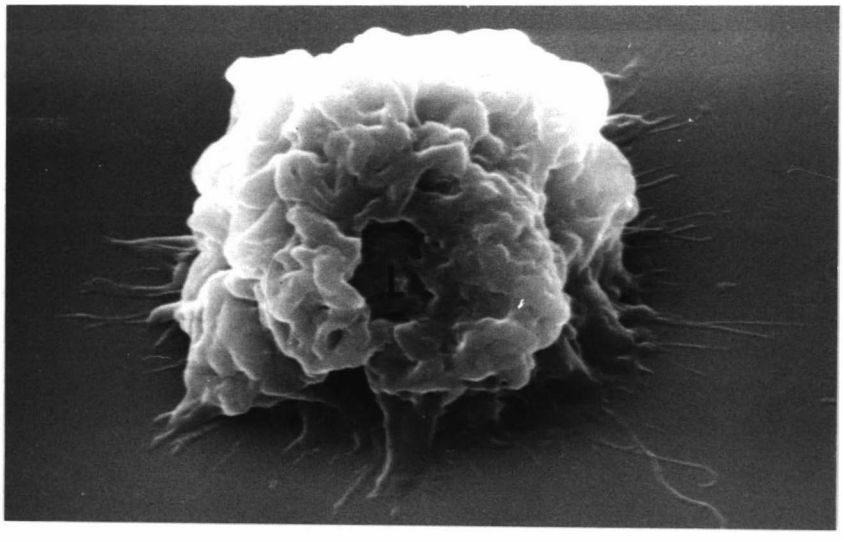
Mitotic division of an alveolar macrophage after one hour of incubation with *M. ovipineumoniae*. x 3,400.

Figure 17

After one hour of incubation with *M. ovipneumoniae* and specific antibody, alveolar macrophages appeared in a contractile position. Note the large extensive opening (L) and the fine pits on the surface membrane. x 6,000







membrane of the macrophages could be differentiated from the ½ hour preparation by two distinct features. Firstly, it was covered by a greater number of ridge-like profiles and secondly; it contained numerous indentations. The indentations consisted of exterior openings measuring about 1 to 2.5 µm in diameter as well as finer pits measuring 160 to 200 µm in diameter (Fig. 17).

M. ovipneumoniae organisms were not seen either attached to the macrophage plasma membrane or over the glass substratum surrounding the macrophage. The absence of micro-organisms and the contractile state of the macrophages indicated that phagocytosis had occurred earlier.

E. TWO HOURS POSTINOCULATION WITH *M. OVIPNEUMONIAE*

After two hours of incubation, macrophages appeared as single cells or small clusters of cells, attached to the glass coverslip surface. They were almost spherical and measured 10.5 to 16 µm in diameter (Fig. 18). A few fine filopodia protruded from these cells in various directions. They had some micro-projections and exhibited very pronounced membrane ruffling (Figs. 18 and 19). At this stage the cells were surrounded by glass adherent *M. ovipenumoniae* randomly distributed either as single organisms, small aggregations or occasional micro-colonies. Contact points between macrophage plasma membrane and *M. ovipneumoniae* organisms were apparent at the base of the cells. The micro-organisms were attached to the cell by fine filopodia and some micro-organisms were clumped together and attached to the base portions of the cell's surface membrane (Figs. 18 and 19). The presence of filopodia extending on the glass substratum gave the impression that no contractile pull was being exerted on the attached micro-organisms.

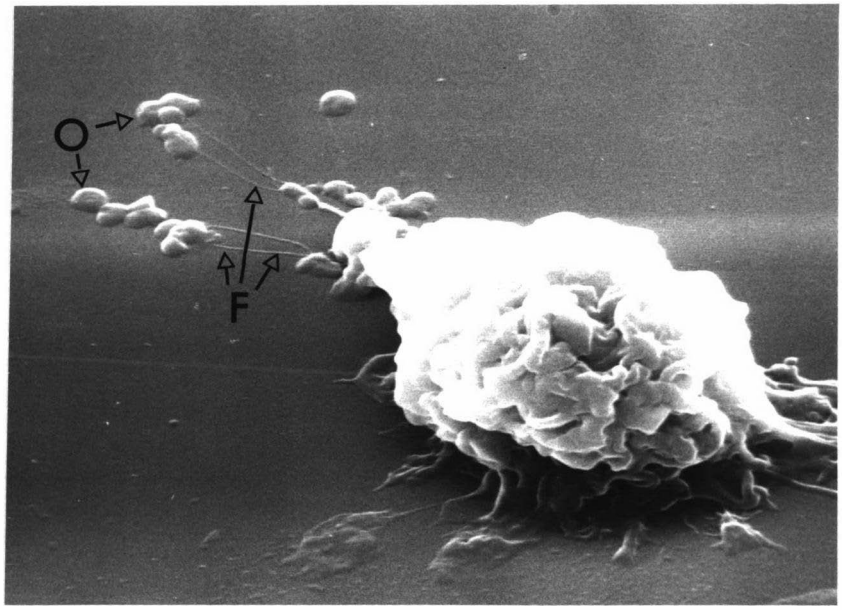
At this stage the *M. ovipneumoniae* organisms were

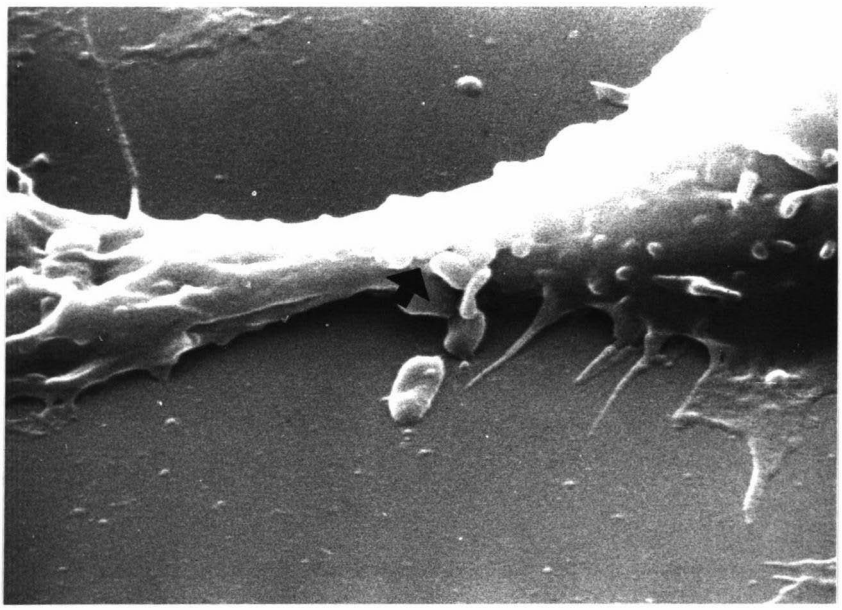
Figure 18

The macrophage in this preparation has sent a few fine filopodia (F) towards some randomly distributed *M. ovipneumoniae* organisms (O). x 5,000.

Figure 19

Attachment between *M. ovipneumoniae* organisms and the base portion of the plasma membrane of an alveolar macrophage (arrow) after 2 hours of incubation without antibody. No space is visible between the macrophage plasma membrane and the cellular membrane of the *M. ovipneumoniae* organism. x 12,000.


O
F



somewhat pleomorphic with a biconcave or spherical surface and measured 1 to 2 μm in diameter. No folding of the macrophage plasma membrane over or around the micro-organisms was seen.

F. TWO HOURS POSTINOCULATION WITH *M. OVIPNEUMONIAE* AND ANTIBODY

After 2 hours of incubation, the macrophages measured between 8 to 12.5 μm in diameter and were more or less spherical in shape. These cells were attached to the glass coverslip by cytoplasmic veils which were smaller than those seen in the ½ hour preparation but wider than those seen at one hour. The cytoplasmic veils did not contain intracytoplasmic spherules but filopodia extended from their margins (Fig. 20). The macrophage plasma membrane exhibited moderate ruffling, displayed a ridge-like profile and contained microprojections and numerous fine surface indentations (Fig. 21).

No *M. ovipneumoniae* organisms were present on either the macrophage plasma membrane or the surrounding substratum in this preparation. In contrast to the well shaped, glistening, surface membrane characteristic of the micro-organisms in the preparations without antimycoplasma antibody, the *M. ovipneumoniae* cells elsewhere in this preparation were dull in appearance and flattened onto the underlying substratum (Fig. 22).

In the specimens fixed for TEM a marked change in the macrophage-mycoplasma association was observed. *Mycoplasma ovipneumoniae* organisms were mainly located intracellularly enclosed by the unit membrane of a phagocytic vacuole, although a few were located on the macrophage plasma membrane. The phagocytic vacuoles were variable in size and measured between 0.7 and 2.7 μm (Figs. 24 and 25). Some vacuoles contained *M. ovipneumoniae* organisms which appeared normal (Fig. 24), whereas in others most of the membrane and

Figure 20

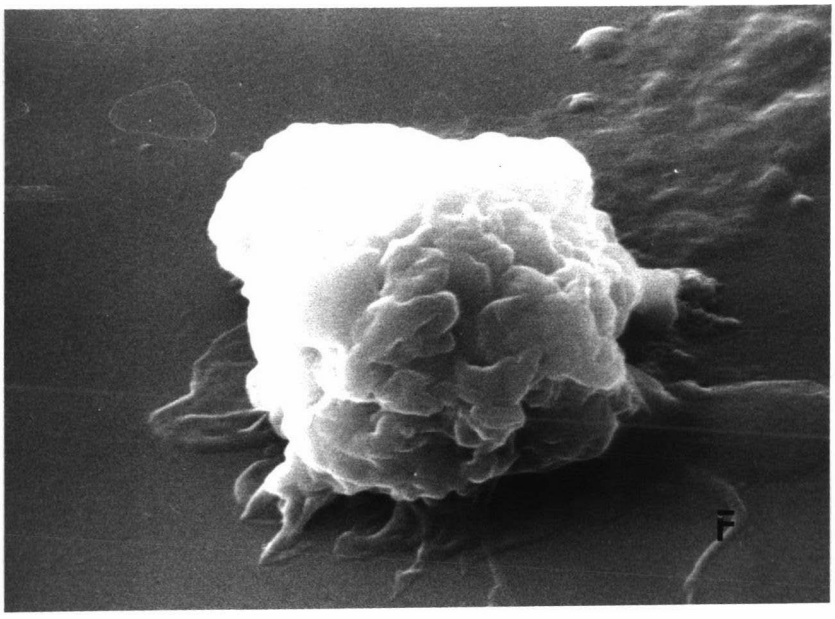
An alveolar macrophage cultured for 2 hours with *M. ovipneumoniae* and specific antibody. It has a relatively smooth surface and occasional filopodia (F) are extending from its margins. x 8,000.

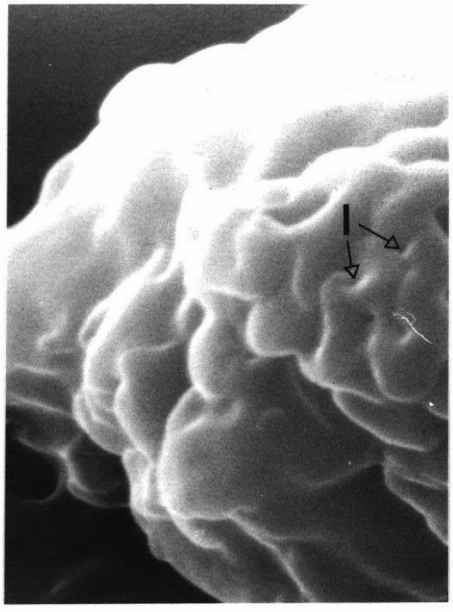
Figure 21 (left)

High magnification of the plasma membrane of the macrophage in figure 20. Note the moderate ruffling and fine surface indentations (I). x 20,000.

Figure 22 (right)

Mycoplasma ovipneumoniae organisms cultured for 2 hours with specific antibody. The organisms have a dull appearance and are flattened onto the surface of the substratum. x 20,000.





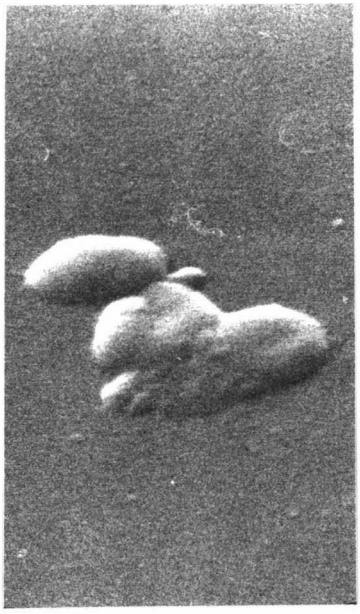
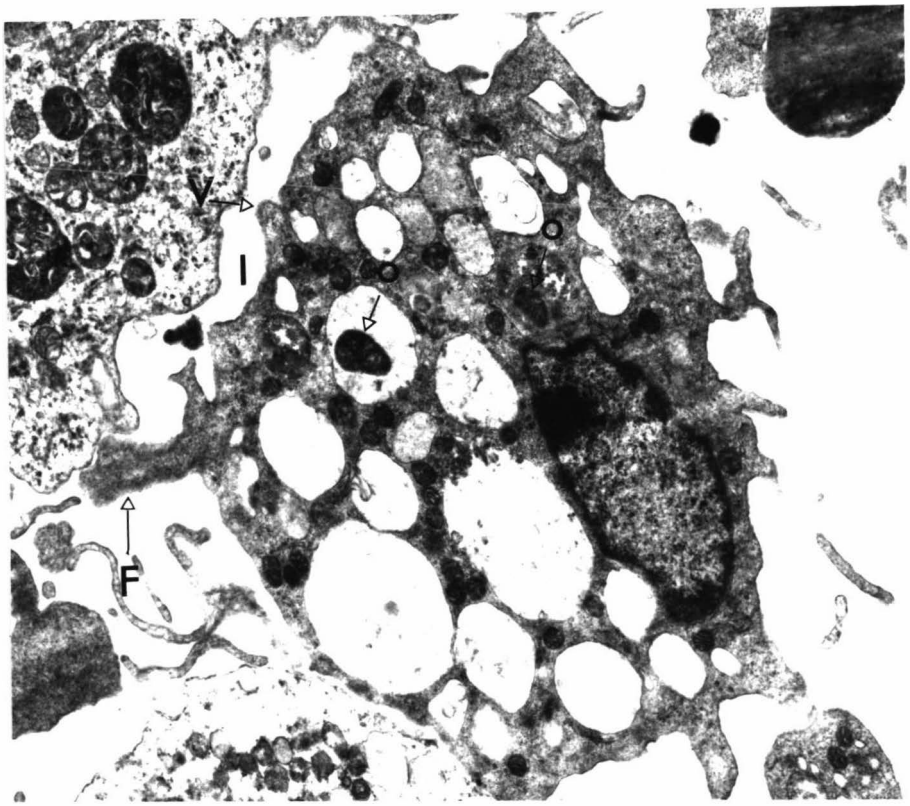


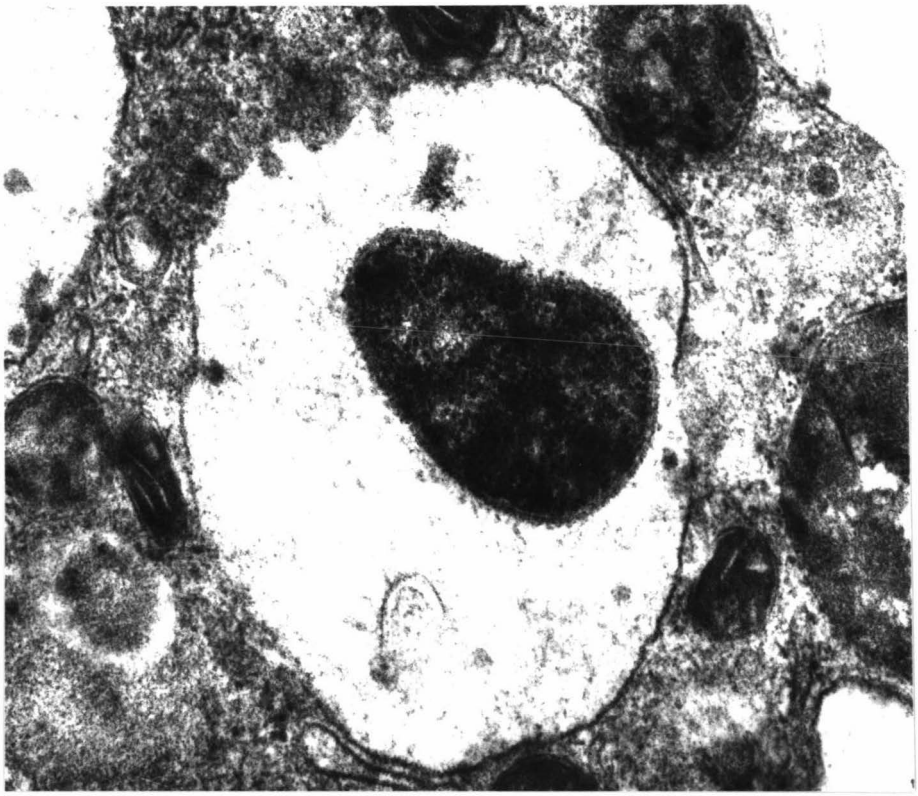
Figure 23

An alveolar macrophage cultured for 2 hours with *M. ovipneumoniae* organisms and specific antibody. The cell possesses a well-defined plasma membrane which displays moderate numbers of microvilli (V), finger-like projections (F) and invaginations (I). The cytoplasm contains a large number of vacuoles of variable sizes. Note that two of vacuoles contain *M. ovipneumoniae* organisms (o). The endoplasmic reticulum is poorly represented and a small number of ribonuclear protein (RNP) particles are attached to their membranes. The cytoplasm contains a large number of free RNP particles. The nucleus contains more heterochromatin than normal. x 10,500.

Figure 24

High magnification of the cytoplasmic contents of the cell in figure 23. A large phagocytic vacuole containing a viable *M. ovipneumoniae* organism is visible. x 61,000.


V
I
F



intracellular structure of the micro-organisms had disappeared or become partially degraded as indicated by a clearing of the contents of the central region (Fig. 25). The phagocytic vacuoles were very large and an obvious space existed between the vacuolar membrane and the *M. ovipneumoniae* cell wall. This space contained varying amounts of moderately stained amorphous material (Figs. 24 and 25).

The macrophages varied in shape and size and measured between 10.6 to 13 μm in diameter. They possessed a well defined plasma membrane which displayed moderate microvilli and finger-like projections. Plasma membrane invaginations were also common (Fig. 23).

The cytoplasm contained a large number of vacuoles and vesicles of variable size (Fig. 23). The endoplasmic reticulum was poorly represented and a small number of RNP particles were seen attached to their membranes although a very large number of free RNP particles were also observed. The Golgi apparatus was extensive and occupied a large area of the cytoplasm. The mitchondria were moderate in number with variable size and shape. The nuclei tended to be oval or kidney shaped and contained more heterochromatin than normal macrophages (Figs. 9 and 27).

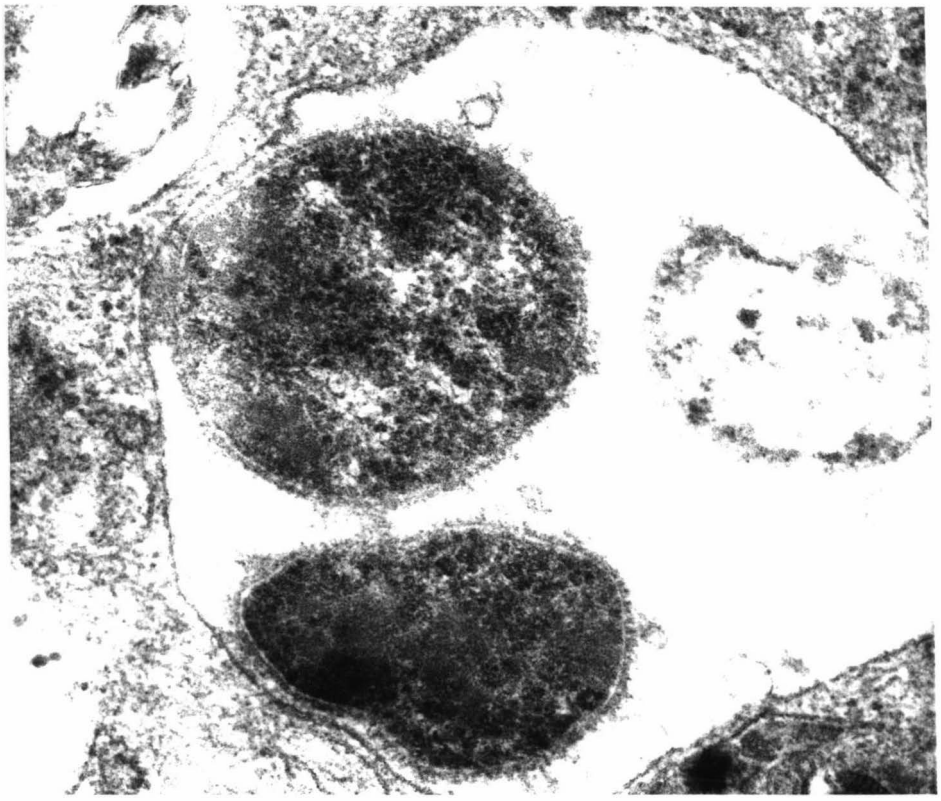
The ultrastructure of normal *M. ovipneumoniae* organisms located on the macrophage plasma membrane was not significantly different from that described by others (76). The micro-organisms were somewhat pleomorphic, but usually appeared as round bodies measuring between 0.7 to 0.8 μm in diameter. The cells were limited by a typical trilaminar membrane consisting of two electron dense zones separated by an electron lucent area. The trilaminar membrane appeared to be covered by micro-projections. The cytoplasm contained many ribosomes, with amorphous densities and electron lucent areas. No specific

Figure 25

A large phagocytic vacuole containing three *M. ovipneumoniae* organisms in different stages of destruction is present within a macrophage 2 hours after the addition of specific antibody. x 82,500.

Figure 26

An *M. ovipneumoniae* organism surrounded by finger-like projections of macrophage plasma membrane 2 hours after addition of specific antibody. Note the clear space between the micro-organisms and the macrophage plasma membrane. x 81,000.



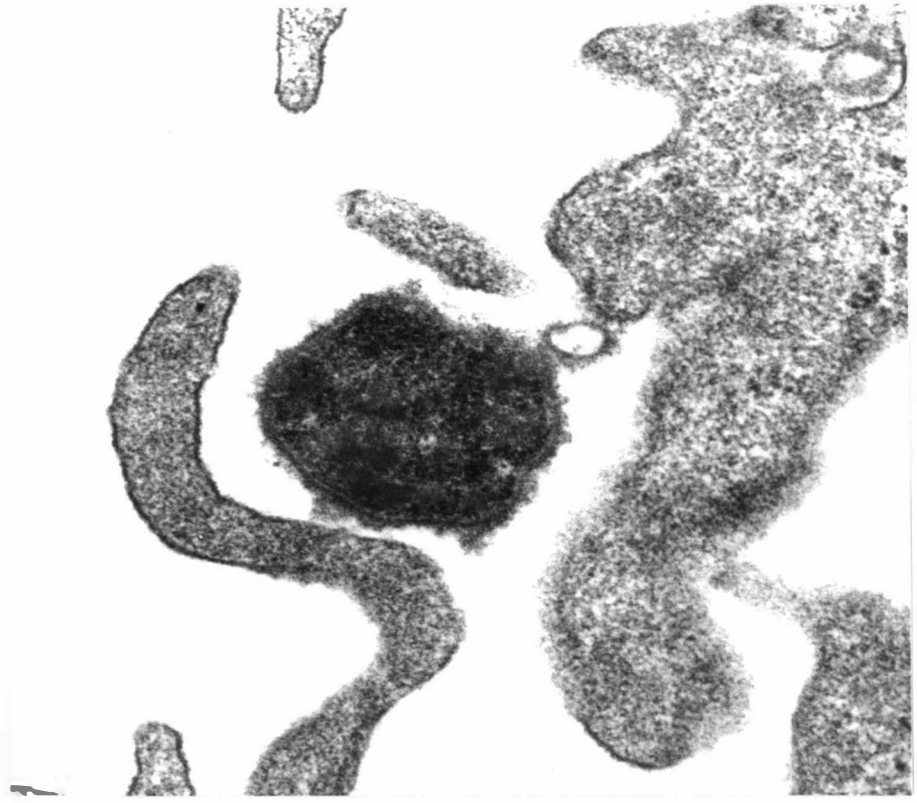
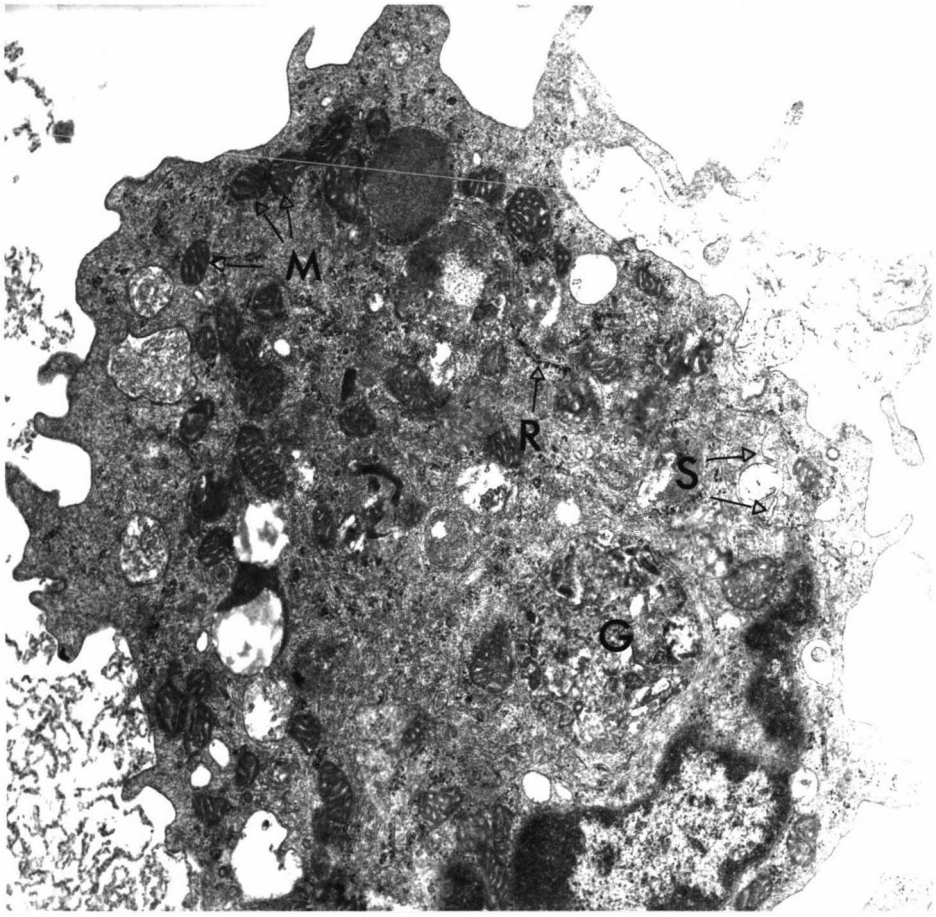


Figure 27

The cytoplasmic contents of an alveolar macrophage cultured for 2 hours with *M. ovipneumoniae* organisms and specific antibody. The cell appears highly active and contains large numbers of mitochondria (M), rough (R) and smooth (S) endoplasmic reticulum and extensive Golgi apparatus (G). x 7,500.


M
R
S
G

organelles were seen. Generally the *M. ovipneumoniae* organisms were seen in close opposition to the plasma membrane of the macrophages, and there was no special morphological feature evident at the site of the attachment. There was a clear space measuring 0.04 µm separating the micro-organism cell membrane and the macrophage plasma membrane but the space between the micro-projections of the organisms and the plasma membrane of macrophages measured 0.016 µm (Fig. 26).

G. SIX AND TWELVE HOURS POSTINOCULATION WITH *M. OVIPNEUMONIAE*

Ovine alveolar macrophages cultured with *M. ovipneumoniae* for 6 hours were either rounded or expanded and flattened onto the glass substratum surface thus displaying a greater variation and irregularity in shape than seen earlier (Figs. 28 and 29). The cytoplasmic membrane of these cells exhibited prominent ridge-like profiles with frequent long filopodia. Pronounced cytoplasmic veils were visible for the first time in the preparations without anti-mycoplasma antibody (Fig. 28). The attachment of macrophages to *M. ovipneumoniae* by means of fine filopodia were developed at this stage (Fig. 29). *Mycoplasma ovipneumoniae* organisms were distributed as micro-colonies, small aggregations and occasional single cells surrounding adherent macrophages. The presence of mycoplasma micro-colonies indicated that *M. ovipneumoniae* replication was occurring in the culture at the time of fixation.

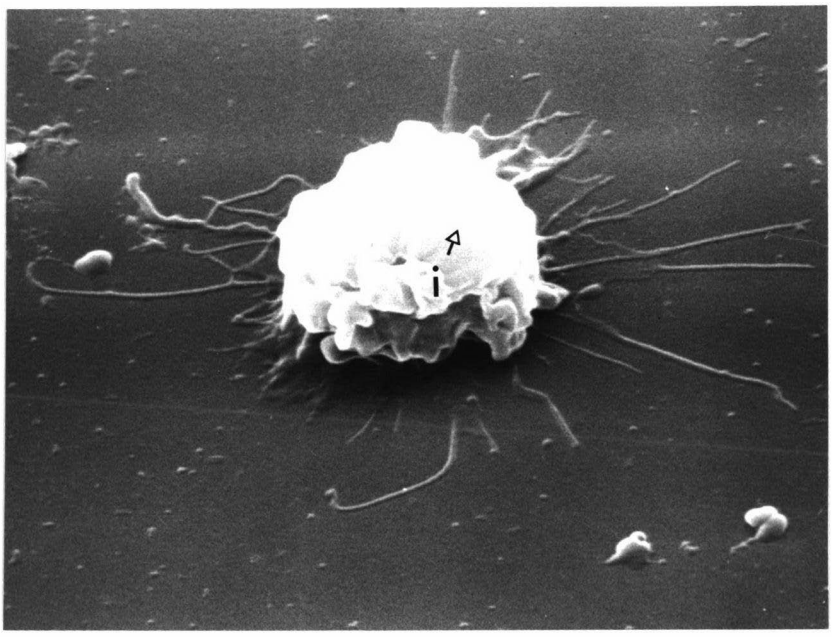
After 12 hours of incubation with *M. ovipneumoniae* organisms, macrophages appeared rounded and measured 12 µm in diameter. The cells in this preparation showed a large number of filopodia projecting in various directions (Fig. 30). The attachment of clusters of micro-organisms to the macrophage membrane and around the base of the cell was very clear (Fig. 31). A few small openings and pits were seen on the macrophage membrane, particularly at the base of the cell. The presence

Figure 28

After 6 hours of incubation with *M. ovipneumoniae* mycoplasma-macrophage attachment has developed and the macrophage plasma membrane displays prominent ridge-like profiles (i). x 7,000.

Figure 29

Striking irregularity in the shape of an alveolar macrophage after 6 hours culture with *M. ovipnuemoniae*. x 8,000.


i

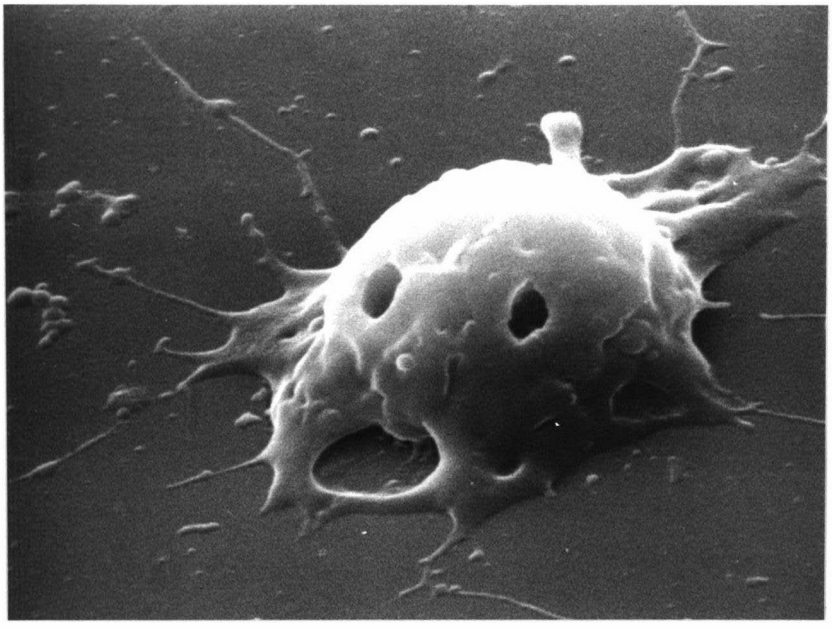


Figure 30

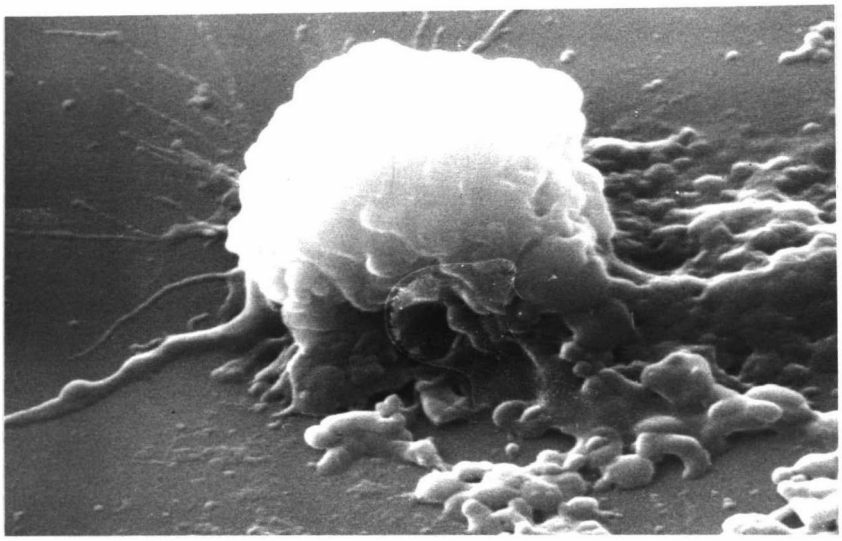
Attachment between a cluster of *M. ovipneumoniae* organisms and the base of a macrophage after 12 hours of incubation in the absence of antibody. x 10,000.

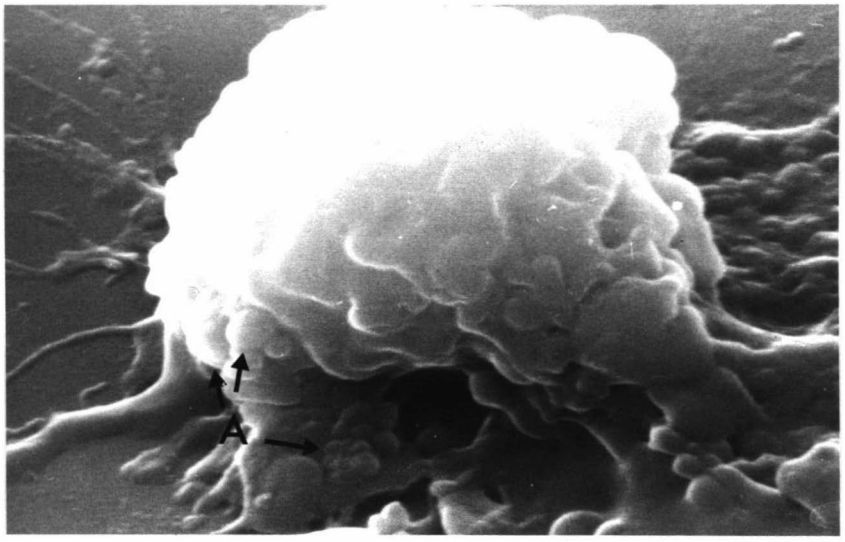
Figure 31

High magnification of the cell in figure 30 showing attachment between the micro-organsism and the alveolar macrophage. Not only are mycoplasmas attached to the base of the cell but also to the macrophage membrane (A). x 16,000.

Figure 32

After 12 hours of incubation, *M. ovipneumoniae* organisms show a high degree of pleomorphism. x 16,000.

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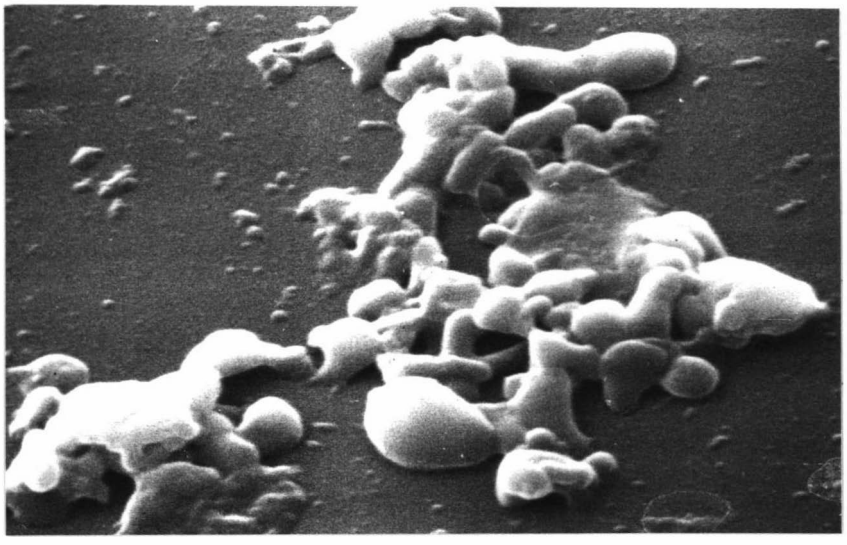


of more filopodia on the cells in this preparation than at 2 hours indicated that the cells were still expanding and strengthened the impression that no contractile pull was being exerted on the attached or surrounding *M. ovipneumoniae* organisms.

At twelve hours postinoculation the *M. ovipneumoniae* organisms showed a high degree of pleomorphism (Fig. 32). These changes suggested that the *M. ovipneumoniae* was in the death phase of its growth curve (76). The presence of large numbers of filopodia and the replication of *M. ovipneumoniae* organisms provided further evidence that little or no phagocytosis of the organisms had taken place in the preceding 12 hours.

H. TWELVE HOURS POSTINOCULATION WITH *M. OVIPNEUMONIAE* AND ANTIBODY

After 12 hours of incubation, the macrophages were moderately expanded and their cytoplasm contained very large numbers of spherules of similar size to *M. ovipneumoniae* organisms (Fig. 33). In contrast to the macrophages in the previous specimens, the nuclear poles did not show ruffles, display ridge-like profiles or form micro-projections (Fig. 34). Typically, they were dome-shaped, covered by a smooth plasma membrane and contained intracytoplasmic spherules of variable size. Surface indentations, large openings and fine pits were frequently seen (Fig. 35). Groups of spherical bodies of similar size to *M. ovipneumoniae* organisms were situated at the base of the cell within the cytoplasm and had the appearance of being pulled toward the nuclear pole (Fig. 34).

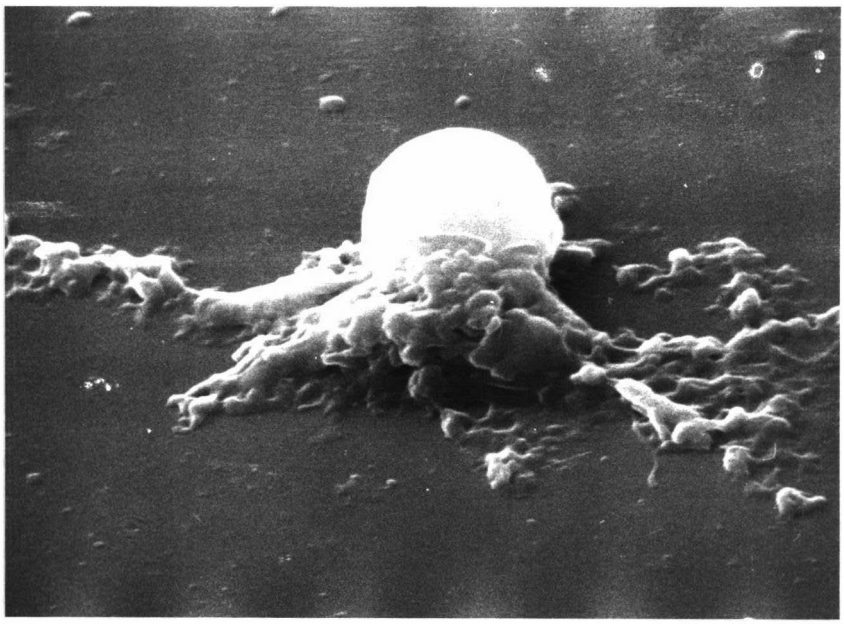
The macrophages in this preparation appeared to form large clusters consisting of large numbers of cells of variable size, shape and density (Fig. 36).

Figure 33

After 12 hours of incubation with *M. ovipnuemoniae* and specific antibody, the cytoplasm of an alveolar macrophage contains a large number of spherules comparable in size with *M. ovipneumoniae*. x 6,000.

Figure 34

High magnification of the nuclear pole of the cell shown in figure 33. The plasma membrane is smooth and contains intracytoplasmic spherules of variable size. The *M. ovipneumoniae* organisms appear to be being pulled towards the nuclear pole. x 16,000.



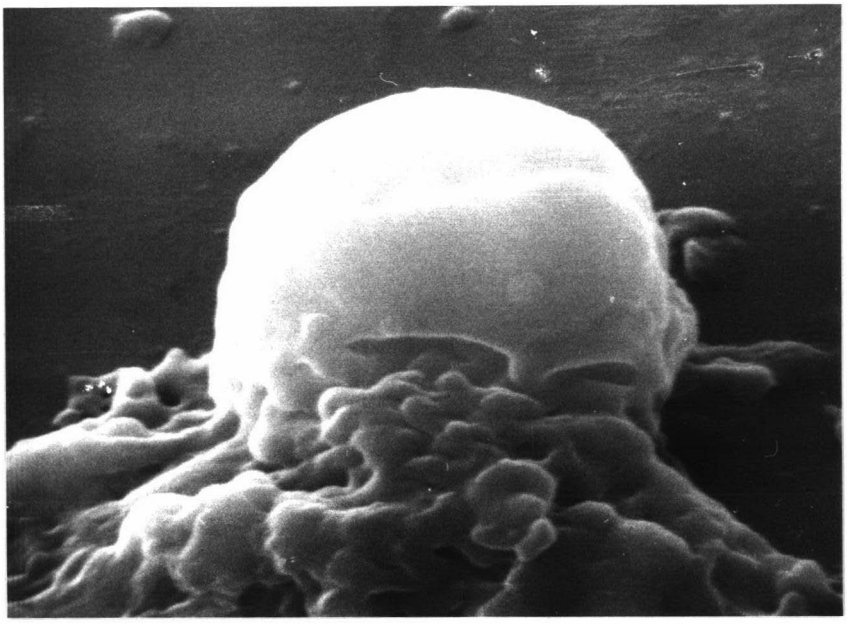
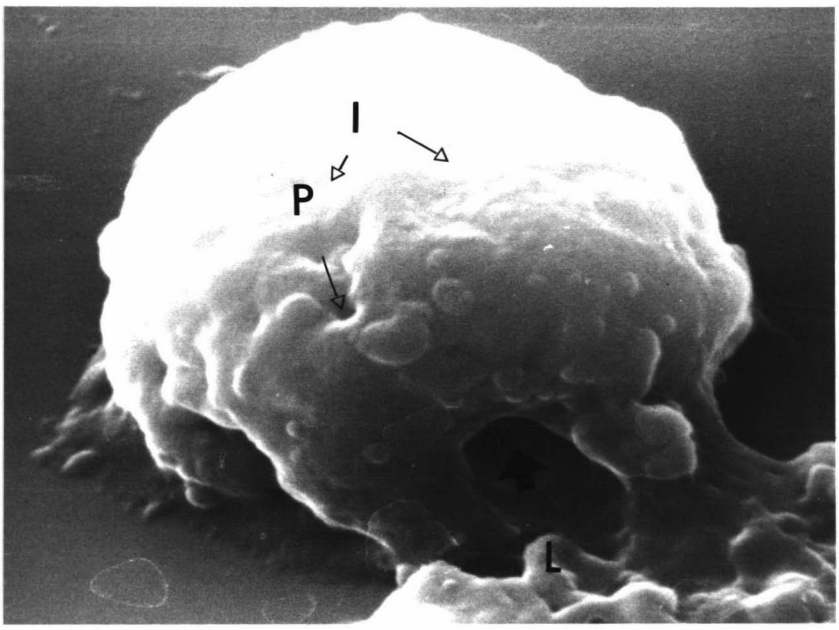


Figure 35

The plasma membrane of an alveolar macrophage cultured for 12 hours in the presence of *M. ovipneumoniae* and specific antibody. It shows surface indentations (I), large openings (L) and numerous fine pits (P) which indicate high pinocytic activity. x 16,000.

Figure 36

An alveolar macrophage culture infected with *M. ovipneumoniae* 12 hours after the addition of specific antibody. Large numbers of cells of variable shape, size and density have accumulated in one area. x 600.


I
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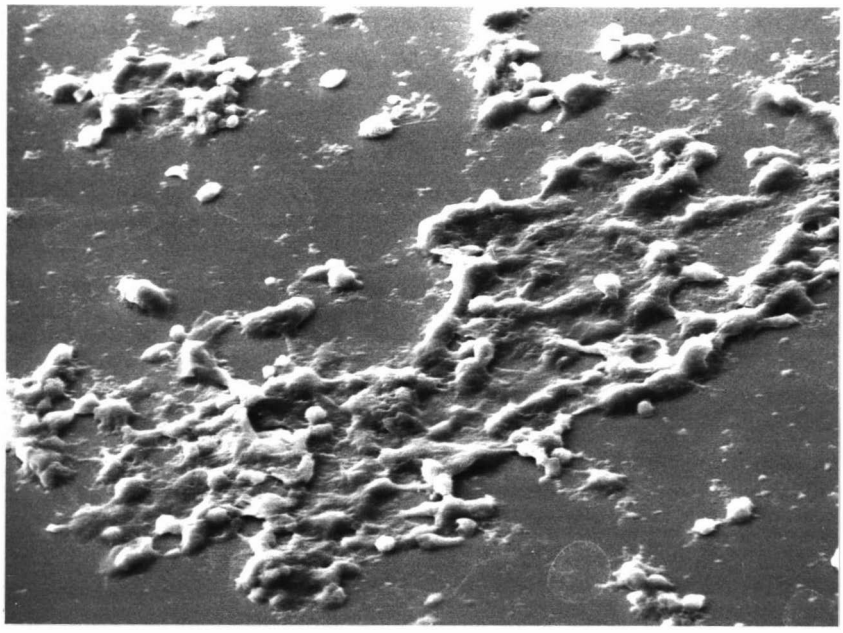
L



I. TWENTYFOUR HOURS POSTINOCULATION WITH *M. OVIPNEUMONIAE*

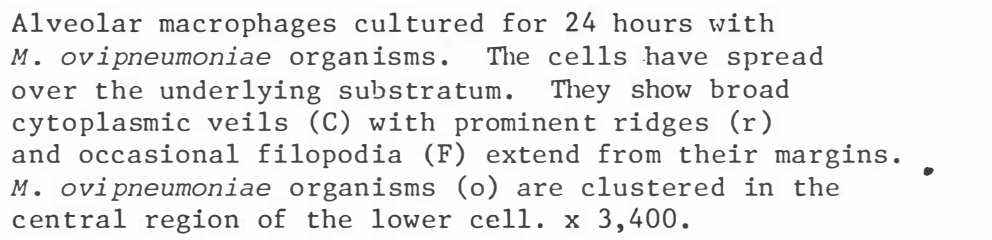
Ovine alveolar macrophages cultivated for 24 hours with *M. ovipneumoniae* were extensively spread and flattened over the underlying glass coverslip frequently reaching 12.4 to 34.7 µm in diameter (Fig. 37). Some cells showed cord-like extensions of their cytoplasm up to 4 µm in length (Fig. 39). Characteristically the cells had broad cytoplasmic transparent veils and some cells projected very long filopodia up to 37 µm (Figs. 37 and 40). The cytoplasmic veils had an undulating surface with very prominent ridges and filopodia sometimes extending from their edges (Fig. 37).

At this time interval, the macrophages also showed prominent pits, ruffles, ridge-like profiles and numerous filopodia. Cytoplasmic perforations and spherules were commonly seen. Interconnections between cells though filopodia were often encountered (Figs. 37 and 39).

A prominent feature of *M. ovipneumoniae* - macrophage relationship not seen in earlier preparations was the clustering of *M. ovipneumoniae* organisms in the central region of the cells (Fig. 37). Under high magnification, healthy mycoplasmas of variable shape and size could be identified clumped together and attached to the surface membrane (Fig. 38). The organisms measured between 0.5 to 1 µm, were round and biconcave, and had the appearance of typical viable mycoplasmas.

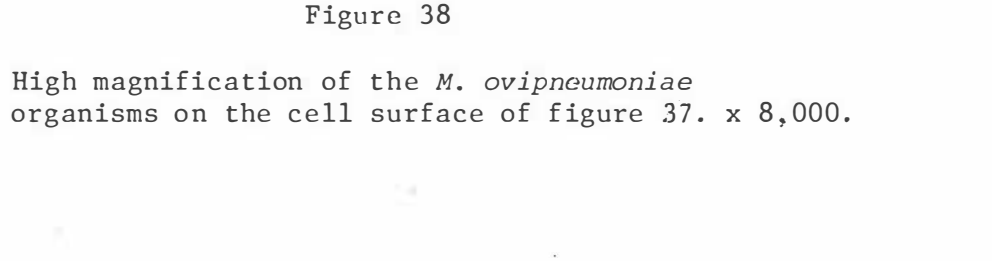
Specimens of macrophage-mycoplasma culture examined by TEM 24 hours postinoculation showed the *M. ovipneumoniae* organisms located on the plasma membrane of the macrophages. There were no special morphological features at the site of attachment except a clear space measuring 0.04 µm between the micro-organism and the macrophage plasma membrane (Fig. 41).

Figure 37



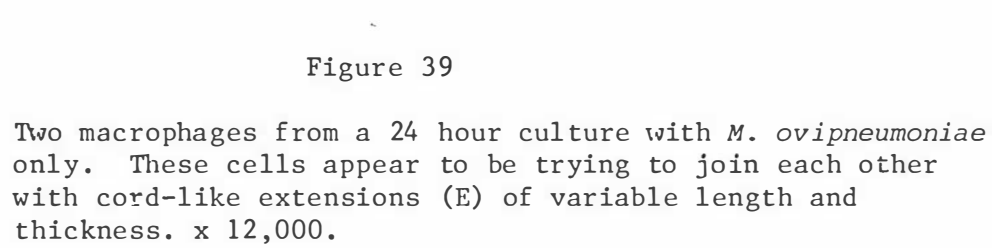
Alveolar macrophages cultured for 24 hours with *M. ovipneumoniae* organisms. The cells have spread over the underlying substratum. They show broad cytoplasmic veils (C) with prominent ridges (r) and occasional filopodia (F) extend from their margins. *M. ovipneumoniae* organisms (o) are clustered in the central region of the lower cell. x 3,400.

Figure 38

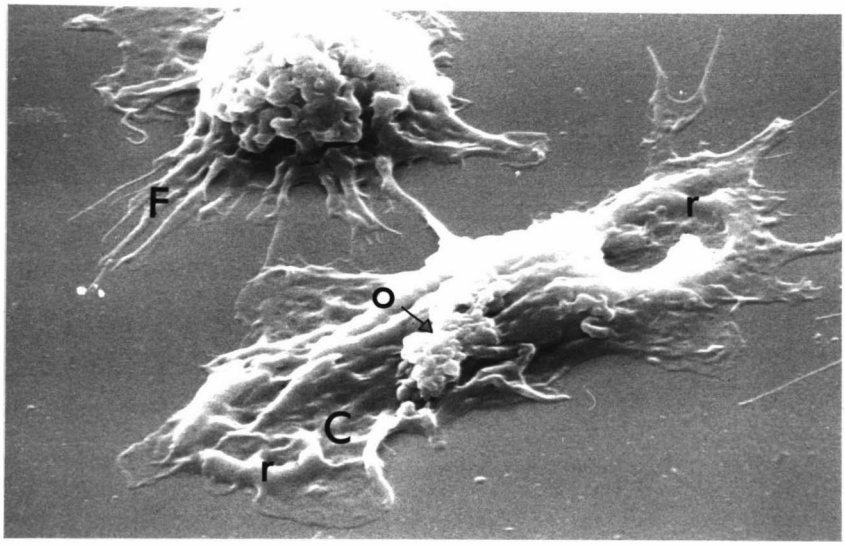


High magnification of the *M. ovipneumoniae* organisms on the cell surface of figure 37. x 8,000.

Figure 39



Two macrophages from a 24 hour culture with *M. ovipneumoniae* only. These cells appear to be trying to join each other with cord-like extensions (E) of variable length and thickness. x 12,000.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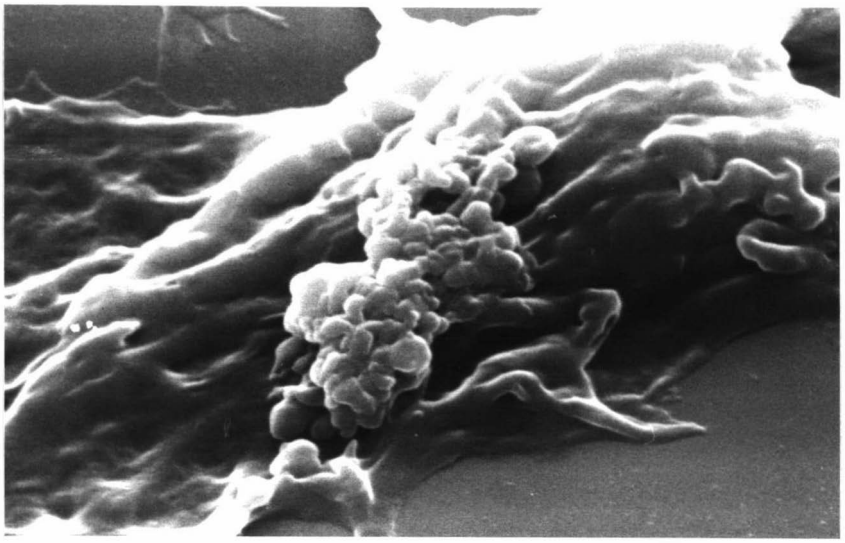

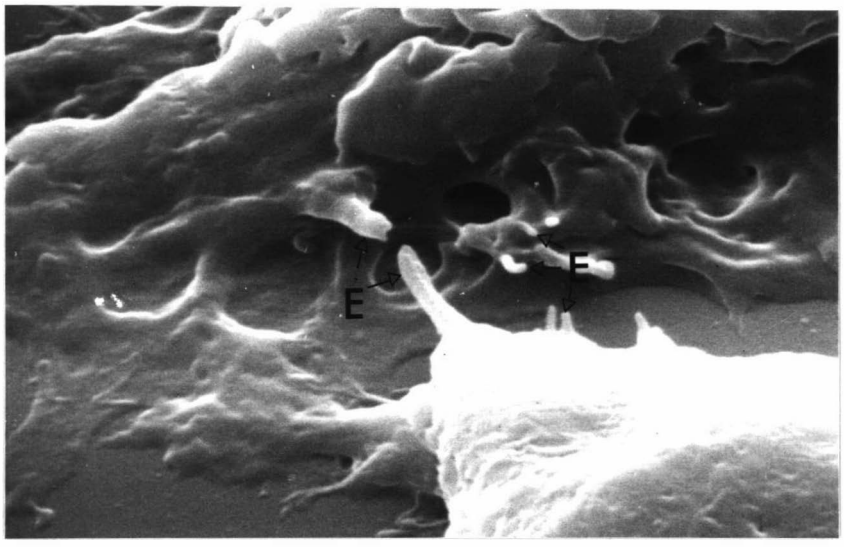
E
E

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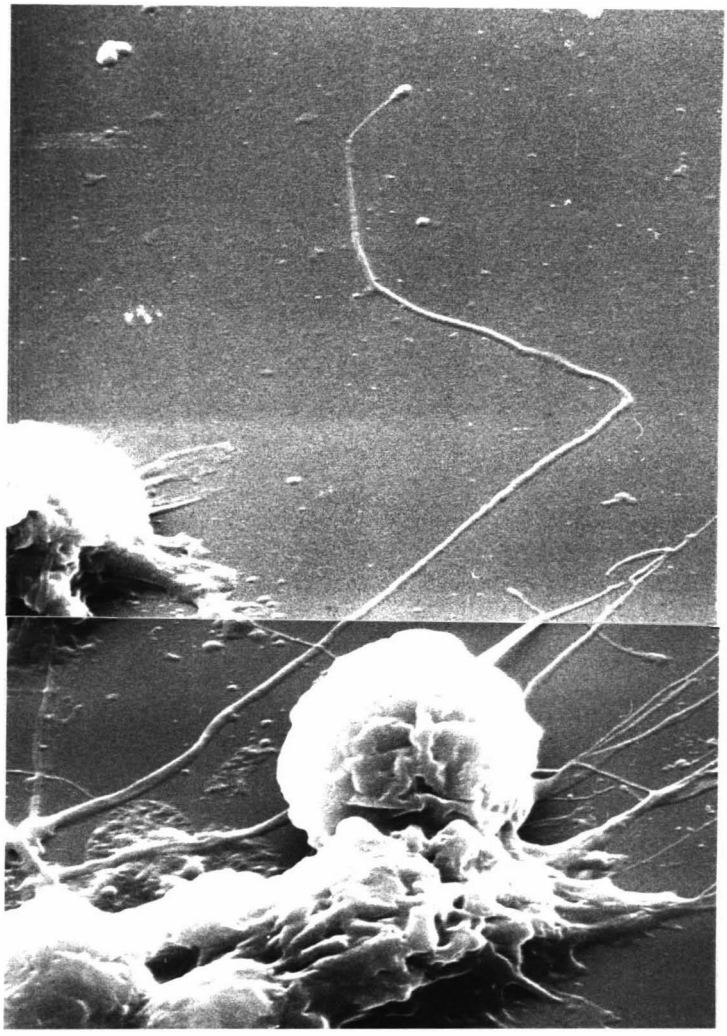
Figure 40

These macrophages are from a culture 24 hours after the addition of *M. ovipneumoniae*. Note the very long filopodia measuring 35.4 µm in length. x 4,800.



The plasma membrane of the macrophages displayed high surface projections and invaginations. These cells appeared more irregular in outline than macrophages from normal preparations (Fig. 9 and 43). The endoplasmic reticulum was poorly represented and had a very small number of RNP particles attached to its membranes, however, the cytoplasm contained very large amounts of free RNP particles (Fig. 42). The mitochondria were few in number and poorly defined. The cytoplasm contained large numbers of membrane-bound vesicles which were lightly stained or sometimes contained very dense material. Large numbers of vacuoles were present and completely occupied by amorphous material. Dark osmiophilic inclusions were also present (Fig. 43).

Ultrastructurally, the *M. ovipneumoniae* organisms were very pleomorphic and had large electron lucent zones which indicated that most of the cytoplasm had been lost. Most of the micro-organisms had a typical trilaminar limiting membrane but the surface projections were poorly defined (Fig. 41).

J. TWENTYFOUR HOURS POSTINOCULATION WITH *M. OVIPNEUMONIAE* AND ANTIBODY

After 24 hours of incubation, macrophages observed by SEM appeared to reshape into spherical forms and cluster in groups, consisting of a small number of cells (Fig. 44). The plasma membrane exhibited moderate ruffling, displayed ridge-like profiles and formed microprojections. Surface indentations of variable size and intracytoplasmic spherules were still evident (Fig. 45). No *M. ovipneumoniae* organisms were seen.

Specimens of macrophage-mycoplasma culture examined by TEM displayed prominent changes. The most significant change was that phagocytic vacuoles containing distinct micro-organisms were no longer detectable (Fig. 46). Surprisingly however,

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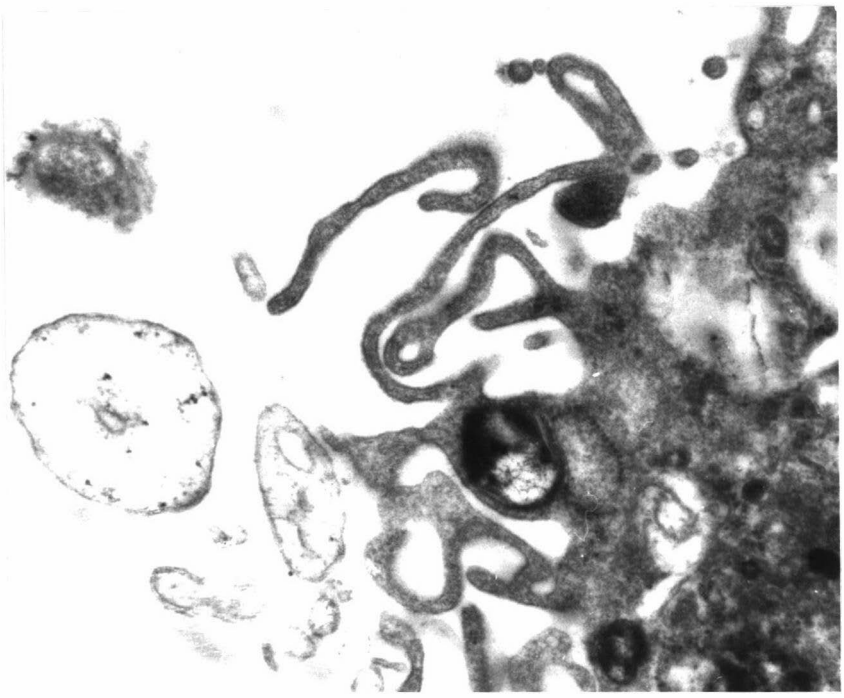
Figure 41

An alveolar macrophage cultured for 24 hours with *M. ovipneumoniae* organisms. Note the clear space between the plasma membrane of the macrophage and the micro-organisms (0.04 μm in width). The *M. ovipneumoniae* organisms are pleomorphic in shape and have a large electron-lucent zone indicating that most of the cytoplasm has been lost. These micro-organisms have a trilaminar membrane but show few surface projections. x 25,000.

Figure 42

High magnification of the cytoplasm of a macrophage after 24 hours culture with *M. ovipneumoniae* organisms. Note that the endoplasmic reticulum has a very small number of RNP particles whereas the cytoplasm contains a large number of free RNP particles. x 34,000.



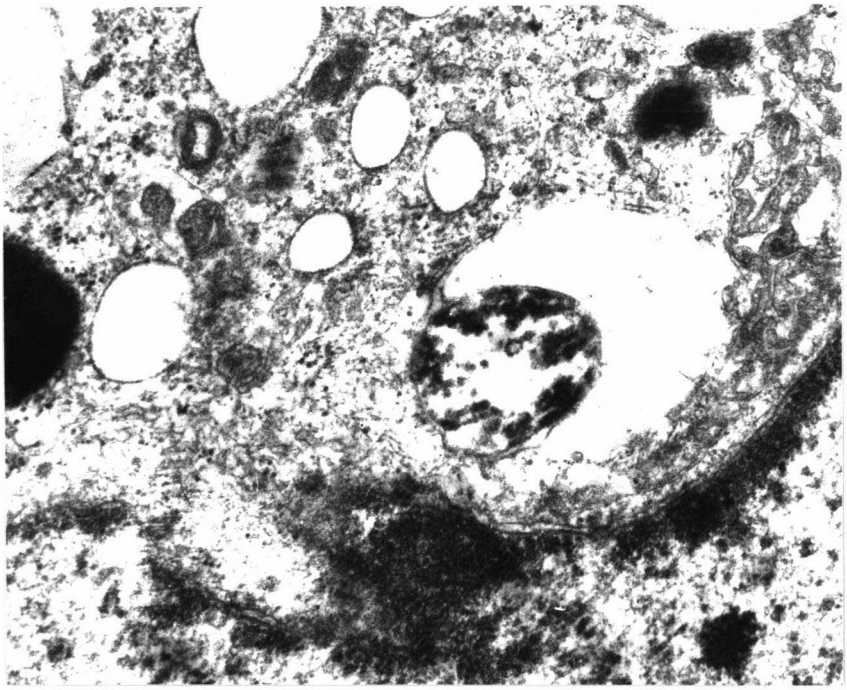


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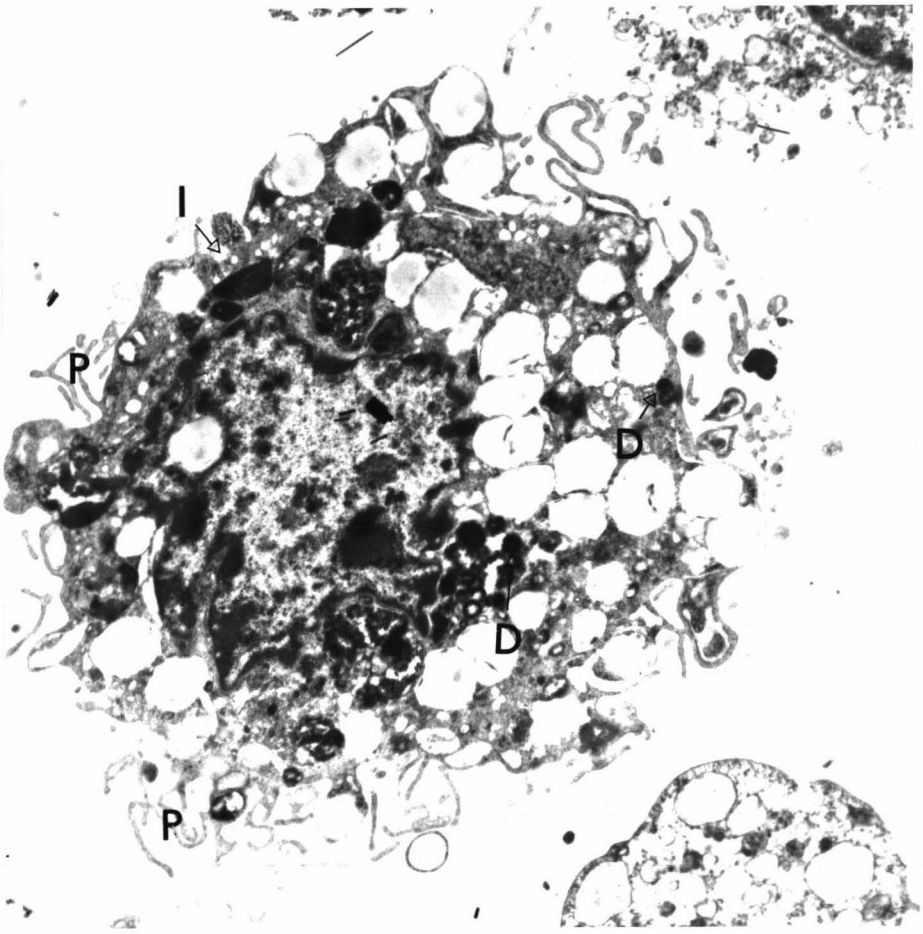
Figure 43

An alveolar macrophage cultured with *M. ovipneumoniae* organisms for 24 hours. The plasma membrane of this cell is well defined and displays high surface projections (P) and invaginations (I). The cytoplasm contains large numbers of vacuoles and vesicles. The vacuoles are completely filled with amorphous material. Note the dark osmiophilic inclusions (D) in the cytoplasm. x 8,100.


I
P
D
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P

some extracellular organisms were still present.

The macrophages in this preparation were rounded in shape and the outline of these cells appeared more regular than macrophages from normal preparations (Fig. 47). The plasma membrane exhibited very few surface projections and invaginations. These cells had large electron lucent areas which suggested that part of the cytoplasm was lost. The intracellular structures were very poorly represented. The cytoplasm contained structureless, round organelles which were variable in size and were probably mitochondria (Fig. 47). Ribonuclear protein particles with variable densities were also scattered throughout the cytoplasm. The nuclei were intact and exhibited large areas of electron opacity which suggested an increase in intranuclear heterochromatin. The nucleolus zone was prominent (FIg. 47).

Figure 44 (Top left)

Alveolar macrophages, 24 hours after the addition of *M. ovipneumoniae* and specific antibody. In contrast to the 12 hour culture, these cells appeared to reshape into spheres and cluster into groups consisting of a small number of cells. x 600.

Figure 45 (Top right)

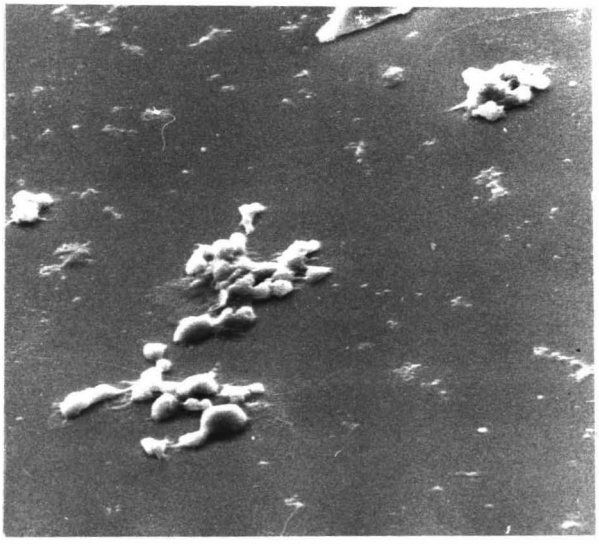
The plasma membrane of an alveolar macrophage cultured for 24 hours in the presence of *M. ovipneumoniae* and specific antibody. The membrane exhibits moderate ruffling, displays ridge-like profiles and microprojections. Note the surface invaginations (I) and intracytoplasmic spherules (s) which are still evident. x 16,000.

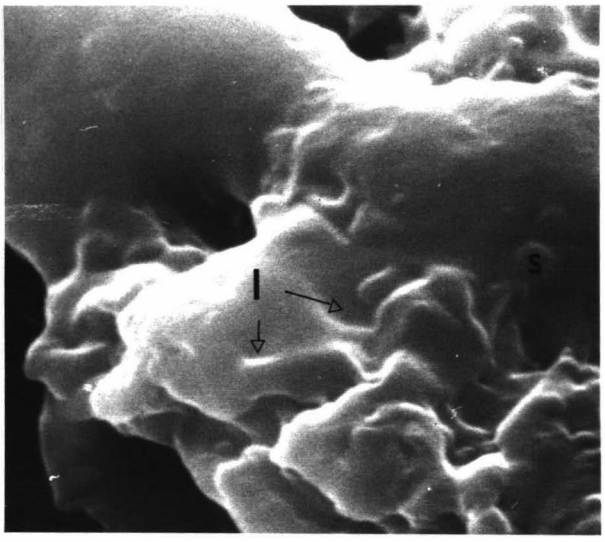
Figure 46 (Bottom left)

Transmission electromicrograph of the cytoplasmic contents of a cell cultured for 24 hours with *M. ovipneumoniae* and specific antibody. In this cell, phagocytic vacuoles containing distinct *M. ovipneumoniae* organisms could not be detected. x 25,000.

Figure 47 (Bottom right)

An alveolar macrophage cultured for 24 hours with *M. ovipneumoniae* and specific antibody. The cell is rounded in shape and the plasma membrane exhibits few projections and invaginations. Distinct intracytoplasmic structures have been lost but some round organelles which are probably mitochondria can be seen. The nucleus contains a large amount of heterochromatin with a very prominent nuclear zone and the nuclear membrane is intact. x 10,500.




I
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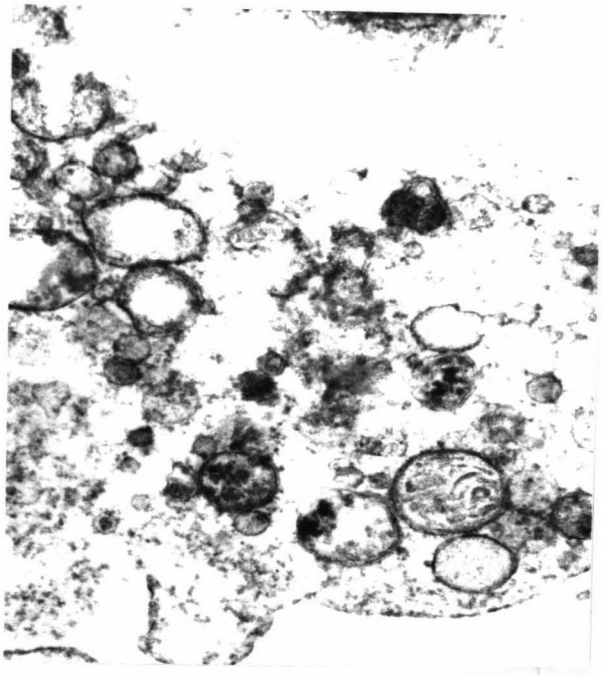

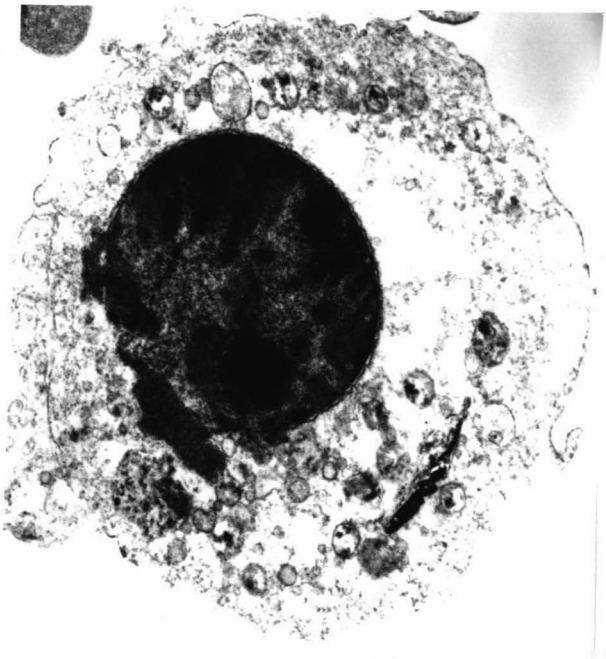

CHAPTER FOUR

DISCUSSION

Macrophages have a characteristic surface and ultrastructural morphology which allows them to be differentiated from other types of cell. In this study, the surface properties of glass adherent ovine alveolar macrophages were observed in striking three dimentional detail by SEM and the intracellular structure by TEM techniques. This provided an opportunity to study and compare resting non-stimulated macrophages with cells stimulated by mycoplasmas with and without the presence of antibody.

The plasma membrane of normal cells studied by SEM showed morphological evidence of a high level of membrane activity including numerous ridge-like profiles, ruffles and long cytoplasmic extensions. The ultrastructure of the ovine alveolar macrophages observed in this study was similar to that described for other mammals but differed from that of rabbit alveolar macrophages by the absence of multilamellated inclusions (89).

Using SEM, the surface morphology of the macrophages stimulated by *M. ovipneumoniae* organisms alone or with the addition of antimycoplasma antibody was found to differ from normal, non-stimulated cells in two general respects. Firstly; the membrane activity was greatly enhanced in the stimulated macrophages as shown by more extensive spreading, a larger surface area with more prominent membrane undulations and the more frequent occurrence of large ruffles and filopodia. Secondly; cellular mitotic divisions were observed in macrophages as early as ½ an hour after the addition of *M. ovipneumoniae* organisms alone, whereas in unstimulated and antibody stimulated cells, mitosis was not observed. In studies by other workers (22), mitotic figures have been observed during the first few days of the cultivation period. Jones (53) found that *M. pulmonis* attaches to the surface of mouse peritoneal macrophages but the ingestion phase of the organism was inhibited. It is possible that attachment of *M. ovipneumoniae*

organisms inhibited the ingestion phase of ovine alveolar macrophages but stimulated cellular division as a compensating action. The division of macrophages in response to *M. ovipneumoniae* and in the absence of antibody has important implications with regard to the development of lesions in chronic non-progressive pneumonia of lambs. It may account for the presence of the clumps of macrophages which are invariably seen in early lesions or the predominance of macrophages in the alveolar exudate of many advanced lesions (1).

There was little evidence of interaction between *M. ovipneumoniae* organisms and ovine alveolar macrophages in the absence of antibody in the specimens cultured for less than two hours. Attachment between the micro-organisms and the plasma membrane of the macrophage was observed by SEM in regions where they were both adherent to the glass coverslip surface, a feature which is similar to the *M. pneumoniae*-macrophage interaction observed by Powell (93). In addition, *M. ovipneumoniae* organisms were found to be attached to the plasma membrane over the whole of the alveolar macrophage surface particularly after 2 and 24 hours of incubation and it seemed that the micro-organisms were randomly distributed.

Under high magnification, SEM showed there was no change in the plasma membrane of macrophages at the site of attachment of *M. ovipneumoniae* organisms. The clear space between the micro-organisms and macrophage plasma membrane which was observed by TEM in both this study and by others (55) was not observed under high magnification with SEM. This observation suggests that the space is an artifact rather than a characteristic feature of the mycoplasma-macrophage interaction as suggested by Jones *et al.* (55). Shrinkage is more likely to occur in the TEM than SEM specimens because of the length of processing and the reagents used for dehydration.

Scanning electron microscopy also provided evidence that *M. ovipneumoniae* organisms attached more readily to the coverslip

surface than to the plasma membrane of the macrophage, a feature which differs from reported observations of *M. pulmonis* (56) (57). The attachment of the strain of *M. ovipneumoniae* organisms used in this study to the glass coverslip was similar to that of *M. pulmonis* (56) (57) and *M. pneumoniae* (93) which are pathogens for mice and man respectively. Since this strain of *M.ovipneumoniae* has been shown to have only mild pathogenicity for sheep (2), this feature may have no significance in relation to mycoplasma pathogenicity. Morphologically, the *M. ovipneumoniae* organisms appeared healthy and were often biconcave particularly in early cultures. Within 6 hours of incubation they replicated, displayed irregularities in shape and formed micro-colonies.

Mycoplasma ovipneumoniae organisms observed by TEM were similar in structure to the earlier description by Major, *et al.* (77). It therefore appears there is no significant difference in the ultra-structure of *M. ovipneumoniae* in cell- associated and broth medium.

Ultrastructurally, the attachment of *M. ovipneumoniae* to the alveolar macrophage plasma membrane in the absence of antibody showed no striking morphological features. The unit membrane of *M. ovipneumoniae* was separated from the plasma membrane of the macrophages by a clear space measuring 0.16 µm. This space was relatively constant, and there was no evidence of fusion or junctional zones between the micro-organisms and macrophage plasma membrane, like that observed with *M. pneumoniae* (55). No *M. ovipneumoniae* organisms were observed free within the cytoplasm ground substance as reported with *M. pneumoniae* (119). In the absence of specific antibody, there were no intracellular micro-organisms or even degenerate forms of *M. ovipneumoniae* seen. This contrasts with the results obtained with *M. pneumoniae* organisms by Jones *et al.*(55). These observations indicate that *M. ovipneumoniae* organisms are not phagocytosed unless antibody is present.

The addition of antimycoplasma antibody to the macrophages

cultured with *M. ovipneumoniae* organisms evokes a sequence of characteristic morphological alterations leading to the phagocytosis of attached and surrounding micro-organisms. The most striking change observed on the macrophage plasma membrane by SEM, was the formation of extensive expanded cytoplasmic veils and the apparent contractility of the cells. These cytoplasmic expansions differed from those of cells cultured with *M. ovipneumoniae* alone by an extensive undulating surface and the present of more spherules. A further notable change was an increase in the number, length and contractility of existing filopodia. This second finding has also been observed in SEM studies of *M. pneumoniae* (93) and in studies of *Bacillus cereus, Staphylococcus aureus* and *Cryptococcus neoformans* (113).

After the addition of antimycoplasma antibody, the macrophages became flattened and developed a marked increase in the number of filopodia. Their appearance contrasted markedly to macrophages cultured with *M. ovipneumoniae* alone, where the cells were spherical and showed regular surface ridges with a few short or long filopodia attached to the glass substratum.

From the time sequence of the observations made by SEM, macrophages in the presence of antibody appeared to be exerting a contractile action on the glass coverslip. Within ½ an hour of incubation the cells were flattened and expanded by the formation of the cytoplasmic veils, but by 1 hour the cytoplasmic veils appeared to be disappearing and filopodia began to develop.

In those cultures to which antibody was added, alveolar macrophages aggregated in small or large groups of cells, the most dramatic of which were seen at 12 hours. This may be due to an offensive by antibody sensitized macrophages against glass adherent micro-colonies of *M. ovipneumoniae* which were well formed in the 12 hour cultures. However, this is a simple explanation for an experimental observation which needs more investigation.

A further effect observed with SEM after the addition of specific antibody, was the appearance of a granular surface on the plasma membrane of the alveolar macrophages, similar to that described in thioglycollate-elicited cells (91). This was assumed to represent enhanced pinocytic activity by these stimulated cells (91). It has been recorded previously that the antigen-antibody interaction at the cell surface is a potent pinocytic stimulus (28).

The first notable event seen in the TEM studies was the presence of a few remaining *M. ovipneumoniae* micro-organisms on the surface of alveolar macrophages 2 hours after the addition of antimycoplasma antibody. In previous studies of mycoplasmas cultured on the surface of mouse peritoneal macrophages, fixed 2 hours after the addition of specific antibody, no organisms were seen on the cell surface (53). There were two major differences between cells at 2 and 24 hours after the addition of specific antibody. Firstly; in the two hour specimens viable *M. ovipneumoniae* organisms were observed intracellularly and secondly; the plasma membrane at 2 hours was very active and displayed greater ruffling and more finger and micro-projections than the 24 hours specimens. By using radiolabelled thymidine, Jones, *et al.* (55) found that after approximately 3 hours of *M. pneumoniae* phagocytosis, there was a rapid movement of the radiolabel from acid-insoluble to acid-soluble material, indicating that degradation of the organisms within phagosomes was occurring. Thus the intercellular survival of *M. ovipneumoniae* organisms seen in this study is likely to be a reflection of recent ingestion.

The projections which cover *M. ovipneumoniae* micro-organisms have not been shown to be part of its structural component but may be host or media constituents. The observations made by TEM and SEM in this study have shown that ovine alveolar macrophages have the ability to phagocytose *M. ovipneumoniae* organisms within 30 minutes after the addition of antimycoplasma antibody directed specifically against the micro-organisms. This provides evidence

that these projections could be part of the structural component of the organisms.

The method used in the preparation of macrophages for SEM in this study differed from those used by others in that alcohol was replaced by acetone as agent for dehydration. The excellent results obtained, with minimal artifact, indicate that this method could perhaps be more widely adopted.

The procedure developed for pulmonary lavage affords an alternative to the methods used by others (85). It allows effective and complete filling of the lungs with sterile fluid in addition to ease of collection by suction and provides sufficient fluid from one filling to obtain adequate numbers of macrophages. The relatively large proportion of washing fluid recovered was probably related to the procedure used and the anatomical structure of the ovine lungs. Information is available on the collection of alveolar macrophages from the lungs of rats (12), mice (21) (79) (50), monkeys (59), rabbits (85), cattle (33) and pigs (117). The procedures used by most of these workers were based on the technique described by Myrvik, *et al.* (85) for rabbits and are probably not practicable for large lungs such as those of ovine and bovine species which require greater filling pressure and fluid volume. This is probably the reason behind the relatively poor proportion of washing fluid recovered from calves by Williams (117) although the distinct lobular anatomy of the bovine lungs may have been a contributing factor.

The monovalent normal saline solution used in this study was found to be a satisfactory pulmonary lavage and cell-transport medium. In the preliminary experiments, it was observed that the number of alveolar macrophages obtained decreased when sterile Hank's balanced salt solution was used. Nevertheless, it has been reported by others (12) that pulmonary lavage solutions

containing divalent cations reduce markedly the number of alveolar macrophages which can be obtained from washing lungs.

In conclusion, the work undertaken has clearly demonstrated that the addition of antimycoplasma antibody to an *M. ovipneumoniae*-macrophage culture stimulates phagocytosis. This suggests that if sheep attain high titres to *M. ovipneumoniae* they will be able to deal quickly and effectively with an *M. ovipneumoniae* infection; a possibility which should be tested *in vivo*. The strain of micro-organisms used in this study was attenuated through laboratory culture over a long period. Further comparative *in vitro* studies with more virulent strains of *M. ovipneumoniae* are therefore essential to gain more complete understanding of the mycoplasma-macrophage interaction.

APPENDIXES

APPENDIX 1.

TC199 medium

TC medium 199 powder (Wellcome)	10	gm
Tricine	1.8	gm
$NaHCO_3$	1.5	gm
Bovine foetal serum*	200	ml
Penicillin	10^5	units
Streptomycin	100	mg
Fungizone (Squibb)	50	mg

Volume made up to 1000 ml with water and sterilized by filtration.

APPENDIX II

Modified FM4 medium

NaCl	5	gm
KCl	0.4	gm
$MgSO_4.7H_2O$	0.2	gm
$NaPHO_4.12H_2O$	4.03	gm
KH_2PO_4	0.1	gm
Glucose	10	gm
Phyto Peptone	10	gm
NAD	0.1	gm
L-Cysteine HCP	0.1	gm
Eagle's vitamin soln x 100 (see below)	25	ml
0.4% phenol red	2.5	ml
Penicillin	10^6	units
1.0 m NaOH to pH	7.8	
Distilled water to	1000	ml

The basal medium was supplemented with 150 ml of unheated bovine serum before use.

* Laboratory Service (Auckland)

Notes on preparation:

A) the medium was clarified by filtration through non sterile 0.45 µm and 0.22 µm pore size filters and subsequently sterilized by filtration through a sterile 0.22 µm filter.

B) The vitamin supplement used (Eagle's Vitamin Solution x 100) was as follows:

D-Biotin	20 mg	Myo-Iodositol	40 mg
Calcium pantothenate	20 mg	Niacinamide	20 mg
Choline chloride	20 mg	Pyridoxine	20 mg
Folic acid	20 mg	Thiamine HCl	20 mg
Riboflavin	2 mg	Distilled water to	200 ml

The solution was sterilized by filtration through a 0.22 µm membrane filter.

APPENDIX III

Modified Karnovsky's fixative

Paraformaldehyde	2.0 gm
Gluteraldehyde	12 ml
$Na_2HPO_4.12H_2O$	2.51 gm
KH_2PO_4	0.41 gm

A) The paraformaldehyde (2.0 g in 80 mls distilled water) was heated to between 60 and 70°C.

B) 1.0N NaOH was slowly added, dropwise until the solution cleared.

C) The buffer salts ($Na_2 HPO_4.12H_2O$) and gluteraldehyde were then added.

D) The solution was made up to 100 mls and stored at 4°C.

APPENDIX IV

Trypsin solution

Trypsin (Difco 1:250) was dissolved in Hank's basic salt solution to make a 0.25% concentrated solution then adjusted to pH 7.6 with $NaHCO_3$.
Penicillin 2 x 10^5 units and Streptomycin 100 mg were added.
The solution was sterilized by filtration and stored at -20°C.

APPENDIX V

Osmium tetroxide

Osmium tetroxide was made up as a 1% aqueous solution in 0.1M phosphate buffer and kept at 4°C in a dark bottle.

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