IMMUNOPATHOLOGICAL STUDIES IN THE OVINE LUNG DURING THE COURSE OF NATURAL AND EXPERIMENTAL PARAINFLUENZA TYPE 3 VIRUS INFECTION

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ABSTRACT OF THESIS

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The pulmonary immunopathology of parainfluenza type 3 (PIV-3) infection in sheep was investigated firstly by isolating the virus from field cases of sheep pneumonia, secondly by experimentally reproducing the disease with the isolated virus, and finally by studying changes in lymphocytes subsets and alveolar macrophages, induced by PIV-3 in vivo and in vitro.

Three ovine virus isolates (270-7, 390-10 and 430-7) were obtained and characterised, as PIV-3, according to virus morphology, transmission electron microscopy (TEM); cytopathic effect (CPE); haemagglutination, of guinea pig erythrocytes; physicochemical properties; serological crossreactivity with antisera raised against PIV-3; and reactivity with monoclonal antibodies to PIV-3 structural proteins, that crossreact with human and bovine strains.

The ability of the virus to induce respiratory disease was investigated by experimental inoculation of ovine PIV-3 isolate, 270-7 in colostrum deprived lambs. Clinical, pathological, bacteriological and virological studies were carried out on days 2, 3, 5 and 7 post infection (p.i.). This PIV-3 ovine strain was able to induce clinical disease. Histopathological findings were interstitial pneumonia with hyperplasia of bronchial associated lymphoid tissue (HALT), degenerative bronchial epithelium with lymphocyte infiltration, areas of atelectasis and increased alveolar septa thickness due to proliferation of type II pneumocytes, lymphocytes, macrophages and later to fibrosis. The large number of lymphocytes, particularly on days 5 and 7 p.i., combined with the minimal to moderate cytolyis of antigen bearing cells suggests that PIV-3-induced pulmonary disease has an immunopathological component. PIV-3 particles were detected more frequently in bronchial epithelium cells than in alveolar septal cell. This was correlated with changes of these cell populations in lung lesions, detected by immunohistology.

After 7 days p.i., virus-induced changes in the leucocyte composition of the lungs were detected using a panel of mAbs to ovine lymphocytes and macrophages. Changes in lung tissue were detected by immunohistology and changes in lungwash fluid (LWF) by flowcytometry. Association between lung cells and virus particles was investigated by double immunostaining. The differential cell count of LWF from PIV-3 infected animals was characterised by a significant increase (p<0.05) in lymphocytes and neutrophils. Lymphocyte phenotyping showed a significant decrease (p<0.05) of CD4+ cells; a significant increase (p<0.05) of CD8+ cells and a significant inversion (p<0.001) of the CD4+/CD8+ ratio. Immunostaining of PIV-3 infected lung sections showed a remarkable increase of lymphocytes, particularly in HALT, and most cells were CD8+. The number of macrophages increased in peribronchial and alveolar septa and some were positive for PIV-3 particles.

Cultures of peripheral blood monocyte-derived macrophages (MDM) and alveolar macrophages (AM) were established. The ability of PIV-3 to infect these cells was studied by CPE, and virus particle immunostaining. Viral replication was detected by TEM and scanning electron microscopy (SEM). PIV-3 induced lytic CPE with intracytoplasmic eosinophilic inclusion bodies and syncytia formation, TEM revealed virus budding at the cell membrane, filamentous cytoplasmic inclusions and clusters of pleomorphic viral particles in the extracellular space.

The expression of MHC class I and MHC class II molecules, which is associated with antigen presenting function, was studied after in vivo infection of MDM and AM with PIV-3, DR and DQ MHC class II expression was moderately high (60-80%) on infected fresh monocytes and AM but, after 3 days in culture the expression of this molecules was dramatically reduced to 5%. Stimulation with γ-IFN was able to promptly restore MHC class II expression in cultured uninfected MDM and AM. This did not occur after PIV-3 infection. The expression of MHC class I molecules was not significantly affected (p<0.05) by culture or PIV-3 infection.

The phagocytic activity of macrophages for FITC-labelled/antibody-coated sheep red blood cells(SRBC) after PIV-3 infection decreased significantly (p<0.05) after 3 days p.i.
DECLARATION

I hereby declare that the composition of this thesis and the experiments described are my own work unless specifically stated in the acknowledgements or text. No part of this work has been or will be submitted for any other degree, diploma or qualification.

October, 1995.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCC</td>
<td>antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AMDGF</td>
<td>alveolar macrophage-derived growth factor</td>
</tr>
<tr>
<td>AMφ</td>
<td>alveolar macrophage</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ARC</td>
<td>acquired immunodeficiency syndrome related complex</td>
</tr>
<tr>
<td>BALT</td>
<td>bronchiolar associated lymphoid tissue</td>
</tr>
<tr>
<td>BAMφ</td>
<td>bovine alveolar macrophage</td>
</tr>
<tr>
<td>BFK</td>
<td>bovine foetal kidney</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BVD</td>
<td>bovine viral diarrhoea</td>
</tr>
<tr>
<td>C3R</td>
<td>complement 3 receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>CTLs</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DI</td>
<td>defective interfering particles</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>EA</td>
<td>rosette erythrocyte antibody coated rosette</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethilenediamine tetra acetic acid</td>
</tr>
<tr>
<td>F</td>
<td>fusion protein</td>
</tr>
<tr>
<td>FA</td>
<td>fluorescent antibody</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc-gamma-receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>forward light scatter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosyl phosphatidyl inositol</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutination</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>H/E</td>
<td>hematoxylin/eosin</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HI</td>
<td>haemagglutination inhibition</td>
</tr>
<tr>
<td>HN</td>
<td>haemoagglutin-neuroaminidase</td>
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HPIV-3 human parainfluenza virus type 3
HRP horseradish peroxidase
HYL lactoalbumin hydrolysate
ICAM intercellular adhesion molecules
IFN interferon
Ig immunoglobulin
IIAP indirect immunoalkaline phosphatase
IIF indirect immunofluorescence
IIP indirect immunoperoxidase
IL interleukine
L large protein
LBP lipopolysaccharide binding protein
LCMV lymphocytic choriomeningitis virus
LFA lymphocyte function associated antigen
LI leucocyte integrins
LPS lipopolysaccharide
LW lung washing
LWF lung wash fluid
M matrix protein
mAb monoclonal antibody
MCMV murine cytomegalovirus
MDMφ monocyte-derived macrophages
2-ME 2-mercaptoethanol
MEM Eagle’s Essential Minimum Media
Mφ macrophage
moi multiplicity of infection
PIV-3 parainfluenza virus type 3
MF mean of fluorescence intensity
MHC major histocompatibility complex
mRNA messanger ribonucleic acid
MW molecular weight
N nucleoprotein
NLS normal lamb serum
NMS normal mouse serum
NP nucleocapside protein
NRS normal rabbit serum
NSE non-specific-esterase
OFK ovine foetal kidney
OPIV-3 ovine parainfluenza type 3 virus
OSF ovine skin fibroblasts
P phosphoprotein
PBA PBS containing 0.1% BSA and 0.01% sodium azide
PBMC peripheral blood mononuclear cells
PBS phosphate buffered saline
PDGF platelet derived growth factor
p.i. post infection
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PID</td>
<td>post-inoculation day</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear neutrophil</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SSC</td>
<td>side or 90 degree light scatter</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SIF</td>
<td>simian immunodeficiency virus</td>
</tr>
<tr>
<td>SN</td>
<td>sero-neutralization</td>
</tr>
<tr>
<td>SRBC</td>
<td>sheep red blood cells</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>tissue culture infective dose&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TxB2</td>
<td>tromboxane 2</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
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CHAPTER ONE

GENERAL INTRODUCTION
Parainfluenza virus type 3 (PIV-3) is an ubiquitous respiratory pathogen of both humans and animals with a worldwide distribution (Chanock & McIntosh, 1990). The antigenically related human and bovine strains (Ito et al., 1987) are important causes of bronchitis, bronchiolitis and pneumonia in their respective hosts (Brysson et al., 1983; Van Wyke Coelingh et al., 1990). PIV-3 is often a factor in causing pneumonia in sheep (Davies et al., 1977; Robinson, 1983). Severe clinical respiratory disease and pneumonia are commonly associated with bacterial infection. However, the attempts to produce pneumonia in sheep with bacteria only have met with variable success. Pasteurella haemolytica is commonly isolated from pneumonic sheep lungs, but attempts to experimentally produce the disease with this microorganism alone has not been successful (Davies et al., 1986). However, experiments in which lambs have been inoculated with combinations of PIV-3 and Pasteurella haemolytica consistently induced severe clinical respiratory disease (Wells et al., 1978; Davies et al., 1981; Davies et al., 1986; Cutlip & Lehmkuhl, 1982). Viral respiratory tract disease is a consequence of mechanical and biochemical injury to epithelial cells and alveolar macrophages (Jakab & Warr, 1983). The lytic effect on epithelial cells leaves a denuded respiratory tract. This also results in impairment of the mucociliary escalator system and reduced surfactant levels (Slauson et al., 1989). Viral impairment of the biochemically mediated killing by macrophages has been thought to enhance bacterial growth and colonization of the respiratory tract (Slauson et al., 1987). As a result, in the most severe cases there is secondary bacterial infection, pneumonia and death (Welliver & Ogra, 1988). PIV-3 in cattle transiently suppresses pulmonary antibacterial defences by causing dysfunctions in the alveolar macrophage phagocytic system (Hesse & Toth, 1983; Liggit et al., 1985; Brown & Ananaba, 1988). Lung pathology induced by human PIV-3 in rats is not associated with the period of time when there is maximum virus proliferation, but rather with the period of time of rapidly declining virus titres and the expression of the antiviral immune response in the lungs (Porter et al., 1991). This temporal relationship suggests that lesions of
interstitial pneumonia caused by PIV-3 might be secondary to the antiviral immune response rather than a direct effect of virus replication. PIV-3 infection is restricted primarily to the respiratory tract where both macrophages and respiratory epithelium are infected (Chanock & McIntosh, 1990). Thus, virally infected macrophages may be an important source of infectious virus or soluble mediators which can enhance or suppress lymphocyte response and function.

The purpose of this work was to study the role of PIV-3 in sheep pneumonia and aspects of the host immune response. Ovine PIV-3 isolates were obtained from field cases of sheep pneumonia. Their antigenic relationship with bovine and human PIV-3 was characterised with monoclonal antibodies. Changes in lung macrophage and lymphocyte populations were studied in experimentally induced interstitial pneumonia. The association of virus particles with these cell populations was examined both in vivo and in vitro. The effect of productive infection on macrophage phagocytic capacity and cell phenotype was studied in vitro.

1.1 Parainfluenza virus type 3 (PIV-3) infection

1.1.1 Epidemiological and clinical aspects of PIV-3 infection

Antibodies to PIV-3 and isolation of the virus have been reported in several species (Brako et al., 1984). In humans, respiratory syncytial virus (RSV) and PIV-3 are responsible for severe viral respiratory tract disease leading to hospitalization of infants and children (Murphy et al., 1994).

Transmission of parainfluenza viruses is by direct contact or by large droplet spread; however, the viruses do not persist long in the environment. The high rate of infection early in life, coupled with the frequency of reinfection, suggests that these viruses spread readily from one individual to another. PIV-3 is the most efficient parainfluenza virus in this aspect. There is no evidence that infection is transmitted from humans to animals or vice versa (Chanock & McIntosh, 1990).

The PIV-3 predominantly induces respiratory problems in lambs but it may also affect adult sheep (Lehmkuhl & Cutlip, 1983; Berrfos et al., 1987). The symptoms include
high body temperature, anorexia, apathy, nasal discharge, respiratory distress, sniffling and coughing (Cutlip & Lehmkuhl, 1982; Lehmkuhl & Cutlip, 1983).

In humans other clinical problems have been associated with PIV-3 infections. This virus has been isolated from the cerebral spinal fluid (CSF) from children and adults. All had fever and signs of meningitis and eventually recovered (Craver et al., 1993).

1.1.2 Pathology of PIV-3 infection

Gross lesions in the upper respiratory tract include hyperaemia of the nasal and turbinate mucosae and small amounts of exudate in nasal passages. In lungs red areas of consolidation are found in the apical lobes. Histopathological examination shows hyperplasia of the bronchial epithelium, acidophilic cytoplasmic inclusions and peribronchial accumulation of lymphocytes with a few PMNs. Also an intense accumulation of lymphocytes and macrophages (Mefs) within interalveolar septa along with proliferation of pneumocytes type II and fibroblasts can be seen. In the alveolar space there are Mefs and epithelial cells (Stevenson & Hore, 1970; Berrios et al., 1991).

1.1.3 Classification of Parainfluenza type 3 virus(PIV-3)

PIV-3 belongs to the Paramyxoviridae family which includes many pathogens of clinical importance for humans and animals. The Paramyxoviridae family has been classified into three genera: Paramyxovirus, Morbillivirus and Pneumovirus. This classification is based on the detection of the viral glycoproteins neuraminidase and haemagglutinin. The Paramyxovirus genus includes the mumps virus, parainfluenza viruses types 1 to 5 and many others of which Sendai virus, Simian 5 and Newcastle disease virus are the most characteristic. The Morbillivirus genus contains the measles, canine distemper and rinderpest viruses which do not show neuraminidase activity. The third genus, Pneumovirus, includes the human respiratory syncytial virus and also those affecting cattle and mice. They lack both neuraminidase and haemagglutinin activity (Bishnu et al., 1990; Galinski, 1991).

The development of gene technology and specific reagents, such as monoclonal antibodies (mAbs), has facilitated a new understanding of the Paramyxoviridae.
Genetic sequencing of paramyxovirus genes has given new insight into the interrelationship between members of the Paramyxoviridae and increased the understanding of the evolutionary diversity of these viruses (Vainionpaa et al., 1989). Based on sequence homology between corresponding genes, Spriggs et al., (1987) divided the Paramyxoviridae into three distinct groups. The first group consists of the Sendai, parainfluenza type 3, measles and canine distemper viruses. The second group contains the simian virus 5 and the Newcastle disease virus. The third group includes the respiratory syncytial virus (RSV).

The paramyxoviruses infect a large variety of mammals and birds, but the individual virus types are very host-specific, and symptoms caused by each virus differ largely. Symptoms can vary from an asymptomatic infection to nearly 100% mortality. (Vainionpaa et al., 1989).

Other biological characteristics of PIV-3 correspond to its ability to agglutinate erythrocytes from guinea pigs, sheep, cattle, pigs, goats, mice, rabbits, monkeys and humans (group O) (Leunen & Wellman, 1966; Nguyen-Ba-Vy, 1967b). Maximum haemagglutinating titres are achieved with guinea pig erythrocytes at 25°C for parainfluenza virus type 1 and 3.

It has been demonstrated that parainfluenza viruses are inactivated by ether, chloroform, UV and extreme pH. On other hand, they show variation in their susceptibility to the temperature of 56°C, for example some strains are destroyed within 20 minutes while others resist for 8 hours (Nguyen-Ba-Vy, 1967b).

PIV-3 multiplies in primary cell culture systems of bovine, ovine and monkey foetal kidney cells and in some cell lines (Chanock et al., 1958; Reisinger et al., 1959; Hore, 1966; St George, 1969; Hawthorne et al, 1982; Lehmkuhl & Cutlip, 1982; Batungbacal & Scott, 1982; Pfeiffer et al., 1983). It has also been reported that some strains of bovine PIV-3 (BPIV-3) proliferate in testicle cells (Nguyen-Ba-Vy, 1967b). On the other hand, some strains can be replicated in embryonated eggs that are inoculated via the amnion (Andrewes et al., 1978).

The cytopathic effect (CPE) that has been observed in cell cultures inoculated with PIV-3 is characterised by the appearance, after 48 hours, of large round cells with increased refractability. This effect extends progressively to the whole monolayer with
consecutive cell degeneration and finally the complete destruction of the cell culture. This may occur after 3 to 5 days post infection (Dennett et al., 1973). Some strains have the ability to produce, in some cell culture systems, a CPE that includes syncytia formation and the presence of inclusion bodies that are mostly intracytoplasmic and eosinophilic (Fenner et al., 1987a,b).

1.1.4 Virus genome and virus structural proteins

Characterisation of the virus antigenic structure is necessary in order to understand the initiation of infection, virus interaction with immune cells and variations in virus strains. The present understanding of the antigenic structure of Paramyxoviruses has been largely derived from the use of mAbs. Gene technology now offers the possibility to analyze and compare antigenic structures of immunologically important proteins at the molecular level as the complete sequences of paramyxovirus genes are known (Vainionpaa et al., 1989). The majority of paramyxoviruses are monotypic, but strain variations may occur as in human PIV-3 (Van Wyke Coelingh et al., 1990).

Paramyxoviruses are pleomorphic, enveloped RNA viruses that replicate in the cytoplasm and are released by budding through the host cell plasma membrane. The size of the virion varies between 100 and 300 nm in diameter. The genome is a single-stranded, nonsegmented RNA molecule with a molecular weight (MW) of about 4.6-7 x10<sup>6</sup> daltons (Vainionpaa et al., 1989). The genome of human parainfluenza virus type 3 (HPIV-3) is a single negative-sense RNA strand (vRNA) that is 15,463 nucleotides in length (Dimock & Collins, 1993). It contains a set of six or more genes covalently linked in tandem, that serve as a template for transcription of six unique mRNAs that encode six structural proteins named nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutini-neuraminidase protein (HN) and large protein (L) (Elango et al., 1986). The genome is surrounded by a nucleoprotein (NP) in the form of a filamentous, herringbone-like nucleocapsid core, which also contains the two minor proteins, L and P. The MW of the NP protein for PIV-3 is 66,000 (Kingsbury et al., 1978; Ray et al., 1985; Vainionpaa et al., 1989; Bishnu & Galinski, 1990). These proteins are analogous to those from other paramyxovirus (Spriggs & Collins, 1987; Panigrahi et al., 1987). A schematic model of a paramyxovirus is shown in Figure 1.1. A transmission electron microscopy
(TEM) photograph of a PIV-3 particle is shown in Fig. 1.2. Some characteristics of the structural proteins are presented in Table 1.1.

According to Chanock and McIntosh (1990) the characteristics of PIV-3 structural proteins are as follows:

- Protein N is abundant and is a structural protein of the nucleocapsid and is tightly complexed with genomic RNA. The carboxy terminus is located outside the nucleocapsid, where it is available for interaction in RNA synthesis or nucleocapsid packaging. The more basic and hydrophobic amino-terminal region probably interacts with RNA.

- Protein P is also located in the nucleocapsid where it is a component of the RNA-polymerase complex. It is phosphorylated and a component of viral transcriptase. It also appears to be mobile on the nucleocapsid and in clusters with protein L.

- Protein M is located on the inner aspect of viral envelope. It is the basic molecule involved in maturation and assembly of virions in infected cells.

- Protein F is located on the surface of viral envelope and mediates viral penetration, haemolysis and syncytium formation. It serves as a major protective antigen that elicits neutralizing and fusion-inhibiting antibodies. Protein F is synthesized as an inactive precursor F₀, which is activated by proteolytic cleavage into disulfide-linked subunits F₁ and F₂.

- Protein HN is located on surface of the viral envelope and takes part in virus attachment and both haemagglutination and neuraminidase activities. It serves as a major protective antigen that elicits neutralizing and neuraminidase inhibiting antibodies. HN is a type II glycoprotein that is inserted into the membrane by a hydrophobic region.

- Protein L is located in the nucleocapsid and forms a crucial part of the virus specific RNA dependent RNA-polymerase activity. It is the least abundant protein produced during infection. Immunogold-labelling indicates that L molecules are distributed in clusters along nucleocapsids isolated from infected cells, suggesting
FIGURE 1. 1 A schematic representation of a paramyxovirus virion. H(N), hemagglutinin (neuraminidase) glycoprotein; F, fusion glycoprotein; RNP, ribonucleoprotein; M, matrix (membrane); P, phosphoprotein; N, nucleoprotein; L, large protein.

FIGURE 1. 2 Transmission electron microscopy (TEM) of a OPIV-3 virion detected in OSF cell culture infected after infection with OPIV-3 chilean viral isolate 270-7. Magnification x630,000.
<table>
<thead>
<tr>
<th>Protein/designation</th>
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<th>Chemical nature</th>
<th>Location</th>
<th>Remarks</th>
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<tr>
<td>Hemagglutinin (HN, H, G)</td>
<td>72-90</td>
<td>Glycosylated</td>
<td>Envelope</td>
<td>Attachment to host-cell receptors; neuraminidase and/or hemagglutinating activities (HN, H); in pneumoviruses both the activities are missing (G)</td>
</tr>
<tr>
<td>Fusion ($F_0 \rightarrow F_1 + F_2$)</td>
<td>$F_0$ 60-70; $F_1$ 40-60; $F_2$ 10-20</td>
<td>Glycosylated</td>
<td>Envelope</td>
<td>Fusion, hemolysis; penetration</td>
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<td>Nucleoprotein (NP)</td>
<td>43-70</td>
<td>Phosphorylated</td>
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<td>Phosphoprotein (P)</td>
<td>32-70</td>
<td>Phosphorylated</td>
<td>Nucleocapsid</td>
<td>Component of polymerase complex</td>
</tr>
<tr>
<td>Large (L)</td>
<td>$\sim$200</td>
<td>-</td>
<td>Nucleocapsid</td>
<td>Component of polymerase complex; posttranscriptional modifications (?)</td>
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<td>Matrix (M)</td>
<td>28-40</td>
<td>Phosphorylated</td>
<td>Inside virion</td>
<td>Assembly; regulation of transcription</td>
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<td>Actin (A)</td>
<td>43</td>
<td>Phosphorylated</td>
<td>Inside virion</td>
<td>Host-cell origin</td>
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</table>

*Estimated by gel electrophoresis.

Taken from Vaninionpaa et al. (1989).
that it acts co-operatively in viral RNA synthesis together with protein P which is also associated in clusters with protein L.

The nucleocapsid structure is surrounded by a lipid-rich envelope containing virus-specific projections (peplomers). The projections are comprised of two glycoproteins, HN and F. They are anchored to the virus membrane by the hydrophobic portion of the molecule. They are synthesised in the rough endoplasmic reticulum and transported through the smooth membranes and Golgi’s apparatus in a similar fashion as other transmembrane proteins (Elango et al., 1986). HN and F glycoproteins are important in the establishment of protection against the disease produced by the virus (Vainionpaa, et al., 1989; Klippmark et al., 1990).

HN protein has six antigenic sites non-topographically overlapped; each one of them contains multiple epitopes. Three of these antigenic sites (A, B, and C) are recognised by mAbs that neutralise viral activity and inhibit the haemagglutination. The role of the other three sites have not been identified yet. Similarly, mAbs against protein F, detect eight different antigenic sites on this protein (Rydbeck, et al., 1986; Van Wyke Coelingh et al., 1990).

Only limited antigenic variations are observed among BPIV-3 strains isolated from different geographical areas and at different times. HN protein of human PIV-3 also has relative antigenic stability, in contrast to the antigenic variability of F protein (Van Wyke Coelingh et al., 1988; Shioda et al., 1988). The human and bovine strains share neutralising epitopes, but show different antigenic characteristics. The differences between both strains are located predominately in HN protein (Ray & Compans, 1986; Klippmark et al., 1990). However, there is no information available on the antigenic relation between bovine and ovine strains.

Although BPIV-3 is clearly distinct from HPIV-3 they are antigenically closely related (Abinanti et al., 1961). The human and bovine PIV-3 HN glycoproteins share two neutralizing epitopes in antigenic site A, whereas three neutralizing epitopes are shared on the F glycoproteins (Coelingh et al., 1986). These two epitopes of HN, as well as two out of the three of F, are completely conserved among HPIV-3 isolates. The other third epitope of F is slightly less conserved (97%). Bovine and human PIV-3 also exhibit significant conservation of amino acid sequence; homology of the N, P, and M proteins varies from 62% to 86%. Homology of the F and HN surface
glycoproteins is 80% and 77%, respectively (Zuku et al., 1987). This makes BPIV-3 a candidate to induce resistance to HPIV-3 as it has been demonstrated in experiments with cotton rats (Coelingh et al., 1987).

1.1.5 Pathogenesis and virus replication

The infection of host cells by PIV-3 begins through the action of two glycoproteins from the virion surface named proteins HN and F. They play a fundamental role in virus adsorption and penetration to the target cell and they present the main immunogenic determinants that are essential for evoking an immune response against the infection. (Rydbeck et al., 1988).

After protein HN binds to the sialic acid contained in host cell receptors, protein F promotes the fusion of the virion envelope with the host cell membrane. This allows the entrance of the viral RNA into the cytoplasm to start the virus replication. Protein F is also responsible for the fusion of infected with non-infected cells during late viral replication resulting in the appearance of syncytia in vivo and in vitro (Van Wyke Coelingh & Tierney, 1989; Moscona & Peluso, 1993a). This event also requires the participation of protein HN (Ebata et al., 1991). Syncytia formation in either CV-1 or HeLa cells requires the recombinant expression of both F and HN glycoproteins from the HPIV-3 (Hemingway et al., 1994). Syncytia formation occurs at the end of the replication cycle, when some changes to the cell membrane take place. The speed of syncytia formation is in relation to high or low multiplicity of infection (Fenner et al., 1987a).

Protein M has been implicated in the establishment or maintenance of a persistent viral infection. The role for the other proteins has not yet been clarified (Vainionpaa et al., 1989).

The negative sense RNA virus genome cannot function directly as a mRNA but must be first transcribed into positive-stranded RNA with viral RNA-dependent RNA-polymerase. The viral proteins N, P, and L are required for this activity (Vainionpaa et al., 1989). The viral RNA is translated to full-size proteins on host cell ribosomes. The fusion protein is synthesized as the inactive precursor F₀ and later cleaved to the
final form by host-cell-specific proteases. Glycoproteins are translated on the rough endoplasmic reticulum and transported to the cell surface via the Golgi complex and glycosylated during the transportation (Sato et al., 1988). PIV-3 replicates in the cytoplasm and is released by budding through the host-cell plasma membrane.

It has been described for Orthomyxovirus and Paramyxovirus that the host cell membrane receptor involved in infection is a neuraminic acid. As these receptors are ubiquitous on the cell surface of different species they can not be considered as determinant factors for virus tissue tropism or host range affinity (Moscona & Peluso, 1992). The cell receptors for PIV-3 are found in different cell types including lung epithelial cells, lymphocytes, monocytes, Mφs and neurones (Vainionpaa et al., 1989).

Evidence has been presented that the receptor requirements for an HPIV-3 particle to infect a cell are different from those for fusion between cells. By treating infected cells in culture with various doses of neuraminidase, Moscona & Peluso (1992) demonstrated that the virus spreads from cell to cell in the complete absence of cell to cell fusion.

The protein F of PIV-3 involved in fusion exists as a precursor and is activated when it is cleaved by host cell proteases located probably on cell membrane surface. The cleavage occurs in the N terminal of the polypeptide in a region that shows homology for Paramyxovirus (Chopin & Scheid, 1980). Although the assembly of the virions occurs normally without this cleavage, the produced virus particles are not infectious. If the infected cell does not have the right protease, the released virus particles are not infectious, leading to a self-limited infection and reduced pathological consequences. Therefore, the susceptibility of a cell to become infected by this virus is determined by the presence of specific proteases together with complex interactions of the virus structure, host cell receptors, cell metabolism and virus replication (Potgieter, 1986). On the other hand, failure to cleave the F protein may also be a contributing factor in the establishment of virus persistence (Moscona & Peluso, 1991).

Virus infections efficiently disrupt the functions of the infected cells. Lytic infection by paramyxoviruses generally leads to rapid destruction and death of the host cells. Adsorption of paramyxoviruses to the cell membrane alters the membrane fluidity and permeability. This leads to an increase in cytosolic calcium (Dyer et al., 1994). Another mechanisms of cell damage is the apparence of hydrophobic pores on the
plasma membrane, which leads to leakage of ions and low-molecular-weight compounds with subsequent loss of cellular functions (Vainionpaa et al., 1989).

The epithelial cells of the respiratory tract are first infected after PIV-3 invasion; however, alveolar macrophages (AM\(\phi\)s) can also become infected at this stage. The involvement of AM\(\phi\)s has important consequences for the pathogenesis of respiratory tract viral disease (Stauber & Weston, 1984).

Some studies carried out in vitro have demonstrated that virus parainfluenza type I replicates more efficiently in cell cultures of pneumocytes type II than in cell cultures of M\(\phi\)s (Castleman, et al., 1989).

The loss of epithelial cells from the respiratory tract along with the impairment of mucociliary movement and a good growth media represented by the exudate promotes the colonisation and growth of bacteria (Davies, et al., 1977; Babiuk et al., 1988).

1.1.6 Replication in macrophages

In pulmonary tissue, apart from antibody and cell mediated immunity, the lung cell defence is derived from the action of AM\(\phi\)s complemented by the influx of monocytes and PMNs from peripheral blood (Polgieter, 1986). The respiratory tract disease induced by PIV-3 is regarded as a consequence of both mechanical and biochemical injuries to epithelial cells and also to AM\(\phi\)s. This can lead in the most severe cases to secondary bacteria infection, pneumonia and death.

The AM\(\phi\) has a critical role in lung resistance to bacteria colonisation, which can take place after the AM\(\phi\) has been affected. This can be a direct consequence of viral replication in this cell or a result of immune mediated cytotoxicity towards virus infected M\(\phi\)s (Davies et al., 1986).

Not only can the number of M\(\phi\)s be reduced by PIV-3 infection but also some functions of this cell type can be impaired. The evidence suggests that such M\(\phi\)s have suppressed immunological and non-immunological activities related to membrane receptor binding, Fc receptor mediated phagocytosis, non-specific receptor
phagocytosis, phagosome-lysosome fusion, intracellular bacteria degradation and killing (Hesse & Toth, 1983; Davies et al., 1986; Slauson, et al., 1987).

BPIV-3 inhibits oxygen-dependent bacterial killing by phagocytes, a key pulmonary defence mechanism, thus predisposing the host to intrapulmonary bacterial superinfection (Dyer et al., 1994). The changes in the production of reactive oxygen species and the release of \( \text{TxB2} \) by AM\( \Phi \)s could account for damage to the airways and bronchial hyperresponsiveness often seen after viral infection. BPIV-3 inhibits \( O_2^- \) generation in AM\( \Phi \)s. The haemagglutinin activity is the essential viral factor required in the inhibition of \( O_2^- \) production by BPIV-3. Increases in cytosolic calcium and activity of PKC signal NADPH oxidase assembly and \( O_2^- \) production in phagocytic cells. Treatment of AM\( \Phi \)s with BPIV-3 selectively and significantly depress the Ca-dependant PKC activity (Henricks et al., 1993).

1.1.7 Immune response

Clinical disease and immunization with paramyxoviruses generally induces permanent protective immunity. For example, infection with either measles or mumps viruses results in lifelong immunity against re-infection (Julkunen, 1984; Ray & Compans, 1986). However, in the case of PIV-3 re-infection occurs with considerable frequency during infancy and early childhood (Chanock & McIntosh, 1990). Even in the presence of circulating neutralizing antibodies adults have been shown to be susceptible to re-infection. It appears that infection with PIV-3 does not result in the establishment of a protective immune response (Sieg et al., 1994).

The antibody response in natural PIV-3 infection is directed against both the surface and internal components of the virion (Kasel et al., 1984). In fact all viral proteins are antigenic and immunogenic but the glycoproteins are most important for eliciting antibody response. The protective antibody response is directed against HN and F proteins. Protein HN is the major immunogenic molecule responsible for the induction of neutralizing antibodies. This protein has four different antigenic sites with variable epitopes (Van Wyke Coeling et al., 1990).

Primary infection usually induces a greater antibody response to the HN protein. Re-infection is often required to stimulate a high level of serum antibody to F protein.
The relative effectiveness of HN and F antibodies in preventing infection has been studied both in vivo and in vitro. Tissue culture studies suggest that F antibodies may be more important because they can neutralize virus infection and prevent cell-to-cell spread by cell fusion. HN antibodies can only neutralize virus infectivity of free virions. However, evaluation of vaccinia virus-PIV-3 glycoprotein recombinants in cotton rats indicated that infection by a recombinant which expressed PIV-3 HN, induced more effective immunity than a recombinant which expressed PIV-3 F (Spriggs et al., 1987).

Production of non-functional local antibodies, as well as poor F antibody response may explain why re-infection with PIV-3 occurs with considerable frequency during infancy and early childhood (Chanock & McIntosh, 1990).

It has been demonstrated that humoral immune mechanisms can destroy PIV-3 infected host cells in the presence of antibody and complement (Hussain & Mohanty, 1984). Paramyxovirus antigens activate the complement pathway not only as immunocomplexes but also via direct activation of the alternative pathway. Strains with higher neuraminidase activity have a greater capacity to activate the complement pathway than strains with lower levels of activity. Activation of the alternative pathway may be an important mechanism of early host response before the immune system has had enough time to mount a specific response and it may contribute to the immunopathogenesis of paramyxovirus infection (Edwards et al., 1986).

The importance of cytotoxic T cells in recovery from PIV-3 infection has not been formally established. Nevertheless, it is likely that this form of cell-mediated immunity is important in the recovery process because type 3 virus infection or children with profound T-cell deficiency can lead to fatal giant-cell pneumonia (Chanock & McIntosh, 1990).

Immunity against PIV-3 seems to be limited. It has been reported and demonstrated that infection reoccurs within short time intervals. There is no vaccine available to prevent the disease in humans, nevertheless several attenuated VPI-3 vaccines have been used in cattle and sheep with favourable results (Well et al., 1976; Lehmkul & Cutlip, 1983; Morein et al., 1983; Ray et al., 1985). Since this virus causes severe disease in early infancy, vaccines must be effective in the presence of maternal
antibodies. Currently, several strategies for immunization against this virus are being explored including peptide vaccines, subunit vaccines, vectored vaccines, live attenuated virus vaccines (Murphy et al., 1994), chimeric glycoproteins (Homa et al., 1993) and microencapsulated virus particles (Ray et al., 1993).

Immunopathology can result from the induction of inappropriate immune responses. The paramyxovirus F glycoprotein acquires its fusion activity following cleavage by a host protease in the membrane of the infected cell. The F protein is responsible for penetration of virions into cells, spread from cell to cell and formation of syncytia. Formaldehyde-inactivated vaccines fail to elicit anti-F antibodies. If vaccinees are subsequently infected, their immune systems fail to control the spread of the virus. This allows large amounts of viral antigen to be produced which react with non-neutralising anti-HN antibodies and give immunopathological changes (Choppin & Scheid, 1980). This provides something of a warning against the possible dangers of inactivated vaccines (Mims & White, 1984).

Cells infected with paramyxoviruses release soluble material such as virus envelope glycoproteins which can produce immunopathological reactions. They also secrete interferon (IFN) and tumour necrosis factor alpha (TNF-α), particularly when they are mononuclear phagocytes (Vainionpaa et al., 1989).

1.1.8 Viral Immunosuppression

Inhibition of the oxygen-dependent bactericidal function of AMΦs and disturbances in signal transduction by PIV-3 may contribute to the immunosuppression and bacterial superinfection accompanying viral respiratory disease (Dyer et al., 1994).

HPIV-3 is a major cause of disease in infants. It also has a striking potential to reinfect individuals throughout their lives, suggesting that HPIV3 does not induce lifelong immunity; however, the operative mechanism for the failure to prevent reinfection is not known. Productive infection of T lymphocytes requires cell activation and results in a marked inhibition of proliferation. Furthermore, exposure to the virus, even without overt expression of viral proteins as detected by immunohistology, profoundly alters the functional capacity of the T cells. The mechanism has not been elucidated however, it is possible that viral proteins interact
with cellular proteins that mediate proliferative responses, thereby inhibiting their function. Alternatively, the presence of the virus may induce the secretion of an inhibitory cytokine, such as transforming growth factor B. Productive viral infection cannot explain the marked functional alteration in the HPIV-3 exposed T cells since detectable viral protein synthesis as detected by immunohistology was not required for profound inhibition of T cell proliferation (Sieg et al., 1994). The ability of the virus to regulate T-lymphocyte function may play an important role in the failure of the virus to induce lifelong immunity (Sieg et al., 1994). This may also contribute to immunosuppression (Sieg et al., 1994).

1.1.9 Persistent viral infection

In this type of viral infection the virus persists inside the host cell as either a complete or incomplete virus particle. In some cases the persistent viral infection is enhanced by interfering defective particles (Barret & Dimmock, 1986; Murphy et al. 1987), which are unable to replicate in absence of standard virus. Such particles not only interfere with the normal virus replication but also expand themselves. They are also involved in the establishment and maintenance of persistently infected tissue cultures (Murphy et al., 1987).

The persistent virus can be either replicative (productive infection) or non replicative (non-productive infection). However, during the non replicative stage some suppressed genes can be reactivated and lead to a new replicative phase (Barret & Dimmock, 1986; Swoveland, 1991).

Persistent viral infections can be associated with chronic and recurrent signs of disease; reduced host immune response by immunosuppressive virus; immune response against infected cells or tissues; and production of pathogenic immune complexes. Such immune complexes can localize in vascular and basal membranes. This leads to a serious damage of these structures, resulting sometimes in death due to glomerulonephritis (Povey, 1986).

It is considered that PIV-3 infection is confined to the lower respiratory tract during the course of disease. However, PIV-3 has been associated in vivo with persistent
viral infection in dogs (Everman et al., 1980) and in humans (Mills et al., 1981; Goswan et al., 1984).

Cells persistently infected with HPIV-3 have also been obtained in vitro. This has been achieved by inoculating tissue cultures at high multiplicity of infection, with high levels of neuraminidase present in the inocula. This produces a rapid destruction of sialic acid from cell surface which prevents the cell fusion and the appearance of CPE (Wechsler et al., 1985; Moscona & Peluso, 1991a). Persistent infection of these cell cultures have been associated with the presence of one or more viral defective-interfering (DI) particles in addition to standard viral genomes (Moscona & Peluso, 1993b).

Cell cultures persistently infected with PIV-3 characteristically do not present cytopathic effect (CPE), but maintain the viral infection within the system. These cells will show cytopathic effect when new non-infected cells are added to the tissue culture (Moscona & Peluso, 1991a). Furthermore, it has been demonstrated that cells persistently infected with PIV-3 fuse only with non-infected cells (Wechsler et al., 1985; Moscona & Peluso, 1991b).

Studies of RNA recovered from cell cultures persistently infected with PIV-3 indicate the existence of a mutational hyperreactivity at the 3' terminal of the viral genome (Murphy et al., 1991).

1.2 Defence mechanisms of the respiratory system

1.2.1 Aerodynamic filtration

The anatomic design of the respiratory tract, with multiple sites of turbulence and bifurcation, is effective in limiting the penetration of larger particles to the upper respiratory tract. Particles of 10 μm or greater usually precipitate in the upper respiratory tract, while particles between 2 and 10 μm are usually trapped in the bronchial tree. Smaller particles may reach the terminal airways and alveoli or may be inhaled and exhaled without deposition. The particle size in which aerolized virus enters the lung is an important determinant of the outcome of infection (Welliver &
Ogra, 1988). During the course of local immunization studies with respiratory viruses, it was observed that large viral particles (100 μm in diameter) precipitate and induce antibody responses in the nasopharynx, with little or no antibody response occurring in the lower respiratory tract. On the other hand, aerolized antigen particles 1.5 μm in diameter produce little or no nasopharyngeal antibody response but are quite effective in inducing antibody responses in the lower portions of the tracheal tree (Waldman & Ganguly, 1974). This pattern of dispersion of droplets may have some significance in infection of a given host. Intranasal inoculation of virulent influenza virus into mice allowed for spread of the virus to the trachea and lungs over three to five days but was rarely lethal. Mice in which the total respiratory tract was inoculated, almost uniformly developed fatal pneumonia (Yetter et al., 1980).

1.2.2 Mucociliary transport

Particles that settle anywhere in the respiratory system tract above the alveoli are subject to clearance by the mucociliary transport system. Particles precipitate on mucous plaques and are swept up to the posterior pharynx by the sweeping action of ciliated respiratory epithelial cells. These ciliated cells are present from the terminal bronchi to the larynx (Yates, 1988). Individuals with defects in ciliary motion are particularly prone to respiratory infections. Viral infections, cigarette smoke and other agents may have a ciliostatic effect that renders the host more susceptible to infection (Welliver & Ogra, 1988).

1.2.3 Polymorphonuclear Neutrophils (PMNs)

PMNs are replete with enzymes and oxidants that could damage either microbial pathogens or lung tissue. It has been shown in vitro that PMNs adhere better to virus-infected cells than to non-infected monolayers (Faden et al., 1984). Immune complexes containing viruses can activate oxidative pathways of PMNs (Kaul et al., 1981), although the importance of this mechanism in pathogenesis of viral diseases is unknown (Welliver & Ogra, 1988). Finally, in the presence of complement, PMNs may exhibit cytotoxicity against virus-infected tissue culture cells in vitro (Kaul et al., 1984).
1.2.4 Complement

Complement may assist in the lysis of virus-infected cells through a number of different mechanisms. Antibody-dependant neutralization of paramyxoviruses is enhanced by the presence of complement. Also virus-infected cells are capable of activating both complement pathways (Welliver & Ogra, 1988). PMN leukocytes are capable of lysing virus-infected cells in the presence of complement (Kaul et al., 1984). Whether or not complement itself can lyse virus-infected cells is unclear (Welliver & Ogra, 1988).

1.2.5 Interferon (IFN)

IFNs are potentially important agents in resistance to viral infection. Exogenously administered α–IFN was shown to have a prophylactic efficacy against rhinovirus upper respiratory infections (Hayden et al., 1986). However, in some cases IFN seems to restrict the spread of virus but does not play a principal role in eradication of infection (Welliver & Ogra, 1988). Most studies to this time have evaluated lymphocyte γ-IFN production in viral infections. RSV appears to be a relatively poor producer of γ-IFN either from lymphocytes or Mφs (Roberts, 1982). In contrast, influenza virus (Roberts, 1982) and PIV-3 (Towsend & Williams, 1988) appear to be potent stimulators of lymphocytic γ-IFN.

1.2.6 Antibodies

The development of local antibody in the respiratory tract to a number of viruses has been investigated. From the nasopharynx to the bronchiolar level, the principal antibody isotype in humans appears to be IgA (Welliver & Ogra, 1988). Strong clinical and experimental evidence is available indicating that the antibody in the respiratory tract is the result of local antibody synthesis in the bronchiolar associated lymphoid tissue (BALT), with a clear contribution from gut-associated lymphoid tissue (Bienenstock & Befus, 1980). In general, naturally acquired respiratory viral infections or immunization by the mucosal route appear to be the most effective means of inducing secretory IgA antibody responses in the respiratory mucosa. However, secretory IgA antibody responses in the respiratory tract may be observed following parenteral inoculation with influenza virus (Waldman et al., 1973).
While IgA is the predominant immunoglobulin isotype in the upper respiratory tract, IgG antibody becomes progressively more predominant in the lower parts of the respiratory tract, become at least equal to concentrations of IgA in the major bronchi and probably predominating over IgA in the alveoli (Reynolds, 1986). IgG1 appears to be the predominant isotype and is much more common than IgG2. IgG3 and IgG4 are found in still smaller quantities. IgG1 and IgG2 are present in the respiratory tract as a result of transudation from serum, while IgG3 and IgG4 are probably synthesized locally (Merrill et al., 1985). Deficiencies of individual IgG subclasses in the human may be seen alone or in conjunction with IgA deficiency and may predispose to more severe illness, in comparison to IgA deficiency alone (Bjorkander et al., 1985).

In ruminants in contrast to other species secretory IgA is a relatively minor component in mucosal surface. Complement-fixing IgG1 is the major immunoglobulin and it is derived almost exclusively from blood serum (Lascelles & McDonnell, 1974).

1.2.7 Natural Killer (NK) cells

NK cells are present in abundance at mucosal surfaces. These cells exhibit HLA-unrestricted cytotoxic activity against cells infected with a number of types of pathogens, including viruses (Welliver & Ogra, 1988). The study of the role of NK cells in recovery from viral respiratory infection has been focused in infection with influenza virus. Their role in PIV-3 infection remains unclear. Natural killer cell response is enhanced after influenza virus infection in mice (Wyde et al., 1977). Nevertheless, "nude" mice, which have NK cell activity but are T-cell deficient, exhibit minimal pulmonary infiltration, no tissue destruction in the lungs, and an increased tendency toward dissemination of influenza virus to the brain in comparison to immunologically competent litter mates (Wyde et al., 1977).

1.2.8 Lung macrophages

As the respiratory tract is continuously exposed to organisms or particles of the inhaled air, there is a need for appropriate defence mechanisms to prevent injurious and infectious processes. In the upper and central respiratory tract (nasopharynx,
trachea and large bronchi), mechanisms such as sneezing, coughing and mucociliary clearance contribute to a large extent to particle removal. By contrast, the distal airways (bronchioles) and the alveoli are devoid of these mechanisms. Therefore, most of the clearance of small inhaled particulate material and microorganisms reaching the periphery of the lung relies on the phagocytic system (Sibille & Reynolds, 1990). The contribution of phagocytes to lung host defence is well illustrated by the high frequency of pneumonia observed in severely neutropenic patients or in cases of lung MØ dysfunction (Reynolds, 1985). In addition to their defensive role, PMNs and AMØs have been implicated in injurious processes associated with both acute and chronic pulmonary diseases (Sibille & Reynolds, 1990).

According to Sibille & Reynolds (1990) the MØ can interact with other cells and molecules through the release of numerous secretory products (Table 1.2) and the expression of several surface receptors (Table 1.3). This strongly supports a central role for pulmonary MØs. In the normal lung, MØs are resident cells, whereas PMNs are almost absent. However, in certain conditions, PMNs can accumulate within the lung structures interacting with MØs (Figure 1.3).

Although all MØs share the same precursor (the circulating monocyte) and share some functions, each type appears to have different characteristics (Sibille & Reynolds, 1990). For example, lung MØs are the only MØs living in aerobic conditions and this environment is likely to influence the behavior of these phagocytes (Sibille & Reynolds, 1990). Pulmonary macrophages are both phenotypically and functionally diverse, even within a single compartment. These differences may reflect the stage of differentiation from blood precursors, but more likely reflect the environment and physiological roles of phagocytes in distinct locations. Important membrane molecules that determine the function of macrophages include complement, Fc, mannose and scavenger receptors, as well as class I and II MHC, adhesion and other signaling molecules (reviewed by Lipscomb et al, 1995).

According to Brain (1988) different kinds of MØs can be recognized in the lung. They include alveolar, interstitial, intravascular and airways MØs.
TABLE 1.2 Major products released by alveolar macrophages.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Enzymes</th>
<th>Biologically active lipids</th>
<th>Biologically active lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-1 alpha and beta</td>
<td>Lysozyme, beta-glucuronidase</td>
<td>Cyclooxygenase metabolites</td>
<td>C2, C4</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>Acid hydrolases</td>
<td>Thromboxane A2</td>
<td>Binding protein</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>Angiotensin converting enzyme</td>
<td>Prostaglandins (E1, D1, 6-keto-PGF-1-alpha and F2-alpha)</td>
<td>Tranferrin, ferritin</td>
</tr>
<tr>
<td>Alpha and gamma interferon</td>
<td>Elastase, serine and metalloenzyme</td>
<td>Lipoxygenase metabolites</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>Colony-stimulating growth factors</td>
<td>Collagenase-like</td>
<td>5-hydroxyeicosatetraenoic acid</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>Transforming growth factor-beta</td>
<td>Fibroblast-like</td>
<td>Leukotriene B4, C4, D4</td>
<td>Antioxidants</td>
</tr>
<tr>
<td>Fibroblast growth factor (insulin growth factor I)</td>
<td>Type V (gelatinase)</td>
<td>Platelet-activating factor</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Neutrophil-activating factor</td>
<td>Plasminogen activator</td>
<td>Oxygen metabolites</td>
<td>Coagulation factors</td>
</tr>
<tr>
<td>Enzyme-releasing peptide</td>
<td>Cysteine proteinase (cathepsin L)</td>
<td>Oxygen metabolites</td>
<td>Factor V</td>
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<tr>
<td>Neutrophil chemotactic factor</td>
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<td></td>
<td>Factor VIII</td>
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<tr>
<td>Platelet-derived growth factor</td>
<td></td>
<td></td>
<td>1,25 dihydroxyvitamin D3</td>
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<tr>
<td>Histamine releasing factor</td>
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</tbody>
</table>

Taken from Sibille & Reynolds (1990).
TABLE 1.3 Surface ligands on alveolar macrophages.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Enzymes</th>
<th>Peptides and phorbol esters</th>
<th>Lectin-binding molecules</th>
<th>Lipid receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulins A, G, M, E</td>
<td>Neutrophil elastase</td>
<td>FMLP, PMA</td>
<td>Manose fucose receptor (163-kD mannose receptor)</td>
<td>Lysokoiene B4</td>
</tr>
<tr>
<td>Ferritin, transferrin</td>
<td>Alpha,-macroglobulin protease complexes</td>
<td></td>
<td>Glucose receptor</td>
<td>Scavenger receptor*</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Cathepsin G</td>
<td></td>
<td>80-kD surface protein</td>
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<tr>
<td>Complement fragments C3, C5a</td>
<td></td>
<td></td>
<td>30-kD smoking associated protein</td>
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<td>Major histocompatibility complex type II molecules: la-like antigen</td>
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<tr>
<td>Hormones</td>
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<td>Glucocorticoids</td>
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<td>Granulocyte-macrophage colony-stimulating factors (CSF)</td>
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<tr>
<td>Histamine</td>
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<tr>
<td>Interleukin-2</td>
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<tr>
<td>Beta-2 adrenergic</td>
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<tr>
<td>Tumor necrosis factor*</td>
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</table>

* These receptors have been described in monocytes and tissue macrophages but not yet in alveolar macrophages.

Taken from Sibille & Reynolds (1990).
FIGURE 1. 3 Interaction between alveolar macrophages (AMφs) and polymorphonuclear neutrophils (PMNs) in the lung. Upon stimulation by various stimuli, AMφs release either chemotactic factors or inhibitory factor for PMNs. These factors will either attract PMNs from the capillary lumen to the alveolar space or prevent PMN migration to the alveoli. Once in the alveolar lumen, the PMNs can be activated by various factors, including AMφ-derived monokines and bioactive lipids. AMφs can also inhibit PMN activity through the release of mediators such as PGE2. Secretory products released by both AMφs and PMNs are likely to influence defense and injury processes.
FIGURE 1. Transmission electron microscopy of sheep lung tissue showing a vacuolated AMΦ in the alveolar space but attaching to the alveolar wall (A). A closer view shows numerous intracytoplasmic vacuoles (v) and granules (g). The nucleus is irregular with aggregated chromatin. Augmentation x3,350 (A) and x10,000 (B).
1.2.8.1 Alveolar Macrophage (AMφ)

Among the different kinds of pulmonary Mφs the AMφ is the best characterised and most extensively studied, because it can be easily recovered by bronchoalveolar lavage (BAL). The AMφ is the resident phagocyte of the alveolar space (Fels & Cohn 1986). As illustrated by the electron microscopy photograph in Figure 1.4, the AMφ is characterised by a lobulated nucleus and a vacuolated cytoplasm containing numerous mitochondria and electron dense secondary lysosomes. The AMφ represent a population morphologically and functionally heterogeneous (Sibille & Reynolds, 1990). The size of the cells varies from 12 μm (similar to monocytes) to 40 μm (often multinucleated cells) (Cohen & Cline, 1971). Taking advantage of these size differences and using gradients of colloidal silica or of concentrated bovine serum albumin solutions, AMφ have been separated into different subpopulations (Shellito & Kaltreider, 1984; Chandler et al., 1986). The membrane receptor expression and cell functions, such as phagocytosis and mediator release, have been shown to vary among these different populations. The distribution of AMφs among the different subpopulations varies according to disease states. For example, in acute inflammation, an increased number of small, monocyte-like Mφs is observed, whereas chronic lung disorders are associated with an increase in larger, more mature Mφs (Brannen & Chandler, 1988).

Particles reaching the alveoli are cleared principally by AMφs. These cells are capable of phagocytosis and enzymatic degradation of substances, may transport substances out of the lung and may activate other immunologic components of the lung defence system (Welliver & Ogra, 1988). Suppression of AMφ function would certainly be expected to predispose individual to more severe respiratory disease, and Mφs secrete numerous products that certainly could damage the lung (Sibille & Reynolds, 1990).

AMφs appear to be able to function as accessory or effector cells in immune reactions and are also capable of initiating the inductive phase of humoral and cellular immune responses by presenting antigens to specific B and T-lymphocytes (Chandler et al., 1986; Fels & Cohn, 1986). However, most studies support the concept that AMφs are poor antigen presenting cells (APCs) for priming T cells even when they express high levels of class II MHC (Holt, 1979; Toews et al., 1984; Lipscomb et al., 1986). This diverse group of cells is now best understood in the context of lung immunity as phagocytes and as regulators of both immunity and nonspecific inflammation (Holt,
1986). The bulk of evidence indicates that lung macrophages are unlikely APCs in the initiation of primary immune responses (reviewed by Lipscomb et al., 1995). However, they may have a role in stimulating recently primed T cells like other class II MHC-positive cells of many types (Steinman, 1991; Croft, 1994). The major role of AMs seems to be to phagocytose and remove potentially dangerous particulates and soluble antigens from the alveoli and to inhibit local lung immune responses. AM suppressive activity is important to prevent the development of hypersensitivity reactions (reviewed by Lipscomb et al., 1995). Mechanisms utilized by populations of AMs to suppress immune responses have been reported in several animal species. They include PGE2 production in dogs (Demenkoff et al., 1980), inhibition of receptor-induced intracellular calcium in humans (Yarbrough et al., 1991), TGFβ (Lipscomb et al., 1993) and nitric oxide (Holt et al., 1993) secretion in mice.

1.2.8.2 Interstitial macrophages

Interstitial MΦs are located in the connective tissue of the lung and, in contrast with AMs, are not directly exposed to airborne particles. Interstitial MΦs are obtained from minced lung explants but are not recovered by BAL (Bowden & Adamson, 1972). Despite some similarities with AMs (phagocytosis, release of oxygen radicals and presence of Fc-gamma-receptor), interstitial MΦs have an increased ability to replicate and to synthesize DNA in vitro compared to AMs (Lehnert et al., 1985). They are most efficient in stimulating the T lymphocyte response against antigens (Holian & Scheule, 1990).

1.2.8.3 Intravascular macrophages

In addition to AMs and interstitial MΦs, pulmonary MΦs resident in intravascular structures have been identified in several animal species (pigs, sheep, cats and humans), but not in rodents (Warner et al., 1986; Dehring & Wismar, 1989). These cells are morphologically different from blood monocytes and adhere firmly to the endothelial cells. Through their clearance function, pulmonary intravascular MΦs may play an important role in the pathogenesis of oedema with acute lung inflammation induced by sepsis. Whether pulmonary intravascular MΦs originate from bone marrow cells and attach to the lung endothelium, or from interstitial or AMs and migrate
back towards the capillary lumen, still remains to be determined (Sibille & Reynolds, 1990).

1.2.8.4 Airways macrophages

MΦs are also present in large and small airways. Some of these airway MΦs are likely to correspond to AMΦs transported by the mucociliary escalator. However, a certain number of these airway MΦs appear to be resident cells of the airways, adhering tightly to the epithelial cells. They could help in the removal of debris present in the large airways (Brain et al., 1984).

1.2.9 Dendritic cells

Also a loosely adherent mononuclear cell present in human lung tissue specimens, but not in BAL, has been characterised (Nicod et al., 1987). This cell is morphologically similar to a dendritic cell (DC), does not express Fc-gamma-receptor and demonstrates a lower phagocytic activity, but a higher T lymphocyte-stimulating accessory function than do AMΦs. These pulmonary DC have also been identified more recently in rat (Holt et al., 1992; Havenith et al., 1993). DCs form an interdigitating network in the airway epithelium similar to the network described for skin Largerhans cells (Holt, 1993). In humans intraepithelial DCs are particularly dense in the trachea and gradually diminish in concentration as the airways branch. DCs also exist in the connective tissue surrounding bronchi and bronchioles, in perivascular connective tissue, in alveolar septa, in the pleura and in very small numbers in alveolar spaces (van Haarst et al., 1994). Like DCs from other sites, lung DC constitutively express both class I and II MHC, are light density, loosely adherent, poorly or nonphagocytic and demonstrate long processes both in tissue sections and in cell suspensions (Nicod et al., 1987). Lung DCs fail to express pan T, natural killer (NK) cell, B cell and many macrophage markers (reviewed by Lipscomb et al, 1995).

1.2.10 Lymphocytes

The lymphocyte system of the lung is capable of functioning independently of, as well as interacting with, the systemic lymphocyte system. According to Welliver & Ogra
The lymphocytic tissue of the lung can be loosely organized into four general types:

- Lymph nodes, which are predominantly found along the upper respiratory tract to the hyla.
- The bronchus-associated lymphoid tissue (BALT) which is concentrated in the areas of bronchial and bronchiolar bifurcations.
- Interstitial lymphocytic nodules.
- Free lymphocytes in both the parenchyma and air spaces.

Interaction with the systemic immune system most probably occurs in the BALT. Lymphocytes from systemic circulation may enter the lung here and be dispersed along the lamina propia, while lung lymphocytes seem to enter the systemic circulation in this area (Welliver & Ogra, 1988).

Considerable evidence has accumulated demonstrating the development of virus-specific cytotoxic T-lymphocytes after viral infections. The development of T-cells with cytotoxic activity specific for influenza virus-infected cells appears to be the critical factor in restricting influenza virus infection to the respiratory tract and eradicating the infection. Passive transfer of immune spleen cells, which were obtained 30 days after infection in mice and restimulated with virus infected cells, enabled mice to recover from pneumonia due to influenza without causing an increase in influenza-specific antibody titres. In contrast, immune spleen cells that were not restimulated enhanced antibody titers but did not aid in recovery from primary pneumonia (Wells et al., 1981a,b). There is evidence that the cytotoxic T-cell response to influenza virus infection may also contribute to the pathogenesis of disease. Immunocompetent mice have more pulmonary lesions than nude mice following infection with influenza virus (Wyde et al., 1977).

The pathways of antigen processing that result in binding of peptides to MHC class I or class II molecules are different. The description of a cytosolic pathway for MHC class I and an endosomal pathway for MHC class II processing and presentation characterises these differences. Whereas proteins added externally to cells are usually processed in the endosomal pathway, biosynthesis of viral proteins (endogenous...
proteins) within cells favours presentation by the cytosolic pathway (Rotzschke et al., 1990). In general but not exclusively CD8⁺ and CD4⁺ T cells have a division of tasks: CD8⁺ CTLs control intracellular pathogens whereas CD4⁺ helper T cells help to eradicate extracellular pathogens by cooperation with B cells and by release of cytokines (Leist et al., 1987). After infection, a virus has achieved control over the cellular protein synthesis machinery. Then presentation of antigenic viral proteins via the cytosolic pathway is clearly favoured. However, even after endogenous synthesis of viral proteins, presentation by MHC class II molecules is not absolutely excluded. Furthermore, after release from infected cells, viruses and viral proteins have to be considered as exogenous sources of antigen. Whether extracellular viral proteins represent a major factor for the generation of an efficient immune response depends on the productivity of the viral infection in a given tissue (Koszinowski et al., 1991).

Paramyxoviruses are enveloped RNA viruses that replicate in the cytoplasm and are released by budding through the host cell plasma membrane (Vainionpaa et al., 1989). Enveloped viruses are thought to enter cells by two mechanisms: by fusion with the cell membrane or by receptor mediated endocytosis followed by fusion with the vesicle (Nagai et al., 1983). These type of viral entry should be able then to load both the MHC class I and the MHC class II pathway of processing and presentation. As a consequence both CD4⁺ and CD8⁺ T cell subpopulations are expected to be stimulated to contribute to protection against viral infection or to exacerbation of disease. However, PIV-3 fuses directly with the plasma membrane at neutral pH without activating endocytic vesicle formation (Chopin & Sheid, 1980).

The degree to which CD8⁺ T-cell induction and function is independant of CD4⁺ Th cell activity apparently varies between viral infections. The clearance of mouse hepatitis virus from the central nervous system follows a pattern that suggests the CD8⁺ T cells are the main effectors, but need CD4⁺ help (Williamsom & Stohlman, 1990). This finding is consistent with the hypothesis that CD4⁺ T cells are critical for the provision of cytokines to help CD8⁺ T cells differentiate and proliferate. However, in influenza A virus infection neither the recruitment of CD8⁺ T cells to lymphoid tissue nor their localization to the site of virus growth is substantially dependant on a primed CD4⁺ helper population. Furthermore, CD4⁺-depleted mice clear influenza A virus from the lungs, and the frequency of antiviral CTL precursors is not reduced (Allan et al., 1990).
When CD8⁺ response is precluded due to a lack of CD8⁺ T cells or an alteration in the presenting MHC molecules the result also seem to vary according to the type of viral infection. In lymphocytic choriomeningitis virus (LCMV)-infected mice this situation leads to persistent infection, which clearly indicates that CD8⁺ T cells are critical for clearance (Pircher et al., 1990). This is different however, for murine cytomegalovirus (MCMV). Although in the immunocompetent mouse protective immunity to MCMV is clearly a function of the CD8⁺ subset, these cells are dispensable for virus control in long-term CD8⁺ depleted mice (Jonjic et al., 1990). In these mice, CD4⁺ T cells have an antiviral function that is absent in fully immuncompetent mice.

1.2.11 Cytokines

1.2.11.1 Macrophage derived cytokines

Among the cytokines secreted by Mφs IL-1α and β and TNF-α are believed to play an important role in inflammatory reactions, according to both in vitro and in vivo studies (Dinarello et al., 1986). The activities of these cytokines on different cells are numerous. The actions of IL-1 and TNF-α often overlap (Nathan, 1986). Both IL-1 and TNF-α are secretory products of AMφs and this secretion appears to be increased in AMφs from patients with sarcoidosis (Hunninghake, 1984; Bachwich et al., 1986). Because sarcoidosis is a granulomatous disease perpetuated by activated Mφs and T lymphocytes accumulating in the lung interstitium and alveoli, the increased IL-1 release may induce the recruitment of T-lymphocytes and their activation, whereas TNF-α may be responsible for monocyte differentiation and release of IL-1 (Thomas & Hunninghake, 1987). The large variety of actions of these two cytokines on lymphocytes, Mφs, endothelial cells, PMN, fibroblasts and tumour cells suggests that both IL-1 and TNF-α are likely implicated in infectious, inflammatory and tumoricidal processes (Le & Vilcek, 1987).

In addition to IL-1 and TNF-α, AMφs produce fibroblast growth factors in vitro. In particular the AMφ-derived fibroblast growth factor (AMDGF) is able to induce fibroblast proliferation in vitro (Bitterman et al., 1986). Among factors promoting fibroblast growth, fibronectin, a glycoprotein present in extracellular matrix and implicated in cell surface interactions, is known to be released by human AMφs.
(Rennard et al., 1983). Furthermore, AM\(\phi\)s release inhibitory factors for both fibroblast growth and IL-1 (Elias et al., 1990).

M\(\phi\)s from different sources have been found to release IL-6. This factor has a broad spectrum of biological activities, including the induction of fever, acute phase proteins and growth and differentiation of T and B cells (Wong & Clark, 1988). Other polypeptide hormones released by AM\(\phi\)s include platelet derived growth factor (PDGF), transforming growth factor-beta (TGF-\(\beta\)) and alpha and gamma-interferon (\(\alpha\) and \(\gamma\)-IFN) (Elias et al., 1990). This secretion of \(\alpha\) and \(\gamma\)-IFN have potentially important implications in modulating the antiviral and immune activities of AM\(\phi\)s (Sibille & Reynolds, 1990).

1.2.11.2 Lymphocyte derived cytokines

There is growing evidence that cytokines delivered locally by T cells can contribute to selective virus elimination. This is achieved by direct antiviral effects and a combination of indirect effects involving specific as well as non-specific effector cells. When tested at the clonal level, mouse CD\(4^+\) T cell clones fall into two main groups, Th1 and Th2. Th1 clones synthesize IL-2 and \(\gamma\)-IFN, whereas Th2 clones synthesize IL-4, IL-5, IL-6 and IL-10 (Mosmann & Coffman, 1989). CD\(8^+\) CTLs synthesize a range of cytokines similar to those that are synthesized by Th1 and CD\(4^+\) CTLs.

CD\(4^+\) and CD\(8^+\) CTLs may operate in vivo by direct cytolysis and by the local release of cytokines such as \(\gamma\)-IFN. In view of the fact that a major effect of \(\gamma\)-IFN is the upregulation of MHC class II gene expression, an enhanced antigen presentation by MHC class II molecules, in combination with the induction of an antiviral state in neighbouring cells, could potentiate the effects of CD\(4^+\) T cells in vivo (Kottonen-Corish et al., 1990).

There is no report of virus control by Th2 cells. Th2 cells produce among other cytokines IL-10, which inhibits the synthesis of \(\gamma\)-IFN by Th1 cells (Pearce et al., 1991). Thus, Th2 cells that synthesize IL-10 may be envisaged to counteract the antiviral function of virus-specific CD\(4^+\) and CD\(8^+\) CTLs, both of which produce \(\gamma\)-IFN (Mosmann & Coffman, 1989).
1.3 AIMS OF THE THESIS

The frequency of infection by PIV-3 virus in sheep, determined by serological tests, is quite high in the south of Chile (68%) where it is seems to produce severe cases of pneumonia in association with *Pasterurella haemolytica* (Berrios *et al.*, 1987; Riedemann *et al.*, 1990). However, no isolations of this virus from field cases of sheep pneumonia has yet been reported.

This study deals with the isolation of a OPIV-3 strain from field cases of sheep pneumonia, from the South of Chile and the experimental reproduction of lung lesions corresponding to viral pneumonia. This will allow the characterisation of histological lesions and changes in cell populations, associated with immune response, that occur during replication of OPIV-3 in lung tissue. The ability of the virus to infect lymphocytes and monocytes/macrophages will be study *in vitro*, as well as the effect of infection on phenotype and functional properties of monocytes/macrophages.

**Specific objectives**

- To isolate and characterise OPIV-3 from field clinical cases of sheep pneumonia in Chile.
- To reproduce and characterise lung lesions of viral pneumonia through the experimental inoculation of colostrum deprived lambs with OPIV-3.
- To detect the presence of PIV-3 particles *in situ* in association with lung lesions of experimental interstitial pneumonia.
- To detect the presence of OPIV-3 particles in association with lymphocytes and macrophages recovered by lung wash (LW) from animals with experimental interstitial pneumonia.
- To investigate the capacity of OPIV-3 to establish a productive infection in lymphocytes, monocytes-derived macrophages and alveolar macrophages *in vitro*.
- To characterise ultrastructural changes of monocytes-derived macrophages and alveolar macrophages during replication of OPIV-3 *in vitro*.
- To characterise phenotypic changes of monocytes-derived macrophages and alveolar macrophages during replication of OPIV-3 *in vitro*.
- To evaluate alterations in the phagocytic capacity of monocytes-derived macrophages and alveolar macrophages during replication of OPIV-3 *in vitro*.
2. MATERIALS AND METHODS

All chemicals were supplied by SIGMA Chemical Co., Poole, Dorset, England or BHD Ltd., Poole, England unless otherwise stated. Tissue culture plasticware was supplied by GIBCO BRL, Paisley, Scotland. Universals and centrifuge tubes were supplied by Bibby Ltd., Stirling, Scotland.

2.1 Cell cultures for virus isolation

2.1.1 Culture of ovine skin fibroblasts (OSF)

Ovine skin fibroblasts (OSF) derived from biopsies as detailed in Bird et al. (1993), were cultured in Dulbecco’s modified Eagle’s medium (GIBCO, BRL) containing 2 mM-L-glutamine, 100U penicillin per ml, 100 µg streptomycin per ml and 10% FCS at 37°C, 5% CO₂.

Cell cultures were detached using 0.05% (w/v) trypsin in 0.02% (w/v) EDTA in PBS and transferred into other tissue culture flasks in a 1:3 proportion.

2.1.2 Ovine foetal kidney (OFK) and bovine foetal kidney (BFK) cells

Primary and secondary OFK and BFK monolayer cultures were used for the isolation and characterisation of OPIV-3 isolates. These cells were derived from biopsies as detailed in Berrios et al. (1991). OFK and BFK cultures were all negative to the presence of bovine diarrhoea virus (BDV) and respiratory sincitial virus (RSV) by ELISA. They were also used for the passage of the virus, used as inocula in the experimental infection of lambs. Cells were grown in Eagle’s Essential Minimum Media (MEM, Sigma Chemical Co USA) supplemented with 2 mM-L-glutamine, 0.08% sodium bicarbonate, 100 units/ml penicillin, 100µg/ml streptomycin, 10% foetal calf serum (FCS, Sigma Chemical Co USA), at 37°C, 5% CO₂.
CHAPTER TWO

MATERIALS AND METHODS
2.1.3 Freezing and thawing of cells

1 to 2 x10^6 cells per ml, in an ice-cold mixture of 90% FCS/10% DMSO and were frozen slowly by placing the cells in a polystyrene box filled with cottonwool at -70°C overnight. Then the vials were transferred to liquid nitrogen. To resuscitate frozen cell lines, an aliquot was warmed quickly at 37°C, gradually diluted with warm DME/10% FCS and then centrifuged at 350 xg for 5 minutes. The cell pellet was resuspended in DME/10% FCS, incubated for 5 minutes at 37°C and then pelleted again. Finally, the cells were resuspended in DME/10% FCS and transferred into 25cm² tissue culture flasks.

2.2 Animals

2.2.1 Animals for virus isolation

Sheep that arrived at different times from the south of Chile to a slaughter house in Santiago showing clinical signs of respiratory disease including coughing, rapid respiration, anorexia, nasal discharge and fever, were selected for virus isolation attempts. They were taken to the Animal Pathology Department of University of Chile, in Santiago for necropsy. Lung samples were taken for virus isolation, bacteriological examination and histopathology. Standard bacteriological procedures were used to isolate and identify bacteria and mycoplasms from the lung.

2.2.2 Animals for experimental infection

Ten lambs were obtained at birth and deprived of colostrum. They were bottled-fed 3 times daily on 300ml of warmed cow’s milk supplemented twice daily with a commercial oral solution of streptomycin sulphate and neomycin sulphate.
2.2.3 Rabbit for OPIV-3 antisera production

New Zealand adult rabbits (Bioterio del Instituto de Salud Publica de Chile, Santiago, Chile), clinically healthy and free from antibodies to PIV-3 were used to raise polyconal sera against the PIV-3 isolated in this study from clinical cases of sheep respiratory disease (see 4.1.1.1.)

2.3 Antibodies

All sera were heat-inactivated at 56°C for 30min. Antibody titres for PIV-3 were determined by a microtitre serum-virus neutralization test (Lehmkuhl and Cutlip, 1982).

- BPIV-3 reference antisera kindly supplied by Dr. Neil Watt (Edinburgh University, Veterinary Pathology Department, Field Station, Edinburgh, UK).

- BPIV-3 reference antisera raised against a bovine strain named La Pintana 84 (Berríos et al., 1990) kindly supplied by Dr. Patricio Berríos (Laboratorio de Virología, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile).

- OPIV-3 antisera raised against the ovine isolate named 270-7 (Berríos et al., 1991) raised in New Zealand rabbits (Laboratorio de Virología, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile).

- Murine monoclonal antibodies anti HPIV-3 proteins that have cross reactivity with the same proteins of BPIV-3 (Klippmark et al., 1990). They were kindly supplied by Dr. Robert Rydbeck, Virology Departament, Karolinska Institute, Stockholm, Sweden (see Table 2.1).
TABLE 2. Monoclonal antibody panel anti HPIV-3 and grouping according epitopes*

<table>
<thead>
<tr>
<th>Viral Protein</th>
<th>MAbs</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN</td>
<td>4771</td>
<td>II**</td>
</tr>
<tr>
<td></td>
<td>4795</td>
<td>III</td>
</tr>
<tr>
<td>F</td>
<td>3283</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>4503</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>4673</td>
<td>II</td>
</tr>
<tr>
<td>NP</td>
<td>4721</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>4794</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>4890</td>
<td>II</td>
</tr>
<tr>
<td>M</td>
<td>2873</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>3471</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>4877</td>
<td>V</td>
</tr>
</tbody>
</table>

*= according to Rydbeck et al., (1986)
**= a different group indicates that the Mab recognizes different epitope

- FITC, HRP and alkaline phosphatase conjugated antibodies against rabbit IgG (Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland) and against mouse IgG (Sigma Chemical Company, St. Louis, MO, USA).

2.4 Reference PIV-3 strains

- Bovine strain of PIV-3 kindly supplied by Dr. Neil Watt (Edinburgh University, Veterinary Pathology Department, Field Station, Edinburgh, UK).

- Bovine strain of PIV-3 named La Pintana 84 (Berrios et al., 1990) kindly supplied by Dr. Patricio Berrios (Laboratorio de Virología, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile).

- Ovine strain of PIV-3 named Moredun kindly supplied by Dr. Peter Nettleton (Moredun Institute, Edinburgh University, Edinburgh, UK)
2.5 Flow cytometry and analysis of cytometric data

Flow cytometry is an analytical technique which in a population of continuously flowing fluorochrome labelled cells permits accurate quantitation of various parameters of individual cells, such as the distribution according to their sizes and fluorescence intensities. Data on these parameters can be stored and analysed in a computer. In this work, flow cytometric acquisition of fluorescein isothiocyanate (FITC) labelled cell data was carried out using a fluorescence-activated cell analyser, FACScan (Becton Dickinson, Mountain View, CA, U.S.A.). The Consort 30 programme was used in the acquisition, storage and analysis of the single colour flow cytometric parameters of the labelled sheep cells. In each case data was derived by analysing 10,000 cells (or events) per sample. The cytometer configurations were calibrated and set using negative control cells stained with normal mouse serum (NMS) and the appropriate immunoconjugates. FITC fluorescence (520 nM) emission was detected with the photomultiplier tube voltage set at 555 mV. Forward (FSC) and side or 90 degree (SSC) light scatter amplifications were linear and set at E00 and 326 respectively, while FITC amplification was logarithmic (all 256 channels). Using dot plot (Figure 2.1a) cells (lymphocytes or monocyte/macrophages) were “live-gated” for acquisition and analysis while cell debris, dead cells, red blood cells (RBCs) and were excluded on the basis of FSC and SSC parameters (Figure 2.1b,c). FSC, SSC and fluorescent data were plotted as frequency histograms with the log fluorescent intensity presented on the x-axis and the frequency of cell numbers presented on the y-axis (Figure 2.1d).

2.6 Immunofluorescence and immunohistochemistry

2.6.1 Indirect immunofluorescence

Cells or sections mounted on coverslips were immediately placed in fresh 24well tissue culture plates and rinsed twice in buffer PBS and then fixed for 5 minutes at 4°C in buffer PBS containing 80% cold acetone. They were rinsed twice in rinsing buffer PBS containing 0.5% bovine serum albumin (BSA), 0.01M sodium azide and 0.05% Tween 20. Next step was to incubate at 4°C for 30 minutes with the same buffer plus 1% normal mouse serum or normal rabbit serum according to the nature of
FIGURE 2.1 Flow cytometry of lung wash fluid (LWF) for live gating of lymphocytes and macrophages (Mφs) using forward (FSC) and side (SSC) angle light scatter.

A. Dot plot display (ungated) of a negative control sample of fresh LWF showing the distribution of distinct cell populations (RBC= red blood cells; Ly= lymphocytes; Mφ= macrophages) based on their physical characteristics.

B. Exclusion of dead cells, RBC, granulocytes and lymphocytes by "live gating" the macrophages.

C. Frequency histogram display of the "live gated" macrophages.

D. Exclusion of dead cells, RBC, granulocytes and macrophages by "live gating" the lymphocytes.
the primary antibody used. Then the buffer was removed and they were incubated with 50μl of anti PIV-3 rabbit polyclonal sera or mouse monoclonal antibody at 4°C for 60 minutes. After this, the primary antibody was removed and they were given three washes in rinsing buffer for 10 minutes each time with constant shaking. Then they were incubated for 30 minutes at 4°C with 50μl of a 1:100 dilution in rinsing buffer of the second FITC conjugated antibody anti-rabbit IgG or anti-mouse IgG according to the nature of the primary antibody. The secondary antibody was removed and they were given three washes in rinsing buffer for 10 minutes each time with constant shaking. Finally, the coverslips were removed and mounted on slides with buffer PBS containing 10% glycerol. The reactivity was immediately observed and photographed under UV microscope (Nikon Labophot II).

2.6.2 Indirect immunoperoxidase

Cells or sections mounted on coverslips were immediately placed in fresh 24well tissue culture plates and rinsed twice in buffer PBS and fixed for 5 minutes at 4°C in buffer PBS containing 80% cold acetone. They were rinsed twice in buffer PBS and endogenous peroxidase activity was blocked by incubating in PBS 1% hydrogen peroxide for 30 minutes at 4°C. Then they were rinsed twice in rinsing buffer PBS containing 0.5% bovine serum albumin (BSA), 0.01M sodium azide and 0.05% Tween 20. Next step was to incubate at 4°C for 30 minutes with the same buffer plus 1% normal mouse serum or normal rabbit serum according to the nature of the primary antibody used. Then the buffer was removed and they were incubated with 50μl of anti PIV-3 rabbit polyclonal sera or mouse monoclonal antibody at 4°C for 60 minutes. After this, the primary antibody was removed and they were given three washes in rinsing buffer for 10 minutes each time with constant shaking. They were incubated for 30 minutes at 4°C with 50μl of a 1:200 dilution in rinsing buffer of the second HRP conjugated antibody anti-rabbit IgG or anti-mouse IgG according to the nature of the primary antibody. The secondary antibody was removed and they were given three washes in rinsing buffer for 10 minutes each time with constant shaking. The reactivity was visualized by incubating for 5 minutes in 50μl of PBS containing 0.1% diaminobenzidine (DAB) and 0.02% hydrogen peroxide. The reaction was stopped by washing in distilled water and then enhanced with 1% osmium tetroxide for 1 minute. Coverslips were washed in distilled water and placed briefly in
hematoxylin for nuclei counterstaining, dehydrated in alcohol cleared in xylol and finally mounted for observation under light microscopy.

2.6.3 Indirect alkaline phosphatase

The coverslips removed from infected and control culture cells were immediately placed in fresh 24well tissue culture plates and rinsed twice in buffer PBS and then fixed for 5 minutes at 4°C in buffer PBS containing 80% cold acetone. They were rinsed twice in buffer Tris/saline (1/10 of 0.5 M Tris-Cl pH 7.6 0.15 M/Saline solution) containing 0.5% bovine serum albumin (BSA), 0.01M sodium azide and 0.05% Tween 20. Next step was to incubate at 4°C for 30 minutes with the same buffer plus 1% normal mouse serum or normal rabbit serum according to the nature of the primary antibody used. Then the buffer was removed and they were incubated with 50 μl of anti-PIV-3 rabbit polyclonal sera or mouse monoclonal antibody at 4°C for 60 minutes. After this, the primary antibody was removed and they were given three washes in rinsing buffer for 10 minutes each time with constant shaking. They were incubated for 30 minutes at 4°C with 50 μl of a 1:30 dilution in rinsing buffer of the second AP conjugated antibody anti-rabbit IgG or anti-mouse IgG according to the nature of the primary antibody. The secondary antibody was removed and they were given three washes in rinsing buffer for 10 minutes each time with constant shaking. The reactivity was visualised by incubating for 15 minutes in 50 μl of freshly made developing solution prepared by mixing 9.8ml of 0.1 M Tris-Cl (pH 8.2), 2mg of Naphthol AS-MX Phosphate dissolved in 0.2ml Dimethylformamide and 10 mg Fast Red TR Salt. The reaction was stopped by washing in distilled water and then coverslips were placed briefly in hematoxylin for nuclei counterstaining, and finally mounted in Farrant’s mounting media for observation under light microscopy.

2.6.4 Double-immunostaining (SA-HRP/IAP) for PIV-3 particles and cell markers

Double-immunostaining was carried out in frozen as well as in formalin fixed sections. Frozen sections cut 8μm thick and mounted on round glass coverslips were placed in 24 well tissue culture plates, air dried at least for 30 minutes and then fixed for 5 minutes at 4°C in buffer PBS containing 80% cold acetone, then processed for double
immunostaining. Formalin fixed, paraffin embedded sections were cut 5μm thick and mounted on round glass coverslips. They were dewaxed, rehydrated in alcohol and then rinsed twice in buffer PBS before being processed for double immunostaining.

Double immunostaining was performed in a humid chamber in two stages according to the method described by Braun et al. (1991). In the first stage (detection of OIV-3 antigens by SA-HRP) the endogenous peroxidase activity was blocked by incubating in PBS 1% hydrogen peroxide for 30 minutes at 4°C. Then the sections were rinsed twice in rinsing buffer PBS containing 0.5% bovine serum albumin (BSA), 0.01M sodium azide and 0.05% Tween 20. The next step was to incubate at 4°C for 30 minutes with the same buffer plus 1% normal rabbit serum in a humid chamber at room temperature. Then the buffer was removed, by three washes in rinsing buffer for 10 minutes each time with constant shaking, and the sections were incubated with 50μl of anti PIV-3 rabbit polyclonal sera at 4°C for 1 or 2 hours at room temperature. After washing as before, second incubation was carried out with biotinylated goat anti-rabbit IgG (Sigma) diluted 1:200 in buffer for 2 hours at room temperature. The third incubation after washing was performed with horseradish peroxidase (HRP) conjugated avidin (Sigma) 5μm/ml in 0.05 M Tris/NaCl-buffer, pH 7.6 for 1 hour at room temperature. Sections were stained with 0.5mg/ml 4-chloro-l-naphtol (4CN)/0.005% hydrogen peroxide for 30 minutes. Positive reaction was detected by the appearance of a blue colour. The reaction was stopped by washing in distilled water, and then enhanced with 1% osmium tetroxide for 1 minute. In the second stage (detection of cell markers by IAP) the sections were rinsed twice in buffer Tris/saline (1/10 of 0.5 M Tris-Cl pH 7.6 0.15 M/Saline solution) containing 0.5% BSA, 0.01M sodium azide and 0.05% Tween 20. The next step was to incubate at 4°C for 30 minutes with the same buffer plus 1% normal mouse serum. Then the buffer was removed and they were incubated with mouse monoclonal antibody SBU-T4, SBU-T8 or VPM32 at 4°C for 60 minutes. After this, the primary antibody was removed and they were given three washed in rinsing buffer for 10 minutes each time with constant shaking. They were incubated for 30 minutes at 4°C with 50μl of a 1:30 dilution in rinsing buffer of the second AP conjugated antibody anti-mouse IgG. The secondary antibody was removed and they were given three washed in rinsing buffer for 10 minutes each time with constant shaking. The reactivity was visualised by incubating for 15 minutes in 50μl of freshly made developing solution prepared by mixing 9.8ml of 0.1 M Tris-Cl (pH 8.2), 2mg of Naphthol AS-MX Phosphate dissolved in 0.2ml Dimethylformamide and 10 mg Fast Red TR Salt. Positive staining
was detected by the appearance of a red colour. The reaction was stopped by washing in distilled water and then coverslips were placed briefly in methylene green for nuclei counterstaining, and finally mounted in Farrant's mounting media for observation under light microscopy.

2.7 Isolation of OPIV-3 chilean strains

Samples from clinical cases of sheep pneumonia were processed to isolate OVPI-3. For this, 0.1ml of obtained inoculum, from either nasal swabs or lung samples, was inoculated into 3 flasks (per sample) containing subconfluent OFK cells in Eagle's Essential Minimum Media (MEM, Sigma Chemical Co. USA) supplemented with 5% foetal calf serum (FCS, Sigma Chemical Co. USA), 0.08% sodium bicarbonate, 200 units/ml penicillin, 100ug/ml streptomycin. As negative controls tissue cultures were inoculated with samples processed from normal lungs and as positive control tissue cultures were inoculated with OPIV-3 reference strain. Virus isolation was based upon the following procedures:

2.7.1 Titration of virus infectivity

The virus infectivity was determined according to titration of cytopathic effect (CPE), haemadsorption and haemagglutination of guinea pig erythrocytes.

For infectivity titrations serial tenfold dilutions of virus were added in aliquots of 0.01ml to tissue culture flasks. Adsorption was allowed to proceed for 30 minutes at 37°C prior to overlaying with maintenance medium. On the 4th or 5th following days the infected cultures were examined for haemadsorption and cytopathic effect.

2.7.1.1 Haemadsorption test

The end point was taken as the highest initial dilution of virus giving detectable haemadsorption of 1% guinea pig erythrocytes suspension following incubation at 4°C for 30 minutes. The titre was expressed as the number of tissue culture infective doses (TCID₅₀) per 0.1ml.
2.7.1.2 Cytopathic Effect (CPE)

The inoculated cell cultures were examined under an inverted light microscope each day after infection to detect the presence of virus induced CPE. The inocula were subpassaged twice if no CPE developed after 5-7 days of incubation.

CPE was further investigated by placing cells in 24 well tissue culture plates with sterile 13 mm diameter round glass cover slips. They were removed at different times post inoculation and fixed in methanol for 5 minutes. Then they were stained with hematoxylin-eosin (H/E) and mounted to observe CPE including inclusion body formation.

2.7.1.3 Haemagglutination (HA) test

Material harvested from infected OFK cell culture was used as the source of haemagglutinin (Sever, 1962). The tests were performed in U shaped bottom well microtitre plates. Haemagglutinating activity was titrated using 0.2 ml volumes of a two-fold dilution series of haemagglutinin and an 0.5% suspension of guinea pig erythrocytes. The plates were kept at room temperature for 40 minutes and the end points were read by the sedimentation pattern method. The titre was expressed as the highest initial dilution of haemagglutinin causing complete agglutination of the erythrocytes. The dilution was considered to contain 1 haemagglutinating unit (HA unit) per 0.2 ml.

2.7.2 Electron microscopy

For negative staining, infected cultures with over 70% CPE were frozen and thawed once and the resulting lysate was processed. Lysates were precleared by centrifugation at 1,500 x g for 30 minutes. The supernatant was centrifuged at 100,000 x g for 2 hours and the pellet was resuspended in PBS pH 7.2. The negative staining was done with 2% phosphotungstic acid on 400 mesh grids covered with Formbar. The observation and photography was carried out in a transmission electron microscope Zeiss EM 109.
Thin sections for electron microscopy were prepared from monolayers of OFK cell cultures. Infected cells were harvested at 24, 48 and 72 hours post inoculation. They were inoculated with virus and processed according to Cutlip & Laird (1976). The cell monolayers were removed from the flasks with a rubber policeman, centrifuged at 120 x g for 5 minutes and resuspended and fixed in glutaraldehyde 2.5% in cacodylate buffer 0.2 M pH 7.4. After 2 rinses in cacodylate buffer for 30 minutes each, the cells were postfixed for 30 minutes in 1% osmium tetraoxide in cacodylate buffer and again rinsed twice in buffer. After fixation and after rinsing the cells were pelleted by centrifugation at 120 x g for 5 minutes. Fixed cells were embedded in 2% agar by centrifugation at 1,300 x g at 40°C for 10 minutes. Pellets thus formed were cut into 1 to 2mm samples and dehydrated in a graded series of ethanol. Then, they were cleared in 3 changes of propylene oxide for 8 minutes each and finally in a solution containing 50% propylene oxide and 50% epoxy resin (Epon 812, Shell Chemical Company, Kansas City Mo.). Later, they were embedded overnight in a solution 1:10 propylene oxide/epoxy resin and then for 24 hours only in epoxy resin and hardened at 50°C for 72 hours. Sections were cut on an ultramicrotome (Sorval MT2 B), stained with lead citrate for 15 minutes and uranyl acetate for 5 minutes according to Bryson et al.(1983) and examined with an electron microscope (Model 200, Phillips Electronics, NY)

2.7.3 Physical and biochemical virus characterisation

2.7.3.1 pH sensitivity

Buffer solutions pH 3.0, 7.0 and 9.0 were prepared, then 10-fold virus dilutions were made with them to be incubated at 40°C for 15 minutes, finally the extreme pHs were neutralized by adding NaOH or HCl 0.25M.

As control virus dilutions were made with culture medium and processed as the others.
Buffer solutions

- pH 3.0

Sodium citrate 1.37 g  
Citric acid 1.12 g  
Distilled water up to 100 ml

- pH 7.0

Sodium phosphate 0.742 g  
Disodium phosphate 0.542 g  
Distilled water up to 100 ml

- pH 9.0

Sodium borate 3.607 g  
Boric acid 0.034 g  
Distilled water up to 100 ml

2.7.3.2 Lipid solvents sensitivity

Ether: The sensitivity to ether was performed according to a method described by Andrewes & Horstman (1949). The virus was suspended in 20% v/v ether in culture media, the control was suspended in 20% v/v Puck's saline solution A. The tubes were hermetically closed and taken to 40°C for 24 hours and shaken constantly. Then, ether was removed by evaporation and the virus suspension was stored at -70°C until titration.

Chloroform: The sensitivity to chloroform was assessed following the method described by Feldman & Wang (1961). Briefly, to 1ml of virus suspension was added 0.5ml of chloroform. Simultaneously, a control was prepared with 0.5ml of
Puck's saline solution A. The tubes were gently shaken for 10 minutes at room temperature.

In order to eliminate the chloroform the virus suspensions were centrifuged at 12 x g for 5 minutes. The chloroform and virus separate in different bands, so this allowed the virus to be collected and kept frozen at -70°C until titration.

2.7.3.3 Trypsin sensitivity

The sensitivity to trypsin was performed according to a method described by Gresser & Enders (1961). 1%, 0.5% and 0.25% solutions of trypsin were prepared with Puck's saline solution A. Then 1 ml of virus suspension was mixed with 1ml of the trypsin dilution. Tubes were incubated at 37°C in a thermoregulated bath for 60 minutes. Trypsin was inactivated by adding 1% cold foetal calf serum.

As controls, 1 ml virus suspension was mixed with 1 ml of Puck's saline solution A. Then they followed the same procedure.

2.7.3.4 Heat sensitivity

Test tubes were previously warmed up to 56°C and to each of them 0.5ml of virus suspension was added and immediately taken to a thermoregulated bath at 56°C.

Each tube was removed from the thermoregulated bath at different times (0, 5, 10, 20 and 30 min), and then transferred to an ice box and finally stored frozen at -70°C until titration.

2.7.4 Serological identification

2.7.4.1 Anti PIV-3 sera production

Anti PIV-3 sera was raised in rabbit immunized with the virus isolates described in this study. Two rabbits were used for each virus isolate, they were free of anti PIV-3
antibodies as determined by III test before immunization. Each rabbit was given four inoculations containing 1ml of virus suspension with an infective titre of $10^{7.5}$ TICD$_{50}$ for isolate 271-7, $10^{8.5}$ for isolate 390-10 and $10^{6.9}$ for isolate 410-7. Each of the four inoculations was given as follows: the first one via the subcutaneous route with complete Freund’s adjuvant (Difco Laboratories Detroit Michigan, USA.); the second one via intramuscular and the third and four ones via intraperitoneal. Each inoculation was given with a week interval.

Ten days after the last inoculation the rabbits were bled to obtain the sera. All sera was inactivated at 56°C for 30 minutes and stored at -70°C until titration. Non specific haemaggultination inhibition factors were eliminated by mixing and incubating the sera with 25% caolin for 30 minutes at room temperature. Then, the sera were centrifuged at 1,500 x g for 10 minutes. The supernatant was treated for heteroagglutinin elimination with a suspension of 5% guinea pig erythrocytes for one hour.

As a reference the following anti PIV-3 sera was used:

- anti bovine PIV-3 sera raised with strain La Pintana 84 (Berrios et al, 1990), kindly supplied by Dr. Patricio Berrios (Lab. Virologia, Facultad de Ciencias Veterinarias y Pecuarias. Universidad de Chile);
- anti bovine PIV-3 sera supplied by Dr. Neil Watt (Veterinary Pathology Department, Edinburgh University, Edinburgh, UK); and anti ovine PIV-3 sera supplied by Dr. Peter Nettleton (Moredun Institute, Edinburgh, UK.)

Prior to testing the serum samples were diluted twofold in MEM and heat inactivated at 56°C for 30 minutes.

2.7.4.2 *Haemagglutination Inhibition (III) test*

For serum III antibody titrations 0.2ml of serial-two fold dilutions of serum were mixed with 0.2 ml of a dilution of haemagglutinin containing 4 HA units and incubated for 60 minutes, then 0.2 ml of a 0.5% suspension of guinea pig erythrocytes was added and incubated for 40 minutes. The plates were kept at room temperature for 60 minutes and the end points were read again by the sedimentation pattern
method. The HI antibody titre of the serum was taken as the highest initial dilution of serum which affected complete inhibition of haemagglutination.

2.7.4.3 Sero-neutralization (SN) test

All samples were tested in triplicate, using twofold dilutions (from 1:2 to 1:512) in a microtitration procedure (Lehmkuhl & Gough, 1977). The test virus (100 median tissue culture infective doses, TCID₅₀) was added to each well. The serum-virus mixture was incubated for 1 hour at room temperature before the addition of 0.05ml of appropriate cells, at concentrations calculated to allow the immediate formation of monolayers. The plates were incubated in a CO₂ incubator at 37°C. The plates were read 2-3 days later. The serum-virus neutralization titres were the highest dilution of serum in which 50% of the wells were protected from the viral CPE.

2.7.5 Immunofluorescence and immunocytochemistry

Immunocytochemistry was carried out in infected cell cultures in order to detect virus particles with the polyclonal sera and also with anti human PIV-3 virus monoclonal antibodies. OFK cells were placed in tissue culture 24 well plastic microplates (Corning Glass Works. Corning NY 14831) containing glass round sterile coverslips measuring 13 mm diameter. Each well received 1 ml of Eagle’s MEM with 5% foetal calf serum and containing 10⁵ cells. They were incubated for 48 hours at 37°C and then inoculated with 0.1ml of virus suspension under study at a multiplicity of infection (m.o.i.) of 0.1. The virus was absorbed at 37°C for 60 minutes. The virus suspension was removed and 1 ml of culture media was added and incubation at 37°C followed. Wells in separate microplates were inoculated with tissue culture media and kept as negative controls. Separate microplates wells were infected with the reference virus as positive controls.

The coverslips were removed after 24, 48 and 72 hours post inoculation to be processed for immunostaining by immunofluorescence, indirect immunoperoxidase and indirect immuno-alkaline-phosphatase staining as described in sections 2.6.1, 2.6.2 and 2.6.3, respectively.
2.7.6 Detection of viral antigens by flow cytometry

Detection of viral antigens expressed on the cell surface and in the cytoplasm was carried out by flow cytometry. The presence of OPIV-3 antigens on the surface of infected cells was detected by indirect immunofluorescence carried out in rhesus tubes. Infected and mock-infected cells were cultured and harvested as described before then blocked with PBA (PBS containing 0.1% BSA and 0.01% sodium azide) including 2% NRS and 2% NMS for 30 minutes at 0°C. The cells were further incubated with anti OPIV-3 sera or mAbs anti HPIV-3 antigens (see table 2.1) at 0°C for 40 minutes, washed three times with PBA and then incubated with FITC-conjugated anti-mouse IgG F(ab')2 1:50 dilution (Dakopatts, Ltd.) at 0°C for another twenty minutes. After three washes with PBA, cells were fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry. In order to detect viral antigen, the infected and mock-infected cells were fixed in 80% methanol in PBS before blocking and incubation with antibodies, according to method described by Lee (1994). Cells were gated according to FSC and SSC parameters. The reactivity of the antibodies was analyzed by flow cytometry using a Becton-Dickinson FACScan with Consort 30 version F Software. Normal mouse serum (1:500 dilution) stained cells were set up as background staining controls. The percentage of positive cells and mean of fluorescence intensity (MF) were determined by using Lysis Version 1.62 and Consort 30 version F Software.

2.8 Experimental interstitial sheep pneumonia

2.8.1 OPIV-3 270-7 inoculation of experimental lambs

The ovine isolate of PIV-3 named 270-7 (see 4.1.1.1) was isolated (Laboratorio de Virologia, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile) and described in this study. This strain was used for the experimental inoculation of colostrum-deprived lambs at the sixth passage in OFK cells grown in Eagle's medium containing 0.5% foetal calf serum. The titre was $10^{7.2}$ TCID$_{50}$ per ml

Fourteen lambs were obtained at birth and colostrum-deprived. They were bottled-fed 3 times daily on 300ml of warmed cow's milk supplemented twice daily by oral
administration of a commercial solution of streptomycin sulphate and neomycin sulphate. Serum samples were obtained from each lamb before inoculation and at necropsy and stored at -70°C. After three days they were divided into two groups. Eleven lambs were inoculated with 5ml tissue culture fluid containing the virus; 2ml were given intranasally and 3ml transtracheally. For intratracheal inoculation a 16 gauge hypodermic needle was inserted into the mid-cervical section of the trachea under local anaesthesia; a polythene catheter (20 cm x 1mm external diameter) was then passed into the trachea to deposit the inoculum in the thoracic part of the trachea while the lamb was being held horizontally. Three control lambs, housed separately, received a similar amount of cell culture fluid from non-inoculated cell cultures. The lambs were observed daily for clinical response. Three lambs were euthanized and necropsied on post-inoculation day (PID) 3, three on day 5 and five on day 7. The control lambs were euthanized and necropsied on PID 8. Rectal temperatures were taken daily from birth. Nasal samples were collected daily with sterile swabs for isolations of virus beginning from birth. Lung tissues were taken for attempts to isolate virus and bacteria at necropsy.

2.8.2 OPIV-3 recovery and identification

Nasal swabs during experiment and lung tissues after necropsy were collected for virus isolations. Nasal secretions or 0.5cm³ of lung tissue, minced in Eagle’s minimal essential medium, were inoculated on triplicate tissue culture flasks of subconfluent OFK cells. The medium was supplemented with 5% foetal calf serum (FCS), 100 μg gentamycin sulphate and 10 μg of amphotericin B per ml. The flasks were observed daily for the onset of viral cytopathic effect. Virus in the 3rd to 6th passages were used for further studies. Cultures for virus isolations were incubated for 14 days, frozen and thawed, subpassaged and incubated for a further 14 days before they were considered to be negative.

2.8.3 Bacteriologic examination

Standard bacteriological procedures were used to isolate and identify bacteria and mycoplasmas from nasal secretions and lung. Samples of the lung were homogenized to give a 10% suspension in Hank’s balanced salt solution (HBSS) containing 0.1%
yeast extract and 0.5% lactic albumin hydrolysate (HYL). Nasal swabs were collected into 2 ml of HYL. Duplicate 0.1 ml volumes of each dilution were spread on plates of 7% sheep blood agar and the number of bacteria colonies was determined after overnight incubation at 37°C.

Samples for mycoplasma isolations were stored frozen at -70°C until processing. Mycoplasma broth (BHS-L) and mycoplasma-agar (BHS-A) were prepared as described by Carmichael et al. (1972). They were thawed and agitated vigorously then the fluid was passed through a 0.65 μm membrane filter to remove gross bacterial contamination. Serial ten fold dilutions of the filtered material were prepared in 1.8 ml volumes of (BHS-L) pH 7.8 and in BHS-L containing 10 mg/ml arginine pH 7.0. The dilutions were incubated for 10 days at 36°C and the titre was recorded as the highest dilution of sample material showing a pH change and expressed as colour changing units (c.c.u.)/0.2 ml of sample. Isolates were identified by colony morphology on (BHS-A) and growth inhibition with specific antiserum.

2.8.4 Serology

Serum samples were collected from all sheep at the beginning of the experiments and before necropsy. Prior to testing, the serum samples were diluted twofold in MEM and heat inactivated at 56°C for 30 minutes.

Neutralization tests: Antibody titres to PIV-3 were determined on paired serum samples from the lambs, using a microtitration serum-virus neutralization test (Lehmkuhl & Gough, 1977). All samples were tested in triplicate, using twofold dilutions (from 1:4 to 1:512) in a microtitration procedure. The test virus (100 median tissue culture infective doses, TCID₅₀) was added to each well. The serum-virus mixture were incubated for 1 hour at room temperature before the addition of 0.05 ml of appropriate cells, at concentrations calculated to allow the immediate formation of monolayers. The plates were incubated in a CO₂ incubator at 37°C. The plates were read 2-3 days later. The serum-virus neutralization titres were the highest dilution of serum in which 50% of the wells were protected from the viral CPE.

All sera were inactivated at 56°C for 30 minutes. Serum neutralisation (SN) tests were carried out by mixing serial two fold dilutions of serum with an equal volume of
virus at a dilution calculated to contain 100 TCID$_{50}$. The mixtures were incubated at 18°C for 1 hour before inoculation of 4 OFK cell cultures per dilution. End points were determined by haemadsorption on the 4th day of incubation. The titre was expressed as the highest dilution of serum preventing growth of virus in 50% of tubes. Haemadsorption inhibition test were performed as described by Davies et al. (1982). Sera for haemagglutination inhibition (HI) tests were adsorbed with guinea pig erythrocytes and titrated as described before (see 4.2.1.2.3.). The HI antibody titre was expressed as the highest initial dilution of serum which completely inhibited agglutination by 4 units of virus. The source of haemagglutinin was tissue culture fluid harvested from OFK cell cultures 5 days after infection.

2.8.5 Histopathology of lung lesions

Samples for lung histopathology were taken from each pneumonic and control lungs. They were fixed in buffered 10% formalin with calcium carbonate. Tissues were formalin fixed and paraffin embedded according to standard techniques (Lopez et al., 1982). 5μm sections were obtained with a conventional rotatory microtome Minot Leitz (Wetzler) and mounted on slides with egg albumin. Mounted sections were dried and then deparaffined in xylol, rehydrated in alcohol and stained. Then they were dehydrated in alcohol, cleared in xylol and mounted.

- **Histological stains** (Lopez et al., 1982):
  - Hematoxilin-eosin for general morphology
  - Van Gieson staining for identification of fibrosis (collagen type I)
  - Giemsa staining for identification of infiltrative leucocytes

2.8.6 Immunofluorescence and immunohistochemistry

Lung samples were taken for immunostaining from each pneumonic and control lambs. They were labelled and placed in plastic bags and immediately immersed in
liquid nitrogen. The frozen samples were then transferred and stored at -70°C until being cut with a cryostat (Lipshaw MFG. Co. Detroit 10, Michigan, USA). The 8μm frozen sections were mounted on round coverslips previously covered with 1% polylysine and then air dried for at least 30 minutes. Sections were processed according to method described by Bancroft (1975). Briefly, they were fixed in 80% cold acetone (-20°C) in buffer PBS and then processed for immunostaining by immunofluorescence, indirect immunoperoxidase, indirect immunoalkaline-phosphatase staining and double-immunostaining as described in sections 2.6.1, 2.6.2, 2.6.3 and 2.6.4, respectively.

2.9 Monocyte-derived macrophages (MDMφ) and alveolar macrophage (AMφ) cultures

2.9.1 Gelatin-plasma coated flasks or plates

Gelatin coated flasks or plates were prepared according to the method described by Jones et al. (1989). 2.5 ml or 0.5 ml of 2% sterile gelatin was added to each 25cm² tissue culture flask or well (of a 24 well plate), respectively. After 1 hr incubation at 37°C, the excess gelatin was removed and the plates were dried in a 37°C incubator.

The dried flasks or plates could be stored at room temperature until they were used. Immediately before use they were incubated with autologous plasma for 30 min at 37°C. The plasma was removed and the flasks or plates were washed twice with PBS. These pre-treated flasks or plates were then ready for the culture of MDMφ or AMφ.

2.9.2 Monocyte-derived macrophage (MDMφ) isolation and culture

Blood was collected in heparin-containing tubes (10 U/ml of blood). Buffy coat cells were obtained by centrifugation of the heparinized blood at 1,250 xg for 15-20 minutes at room temperature. Autologous plasma was collected and used in the preparation of gelatin-plasma coated flasks or plates. The buffy coat was diluted in sterile phosphate buffered saline (PBS=0.85% NaCl, 0.02% KCl, 27mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2), overlaid on lymphoprep (Nyegaard, Oslo, Norway) and then centrifuged at 1,200 xg for 20 minutes at room temperature. Peripheral blood
mononuclear cells (PBMC) were harvested at the interface and then washed three times (500 x g for 5 minutes) with PBS. The PBMC were finally resuspended in RPMI-1640 medium containing 2 mM L-glutamine, 20 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid), 100 U penicillin per ml, 100 μg streptomycin per ml and 5 x 10^-1 M 2-mercaptoethanol (2-ME) (RPMI) with 10% foetal calf serum (FCS) and 10% normal lamb serum (NLS) (Gibco) (RPMI/10% FCS/10%NLS).

The PBMC were counted and the concentration adjusted to 5 x 10^6 cells per ml. Then 0.5 ml, 5 ml or 15 ml of the cell suspension was plated into each well (of 24 well plates), 25 cm² or 75 cm² flasks, respectively. The cells were incubated from 1 hour to overnight at 37°C, 5% CO₂, depending on the experiment. The non-adherent cells were washed off with warm RPMI/2% FCS. Fresh RPMI/10% FCS/10% NLS was added for continuous culture. The culture medium was changed every 5 days. After 5 to 7 days of culture most non-adherent lymphocytes could be removed as above and the cultured adherent monocyte-derived Mφs (MDMφ) were usually confluent. Over 95% of these adherent cells were non-specific-esterase (NSE) positive.

After 7 days in culture, normal cultured MDMφ showed proliferation and some detached and became suspended in the supernatant. The suspended cells could be passaged to other flasks where they became adherent.

2.9.3 Lymphocyte isolation in culture

PBMC from buffy coat were obtained and fractionated in lymphoprep (Nyegaard, Oslo, Norway) as described in section 2.9.2. Then lymphocytes were separated from adherent cells. Briefly, PBMC to be depleted of adherent cells were resuspended in lymphocyte complete RPMI-1640, containing 20% FCS. These cells were incubated in gelatin-coated prewarmed polystyrene flasks for 3 hrs. at 37°C, 5% CO₂. Non adherent cells were aspirated and the cycle repeated. Following the second incubation lymphocytes were aspirated, washed and resuspended in lymphocyte complete RPMI-1640 medium containing 2 mM L-glutamine, 20 mM HEPES, 100 U penicillin per ml, 100 μg streptomycin per ml and 5 x 10^-1 M 2-mercaptoethanol (2-ME) (RPMI) with 10% FCS (Gibco). Lymphocytes were resuspended to a concentration of 2 x 10^6 cells/ml.
2.9.4 Alveolar macrophage (AMφ) isolation and culture

Lungs were collected from normal healthy sheep. AMφ were obtained by lung washing (LW) with cold Hank's balanced salt solution (HBSS) according to method described by Mayer & Lam (1984). Firstly, the thoracic cavity was opened and the trachea, lungs and heart dissected out as a single package, with the upper part of the trachea clamped shut to avoid entrance of blood into the lungs. The exterior of this package was carefully washed with PBS at 37°C to eliminate any remaining blood. Secondly, the lumen of the trachea was opened and approximately 50ml of HBSS at 37°C were injected to reach every lobe. The trachea was clamped off and the lungs gently shaken for several minutes. The fluid was drained into a container and the same procedure was repeated ten to fifteen times. Finally, after removing the floating cell debris and mucus at the top of the cell suspension, the collected fluid was pelleted by centrifugation at 250 x g at 4°C for 10-20 minutes, depending on the volume of LW fluid. Cell pellets were washed with RPMI/2% FCS three times. The cell number was counted and adjusted to 5 x10⁵ cells per ml in RPMI/20% FCS. Specimens contaminated with blood were not used. Five ml of cell suspension were seeded into 25cm² flasks and then incubated at 37°C, 5% CO₂. Non-adherent cells were removed 3-4 hours later and the flasks re-fed with fresh medium. The purity of AMφ cultures was over 95% as determined by NSE staining. AMφ were cultured in a similar manner to monocytes but with RPMI/20% FCS.

In order to harvest adherent MDMφ or AMφ, cultures were washed twice with PBS once and 2.5 ml of 5mM EDTA in PBS was added to 25cm² flasks. After 2-5 minutes incubation the flasks were knocked to dislodge the cells and a rubber policeman was used to scrape off any adherent remaining cells. The cells were washed once with warm RPMI/2%FCS and then used.

2.9.5 Non-specific esterase (NSE) staining

Cytospin smears or cell cultures on glass coverslips were stained for NSE according to Hudson and Hay (1989). Then cells were fixed in cold 45% v/v formaldehyde, 25% v/v acetone in 0.1 M phosphate buffer (pH 6.6) for 40 seconds. The fixed cells were washed in running water and the nuclei were counterstained with 0.4%
methylene green for 5 minutes. Cells were classified as positive by the appearance of a brown colour.

2.9.6 Giemsa staining

Fibroblasts were fixed in a cold methanol/acetone mixture (1:1) for 5 minutes and then treated with 1% dichromate for 30 minutes. The treated cells were further fixed with the cold methanol/acetone mixture for 5 minutes. The cells were stained with Giemsa’s solution (2-fold dilution made fresh) for 5 minutes. The stained cells were washed in running water, air-dried and then mounted in DPX. Macrophages were stained as above but after washing in water they were incubated in Giemsa’s buffer (0.1 M acetic acid, 0.2 M disodium hydrogen phosphate pH 5.75) for 5 minutes, washed and mounted.

2.10 infection of Monocyte-derived macrophage (MDMφ) and alveolar macrophage (AMφ) cultures with OPIV-3

MDMφ and AMφ were cultured in 25 cm² flasks and infected with OPIV-3 270-7 virus at 1 TCID₅₀ per cell on days 5 and 3 of culture, respectively. Then infected and mock-infected cells were harvested on different days post inoculation by treatment with 5mM EDTA in PBS.

2.10.1 Transmission electron microscopy (TEM) of OPIV-3 infected monocyte-derived macrophage (MDMφ) and alveolar macrophage (AMφ) cultures

MDMφ and AMφ cultured in 75cm² were infected with OPIV-3 270-7 as described. On days 1 to 7 p.i. infected and mock-infected cells were gently scraped off the flask surface with a rubber policeman. The cells were washed once with PBS and then immediately fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 2 hours. The fixed cells were washed with cacodylate buffer and then post-fixed in 1% Osmium tetraoxide for 1 hour at 4°C. The cells were dehydrated through a gradient of acetone and then embedded in araldite. Ultrathin sections (60nm) were cut and stained with uranyl acetate and lead citrate. The stained sections were examined under a TEM Philips 400 electron microscope.
2.10.2 Phenotypic analysis of monocyte-derived macrophage (MDMφ) and alveolar macrophage (AMφ) cultures by flow cytometry

2.10.2.1 Indirect immunofluorescence staining for flow cytometry

Cultured MDMφ, AMφ or fresh peripheral blood monocytes were washed twice with PBA (PBS containing 0.1% BSA and 0.01% sodium azide). Then they were aliquoted into Rhesus tubes at 1x 10^5 cells per tube. Cells were pelleted by centrifugation at 250 x g for 3 minutes, 0°C and then resuspended in 25 μl of primary antibody (mouse monoclonal antibody) for 40 minutes at 0°C. As a control of staining, cells were incubated with NMS or NRS (1:500 dilution) as primary antibodies. The panel of monoclonal antibodies used is shown in Table 2.2. The cells were washed three times in cold PBA including 2% NRS and 2% NMS and then incubated with 25 μl of anti-mouse or anti-rabbit (according to primary antibody) IgG F(ab')2 1:50 dilution (Dakopatts, Ltd.) for 20 minutes at 20°C. After 3 washes in cold PBA cells were resuspended in 200 μl of PBA and immediately analyzed by flow cytometry. Cells were gated according to FSC and SSC parameters on Mφ or lymphocyte areas. The reactivity of the antibodies was analyzed by flow cytometry using a Becton-Dickinson FACScan with Consort 30 version F Software. Normal mouse serum (1:500 dilution) stained cells were set up as background staining controls. The percentage of positive cells and mean of fluorescence intensity (MF) were determined by using Lysis Version 1.62 and Consort 30 version F Software.

2.10.3 Effect of OPIV-3 infection on monocyte-derived macrophage (MDMφ) and alveolar macrophage (AMφ) phagocytic capacity

MDMφ and AMφ were cultured in 24 well plates. The cultured MDMφ were infected with OPIV-3 270-7 at 1 TCID_{50} per cell on days 3, 5, 7 and 9 of culture. The phagocytic assay was performed on day 10 of culture.
TABLE 2.2 Monoclonal antibody panel to sheep leukocyte antigens.

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Isotype</th>
<th>Antigen</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBU-T4</td>
<td>IgG2a</td>
<td>CD4</td>
<td>MHC Class II restricted T cells</td>
<td>Maddox et al., (1985)</td>
</tr>
<tr>
<td>SBU-T8</td>
<td>IgG2a</td>
<td>CD8</td>
<td>MHC Class I restricted T cells</td>
<td>Maddox et al., (1985)</td>
</tr>
<tr>
<td>VPM5</td>
<td>IgG2a</td>
<td>CD1</td>
<td>Dendritic cells (DC)</td>
<td>Budjoso et al., (1989)</td>
</tr>
<tr>
<td>VPM8</td>
<td>IgG1</td>
<td>Ig light chain</td>
<td>B cells</td>
<td>Bird et al., (1993)</td>
</tr>
<tr>
<td>VPM19</td>
<td>IgG1</td>
<td>MHC class I</td>
<td>Nucleated cells</td>
<td>Hopkins &amp; Dutia (1990)</td>
</tr>
<tr>
<td>VPM32</td>
<td>IgG2a</td>
<td>not defined</td>
<td>Tissue Mφs</td>
<td>González (1989)</td>
</tr>
<tr>
<td>VPM36</td>
<td>IgG1</td>
<td>MHC class II DQ-α</td>
<td>B cells, activated T cells, DC, monocytes, Mφs</td>
<td>Dutia et al., (1993)</td>
</tr>
<tr>
<td>VPM38</td>
<td>IgG1</td>
<td>MHC class II DR-α</td>
<td>B cells, activated T cells, DC, monocytes, Mφs</td>
<td>Dutia et al., (1993)</td>
</tr>
<tr>
<td>VPM65</td>
<td>IgG1</td>
<td>CD14</td>
<td>Monocytes, Mφs, DC, PMNn</td>
<td>Gupta et al., (1994)</td>
</tr>
<tr>
<td>ST197</td>
<td>IgG2b</td>
<td>T19</td>
<td>γδT cells</td>
<td>MacKay et al., (1989)</td>
</tr>
<tr>
<td>F10-150-39</td>
<td>IgG1</td>
<td>LFA-1</td>
<td>B and T cells, monocytes, Mφs</td>
<td>MacKay et al., (1990)</td>
</tr>
<tr>
<td>180-1</td>
<td>IgG1</td>
<td>LFA-3</td>
<td>B and T cells, monocytes, Mφs</td>
<td>Hunig (1985)</td>
</tr>
</tbody>
</table>
2.10.3.1 **Opsonization of sheep red blood cells (SRBC)**

FITC-labelled SRBC were opsonized by a sub-haemagglutinating dilution (1:40 in PBS) of anti-SRBC serum developed in rabbits\(^1\). SRBC were incubated with this antiserum for 30 minutes at 37°C. The opsonized SRBC were washed twice with PBS and then resuspended to 1% in RPMI/2% FCS.

2.10.3.2 **FITC-labelling of sheep red blood cells (SRBC)**

The method described by Tuijman *et al.* (1990), was used to FITC-conjugate SRBC. A 5% suspension of SRBC in microtube was washed 3 times with PBS using a microcentrifuge (Sanyo) at 3,000 r.p.m. for 2 minutes. The SRBC were incubated with FITC at a final concentration of 100 μg/ml in PBS for 30 minutes at 37°C. The FITC labelled SRBC were washed twice in PBS and then opsonized with a pooled antiserum.

2.10.3.3 **Rosetting of opsonized SRBC with monocyte-derived macrophages (MDMφs) and alveolar macrophage (AMφs) in culture.**

The rosette method consists in coupling specific proteins to the surface of erythrocytes. The red blood cells act simply as inert support and the added protein provides the specificity for all binding. Sheep erythrocytes, coated with immunoglobulins, by addition of rabbit anti SRBC serum, will form rosettes with sheep Mφs that carry Fc receptors.

1 x10^6 AMφs in 100 μl were pipetted into Rhesus tubes and incubated with 100 μl of 1% suspension of antibody coated FITC-conjugated SRBC in PBS. After being spun at 1,500 rpm for 5 minutes, the mixed cell suspension was incubated for 10 minutes at room temperature and then resuspended and analyzed by flow cytometry.

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\(^1\) Kindly given by Dr. John Hopkins, Veterinary Pathology Department, Edinburgh University.
2.10.3.4 Phagocytosis of opsonized SRBC by cultured monocyte-derived macrophages (MDMφs) and alveolar macrophage (AMφs)

MDMφ and AMφ were cultured in 24 well plates and infected with OPIV-3 270-7 as described in section 2.7.7. The cultures were washed with RPMI/2% FCS to remove non-adherent cells. Assays were then undertaken on adherent cells. The cell cultures were fed with 250 μl RPMI/2% FCS containing 50 μl of FITC-opsonized-SRBC and incubated for 1 hour at 37°C, 5% CO₂.

Non-ingested SRBC were removed with PBS and any of them remaining on the cell surfaces of the macrophages was lysed hypotonically. This was carried out by adding distilled water for 30 seconds. The isotonicity of the medium was restored by the addition of x2 PBS.

The Mφ cultures were treated with 5mM EDTA in PBS for five minutes and then gently scraped off with a rubber policeman. The cells were transferred into rhesus tubes, washed with PBA and immediately processed for flow cytometry analysis. Macrophages that had not been fed with FITC-SRBC were used as negative control of staining.
CHAPTER THREE

ISOLATION OF PARAINFLUENZA TYPE 3 VIRUS FROM SHEEP: LUNG PATHOLOGY, VIRUS ISOLATION AND VIRUS CHARACTERISATION
3.1 INTRODUCTION

Parainfluenza virus type 3 (PIV-3) is one of the most widespread cause of interstitial pneumonia and it has been isolated in different countries around the world, including Great Britain (Hore, 1966). The virus is generally associated with benign respiratory disease. However, PIV-3 infection has been demonstrated to predispose animals to acquiring pneumonia produced by bacteria such as Pasteurella haemolytica, Corynebacterium sp. and rickettsias (Davies et al., 1977, 1986; Sharp et al., 1978; Yates, 1988). Also, it can increase the severity of bacterial pneumonia (Yates, 1988).

In humans, almost two-thirds of infants can be infected with this virus in the first year of life; 92% of the children are infected at least once with human PIV-3 (HPIV-3) by two years of age and 37% have been infected more than once. Most of the lower respiratory tract disease associated with HPIV-3 infection is found during the first infection (Glezen et al., 1984).

Reports on PIV-3 affecting sheep (OPIV-3) from various parts of the world, have included viral isolations and antibody surveys. OPIV-3 has been isolated, from the lungs of sheep that had pneumonia, in the United Kingdom (Hore & Stevenson, 1969), in the U.S.A. (Lehmkhul & Cutlip, 1982), Canada (Ditchfield, 1966), Australia (St.George, 1969), New Zealand (Carter & Hunter, 1970), and Argentina (Epstein, B. 1974) among many other countries. In addition, viral antibodies to OPIV-3 have been detected in 80% of sheep in France, 90% in South Africa, 32.5% in Iran, 15.6% in Egypt, 53% in Britain, 60% in Nigeria and 40.9% in India (Brako et al., 1984). These are the results from the haemagglutination inhibition (HI) test. These numbers would probably be found to be higher with the virus neutralization test which is more sensitive than HI (St. George, 1971). These results indicate that OPIV-3 virus infection is widespread throughout the world.

Different PIV-3 strains have been identified in human and cattle producing respiratory tract disease (Klippmark et al., 1990) and their antigenic relationship has been investigated (Ray & Compans, 1986). Strains of this virus have also been identified in sheep (Ditchfield 1966; St George 1969; Carter & Hunter, 1970; Epstein, 1974;
Davies et al., 1977; Sharp et al., 1978; Lehmkuhl & Cutlip, 1982), however, the antigenic relation between ovine and the human or bovine strains is not clear.

The frequency of infection by OPIV-3 virus in sheep, determined by serological tests, seems to be quite high in Chile (68%), particularly in central and southern areas of the country (Berríos et al., 1987; Riedeman, et al., 1990). Here infection produces economic losses because of treatment costs incurred, delayed sale and death. PIV-3 has been isolated from cattle (Berríos et al., 1990), but there are no reports on isolations from sheep.

PIV-3 causes clinical disease in cattle and sheep, independent of its role in leading to secondary bacterial infections of the respiratory tract. In calves and lambs infection is marked by fever, lacrymation, serous nasal discharge, depression, dyspnea and coughing. Many animals may exhibit minimal clinical signs, but some may develop interstitial pneumonia. In the latter cases, lesions consisting of inflammatory consolidation are usually present only in the anterior lobes of the lungs. Because of the variety of agents that can cause these kinds of clinical and pathological manifestations, etiological diagnosis can only be achieved by virus isolation from nasal swabs or postmortem material. Virus isolation is usually done in cell cultures of bovine origin and virus is identified serologically by immunofluorescence, haemagglutination inhibition or virus neutralization (Fenner, 1987a,b).

The objective of this work was to isolate and characterise OPIV-3 from field cases of sheep pneumonia in Chile. The infectivity of the viral isolates was titrated in order to use them in experimentally reproducing respiratory disease in sheep. This allowed the production of pathological lung material, for studies on the immune cell response to PIV-3 infection that were carried out later during this study.
3.2 RESULTS

3.2.1 Isolation of ovine PIV-3 from clinical cases

Ten sheep, from southern areas in Chile, showing respiratory disease symptoms were necropsied and samples from nasal secretions and lung lesions were taken for OPIV-3 isolation. Viral isolates were obtained from three animals and identified as 430-7, 392-10 and 270-7. These three viral isolates showed the same characteristics as described for PIV-3.

The animals showed similar symptoms including increased respiratory rate, mouth breathing, mucopurulent rhinitis, anorexia and some weight loss.

3.2.2 Pathology

3.2.2.1 Gross pathology findings:

Gross lesions consisted of well demarcated, homogenous red or red-gray foci of consolidation scattered in all lobes but affecting in particular the anteroventral lung areas. Atelectasis was generally observed at the margins of the anterior lobes. In cases 392-10 and 430-7 a few small pulmonary abscesses were found with associated fibrous pleural adhesions. Most consolidated areas were dark red, slightly depressed, rounded or irregular in shape from 1 to 5cm in diameter. A fibrinopurulent exudate was present in nasal mucosa of sheep 430-7 (data not shown).

3.2.2.2 Histopathology findings:

Common microscopic lesions included diffuse subacute interstitial pneumonitis and secondary bronchopneumonia. There was bronchial and bronchiolar epithelium damage with swollen, defoliating and necrotic cells. Areas of consolidation were present in the parenchyma, due to extensive proliferation of septal cells and Mφs. A variable degree of peribronchial and peribronchiolar lymphocyte and Mφ accumulation
was present in all lungs (Fig.3.1a). A marked bronchiolar associated lymphoid tissue (BALT) hyperplasia was frequently observed. These lymphoid structures were surrounded by both atelectasis and emphysema (Fig.3.1b). Sometimes, bronchioles and small bronchi were occluded by necrotic debris and PMN infiltration (Fig.3.1c). In addition, perivascular lymphocytes cuffs were often observed. More severe lesions were found in cases 392-10 and 430-7 and consisted of areas of necrosis, focal abscessation, perivascular fibrosis and pleural inflammation and fibrosis (Fig. 3.1d).

3.2.2.3 **Bacteriology findings**

Material from lung specimens was inoculated into 5ml amounts of infusion broth and into 7% sheep blood agar, then incubated overnight at 37°C. Broth cultures were subcultured to blood agar the following day and suspected colonies of *Pasteurella haemolytica* were detected in samples from two lungs (392-10 and 430-7) and subcultured for further examination. Isolates of *Pasteurella haemolytica* biotype A, serotype 2 were obtained from these two lungs, according to the method described by Biberstein & Thompson (1966). No *Pasteurella spp* was isolated from the third lung (270-7). *Mycoplasma* was not detected in any of these three cases of sheep pneumonia.

3.2.2.4 **Serology**

Antibody titres to PIV-3 were determined in serum samples from the lambs (270-7, 392-10 and 430-7), taken before necropsy, using a microtitration serum-virus neutralization test (Lehmkuhl & Gough, 1977). A reference strain of OPIV-3\(^1\) and its antiserum, were used to carry out the test. The three lambs 270-7, 392-10 and 430-7 had positive SN titres of 640, 320 and 320, respectively.

---

\(^1\) Ovine isolate of PIV-3 named Moredun kindly supplied by Dr. Peter Nettleton (Moredun Institute, Edinburgh, UK)
FIGURE 3.1  Formalin fixed and paraffin embedded tissue sections of pneumonic lungs taken from lambs 270-7, 392-10 and 430-7.

a) A marked accumulation of mononuclear cells with lymphocytes (L) and Mφs is observed around the bronchioles (B) in lamb 270-7. H/E stain. Magnification x100.

b) Hyperplasia of bronchiolar associated lymphoid tissue (BALT) (arrow) surrounded by emphysema (E) and atelectasis (A) in lamb 270-7. Giemsa’s stain. Magnification x40.

c) Necrotic cell debris and PMNs (P) occluding the lumen of a small bronchiole. Intense accumulation of lymphocytes (L) around and beneath the epithelium in lamb 430-7. Giemsa’s stain. Magnification x100.

d) Pleural inflammation with an accumulation of lymphocytes (L) and a marked increase in thickness due to fibrosis (F). Van Gieson’s stain. Magnification x40.
3.2.3 Virus Isolation

3.2.3.1 Inoculation of cell cultures

The isolation and characterisation of the viral isolates were carried out in ovine foetal kidney (OFK), bovine foetal kidney (BFK) and ovine skin fibroblast (OSF) cell cultures. All cell culture were assessed as free of bovine viral diarrhea (BVD) virus. For comparison purposes and as positive controls reference virus strains\(^2\) were used including two bovine isolates of PIV-3 (BPIV-3) and one ovine isolate of PIV-3 (OPIV-3). Cell cultures were carried out in 24 well tissue culture plates with round coverslips and inoculated with 0.1 ml of inoculum from lung samples. Coverslips were removed after 1,2,3,4, and 5 days p.i.

3.2.3.2 Cytopathic effect (CPE)

The CPE induced by the three ovine isolates was studied in primary and secondary OFK BFK and OSF cell cultures. Observations by inverted light microscopy showed disruption of the cell monolayer with presence of large swollen round cells, as the earliest indication of CPE. This started from 24hrs. post inoculation (p.i.) and increased in severity through 48 hours p.i., when syncytia formation appeared. After 72 hours the virus had destroyed most of the cell culture, achieving rapidly 100% CPE. Syncytia began to shrink, became pyknotic and eventually sloughed into the medium (data not shown).

After 24 hrs. p.i., cell cultures stained with haematoxylin/eosin revealed the appearance of large round refractive cells, some of them in mitosis (Fig.3.2a). Whereas, the formation of cytoplasmic eosinophilic inclusions, irregular in shape and

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\(^2\) Bovine isolate of PIV-3 kindly supplied by Dr. Neil Watt (Edinburgh University, Veterinary Pathology Department, Field Station, Edinburgh, UK).

Bovine isolate of PIV-3 named La Pintana 84(Berrios et al., 19) kindly supplied by Dr. Patricio Berrios (Laboratorio de Virología, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile).

Ovine isolate of PIV-3 named Moredun kindly supplied by Dr. Peter Nettleton (Moredun Institute, Edinburgh, UK)
size and surrounded by a halo was most frequently observed after 48 hrs. p.i. (Fig.3.2b). At this time p.i. there was also syncytia formation (Fig.3.2c,d), that was produced by isolates 392-10 and 270-7, but not by isolate 430-7. Nuclear inclusions were rarely observed.

The CPE produced by the three isolates was similar to that observed after inoculation with two BPIV-3 and one OPIV-3 reference viruses.

3.2.3.3 *Transmission electron microscopy (TEM) of viral particles*

Material from cell cultures infected with the three ovine isolates was processed for negative staining and observed under transmission electron microscopy (TEM). Predominantly pleomorphic spherical viral particles of 150 to 320nm in size were found. Nucleocapsids with the typical herringbone arrangement could be seen within and spilling out of disrupted virus (data not shown).

Cell cultures were grown in 75 cm² tissue culture flasks and inoculated with ovine viral isolates 270-7, 430-7 and 392-10. Cells were collected by scraping them off after 1, 2, and 3 days p.i. Ultrathin sections were prepared from infected and mock-infected cultures and observed by TEM. The first sign of progeny viral development was the appearance of small filamentous cytoplasmic inclusions, after 24 hrs. p.i. These were usually located in a perinuclear position (Fig.3.3a). Inclusion material in the perinuclear region tended to be more tightly packed than the one located in other parts of the cytoplasm. At 48 hrs. p.i. cytoplasmic inclusions were larger and more numerous (Fig.3.3b). This material was only occasionally seen inside the nucleus (Fig.3.3c). Other changes in the cytoplasm included an increased number of polyribosomes, swollen mitochondria and displacement of cytoplasmic organelles by filamentous inclusions. This was concurrent with the first signs of viral maturation which included both presence of immature virus particles in cytoplasm and budding and release of virus particles inside cytoplasmic vesicles (Fig. 3.3d). Then budding was also observed at the cell membrane (Fig.3.3e). In isolated areas the cell membrane appeared thickened with the outer aspect being covered with a fringe (Fig.3.3f). Along the inner surface of the cell membrane lay a row of tubules cut in cross section. Adjacent to these areas and outside the cell there were roughly spherical particles (Fig.3.3g) completely surrounded by a similar fringed membrane
FIGURE 3.2 Time dependent cytopathic effect (CPE) in ovine foetal kidney (OFK) and ovine skin fibroblasts (OSF) cell cultures inoculated with ovine isolates 270-7, 430-7 and 392-10. Cell cultures were carried out in 24 well tissue culture plates with round coverslips and inoculated with 0.1 ml of inoculum from lung samples. Coverslips were removed after 1, 2, 3, 4, and 5 days p.i.

a) OFK cell culture after 24 hrs. p.i. with an inoculum of lung sample 430-7. Formation of large round cells which later detach from the cell culture monolayers is observed. H/E stain. Magnification x100.

b) BFK cell culture after 48 hrs. p.i. with inoculum of lung sample 270-7. There are numerous pleomorphic cytoplasmic eosinophilic inclusion bodies (arrows). H/E stain. Magnification x400.

c) OSF cell culture after 48 hrs. p.i. with inoculum of lung sample 392-10. Syncytia formation together with a cytoplasmic eosinophilic inclusion body are observed. H/E stain. Magnification x200.

d) OSF cell culture after 48 hrs. p.i. with inoculum of lung sample 270-7. Syncytia formation together with numerous pleomorphic cytoplasmic eosinophilic inclusion bodies are observed. H/E stain. magnification x200.
(Fig. 3.3h). These particles were pleomorphic and had an overall diameter of 150 to 320 nm. They were observed first inside the cytoplasm then budding through the cell membrane and finally detaching from the cell surface.

Cytoplasmic inclusions containing filamentous material were seen as early as 24 hours p.i., but were larger and more numerous after 48 and 72 hours p.i. Virus buds being most evident after 48 hours p.i. of cell cultures. Beneath the virus buds, ribonucleoprotein appeared to follow a helical configuration or as dots in cross section. The cytoplasmic membrane in some areas was covered with virus spikes (peplomers).

The ultrastructure of the three reference virus particles showed similar morphological characteristics, although BPIV-3 particles had a larger range for virus particle size of 210-520 nm (data not shown).

3.2.4 Titration of the ovine viral isolates

OFK cell cultures were infected separately with the three ovine isolates 430-7, 392-10 and 270-7. Culture fluid from the 5th passage was used to titrate virus infectivity. The titration considered the CPE induced in OFK cell cultures and the mean titre was expressed as mean TCID$_{50}$/0.1ml of cell culture fluid ±S.E. The haemagglutinating activity was assessed with guinea pig erythrocytes and the titre was expressed as the highest initial dilution of haemagglutinin causing complete agglutination of the erythrocytes (Table 3.1).

3.2.5 Viral physical and chemical properties

3.2.5.1 Sensitivity to extreme pH.

Buffers solutions pH 3.0, 7.0 and 9.0 were prepared (see section 2.2.1.4.1), then 10 fold virus dilutions were made with them to be incubated at 40°C for 15 minutes, finally the extreme pHs were neutralized by adding NaOH or HCl 0.25M. After being exposed to pH 3.0 and 9.0 media conditions for 15 minutes, the three ovine viral isolates as well as the reference virus, lost infectivity. In contrast, they did not show
FIGURE 3.3 Transmission electron microscopy (TEM) of OFK and OSF cell cultures infected after OPIV-3 infection. Cell cultures were grown in 75 cm$^3$ tissue culture flasks and inoculated with ovine viral isolates 270-7, 430-7 and 392-10. Cells were collected by scraping them off after 1, 2, and 3 days p.i.

a) OSF cell culture after 24 hrs. p.i. with ovine viral isolate 392-10. There is an accumulation of filamentous material (arrow) in the cytoplasm close to the perinuclear area. TEM. Magnification x46,000.

b) OSF cell culture after 48 hrs. p.i. with ovine viral isolate 270-7. A large inclusion of filamentous material tightly packed can be observed close to the nucleus. TEM. Magnification x16,500.

c) OSF cell culture after 48 hrs. p.i. with ovine viral isolate 430-7. A large inclusion of filamentous material is observed inside the nucleus. TEM. Magnification x16,500.

d) OSF cell culture after 48 hrs. p.i. with ovine viral isolate 430-7. Virus budding in the membrane of the cytoplasmic vesicles can be seen with an accumulation of virus particles inside these vesicles. TEM. Magnification x215,000.

e) OSF cell culture after 48 hrs. p.i. with ovine viral isolate 430-7. Virus budding can be seen from the cell membrane with viral particles detaching from it. TEM. Magnification x215,000.

f) OSF cell culture after 48 hrs. p.i. with ovine viral isolate 270-7. An incomplete virus particle can be seen in an early stage of virus budding. The virus particle is inside the cytoplasm close to the cell membrane. The cell membrane appears thickened at this point with the outer aspect being covered by a fringe. TEM. Magnification x315,000.

g) OSF cell culture after 48 hrs. p.i. with ovine viral isolate 270-7. A group of pleomorphic virus particles (150-320nm) can be seen after having been released from the cell membrane. TEM. Magnification x215,000.

h) OSF cell culture after 48 hrs. p.i. with ovine viral isolate 270-7. Virus particle surrounded by a fringed membrane after being released. TEM. Magnification x315,000.
alterations in infectivity when maintained in standard MEM culture media or exposed to pH 7.0 media conditions for 15 minutes (Table 3.2).

### 3.2.5.2 Sensitivity to lipidic solvents

The sensitivity to ether was performed according to a method described by Andrewes & Horstman (1949). The sensitivity to chloroform was assessed following the method described by Feldman & Wang (1961). The virus was suspended in 20% v/v ether or 33% chloroform, in culture media (the control was suspended in 20% v/v Puck’s saline solution A). The three ovine isolates together with the reference viruses lost their infectivity after being exposed to 20% ether for 24 hrs. and to 33% chloroform for 10 minutes (Table 3.3).

### 3.2.5.3 Sensitivity to trypsin

The sensitivity to trypsin was shown using a method described by Gresser & Enders (1961). 1%, 0.5% and 0.25% solutions of trypsin were prepared with Puck’s saline solution A. Then, 1 ml of virus suspension was mixed with 1ml of the trypsin dilution. Tubes were incubated at 37°C in a thermoregulated bath for 60 minutes. As control, 1 ml virus suspension was mixed with 1 ml of Puck’s saline solution A. Then they followed the same procedure.

The three ovine PIV-3 isolates along with the reference virus lost their infectivity after being treated with high concentrations of trypsin. Virus isolate 392-10 and the reference virus showed decreased infectivity after being treated with 0.25% and 0.5% trypsin dilutions and lost all infectivity after 1% trypsin treatment. Isolates 430-7 and 270-7 did not show change in infectivity titre after 0.25% trypsin treatment but lost infectivity after being treated with both 0.5% and 1% trypsin (Table 3.4).

### 3.2.5.4 Heat sensitivity

Test tubes were previously warmed up to 56°C and then 0.5ml of virus suspension was added to each of them. They were immediately taken to a thermoregulated bath at 56°C. Each tube was removed from the thermoregulated bath at different times
TABLE 3. 1 Titration of both virus infectivity in ovine foetal kidney (OFK) cell cultures and haemagglutinating activity (HA) of guinea pig erythrocytes for ovine viral isolates.

<table>
<thead>
<tr>
<th>Viral isolate</th>
<th>TCID$_{50}$/ml$^1$</th>
<th>IHA titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>392-10.</td>
<td>6.5$^1$</td>
<td>256</td>
</tr>
<tr>
<td>430-7</td>
<td>6.2</td>
<td>128</td>
</tr>
<tr>
<td>270-7</td>
<td>6.9</td>
<td>1024</td>
</tr>
<tr>
<td>Reference OPIV-3</td>
<td>5.8</td>
<td>512</td>
</tr>
</tbody>
</table>

1 Calculated according to method by Reed & Muench (1938).

2 Titre expressed as Log$_{10}$ TCID$_{50}$/1ml of virus.

TABLE 3. 2 Sensitivity of three ovine viral isolates to extreme pH conditions during 15 minutes.

<table>
<thead>
<tr>
<th>Viral isolate</th>
<th>pH</th>
<th>Control( MEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.0</td>
<td>7.0</td>
</tr>
<tr>
<td>392-10</td>
<td>0.0</td>
<td>6.5*</td>
</tr>
<tr>
<td>430-7</td>
<td>0.0</td>
<td>6.2</td>
</tr>
<tr>
<td>270-7</td>
<td>0.0</td>
<td>6.9</td>
</tr>
<tr>
<td>Reference OPIV-3</td>
<td>0.0</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* Titre expressed as Log$_{10}$ TCID$_{50}$/1ml of virus.
**TABLE 3.3** Sensitivity of three ovine viral isolates of PIV-3 to lipidic solvent exposure.

<table>
<thead>
<tr>
<th>Viral isolate</th>
<th>Ether 20%</th>
<th>Chloroform 33%</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>392-10</td>
<td>0</td>
<td>0</td>
<td>6.5*</td>
</tr>
<tr>
<td>430-7</td>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td>270-7</td>
<td>0</td>
<td>0</td>
<td>6.9</td>
</tr>
<tr>
<td>Reference OPIV-3</td>
<td>0</td>
<td>0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Titre expressed as $\log_{10} \text{TCID}_{50}/\text{ml}$ of virus suspension.

**TABLE 3.4** Sensitivity of the three ovine viral isolates to 60 minutes trypsin treatment.

<table>
<thead>
<tr>
<th>Viral isolate</th>
<th>Trypsin solution</th>
<th>Saline solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25%</td>
<td>0.5%</td>
</tr>
<tr>
<td>----------------</td>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>392-10</td>
<td>5.8</td>
<td>2.1</td>
</tr>
<tr>
<td>430-7</td>
<td>6.5</td>
<td>0</td>
</tr>
<tr>
<td>270-7</td>
<td>6.9</td>
<td>0</td>
</tr>
<tr>
<td>Reference OPIV-3</td>
<td>5.1</td>
<td>2.6</td>
</tr>
</tbody>
</table>

* Titre expressed as $\log_{10} \text{TCID}_{50}/\text{ml}$ of virus suspension
(0, 5, 10, 20 and 30 min.). The three viral isolates and the reference virus were sensitive to exposure to 56°C for 30 minutes. After this exposure they lost their infective capacity. Virus isolate 392-10 lost its infectivity completely after 30 minutes exposure whereas the other two lost all infectivity after 20 minutes of exposure to 56°C (Table 3.5).

3.2.6 Antigenic relationships.

3.2.6.1 Comparison of three ovine isolates of PIV-3 (270-7, 392-10 and 430-7) and reference strain of OPIV-3 by reciprocal cross inhibition of haemagglutination.

The antigenic relationships among the three ovine isolates and reference strain of OPIV-3 were studied by a two-way cross-inhibition of haemagglutination test. The HI antibody titre of the serum was taken as the highest initial dilution of serum which affected complete inhibition of haemagglutination.

Homologous titres ranged from 1280 to 640 and were higher than, or equivalent, to those obtained with any of the heterologous strains (Table 3.6). The three ovine isolates (270-7, 290-10 and 430-7), appeared to be closely related to each other, less so to reference OPIV-3, although none of the differences could be considered notable. Furthermore, there was no evidence that any of these ovine isolates was serologically distinct from the others or from the reference strain. There was, however, a distinct one-sided relationship between reference OPIV-3 and sera raised against the other ovine isolates.

3.2.6.2 Comparison of three ovine isolates of PIV-3 (270-7, 392-10 and 430-7) and reference strains of OPIV-3, BPIV-3 and HPIV-3 by reciprocal cross serum-virus neutralisation test.

In view of the results of the cross-haemagglutination test using OPIV-3 reference strain, it was considered worthwhile to investigate further the antigenic relationship between these ovine isolates of PIV-3 (270-7, 392-10 and 430-7) and reference PIV-3 from different species. This time a two-way cross serum virus neutralisation test was
TABLE 3.5 Sensitivity of three ovine viral isolates to different times of exposure at 56°C.

<table>
<thead>
<tr>
<th>Viral isolate</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>392-10</td>
<td>6.5</td>
<td>5.5</td>
<td>3.5</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>430-7</td>
<td>6.2</td>
<td>4.5</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>270-7</td>
<td>6.9</td>
<td>5.6</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Reference OPIV-3</td>
<td>6.2</td>
<td>5.0</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Titre expressed as Log_{10} TCID_{50}/ml of virus suspension.

TABLE 3.6 Comparison of antigenic relationships among three ovine PIV-3 isolates and reference OPIV-3 (OPIV-3r) strain in a two-way cross-haemagglutination inhibition test.

<table>
<thead>
<tr>
<th>Viral isolate</th>
<th>392-10</th>
<th>430-7</th>
<th>270-7</th>
<th>OPIV-3r</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>392-10</td>
<td>640&lt;sup&gt;1&lt;/sup&gt;</td>
<td>320</td>
<td>640</td>
<td>160</td>
<td>&lt;5</td>
</tr>
<tr>
<td>430-7</td>
<td>640</td>
<td>640</td>
<td>320</td>
<td>160</td>
<td>&lt;5</td>
</tr>
<tr>
<td>270-7</td>
<td>640</td>
<td>640</td>
<td>1280</td>
<td>320</td>
<td>&lt;5</td>
</tr>
<tr>
<td>OPIV-3r</td>
<td>320</td>
<td>320</td>
<td>640</td>
<td>640</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Control&lt;sup&gt;2&lt;/sup&gt;</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>640</td>
</tr>
</tbody>
</table>

<sup>1</sup> Median titre is expressed as reciprocal of serum dilution

<sup>2</sup> BVDV-161 kindly supplied by Dr. Patricio Berrios (Laboratorio de Virologia, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile).
used (Lehmkuhl & Gough, 1977) and reference strains including ovine PIV-3 (OPIV-3), bovine (BPIV-3) and human (HPIV-3) strains. All samples were tested in triplicate, using twofold dilutions (from 1:2 to 1:512). The serum-virus neutralization titres were the highest dilution of serum in which 50% of the wells were protected from the viral CPE.

Homologous titres ranged from 1280 to 640; however the titres in general were higher in the SN test. Homologous titres were higher than, or equivalent, to those obtained with any of the heterologous strains (Table 3.7). The three ovine isolates (270-7, 290-10 and 430-7), appeared to be more closely related to each other than to any of the reference PIV-3 strains. They appeared to be less related to HPIV-3 than to OPIV-3 or BPIV-3. However, again none of the differences could be considered significant. Also, there was no evidence that any of these ovine isolates was serologically distinct from the others or from the reference strains. Similar to that observed with HI there was a distinct one-sided relationship between reference OPIV-3 and sera raised against the three ovine isolates. Furthermore, the same pattern of relation was observed between the BPIV-3 reference strains and the three ovine isolates.

3.2.7 Identification of OPIV-3 particles in infected cell cultures by immunofluorescence and immunocytochemistry

Every staining was carried out in triplicate using the rabbit anti-sera against both the ovine viral isolates and the reference virus. Monoclonal antibodies raised against HN (4.795) and F (3.283) proteins of human PIV-3 and showing reactivity with bovine PIV-3 (Klippmark et al., 1990) were also used to detect viral particles in lung lesions of infected lambs. Previously the sera and monoclonal antibodies were titrated in order to determine optimal dilutions for immunostaining.

3.2.7.1 Indirect immunofluorescence (IF)

OFK and OSF cell cultures were infected separately with each one of the three ovine isolates and also with the reference virus. Positive fluorescence was detected in all these infected cultures after incubation with either homologous or heterologous rabbit anti-sera, followed by incubation with FITC-conjugated anti-rabbit Ig. The
TABLE 3. 7 Comparison of antigenic relationships between three ovine PIV-3 isolates and reference PIV-3 strains from different species by two-way cross serum-virus neutralization test.

<table>
<thead>
<tr>
<th>Virus</th>
<th>BPIV-3/CH</th>
<th>BPIV-3/UK</th>
<th>HPIV-3</th>
<th>OPIV-3</th>
<th>392-10</th>
<th>430-7</th>
<th>270-7</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>392-10</td>
<td>320³</td>
<td>320</td>
<td>160</td>
<td>320</td>
<td>1280</td>
<td>640</td>
<td>640</td>
<td>&lt;5</td>
</tr>
<tr>
<td>430-7</td>
<td>320</td>
<td>640</td>
<td>160</td>
<td>160</td>
<td>640</td>
<td>1280</td>
<td>640</td>
<td>&lt;5</td>
</tr>
<tr>
<td>270-7</td>
<td>640</td>
<td>320</td>
<td>320</td>
<td>640</td>
<td>1280</td>
<td>640</td>
<td>1280</td>
<td>&lt;5</td>
</tr>
<tr>
<td>BPIV-3/CH</td>
<td>1280</td>
<td>640</td>
<td>320</td>
<td>640</td>
<td>160</td>
<td>160</td>
<td>320</td>
<td>&lt;5</td>
</tr>
<tr>
<td>BPIV-3/UK</td>
<td>320</td>
<td>640</td>
<td>320</td>
<td>320</td>
<td>160</td>
<td>160</td>
<td>320</td>
<td>&lt;5</td>
</tr>
<tr>
<td>HPIV-3</td>
<td>ND</td>
<td>ND</td>
<td>ND³</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>OPIV-3</td>
<td>640</td>
<td>640</td>
<td>160</td>
<td>1280</td>
<td>640</td>
<td>640</td>
<td>1280</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>640</td>
<td></td>
</tr>
</tbody>
</table>

1 BPIV-3/CH=antiserum to bovine isolate of PIV-3 named La Pintana 84 (Berrios et al., 1991) kindly supplied by Dr. Patricio Berrios (Laboratorio de Virología, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile).
BPIV-3/UK=antiserum to bovine isolate of PIV-3 kindly supplied by Dr. Neil Watt (Edinburgh University, Veterinary Pathology Department, Field Station, Edinburgh, UK).
HPIV-3=antiserum to human isolate of PIV 3 used to raise monoclonal antibodies anti HPIV 3 viral proteins (Klipppmark et al., 1990), kindly supplied by Dr. Robert Rydbeck, Virology Department, Karolinska Institute, Stockholm, Sweden.
OPIV-3=antiserum to ovine isolate of PIV-3 named Moredun kindly supplied by Dr. Peter Nettleton (Moredun Institute, Edinburgh University, Edinburgh, UK).
2 BVDV-161 kindly supplied by Dr. Patricio Berrios (Laboratorio de Virologia, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile).
3 Median titre is expressed as reciprocal of serum dilution.
4 Not done for this experiment. Reference titre with homologous virus=640.
fluorescence staining pattern was of high intensity and involved both the cell membrane and cytoplasm (Fig. 3.4a). Immunostaining of the same infected cultures with monoclonal antibody HN4.795 (against HPIV-3 HN protein), showed a more scattered and discontinuous staining pattern of cell membrane and cytoplasm. Furthermore, the intensity of staining was lower (Fig. 3.4b). On the other hand, no reaction was observed after incubation with monoclonal antibody F3.283 (against HPIV-3 F protein).

In general, the majority of cells in the infected cultures showed some degree of fluorescence, particularly large round cells that were detaching from the cell monolayers (Fig. 3.4a,b). Although fluorescence was detected from 24 hrs. p.i. the fluorescence intensity was greatest at 48 hrs. p.i. Non-infected control cultures and infected cultures incubated with normal rabbit sera did not reveal fluorescence. The specific fluorescence seen in infected cultures first appeared in occasional cells by 24 hrs. p.i. By 48 hrs. small groups of cells were positive and by 72 hrs. p.i. most of the cell monolayer was disrupted and the remaining cells were reacting intensely for PIV-3 staining. Specific fluorescence first appeared in the perinuclear area, usually as a diffuse fluorescence which spread to larger areas of the cytoplasm. In a few cells early fluorescence appeared as bright pin-points on the nuclear membrane. At 48 to 72 hrs. p.i. the whole cytoplasm including the long cytoplasmic processes of spindle-shaped cells was positive. Very bright fluorescence occurred occasionally at the margins of the cytoplasm (Fig. 3.4a). No specific nuclear fluorescence was detected, although a hazy and somewhat diffuse fluorescence was seen in some nuclei in the later stages of infection.

3.2.7.2 Immunocytochemistry

OFK cell cultures, infected with either the ovine isolates or the reference virus were stained by both indirect immunoperoxidase (IIP) and indirect immunoalkaline phosphatase (IAP) methods. These techniques were carried out using rabbit antisera to OPIV-3 and monoclonal antibodies to HPIV-3. The virus-antibody reaction was visualized by the appearance of a brown colour in the case of IIP and of a red colour in the case of IAP. The reactivity with the antibodies showed a similar pattern as for IIF in that positive staining was achieved with anti OPIV-3 polyclonal sera and monoclonal antibody 4.795 (anti HPIV-3 HN protein) but not with monoclonal
antibody 3.283 (anti HPIV-3 protein F). The staining pattern was similar to that observed after IIF. This included the staining of the cell membrane and cytoplasm but not of nuclei (Fig.3.4c,d). After IIP and IIAP it is possible to counterstain nuclei and observe under light microscope; this gives samples with a better morphology and allows identification of cell types and their characteristics. Here a marked staining of cytoplasm of large round cells was also observed. Many of these cells were undergoing mitosis (Fig.3.4c,d). Along with the positive staining of cell membrane and cytoplasm a number of multiple intracytoplasmic inclusion bodies were observed (Fig.3.4c). There were no significant differences between staining with homologous and heterologous rabbit anti-sera. Some background was detected with control staining by IIP, but it did not interfere with interpretation of positive staining. The staining with monoclonal antibody showed no background after either IIP or IIAP staining.

3.2.8 Reactivity of a panel of monoclonal antibodies anti-HPIV-3 with OPIV-3 isolates by flow cytometry.

Monoclonal antibody F3.283 recognizes an epitope present in protein F of both HPIV-3 and BPIV-3 (Klippmark et al., 1990). OFK and OSF cell cultures, infected with either the ovine isolates or the reference OPIV-3, were incubated with this mAb for virus detection by immunostaining (see section 3.2.7). No expression for this epitope of protein F was detected in either cell membrane or cytoplasm. This was thought to be due to antigenic differences between the ovine isolates and both HPIV-3 and BPIV-3. In order to investigate if there were any further antigenic differences, a panel of murine mAbs anti HPIV-3 proteins (see Table 3.8) was tested with both the three ovine isolates and the reference OPIV-3. All these mAbs have cross reactivity with epitopes on the same proteins of BPIV-3 (Klippmark et al., 1990). Reference BPIV-3 was also used as a control for the reactivity of the mAb panel and for comparison.

The reactivity of this mAb panel with viral proteins expressed either on the cell surface or cytoplasm of infected OSF cells was detected by indirect immunofluorescence (IIF). OSF cells were cultured in 75cm3 flasks and were infected

3 Kindly supplied by Dr. Robert Rydbeck, Virology Department, Karolinska Institute, Stockholm, Sweden.
FIGURE 3.4 Immunostaining of OFK and OSF cell cultures infected with ovine viral isolates 270-7, 430-7 and 392-10. Cell cultures were grown in 24 well plates with round coverslips. After 1, 2, 3 and 5 days p.i. coverslips were removed and immunostained by IIF, IIP or IIAP using anti OPIV-3 rabbit antisera or monoclonal antibodies (HN 4.795 and F3.283).

a) OFK cell culture after 48 hrs. p.i. with ovine viral isolate 430-7. After staining with OPIV-3 rabbit antisera there is a high intensity of fluorescence of the cell membrane and the cytoplasm. Very bright fluorescence can be observed sometimes at the margins of the cytoplasm. IIF. Magnification x400.

b) OFK cell culture after 48 hrs. p.i. with ovine viral isolate 430-7. After staining with monoclonal antibody HN 4.795 there is an intermediate intensity of fluorescence of the cell membrane and the cytoplasm. The photograph was taken with the same exposure time as in a). Magnification x400.

c) OFK cell culture after 48 hrs. p.i. with ovine viral isolate 392-10. After staining with rabbit antisera there is stronger staining (brown colour) of the cell membrane and the cytoplasm of round cells(large arrows) than in cells showing pleomorphic cytoplasmic inclusions (small arrows). IIP. Magnification x400.

d) OFK cell culture after 48 hrs. p.i. with ovine viral isolate 270-7. After staining with rabbit antisera there is strong staining (red colour) of the cell membrane and the cytoplasm of round cells. In the other cells the staining is seen mostly in the cell membrane (small arrows). IIP. Magnification x400.
with either of the three ovine isolates (270-7, 392-10 or 430-7) or reference strains (OPIV-3 or BPIV-3). Cell cultures were infected with the virus at 0.5 TCID₅₀ per cell or mock infected 1, 2 or 3 days before immunostaining by IIF. Viral proteins expressed on the cell surface were detected on non-fixed cells as previously described (section 2). IIF was carried out in rhesus tubes as described in section 2. and analyzed by flow cytometry. OSF cells infected with the BPIV-3 reference strain expressed all proteins epitopes detected by this mAb panel in a moderate to high amount, according to the mean fluorescence intensity. Fig. 3.5. shows a profile with the time dependent expression of different viral protein epitopes in OSF cultures after infection with BPIV-3.

The reactivity of the mAb panel with OSF cell cultures infected with either of the ovine isolates or OPIV-3 reference virus was very similar in each case. Generally no significant differences (at p≤0.05) were detected in comparing the reactivity among the three ovine isolates with this mAb panel against HPIV-3 antigenic proteins. In contrast, some significant differences were found when their reaction was compared to that shown by OSF cells infected with the BPIV-3 reference strain. The expression of the PIV-3 protein epitopes, detected by the monoclonal panel, was analysed in OFS cultures after 3 days of infection. Table 3.8. shows the comparison of expression between cultures infected with either of the three OPIV-3 isolates and cultures infected with BPIV-3 or OPIV-3 reference strains.

The reactivity of mAbs HN4771 and HN 4795, against two different epitopes of HN protein from both HPIV-3 and BPIV-3 (see Table 3.8), was tested. These two mAbs reacted with the three ovine isolates and also with reference strains of OPIV-3 and BPIV-3. No highly significant differences (at p≤0.05) in the expression of the epitopes detected by these mAbs were observed among the ovine isolates. However, in cells infected with either of the ovine derived virus strains there was a significantly lower intensity of expression for both HN epitopes 4.771 and 4795 (p<0.05 and p<0.001, respectively) when compared to that shown by BPIV-3 infected cells (Fig.3.6).

Monoclonal antibodies F3263, F4503 and F4673 react with two different epitopes of F protein from HPIV-3 and BPIV-3 (see Table 3.8). None of these mAbs reacted with any of the ovine derived virus strains (Fig.3.7). In contrast, the reactivity of the three mAbs against HPIV-3 F protein with reference BPIV-3 was of very high intensity. Staining on fixed cells in PBS 80% methanol was carried out to detect
TABLE 3.8 Reactivity of a monoclonal antibody panel anti HPIV-3/BPIV-3 viral proteins with OSFa cultures after 3 days post infection with ovine OPIV-3 isolates (270-7, 392-10 and 430-7), by flowcytometry.

<table>
<thead>
<tr>
<th>Viral Protein</th>
<th>MAb</th>
<th>Epitope</th>
<th>OPIV-3 Virus isolates</th>
<th>PIV-3 reference strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td></td>
<td>270-7</td>
<td>392-10</td>
</tr>
<tr>
<td>HN</td>
<td>4771</td>
<td>II</td>
<td>40.5±3.8c,d</td>
<td>35.1±5.2d</td>
</tr>
<tr>
<td></td>
<td>4795</td>
<td>III</td>
<td>40.3±4.4e</td>
<td>39.3±4.5e</td>
</tr>
<tr>
<td>F</td>
<td>3263</td>
<td>I</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4503</td>
<td>I</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4673</td>
<td>II</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>NP</td>
<td>4721</td>
<td>III</td>
<td>40.6±4.3d</td>
<td>43.7±3.9d</td>
</tr>
<tr>
<td></td>
<td>4794</td>
<td>V</td>
<td>56.2±4.7</td>
<td>49.2±5.1</td>
</tr>
<tr>
<td></td>
<td>4890</td>
<td>II</td>
<td>62.7±5.2</td>
<td>59.5±5.8</td>
</tr>
<tr>
<td>M</td>
<td>2873</td>
<td>III</td>
<td>07.5±3.5e</td>
<td>08.1±5.0e</td>
</tr>
<tr>
<td></td>
<td>3471</td>
<td>III</td>
<td>10.3±4.0e</td>
<td>13.5±3.8e</td>
</tr>
<tr>
<td></td>
<td>4877</td>
<td>V</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

a=Ovine skin fibroblasts (OSF) cultures were infected with the virus at 0.5 TCID50/cell
b=different group indicate that the mAb recognizes different epitope (Rydbeck et al., 1986)
c=reactivity expressed as mean of fluorescence±SD from 5 separate experiments. Fluorescence of negative control (NMS) has been subtracted.
d=indicates a significant difference (p<0.05) from BPIV-3 strains (Mann-Whitney non-parametric rank test).
e=indicates a significant difference (p<0.001) from BPIV-3 strains (Mann-Whitney non-parametric rank test).
FIGURE 3.5 Frequency histograms showing the time dependent reactivity of mAbs, against distinct HPIV-3 protein epitopes, with BPIV-3 infected OSF cell cultures. Ovine skin fibroblast (OSF) cultures were infected with BPIV-3 reference strain at 0.5 TCID<sub>50</sub>/cell. Cell cultures were infected 1, 2 or 3 days before immunostaining. All cells were harvested and incubated with mAbs anti HPIV-3 proteins on the same day. The profiles show the reactivity after 1 day p.i. (red line); 2 days p.i. (green line) and 3 days p.i. (blue line). The red shaded area shows the reactivity with mock-infected cells.
Reactivity of monoclonal antibodies HN4771 and HN4795 with OPIV-3 and BPIV-3 strains

FIGURE 3.6 Reactivity of monoclonal antibodies HN4771 and HN4795, against HN protein of HPIV-3, with OPIV-3 and BPIV-3 strains. Both mAbs recognize distinct epitopes. OFS cell cultures were infected with ovine viral isolates (270-7, 430-7 and 392-10) and OPIV-3 and BPIV-3 reference strains. Cells were collected 3 days p.i. and stained with mAbs HN4771 and HN4795 and analysed by flow cytometry. Data from five separate experiments is expressed as the mean of fluorescence intensity ± SD with negative control fluorescence (NMS) subtracted. (*) indicates a significant difference (p<0.05) from OPIV-3 strains; (**) indicates a significant difference (p<0.001) from OPIV-3 strains (Mann-Whitney non parametric rank test).
Reactivity of monoclonal antibodies F3263, F4503 and F4673 with OPIV-3 and BPIV-3 strains.

**FIGURE 3.** Reactivity of monoclonal antibodies F3263, F4503 and F4673, against F protein of HPIV-3, with OPIV-3 and BPIV-3 strains. Monoclonal antibodies F3263 and F4503 recognize the same epitope. OSF cell cultures were infected with ovine viral isolates (270-7, 430-7 and 392-10) and OPIV-3 and BPIV-3 reference strains. Cells were collected 3 days p.i. and stained with mAbs F3263, F4503 and F4673 and analysed by flow cytometry. Data from five separate experiments is expressed as the mean of fluorescence intensity ±SD with negative control fluorescence (NMS) subtracted.
intracytoplasmic antigen. No reaction was detected with any of these three mAbs against HPIV-3 F protein epitopes, after 1, 3, 5 or 7 days p.i. in cells infected with OPIV-3 isolates (data not shown).

Monoclonal antibodies NP4721, NP4794 and NP4890 react with three distinct epitopes of NP protein from HPIV-3 and BPIV-3 (see Table 3.8). The reactivity of these three mAbs with the ovine derived virus strains and also with the reference BPIV-3 was of high to moderate intensity. Cells infected with ovine isolate 430-7 and stained with mAb NP4721 showed significantly lower (p<0.05) intensity of fluorescence when compared to cells infected with the other ovine derived isolates. Likewise, the staining with this mAb was significantly lower (p<0.05) in cells infected with either the ovine derived isolates 270-7, 392-10 or reference OPIV-3 when compared to cells infected with BPIV-3. This difference was more marked (p<0.001) between ovine viral isolate 430-7 and BPIV-3. In general, no significant differences (at p<0.05) were observed in intensity of fluorescence after staining with mAb NP4794, except for the significantly lower (p<0.05) staining of cells infected with ovine isolate 430-7 in comparison to the staining with any of the other strains tested. Finally, the reactivity of mAb NP4890 with all strains tested was very similar in each case (Fig. 3.8).

Monoclonal antibodies M2873, M3471 and M4877 react with two distinct epitopes of M protein from HPIV-3 and BPIV-3 (see Table 3.8). Both mAbs M2873 and M3471 showed similar low reactivity with all ovine derived virus strains (Fig. 3.9). This reactivity was significantly lower (p<0.001) in comparison to the reactivity observed with BPIV-3. Staining with mAb M4877 showed no reactivity with any of the ovine derived virus strains. In contrast, cells infected with reference BPIV-3 showed a moderate but much higher intensity of fluorescence after staining with any of these three mAbs against HPIV-3 M protein. A representative profile is shown in Fig. 3.10 with the reactivity of mAbs that showed major differences of staining between cells infected ovine derived viral isolates and BPIV-3 reference strain.
Reactivity of monoclonal antibodies NP4721, NP4794 and NP4890 with OPIV-3 and BPIV-3 strains

FIGURE 3.8 Reactivity of monoclonal antibodies NP4721, NP4794 and NP4890 against NP protein of HPIV-3, with OPIV-3 and BPIV-3 strains. These three mAbs recognize distinct epitopes. OFS cell cultures were infected with ovine viral isolates (270-7, 430-7 and 392-10) and OPIV-3 and BPIV-3 reference strains. Cells were collected 3 days p.i. and stained with mAbs NP4721, NP4794 and NP4890 and analysed by flow cytometry. Data from five separate experiments is expressed as the mean of fluorescence intensity ±SD with negative control fluorescence (NMS) subtracted. (a),(b) and (c) indicate a statistically different group. (a) is significantly different from (b) at p<0.05; b is significantly different from (c) at p<0.001 and (a) is significantly different from (c) at p<0.05 (Mann-Whitney non parametric rank test).
Reactivity of monoclonal antibodies M2873, M3471 and M4877 with OPIV-3 and BPIV-3 strains.

**FIGURE 3.9** Reactivity of monoclonal antibodies M2873, M3471 and M4877 against M protein of HPIV-3, with OPIV-3 and BPIV-3 strains. Monoclonal antibodies M2873 and M3471 recognize the same epitope. OFS cell cultures were infected with ovine viral isolates (270-7, 430-7 and 392-10) and OPIV-3 and BPIV-3 reference strains. Cells were collected 3 days p.i. and stained with mAbs M2873, M3471 and M4877 and analysed by flow cytometry. Data from five separate experiments is expressed as the mean of fluorescence intensity ±SD with negative control fluorescence (NMS) subtracted. (*) indicates a significant difference (p<0.001) from OPIV-3 strains (Mann-Whitney non parametric rank test).
FIGURE 3. Frequency histograms showing the reactivity of mAbs against distinct HPIV-3 viral protein epitopes (—) with OSF cultures infected with BPIV-3 or OPIV-3 isolates (270-7, 392-10 and 430-7). The reactivity of rabbit OPIV-3 antisera is shown for comparison. Ovine skin fibroblasts (OSF) cultures were infected with the virus at 0.5 TCID_{50}/cell, 3 days before immunostaining. The reactivity with NMS was used as negative control of staining (....).
BPIV-3

HN 4795

F 3263

NP 4721

M 4877

OPIV-3 antisera

Log10 Fluorescence Intensity →
OPIV-3 270-7

HN 4795

F 3263

NP 4721

M 4877

OPIV-3 antisera

Log10 Fluorescence Intensity
OPIV-3 392-10

HN 4795

F 3263

NP 4721

M 4877

OPIV-3 antisera

Log10 Fluorescence Intensity →
OPIV-3 430-7

HN 4795

F3263

NP 4721

M 4877

OPIV-3 antisera

Log10 Fluorescence Intensity
In this study, three viral isolates (270-7, 392-10 and 430-7) were obtained from field cases of sheep pneumonia in Chile. According to characteristics of their cytopathic effect (CPE) in OFK and BFK cell cultures; morphology under TEM; physicochemical properties; serological relationship with PIV-3 reference viruses and associated lung pathology, they were classified as ovine strains of PIV-3. All the characteristics described for these viral isolates, in this study, are compatible with those reported for PIV-3 (Kingsbury et al., 1978; Andrewes & Pereira, 1978).

The cytoplasmic inclusions found in this study, in OFK cell cultures infected with the ovine viral isolates, are morphologically similar to those previously reported for PIV-3 isolated from sheep (Hore, 1966; Lehmkuhl & Cutlip, 1982); cattle (Omar, 1965; McLean & Doane, 1971) and humans (Porter et al., 1991). These characteristic inclusions have been described as large, irregular but homogeneous eosinophilic and “halo-bound” (see Fig.3.2b). Furthermore, the presence of these cytoplasmic inclusions together with syncytia formation have been, since long ago, regarded as the typical features of the cellular pathology of paramyxoviruses (Omar, 1965).

Intranuclear viral inclusions were rarely observed as part of the CPE produced by the OPIV-3 isolates. This low incidence of intranuclear inclusions has been observed also by Singh & Pathak (1977) and by Ditchfield (1966) who isolated OPIV-3, from sheep, in BFK cell cultures. In fact these intranuclear viral inclusions may not be observed at all in some cases. This could be explained by differences in m.o.i., type of cell culture system used or virus infectivity properties (Lehmkuhl & Cutlip, 1982). Also, this may be related to the origin of the virus. One the most striking differences between bovine strains of PIV-3 and other parainfluenza viruses (included OPIV-3) is seen in the ability of BPIV-3 to cause both abundant nuclear and cytoplasmic inclusions (McLean & Doane, 1971). This, together with some viral morphological characteristics, made McLean & Doane (1971) suggest setting BPIV-3 apart as a unique member of parainfluenza viruses, closer to the antigenically unrelated measles-rinderpest-distemper subgroup of paramyxoviruses. In contrast, OPIV-3 in its cytopathology and morphology more closely resembles the parainfluenza viruses.
Ovine PIV-3 isolates 392-10 and 210-7 produced a CPE which included syncytia formation. This effect was not observed with ovine isolate 430-7 or the bovine reference virus, La Pintana 84 (Berrios et al., 1990). It has been reported that there are differences in syncytia formation capacity by different PIV-3 isolates according to the type of cell culture used for virus replication. Thus, Ebata et al. (1991), observed that the same PIV-3 human strain showed different fusion activity in different cell culture systems. They attributed this to the magnitude of HN and F glycoprotein expression. It is also possible that syncytia formation is affected by the number of virus receptor molecules expressed on the host cell membranes.

In this study the ovine isolates induced 100% CPE in OFK and BFK cell cultures after 3 days p.i. This agrees with the time required to achieve 100% CPE reported by Lehmkuhl & Cutlip (1982). They also isolated ovine PIV-3 BFK cell cultures. However, Giauffret & Russo (1972) reported that an ovine PIV-3 isolate produced 100% CPE only after 5 to 8 days p.i., in MBDK cells. Similarly, Reisinger, et al. (1959) described 100% CPE after 5 days in MBDK cell culture, infected with a bovine PIV-3 isolate. Apparently, the time required to achieve 100% CPE in infected cell cultures by PIV-3 is variable and not only depends on the viral strain but also on the type of cell culture inoculated.

Transmission electron microscopy (TEM) of infected OFK cell cultures, with the ovine PIV-3 isolates, revealed the presence of virus particles budding from or attached to the cell membrane. Similar but incomplete particles were seen in cytoplasm. This agrees with previously reported TEM observations of PIV-3 (Bryson et al., 1983). Likewise, aggregations of virus nucleoproteins in the cytoplasm were observed in this study and they were similar to those described in cell cultures of lung epithelial cells AMs infected with PIV-3 (Cutlip & Lehmkuhl, 1982; Bryson, et al., 1983). According to Elango, et al. (1986), these TEM features indicate that PIV-3 replicates in the cytoplasm of the host cells.

The generally diffuse fluorescence pattern observed in the present study is compatible with the manner in which the antiserum was produced. This was by using non-purified whole virus. The antibody produced in this way is against the outer components of the virus particles or the HN. Whereas, fluorescence with monoclonal antibody anti NP produced discrete fluorescent foci (data not shown). The presence of strong fluorescence at the cytoplasmic margins in some cells during the later stages of
infection may be related to the marginal cytoplasmic virus assembly described for PIV-3 (Chanock & McIntosh, 1990).

The three ovine PIV-3 isolates exhibited physicochemical characteristics that have been described for paramyxoviruses, some in particular for PIV-3. Accordingly, all of them lost infectivity after being exposed to pH 3.0 and 9.0. This characteristic has been previously described for paramyxoviruses (Andrewes, et al., 1978). The sensitivity to exposure for 30 minutes at 56°C has also been reported by Orfei, et al.(1966) and Lehmkuhl & Cutilip (1982) as an attribute of PIV-3. The three ovine virus isolates in this study lost infectivity after this treatment. The lipid envelope exhibited by PIV-3 makes this virus sensitive to lipid solvents (Orfei, et al., 1966; Lehmkuhl & Cutilip 1982). This feature was also observed when the ovine PIV-3 isolates were treated with ether and chloroform. Finally, when the isolates underwent trypsinisation at 37°C for 1 hour all of them showed sensitivity to this treatment and lost infectivity after being treated with 1% trypsin solutions. This has been previously reported for bovine PIV-3 (Gresser & Enders, 1961; Berrios et al., 1990).

The haemagglutination test with guinea pig erythrocytes along with the haemagglutination inhibition test with rabbit anti-sera have been suggested as tests of choice in PIV-3 identification (Leunen & Wellemans, 1966; Nguyen-Ba-Vy, 1967b; Lehmkuhl and Cutilip, 1982). The results of these tests, for the ovine viral isolates described in this study, implied that they are in fact strains of PIV-3. The three isolates produced haemagglutination of guinea pig erythrocytes with different titre when compared with the reference virus. This variability can be related to levels of the glycoprotein haemagglutinin, which is determined by genetic factors (Fenner et al., 1987a).

The ovine PIV-3 isolates described in this study showed serological cross-reactivity with bovine and human PIV-3 reference strains by the serum-virus neutralization test, although reference anti-sera were shown to have higher reactivity with the homologous strains. These findings are similar to other reports on antigenic relationship between HPIV-3 and BPIV-3. This relationship has been demonstrated by hemagglutination inhibition (HI), neutralization (NT), complement fixation and the immunodiffusion test (Abinanti et al., 1961; Ray & Compans, 1986).
The reactivity with a panel of monoclonal antibodies cross-reacting with HPIV-3 and BPIV-3 viral proteins showed that the OPIV-3 strains can be distinguished from HPIV-3 and BPIV-3. They also showed that only limited variation occurs among OPIV-3 strains recovered at different geographical locations, in a similar fashion to that observed with BPIV-3 (Klippmark et al., 1990). With the reagents employed here, epitopes of viral proteins common to HPIV3 and BPIV-3 were found to be absent or to be expressed in a lower proportion in OPIV-3 strains. Antigenic differences were found predominantly in the F protein and to a lesser extent in the M protein. F protein seems to have a conserved character among OPIV-3 strains. This has also been observed in HPIV-3 and BPIV-3 strains (Klippmark et al., 1990). In this case three distinct epitopes of F protein that are found in HPIV-3 and BPIV-3 strains were absent in OPIV-3.

These differences should be considered when using vaccines produced with PIV-3 from other species. Antigenic differences suggest that a bovine strain may well induce important neutralization antibodies in sheep but a sheep strain may induce a wider spectrum of neutralizing antibodies against OPIV-3. Production of nonfunctional local antibodies, as well as poor antibody response to F protein, has been suggested to explain why reinfection with PIV-3 occurs with considerable frequency during infancy and early childhood (Chanock & Mcintosh, 1990). Furthermore, immunopathology can result from the induction of inappropriate immune responses. The F protein is responsible for penetration of virions into cells, spread from cell to cell and formation of syncytia. If vaccinees fail to elicit anti-F antibodies and are subsequently infected, they fail to control the spread of virus. This allows large amounts of viral antigen to be produced which reacts with non-neutralising antibodies and causes immunopathological changes (Choppin & Scheid, 1980).

On the other hand, two mAbs against two distinct epitopes of HN protein, common to HPIV-3 and BPIV-3 strains, reacted with the three ovine isolates. These results show that these two epitopes of HN protein seem to be conserved among PIV-3 strains isolated from different species. Antigenic variations in HN proteins among HPIV-3 strains have been found to be limited (Van Wike Coelingh et al., 1988). In contrast, it has been demonstrated that extensive differences are found between HN proteins from HPIV-3 and BPIV-3 strains, although there are some epitopes in common (Ray & Compans, 1986; Klippmark et al., 1990). It is necessary to consider that the two monoclonal antibodies against HPIV-3 HN protein, used in this study, come
from a group of 7 mAbs that crossreact with HPIV-3 and BPIV-3 strains. They were selected out of 16 mAbs raised against HPIV-3 and reacting with HN protein. Therefore, the detection of these two HN epitopes common to PIV-3 strains from different species and from different geographical areas seems relevant. The study of conserved epitopes could be useful for choosing mAbs for diagnostic purposes and production or usage of vaccines.

It is necessary to carry out further antigenic characterisation with a more complete panel of monoclonal antibodies anti-PIV-3, for further characterisation of these and other OPIV-3 strains. This kind of studies have been accomplished so far for human and bovine PIV-3 strains only (Klippmark et al., 1990).

The three ovine PIV-3 isolates described in this study were obtained from lungs showing histopathological lesions that corresponded to those described as bronchointerstitial pneumonia (Yates, 1988). In the bronchiolar epithelium there was hyperplasia, vacuolation, necrosis and less frequently eosinophilic intracytoplasmic inclusions. Also, in the peribronchiolar area there was lymphocyte infiltration and in the lumen there were PMNs, along with epithelial cell debris. In alveolar walls there was intense lymphocyte and Mφ infiltration. Proliferation of fibroblasts and pneumocytes type II was also found at this level.

The presence of PMNs in bronchiolar lumen is a characteristic of bronchointerstitial pneumonia and represents the combined action of virus and bacteria (Yates, 1988). In this study PIV-3 was isolated from two field cases of sheep pneumonia (430-7 and 392-10) where Pasteurella haemolytica was found. It has been reported that experimental and sequential infection with both PIV-3 and Pasteurella haemolytica regularly produces pneumonia in either SPF (Sharp et al., 1978), colostrum-deprived (Davies et al., 1986) or conventionally-reared lambs (Davies et al., 1977), regardless of age. According to Davies et al. (1977), the lesions fall into two main categories: necrotic lesions (demarcated by a zone of PMN infiltration) and a milder purulent bronchopneumonia. Both types of lesion were observed in sheep 430-7 and 392-10 where Pasteurella haemolytica was isolated. The necrotic lesions appeared to develop as a result of bacteria proliferation within the bronchial tree. A necrotising bronchitis and bronchiolitis develop, then infection seems to spread. This occurs either endobronchially or through the partly denuded epithelium, into the loose peribronchiolar connective tissue. The combination of PIV-3 and Pasteurella
*haemolytica* can produce a variety of pathological changes. These range from an acute fatal bronchopneumonia with septicaemia, to a mild purulent bronchopneumonia which resolves rapidly. The lesions produced depend both on the degree of bacterial multiplication in the lung and the efficiency of pulmonary bacteria clearance. The extent of virus-induced damage in the lung may be reflected in the amount of virus recovered (Davies *et al.*, 1986).

Some characteristics of the histopathology of these lungs, suggested that the lesions corresponded to intermediate or late reactions against PIV-3 virus, probably around 7 days post infection. For instance, the Mϕs that predominated in inflammatory areas were mostly of large size. These cells have been associated to late stages of this infection (Brannen & Chandler, 1988). Also, both simultaneous epithelial cell destruction and regeneration, observed in these lesions, have been found during experimental infections after 7 days p.i. (Cutlip & Lehmkuhl, 1982). Furthermore, eosinophilic cytoplasmic inclusions were rarely observed in bronchiolar epithelium. This is normally seen during late stages of the virus replication cycle.
CHAPTER FOUR

EXPERIMENTAL PARAINFLUENZA TYPE 3 INFECTION:
HISTOPATHOLOGY OF LUNG LESIONS AND
IMMUNOHISTOCHEMICAL DETECTION OF VIRUS PARTICLES.
4.1 INTRODUCTION

Historically, strains of PIV-3 have been identified that affect man (Chanock & Parrot, 1958) and cattle (Reisenger et al., 1959). They are known to produce respiratory tract disease. The ovine species can also be affected by this virus and there are reports of its isolation from many countries (Hore, 1966; Ditchfield, 1966; St George, 1969; Carter & Hunter, 1970; Epstein, 1974; Davies et al., 1977; Sharp, et al., 1978; Lehmkuhl & Cutlip, 1982).

Only minimal quantitative data on the course of PIV-3 replication in humans is available. Pathological descriptions indicate that PIV-3 may cause bronchiolitis or interstitial pneumonia (Aherne et al., 1970). Animal models of infection with human PIV-3 (HPIV-3) have been proposed. Hamsters, after infection with HPIV-3, may develop a mild bronchiolitis with occasional giant cells, but generally they show no clinical illness or lesions of interstitial pneumonia. Whereas cotton rats seem to be more appropriate for HPIV-3 studies as the genus *Sigmodon hispidus* develops bronchiolitis and *Sigmodon fulviventer* develops interstitial pneumonia (Porter et al., 1991).

Antibody to PIV-3 may be found in deer, pigs, dogs, cats, monkeys and rats (Fenner et al., 1987), but it is not known whether such infections cause disease or, indeed, whether such species represent natural hosts for PIV-3 or if they have their own distinct strains of PIV-3 (Porter et al., 1991). The cattle virus (BPIV-3) is species specific but is quite similar immunologically and in nucleic acid sequence to HPIV-3 (Coehling et al., 1986). Infection of cattle has been associated with the respiratory illness known as shipping fever. The sheep virus (OPIV-3) has been demonstrated to cause bronchiolitis and interstitial pneumonia in this species (Cutlip and Lehmkuhl, 1982).

Both the inoculation of colostrum-deprived lambs with a BPIV-3 strain and the inoculation of colostrum-deprived calves with an OPIV-3 strain resulted in macroscopic and histopathological lesions of interstitial pneumonia (Stevenson & Hore, 1970). However, the ovine strain of PIV-3 failed to produce clinical signs of pneumonia in calves.
Pathogenicity studies have been done in sheep using OPIV-3 (isolated from the upper respiratory tract of spontaneously infected sheep), inoculated intranasally and/or intratracheally (Lehmkuhl & Cutlip, 1983). This was carried out in both specific pathogen free (SPF) lambs (Sharp et al., 1978) or colostrum-deprived lambs (Lehmkuhl & Cutlip, 1983). However, the clinical and pathological response of sheep to experimental infection, with different OPIV-3 isolates, has been variable and ranged from subclinical infection to severe respiratory tract disease. This may be due to some extent to variations in virulence of different strains.

In this study colostrum-deprived lambs were inoculated with the ovine isolate 270-7 isolated and characterised as PIV-3 in this study (see Chapter 2). The pathogenicity of this virus strain was assessed in vivo through the induction of clinical disease and lung lesions. The relation between these lesions, corresponding to viral pneumonia and the presence of virus particles is discussed. Virus particles were detected by immunostaining with polyclonal and monoclonal antibodies. Finally, this allowed the production of lung pathological material for studies on the immune cell response to PIV-3 infection that are described in the following chapter.
4.2 RESULTS

4.2.1 Clinical findings

Sixteen lambs were obtained at birth and deprived of colostrum. They were divided into two groups. The first group of eleven lambs was inoculated with 5ml tissue culture fluid containing virus (OPIV-3 270-7); 2ml were given intranasally and 3ml transtracheally. The second group, of five control lambs was housed separately and mock-infected with the same amount of cell culture fluid from non-inoculated cell cultures. The lambs were observed daily for clinical response. Three lambs were euthanatised and necropsied on day 3 post-inoculation (p.i.), three on day 5 and five on day 7. The control lambs were euthanatised and necropsied on day 8 p.i.

The eleven lambs inoculated transtracheally with the ovine isolate of OPIV-3 270-7, but not the five control lambs developed clinical signs of respiratory infection that peaked on post inoculation day 4 and 5. After 2 to 5 days p.i. the lambs developed a biphasic febrile response, with increased respiratory rate, mouth breathing and became lethargic. By days 4 to 5 p.i. some of them were mostly recumbent with moderate rhinitis and showed some weight loss. The lambs either refused to suck or had reduced milk intake after days 3 p.i. From days 6 to 7 p.i. the remaining lambs showed some improvement in clinical signs and food intake.

4.2.2 Bacteriological findings

Neither Pasteurella haemolytica nor Mycoplasma or other pathogenic bacteria were isolated from the nasal secretions of any of these lambs on the days preceding the inoculation nor was it isolated after inoculation from any of the swabs collected from animals receiving either the virus or media alone.

4.2.3 Serological findings

Antibody titres to PIV-3 were determined on paired serum samples from the lambs, using a microtitration serum-virus neutralization test (Lehmkuhl & Gough, 1977).
All lambs were seronegative at the beginning of the experiment and the three control lambs remained seronegative. Thus, no virus neutralizing antibody was detected before inoculation of the virus or during the first 2 days p.i. The lambs necropsied on p.i. day 3 had reciprocal serum antibody titres ranging from 4 to 8 whereas the lambs necropsied on p.i. days 5 and 7 had reciprocal titre ranges of 16 to 32 and 16 to 64, respectively. Antibody titres were detected by both HI and SN (Table 4.1), higher antibody titres were observed with SN test (Table 4.2).

4.2.4 Virus recovery

Parainfluenza type 3 virus, as confirmed by virus titration and immunocytochemistry of infected tissue cultures with nasal secretions, was isolated from all inoculated lambs. Virus was recovered from nasal secretions beginning on p.i. day 2. Virus isolations from nasal secretions are summarised in Table 4.3. Titres reached their peak at 4 to 6 p.i. At necropsy, virus was also recovered from lung tissues (Table 4.4) of all inoculated lambs. No virus was recovered from nasal secretions or lungs from control lambs.

4.2.5 Pathology

4.2.5.1 Gross pathology findings

The upper respiratory tract of the infected group showed generally nasal and turbinate hyperaemic mucosae and contained a mucoid or mucopurulent exudate. No significant tracheal lesions were observed in any lambs. No lesions at this level were observed in control lambs.

In lungs of infected lambs the main lesion consisted in areas of consolidation. Initially these areas, which were dull red, atelectatic and consolidated, showed small linear lesions similar to the ones observed in lambs necropsied on p.i. day 3. These consolidated areas gradually increased in size in lambs necropsied on p.i. day 5 and on cross section of the lung they were found to be distributed extensively throughout affected lobes and appeared to follow the smaller bronchi and bronchioles. Maximal lesions were observed in lambs necropsied on p.i. day 7, when the consolidated areas
**TABLE 4.1** Serum antibody conversion for PIV-3 after experimental inoculation of lambs expressed as HI titre.

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<thead>
<tr>
<th>Group</th>
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<th>Days of experiment</th>
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<td>Infected</td>
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</table>

a= Pre-inoculations serum samples were all negatives at a dilution of 1/4
b= Titre is expressed as median of reciprocal of serum dilution.
TABLE 4.2  Serum antibody conversion for PIV-3 after experimental inoculation of lambs expressed as serum-virus neutralization titre

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a=The serum-virus neutralization titres were the highest dilution of serum in which 50% of the wells were protected from the viral CPE. Titre is expressed as median of reciprocal of serum dilution
### TABLE 4.3 PIV-3 recovery from nasal secretions of lambs after experimental inoculation.

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<th>Control</th>
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<th>Control</th>
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</tr>
<tr>
<td>8</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0/5</td>
</tr>
</tbody>
</table>

<sup>a</sup>= Mean titre expressed as mean TCID<sub>50</sub>/0.1ml of nasal secretions ±S.E.

<sup>b</sup>= Number of positive animals v/s animals tested

<sup>c</sup>= Post inoculation day

### TABLE 4.4 PIV-3 recovery from lungs at necropsy after experimental inoculation.

<table>
<thead>
<tr>
<th>PID&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Inoculated</th>
<th>Control</th>
<th>Inoculated</th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td>3</td>
<td>5.0±0.4</td>
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<td>5</td>
<td>6.8±0.4</td>
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<tr>
<td>8</td>
<td>-</td>
<td>0</td>
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</tbody>
</table>

<sup>a</sup>= Mean titre expressed as mean TCID<sub>50</sub>/g of lung tissue ±S.E.

<sup>b</sup>= Number of positive animals v/s animals tested

<sup>c</sup>= Post inoculation day
showed a reddish brown colour and contained small grey foci on their cut surface. These areas were confined only to the anterior lobes of the lungs (Fig. 4.1a).

The mediastinal and bronchial lymph nodes were hyperaemic and slightly enlarged only in lambs necropsied on p.i. day 7, but looked normal in infected lambs necropsied previously. No significant lesions were observed in the kidneys, liver or spleen.

No significant lesions were observed in lungs or other organs in control lambs.

4.2.5.2 Histopathology findings

There were focal areas of ulceration as well as erosion and neutrophil infiltration of the nasal and turbinate mucosae in lambs of the infected group. No significant lesions were found in the trachea.

On p.i. day 3 the bronchi were generally normal but the bronchioles contained a small amount of exudate. There was a slight epithelial and BALT hyperplasia in comparison to controls where BALT structures were not usually visible (Fig. 4.1b,c). Occasionally epithelial cells showed ballooning degeneration and a moderate number of pyknotic cells were seen in the epithelium or in the process of extrusion from the epithelium into the lumen. In no cases was there denudation or severe damage to the epithelium and only in one lamb was there some loss of cilia. From p.i. 3 there were scattered PMN in and beneath the epithelium of bronchi and bronchioles. Lymphocytes appeared in and around the walls of bronchi and bronchioles as early as 3 days p.i. (Fig. 4.1c,d) with a maximum response 6 to 7 days p.i. There were scattered foci of interstitial pneumonia, usually around or near small bronchioles with infiltration of lymphocytes and Mφs and septal alveolar cell proliferation (Fig. 4.2a,b), showing scattered foci of pseudo-epithelialisation. Thickening of the alveolar walls was minimal to moderate when compared with the lungs of non-infected animals. Mφs were also attached to the alveolar wall, while others were found lying free in alveolar space along with detached epithelial cells. The Mφs had pale staining, oval or indented nuclei and abundant cytoplasm which showed varying degree of vacuolation.

In lambs killed on p.i. day 5 the exudate of the bronchioles was more abundant and exudate was also observed in bronchi. In many of the smaller bronchioles there was
Lesions of interstitial pneumonia induced by experimental inoculation with OPIV-3 270-7 isolate. Lambs were obtained at birth and colostrum-deprived. They were inoculated with 5ml tissue culture fluid containing the virus (OPIV-3 270-7); 2ml were given intranasally and 3ml transtracheally. Lung biopsies were taken from lambs euthanised and necropsied on post-inoculation (p.i.) days 3, 5 and 7. Samples were fixed in buffered formalin and processed for staining with hematoxylin/eosin (H/E), Giemsa's or Van Gieson's stains.

a) Photograph of a lung showing pneumonic consolidation areas (arrow) in the anterior lobes. These lesions are surrounded by emphysema. This lung was taken from a lamb that was necropsied after 7 days of experimental inoculation with OPIV-3 270-7 isolate.

b) Histological section of a lung from one of the mock-infected lambs. A bronchiole surrounded by alveoli is shown. No accumulation of inflammatory cells is observed around the bronchiolar epithelium, bronchiolar lumen, alveolar walls, alveolar space or any other histological area. Also, there is no BALT visible close to the bronchiole. H/E stain. Magnification x40.

c) Lung section of a lamb after 3 days p.i. with OPIV-3 270-7. There is a moderate hyperplasia of BALT (large arrow). Also mononuclear cell accumulation, around the bronchiolar epithelium, is seen. Alveoli close to the bronchiole show a slight thickening of their walls (small arrows). H/E stain. Magnification x40.

d) Lung section of a lamb after 3 days p.i. with OPIV-3 270-7. There is an intense accumulation of lymphocytes around the epithelium. They are migrating towards the lumen (small arrows). Presence of cell debris and PMNs in the bronchiolar lumen can be observed. Giemsa's Stain. Magnification x400.
hyperplasia of the epithelium with foci of giant cell bronchiolitis and acidophilic cytoplasmic inclusions (Fig. 4.2c). Mitotic figures were occasionally observed. The areas of interstitial pneumonia were larger and well established and reached their maximal extent by day 6 to 7 p.i. They were similar in composition to those found in lambs killed on p.i. day 3 but the thickening of the alveolar walls showed a two-to-fivefold increase (Fig. 4.2d). There was mononuclear infiltration of alveolar walls with pseudo-epithelialisation of alveoli and small syncytia attached to alveolar walls in some cases (Fig. 4.2d). Compensatory vesicular emphysema and evidence of slight perivascular oedema were seen in some lobules, while in others there was partial atelectasis. Some Mφs and occasionally some epithelial cells were free in alveolar spaces.

By p.i. day 7 most of the above described changes had reached their maximum expression. There was an increased amount of exudate in bronchi and bronchioles, hyperplasia of the bronchial epithelium and less frequently acidophilic cytoplasmic inclusions. There was a remarkable enlargement of bronchiolar associated lymphoid tissue (BALT) in comparison to control lungs (Fig. 4.3a). The interstitial reaction was now lobular in distribution with widespread lymphocyte interstitial infiltration and atelectasis (Fig. 4.3b). Also syncytia and pseudoepithelialisation were seen. Scattered foci of alveolar necrosis with PMN infiltration and areas of atelectasis were present. There was also intense hyperemia in lung blood vessels with lymphocyte perivascular infiltration (Fig. 4.3c) and interlobular oedema with lymphatic vessel dilatation (Fig. 4.3d). Increased thickness of pleura was detected in some areas due to infiltration of lymphocytes and Mφs and proliferation of type I collagen fibres.

There were no marked differences in the composition of the lymph nodes from control and infected lambs. No significant changes were observed in lungs of control lambs or in kidneys, liver, spleen, intestine, brain, and other organs studied, of any lamb.

The histopathology findings are summarized in Table 4.5. The most noticeable lesion found in PIV-3 infected lungs corresponded to interstitial pneumonia. The most characteristic features were the hyperplasia of BALT and degenerative changes of bronchiolar epithelium with lymphocyte infiltration. Other frequent lesions were areas of atelectasis and increase in alveolar septa thickness. The latter were due to
FIGURE 4. Lesions of interstitial pneumonia induced by experimental inoculation with OPIV-3 270-7 isolate. Lambs were obtained at birth and colostrum deprived. They were inoculated with 5ml of tissue culture fluid containing the virus (OPIV-3 270-7); 2ml were given intranasally and 3ml transtracheally. Lung biopsies were taken from lambs euthanised and necropsied on post-inoculation (p.i.) days 3, 5 and 7. Samples were fixed in buffered formalin and processed for staining with haematoxylin/eosin (H/E), Giemsa’s or Van Gieson’s stains.

a) Lung section of a lamb after 3 days p.i. with OPIV-3 270-7. A slight thickening of the alveolar walls can be seen. Inside these walls or attached to them it is possible to observe Mφs (arrows). No cells are present in the alveolar spaces. H/E. Magnification x100.

b) Histological lung section of a lamb after 3 days p.i. with OPIV-3 270. Intense infiltration of lymphocytes (large arrow) is seen around the bronchiolar epithelium. A slight thickening of the alveolar walls and areas of emphysema (small arrows) are shown. Also, some cell debris can be seen inside the bronchiolar lumen. H/E stain. Magnification x40.

c) Lung section of a lamb after 5 days p.i. with OPIV-3 270-7. Many mucus cells (a) are present in the bronchiolar epithelium. There are also some halo bound eosinophilic cytoplasmic inclusions (b). H/E stain. Magnification x600.

d) Lung section of a lamb after 5 days p.i. with OPIV-3 270-7. There is a marked increase in the alveolar wall thickness due to an accumulation of lymphocytes (L), Mφs (M) and the presence of fibrosis (F). There are also some groups of pneumocytes showing epithelialisation. Van Gieson’s stain. Magnification x100.
FIGURE 4.3 Lesions of interstitial pneumonia induced by experimental inoculation with OPIV-3 270-7 isolate. Lambs were obtained at birth and colustrum deprived. They were inoculated with 5ml tissue culture fluid containing the virus (OPIV-3 270-7); 2ml were given intranasally and 3ml transtracheally. Lung biopsies were taken from lambs euthanised and necropsied on post-inoculation (p.i.) days 3, 5 and 7. Samples were fixed in buffered formalin and processed for staining with hematoxylin/eosin (H/E), Giemsa’s or Van Gieson’s stains.

a) Lung section of a lamb after 7 days p.i. with OPIV-3 270-7. Marked hyperplasia of a BALT can be seen (large arrow). Also there is alveolar wall thickening and atelectasis (small arrow). Van Gieson’s stain. Magnification x100.

b) Lung section of a lamb after 7 days p.i. with OPIV-3 270-7. Widespread atelectasis (A) and intense infiltration of mononuclear cells (Y) are present. H/E stain. Magnification x40.

c) Lung section of a lamb after 7 days p.i. with OPIV-3 270-7. A lymphocyte cuff around an arteriole is shown (arrow). Giemsa’s stain. Magnification x400.

d) Lung section of a lamb after 7 days p.i. with OPIV-3 270-7. There is interlobular oedema and hyperemia. Van Gieson’s. Magnification x100.
proliferation of pneumocytes type II (known as epithelialisation), lymphocyte and Mφ infiltration and later to fibrosis.

Small Mφs were more frequent during early inflammation, while large Mφs predominated later. Van Gieson staining confirmed that around p.i. 7 part of the increase in alveolar septae thickness was due to fibrosis with proliferation of type I collagen fibres. Giemsa staining confirmed that the morphology of the cell infiltrate in alveolar septa, around bronchi and bronchioles, pleura and blood vessels corresponded mainly to lymphocytes and to a lesser extent Mφs and plasma cells.

4.2.6 Identification of virus particles in situ by immunohistochemistry

After experimental infection, virus particles were detected in lung tissue by immunohistochemistry. For this purpose, lung samples were taken simultaneously with those for viral isolation and histopathology and stored frozen at -70°C. The immunostaining was carried out with rabbit anti PIV-3 sera raised against both the ovine isolates and reference virus. Also mouse monoclonal antibody anti human PIV-3 protein HN was used (mAbs HN4771 and HN4795).

Attempts were made in order to detect OPIV-3 particles in formalin fixed, paraffin embedded tissue sections, by immunohistochemistry. Positive staining was achieved with rabbit antisera, however the staining was less intense when compared with the one obtained in frozen sections (data not shown). On the other hand, staining with the monoclonal antibodies did not give positive reaction.

Antibodies were previously titrated for indirect immunostaining techniques which included indirect immunofluorescence (IIF), immunoperoxidase (IIP) and immunoalkaline phosphatase (IIAP). All staining was carried out in triplicate. Normal rabbit and mouse serum (NRS and NMS) replaced primary antibodies in control sections for rabbit antisera and monoclonal antibody, respectively. Table 4.6 summarizes the results obtained after analyzing sections immunostained with any of these three techniques.

Positive fluorescence staining in pneumonic lung sections, on p.i. days 3 to 5, was detected on cell membrane and cytoplasm of a great proportion of bronchiolar and
TABLE 4.5 Histopathological findings during experimental infection of colostrum deprived lambs with OPIV-3 270-7.

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</tr>
<tr>
<td>degenerative changes</td>
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<tr>
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<td>PMNs</td>
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<tr>
<td>Møs</td>
<td>+</td>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>Møs</td>
<td>+</td>
</tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>-</td>
</tr>
<tr>
<td>Møs</td>
<td>+</td>
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<td>fibrosis</td>
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<tr>
<td>VESSELS</td>
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<tr>
<td>lymphocytic cuffing</td>
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* Changes are expressed according to frequency of appearance as follows:
- = absent       +=scarce       +++=highly frequent
++=moderate     +++=frequent
alveolar epithelial cells. Also small groups of Mφs showed specific fluorescence (Fig. 4.4a). On p.i. day 7 fluorescent epithelial cells were far less frequent, whereas more cells in alveolar walls, apparently some Mφs among them, showed a positive reaction. In pneumonic lung sections on p.i. days 3 to 5, immunostained by IIP, there was positive staining (brown colour) of cell membrane and cytoplasm in a great proportion of bronchiolar and alveolar epithelial cells and in some Mφs. PIV-3 antigens were more easily demonstrable in the cytoplasm of alveolar epithelial cells. On p.i. day 7 the staining for epithelial cells in bronchi and bronchioles was markedly reduced, whereas more staining was detected in alveolar walls with more Mφs showing positive reaction (Fig. 4.4b).

Immunostaining by IIAP of pneumonic lung sections on p.i. days 3 to 5, showed a similar staining pattern to that observed with IIP. OPIV-3 antigens were demonstrable (red colour) more frequently in cell membrane and cytoplasm in a high proportion of bronchiolar and alveolar epithelial cells (Fig. 4.4c). On p.i. day 7 the staining for epithelial cells was less frequent and it was detected in Mφs and pneumocytes, in alveolar walls. Staining of bronchiolar epithelium was not observed at this stage of infection (Fig. 4.4d).

No reactivity was detected in endothelium, fibroblasts, lymphocytes or any other type of lung tissue cell.
TABLE 4.6  Histological distribution of OPIV-3 particles in lung tissue, by immunohistochemistry, after experimental infection with ovine isolate 270-7.

<table>
<thead>
<tr>
<th>Histological area</th>
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<th></th>
<th></th>
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<td></td>
<td>3</td>
<td>5</td>
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<td>Control</td>
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<tr>
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</tr>
<tr>
<td>Mφs</td>
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<td>++</td>
<td>+++</td>
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<td>BALT</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mφs</td>
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</tr>
<tr>
<td>epithelium</td>
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<td>++</td>
<td>+++</td>
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<td>lymphocytes</td>
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<td>-</td>
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</tr>
<tr>
<td>Mφs</td>
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<td>++</td>
<td>+++</td>
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<td>Alveolar space</td>
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<td>Mφs</td>
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<td>+</td>
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</table>

* Changes are expressed according to frequency of appearance as follows:
- = absent
+ = scarce
++ = moderate
+++ = frequent
++++ = highly frequent
FIGURE 4. 4 Immunostaining of OPIV-3 virus particles in tissue sections of pneumonic lungs taken from lambs after experimental infection with OPIV-3 270-7. Lung biopsies were collected from lambs that were euthanised and necropsied on post-inoculation (p.i.) days 3, 5 and 7. Samples were stored frozen at -70°C until being processed by indirect immunofluorescence (IIF), immunoperoxidase (IIP) or alkaline phosphatase (IIAP).

a) Lung section of a lamb after 3 days p.i. with OPIV-3 270-7. Fluorescence staining of a group of Mφs present in a peribronchiolar area. Positive staining is observed in the cytoplasm and the cell membrane. IIF with rabbit antisera. The nuclei were counterstained with H/E. Magnification x400.

b) Lung section of a lamb after 5 days p.i. with OPIV-3 270-7. In an atelectatic area, positive staining (brown colour) is observed in the cell lining of the alveolar walls. IIAP with rabbit antisera. The nuclei were counterstained with H/E. Magnification x400.

c) Lung section of a lamb after 3 days p.i. with OPIV-3 270-7. There is strong immunostaining (red colour) of the bronchiolar epithelium. IIAP with rabbit antisera. The nuclei were counterstained with H/E. Magnification x100.

d) Lung section of a lamb after 7 days p.i. with OPIV-3 270-7. Positive staining (red colour) of a few scattered Mφs attached to alveolar walls can be seen. IIAP with monoclonal antibody HN4795. The nuclei were counterstained with H/E. Magnification x100.
The present study confirms the pathogenicity of ovine PIV-3 strain 270-7 and shows that this virus is able to induce clinical and pathological symptoms corresponding to interstitial pneumonia. The pulmonary lesions observed here correspond closely to those seen in experimental inoculation of lambs with other PIV-3 ovine strains (Hore & Stevenson, 1969; Stevenson & Hore, 1970; Cutlip & Lehmkuhl, 1982).

Previous investigations, on experimental reproduction of interstitial pneumonia by PIV-3, report variability in the presence of eosinophilic cytoplasmic inclusions. During this study these viral inclusions were observed in bronchiolar epithelium in the early stages of infection. Likewise, Dawson & Crickshanik (1965) & Omar (1966) report similar findings in bronchiolar epithelium in cattle. In contrast, Stevenson & Hore (1970) did not find virus inclusions in lung tissue, during experimental infection with an OPIV-3 isolate. However, these inclusions are usually observed during in vitro replication of OPIV-3 (Hore, 1966; Ditchfield, 1966; Singh & Pathak, 1977; Lehmkuhl & Cutlip, 1982).

Another infrequent feature observed as a result of the experimental infection with OPIV-3 270 strain was syncytia formation in alveolar epithelium. This has been previously reported as being part of in vivo cell pathology induced by OPIV-3 infection both in natural and experimental infections (Hore & Stevenson, 1969; Stevenson & Hore, 1970).

The low seroconversion rate may reflect a rather low level of enzootic infection in these lambs as there was no maternal antibody interference. In fact, the lungs lesions found at necropsy were confined to small portions of the anterior lobes. Furthermore, after 7 days post infection the experimentally induced respiratory disease started to show signs of recovery. Similar antibody seroconversion findings have been observed in studies of naturally occurring respiratory disease, associated with PIV-3 infection in calves (Lehmkhul & Gough, 1977). Moreover, the serological tests HI and SN, used in this study, may have a low sensitivity and underdetect antibody titres. In fact, in this study these tests had different sensitivities in that SN gave higher titres than HI. Similarly, St. George (1971), in a PIV-3 serological survey, observed that HI is less sensitive than the virus neutralization test. Furthermore, Adair (1986) compared the
sensitivity for antibody detection of HI, SN and fluorescence antibody (FA) test in experimental infection of colostrum deprived calves, with PIV-3 and SRV. He obtained very similar low titres for seroconversion by HI and SN. In contrast, serum titres for the same samples detected by FA test were much higher. For example, seroconversion after day 9 p.i., with PIV-3, was 32 according to the HI and 256 according to the FA test.

An increase in the population of fibroblasts of the alveolar walls was observed in lung lesions of lambs inoculated with the OPIV-3 270-7 strain. This change, which has been associated with regeneration (Crouch, 1990), partly explains the increase in thickness of this histological structure during late PIV-3 infection. Thus, PIV-3 infection in sheep may induce pulmonary fibrosis and its severity should be related to the extent of damage induced by the inflammatory process.

OPIV-3 particles were detected, by immunohistochemistry, in greater proportion in alveolar septal cells and less in both bronchiolar epithelium cells and MΦs. This may be associated with the rapid epithelial regeneration and agrees with TEM observations by Bryson et al. (1983) and Tsai (1977) in PIV-3 experimentally infected calves.

OPIV-3 particles, detected by the immunohistochemistry of infected lung tissues, were found more frequently in areas displaying an early rather than late inflammatory process. During the early inflammatory process these virus particles were found mostly in bronchiolar epithelium cells. On the contrary, during intermediate to late stages of infection the highest proportion of virus particles was found in alveolar epithelial cells. These cells were most probably pneumocytes type II, as these are the cells that predominate in the alveolar walls during these stages of PIV-3 infection (Bryson et al., 1983). Furthermore, it has been demonstrated that Paramyxoviruses infect and replicate more easily in pneumocytes type II cell cultures (Castleman et al., 1989).

In this study no virus particles were detected in lung endothelial cells, fibroblasts or lymphocytes, by immunohistochemistry. Similarly, Bryson et al.,(1983) did not find PIV-3 replication in these cells by TEM.

Immunohistochemical tests are rapid and can be a sensitive and reliable method for viral diagnosis (Haines et al., 1992), however this method is dependant upon the
availability of a high quality antiserum that will bind to specific antigens in tissue sections. Traditionally, polyclonal antisera have been developed for this purpose; however, in recent years the polyclonal antisera are being replaced by monoclonal antibodies (mAbs). Both types of antibody preparations have advantages and disadvantages for use in immunohistochemical stains. Polyclonal antisera recognize many epitopes on the infecting virus and thus are likely to detect more isolates of the virus and viral epitopes retained in formalin-fixed specimens. Alternatively, mAbs are less likely to contain contaminating antibodies to other pathogens or to normal tissue constituents that result in nonspecific staining. Also, monoclonal antibodies are generally available in unlimited quantities, in contrast to the finite quantities of polyclonal antisera, which enables standardization of the test methods within and between laboratories. However, several mAbs rather than a single mAb may be preferable for diagnostic testing because individual mAbs binding single epitopes may fail to detect some antigenic variants of the virus.

Both the immunoperoxidase and immunoalkaline phosphatase staining methods, in contrast to immunofluorescence, do not require a special microscope for observation of results and the sample sections can be stored for a much longer period of time. The sections can be counterstained for nuclei identification, thus allowing histological details to be clearly distinguished, particularly in formalin fixed sections. However, the fixation of tissues by formalin produces protein denaturation that may lead to a failure in their identification by antibodies, due to alterations in the epitopes needed to be recognized. This is particularly critical for monoclonal antibodies as they recognize only one epitope. Thus, when using formalin fixed sections for OPIV-3 identification by immunohistochemistry, in this study, the monoclonal antibodies (HN4771 and HN4795) did not react, while the rabbit antiserum gave a weaker reaction. In contrast, both reagents immunostained OPIV-3 particles in frozen sections. This means that the epitopes recognized by the monoclonal antibodies were modified by formalin fixation. Besides, the OPIV-3 anti-serum, used in this study, seems to recognize a different group of epitopes, some of which are modified by formalin and some are not.

One of the most striking histopathological findings in lambs inoculated with OPIV-3 270-7 strain was an intense proliferation of lymphocytes. This was most noticeable in the resulting BALT hyperplasia. This can be associated with the immune response elicited against OPIV-3. In the pulmonary tissue, the lung cell immune response arises from the action of lymphocytes which are partly derived from BALT (Potgieter, 1986;
Yates, 1988). Accumulations of lymphocyte were also seen around the peribronchiolar walls, within the interalveolar septa and as perivascular lymphocytic cuffing.

No virus particles were detected in lymphocytes in this study. Observations in lymphocytes cultures \textit{in vitro} infected with PIV-3 showed that despite the presence of lymphocyte-associated virus antigen detected by direct immunofluorescence, no increase in PIV-3 titre above baseline was seen from infected lymphocytes, irrespective of mitogen stimulation. Likewise, lymphocytes did not contribute to the extracellular virus pool in lymphocyte/AM\(\phi\) cultures as the increases in viral titre above basal levels in supernatants were equal to levels released by M\(\phi\)s alone. The \textit{in vitro} expression of viral antigen on lymphocytes stimulated in the presence of PIV-3-infected AM\(\phi\) suggests a non-productive or abortive infection of lymphocytes mediated through contact with infected AM (Basaraba \textit{et al.}, 1993).

The host cell membrane receptor involved in infection by Orthomyxovirus and Paramyxovirus is a neuraminic acid contained in a glycoprotein. These cell receptors for PIV-3 are found in different cell types including lung epithelial cells, lymphocytes, monocytes, M\(\phi\)s and neurones (Vainionpaa \textit{et al.}, 1989). However, the ultimate susceptibility of a cell to become infected by this virus is determined by the presence of specific proteases together with characteristics of the virus structure, host cell receptors, cell metabolism and virus replication (Potgieter, 1986).

The presence of a large number of lymphocytes, particularly on days 5 and 7 p.i., combined with the minimal to moderate cytolysis in antigen bearing cells, suggests that PIV-3 induced pulmonary disease may have an important immunopathological component. Lymphocytes were first seen at 3 days p.i. and reached a maximum after 7 days p.i., an observation consistent with a specific T-cell response to the virus (Porter \textit{et al.}, 1991).
CHAPTER FIVE

CELLULAR ASPECTS OF THE IMMUNE RESPONSE IN OVINE LUNGS
EXPERIMENTALLY INFECTED WITH PARAINFLUENZA TYPE 3 VIRUS
5.1 INTRODUCTION

Histopathological examination of lung lesions induced by OPIV-3 270-7 strain, (as described in section 4.2.5.2), showed both epithelial damage and inflammatory infiltrate. This included intense peribronchial infiltration by lymphocytes with few PMNs; hyperplasia of BALT; infiltration of lymphocytes and Mφs within interalveolar septa; perivascular lymphocytic cuffing and presence of some PMNs and cell debris in bronchial lumen. The inflammatory cell infiltration in the alveolar space was far less severe with some Mφs and detached epithelial cells.

In pulmonary tissue, the lung cell defence is primarily derived from the action of lymphocytes from bronchiolar associated lymphoid tissue (BALT). They are involved in antibody and cell mediated immunity. The lung immune defence is complemented by the activation of AMφs and the influx of monocytes and PMNs from peripheral blood (Potgieter, 1986).

Cytotoxic T cells are vital for effective recovery from PIV-3 infection. This is illustrated by PIV-3 induced giant-cell pneumonia in T-cell deficiency children which can often prove fatal (Chanock & McIntosh, 1990). Reports of productive infection of PIV-3 by lymphocytes are controversial (Basaraba et al., 1993). However, it has recently been reported that T cells can be infected with PIV-3 \textit{in vitro} and release virus (Sieg et al., 1994). Apparently, the productive infection requires activation of the T cells and results in a marked inhibition of cell proliferation. Furthermore, exposure to PIV-3, even without overt expression of viral proteins as detected by immunohistology, seems to alter the functional capacity of T cells profoundly. The capacity of the virus to regulate T-lymphocyte function may play an important role in the failure of the virus to induce lifelong immunity (Sieg et al., 1994).

It has been demonstrated that cytotoxic T cells react weakly with individual glycoproteins HN or F, but cytotoxic activity is far more effective when both glycoproteins are present. Furthermore, cytotoxic T cells can also react with cell lines persistently infected with PIV-3 (Spriggs et al., 1987).
The infection of AMφs has important consequences for the pathogenesis of respiratory tract viral disease (Stauber & Weston, 1984). These cells are critically important in lung resistance to bacterial colonisation. This can take place after the AMφ has been affected as a direct consequence of the virus replication or as a result of immune mediated cytotoxicity towards virus infected Mφs (Davies et al., 1986).

Pulmonary Mφs can be classified into three different groups according to their localization. They are: AMφs, interstitial Mφs and respiratory airway Mφs (Holian & Scheule, 1990). The AMφ, is a phagocyte residing in the alveolar space and is in direct contact with epithelial cell types I and II; its nucleus is lobulated and has vacuolated cytoplasm. It is morphologically and functionally heterogeneous and its size varies between 12 to 40μm (Pratt et al., 1971; Cohen & Cline, 1971). There are different AMφ subpopulations according to membrane receptor expression, phagocytic function and capacity of releasing mediators (Shellito & Kaltreider, 1984). Variability has also been observed in cell morphology and may have some functional implications. It has been observed that small AMφs predominate in acute diseases while, those of a larger size predominate in chronic disease (Brannen & Chandler, 1988). AMφs can interact with other cells and molecules in their environment through both numerous secretion products and expression of specific receptors on the cell membrane (Fels & Cohn, 1986).

Interstitial Mφs are located in the connective lung tissue in contact with fibroblasts, lymphocytes and endothelial cells and have a great capacity for replication. They are most efficient in stimulating the T lymphocyte response against antigens (Holian & Scheule, 1990).

PMNs participate in both enzymatic degradation and phagocytosis events. They contain enzymes and oxidative elements that can damage not only microbial pathogenic agents, but also the pulmonary tissue where the inflammatory process takes place (Welliver & Ogra, 1988). According to the lesions observed in experimental infection with OPIV-3 in this study (section 4.2.5.2) they seem to be a minor component of the inflammatory reaction induced in lung tissue by this virus.

The organization of the immune response is a biological event that is mounted in an orchestrated fashion in the presence of an antigen. Different cell types and populations are involved such as Mφs, and apart from displaying phagocytic function, they can
interact with other effector cells of the immune system, the lymphocytes. Therefore, it is the aim of this work to deal with changes in proliferation and histological distribution of Mφs as well as lymphocyte subsets after PIV-3 experimental infection.

In the previous chapter, the presence of a large number of lymphocytes was detected, particularly on days 7 p.i. The lymphocytic infiltrate was part of marked bronchi/bronchiolar associated lymphoid tissue (BALT) hyperplasia and alveolar wall thickening. This, combined with the minimal to moderate cytolysis of virus infected cells, suggests that PIV-3 induced pulmonary disease may have an important immunopathological component. Lymphocytes were first seen at 3 days p.i. and reached a maximum 7 days p.i., an observation consistent with a specific T-cell response to the virus (Porter et al., 1991).

As it has been described in the previous chapter (section 4.2.5.2) the lesions of interstitial pneumonia had reached their maximum expression by day 7 post experimental inoculation. In this chapter, aspects of the cell immune response against OPIV-3, in the sheep lung, were studied by detecting changes after 7 days post experimental inoculation with OPIV-3 isolate 270-7. The presence and proportion of lymphocyte subsets and Mφs were evaluated both in lung wash fluid (LWF) and in histopathological lung lesions. Also, the association between these cell populations and virus particles was studied. Both the detection of immune cells and virus particles were carried out by single and double immunostaining with monoclonal antibodies.
5.2 RESULTS

Samples of lung tissue and lung wash fluid (LWF) were collected from 5 infected (OPIV-3 270-7) and 5 mock-infected lambs. These animals were part of the experimental infection described in Chapter 4. Infected animals were necropsied on day 7 p.i. and control animals on day 8 p.i. After dissecting the lungs and taking biopsies for immunohistochemistry LWF was immediately collected. Biopsies for immunohistochemistry were either snap frozen in liquid nitrogen and then stored at -70°C or fixed in buffered formalin. LWF was immediately processed for differential cell counting and staining for flow cytometry.

5.2.1 Total and differential leucocyte cell count in lung wash fluid (LWF) from experimentally infected lambs with OPIV-3 270-7.

LWF from OPIV-3-infected lambs after 7 days p.i. presented an increased cellularity when compared with controls. OPIV-3 =4.3x10^8 (range 0.2-6.3); controls =3.2x10^8 (range 1.6-6.9) (values expressed as median of cells per millilitre) although this difference was not significant at p≤0.05. However, significant changes in most cell types were observed after differential cell counts of LWF (Table 5.1). PIV-3 infected animals had significantly increased proportion of (p<0.05) lymphocytes and PMNs and a significantly decreased proportion of Mφs (p<0.05), when compared with controls. Absolute numbers showed similar changes with a significant increase of lymphocytes (p<0.001) and PMNs (p<0.001). Mφ numbers were slightly higher but not significantly different from the controls (data not shown).

5.2.2 Changes in lymphocyte subsets and in the CD4+/CD8+ lymphocyte ratio in LWF from OPIV-3 experimentally infected lambs.

The different ovine lymphocyte subsets were studied by flow cytometry in LWF from animals necropsied after 7 days p.i. with OPIV-3 270-7. Lymphocyte subsets CD4+, CD8+, T cells and B cells were immunostained with mAbs SBU-T4, SBU-T8, ST-197
TABLE 5.1 Differential cell count in lung wash fluid (LWF) from mock-infected and OPIV-3 infected animals after 7 days post inoculation.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Control (n=5)</th>
<th>PIV-3 infected (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMNs</td>
<td>1.8±1.5$^1$</td>
<td>6.4±2.3$^2$</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>6.1±2.4</td>
<td>14.8±4.2$^2$</td>
</tr>
<tr>
<td>MΦs</td>
<td>85.2±12.2</td>
<td>67.7±9.4$^2$</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>3.2±2.9</td>
<td>4.0±3.7</td>
</tr>
<tr>
<td>Mast cells</td>
<td>2.3±1.9</td>
<td>1.5±1.4</td>
</tr>
</tbody>
</table>

1 Results are expressed as a median of percentages ±SD.
2 Significantly different from control at p<0.05 (Mann-Whitney non-parametric rank test).
and VPM32, respectively. Using FSC and SSC parameters a gate was set on small lymphocytes. Data was analyzed with 1% threshold set on negatively stained cells.

By using the percentage of positive lymphocytes to calculate the CD4'/CD8' ratio it was shown that there was a statistically significant decrease in the CD4':CD8' ratio in PIV-3 infected lambs with lung lesions compared to non-infected lambs (Table 5.2). Most PIV-3 infected lambs with pulmonary lesions showed a decrease in the CD4':CD8' ratio(<1). In contrast, the CD4':CD8' ratio of lymphocytes from normal control lambs was consistently higher (2.8±0.5).

In LWF of PIV-3 infected animals, the most noticeable changes were a significant decrease (p<0.001) of CD4' lymphocytes and a significant increase (p<0.05) of CD8' T lymphocytes ratios, leading to a significant inversion (p<0.001) of the CD4'/CD8' ratio, when compared with controls. The percentage of γδT cells and B cells in LWF from PIV-3 infected animals did not show statistically significant variations, although values were higher for infected animals.

5.2.3 Detection of OPIV-3 infected alveolar macrophages (AMφs) and lymphocytes in LWF from experimentally infected lambs by flow cytometry immunocytochemistry.

LWF cells were obtained from mock-infected and OPIV-3 infected lambs after 7 days p.i. Using FSC and SSC parameters to gate on small lymphocytes or Mφs, these cell populations were studied by flow cytometry after immunofluorescence staining with rabbit anti OPIV-3 sera. No positive lymphocytes were found in either mock-infected or OPIV-3 infected animals (data not shown). In contrast, 18% (range=15.12% to 36.68%) of Mφs from experimentally infected lambs were positively stained with the anti OPIV-3 serum. They showed a low variable intensity of virus particle staining which ranged from 9.8 to 26.73 (with NRS staining subtracted). Figure 5.1 shows the flow cytometry profiles of AMφs obtained from the OPIV-3 infected animals. The rabbit anti OPIV-3 serum showed no reactivity with AMφs from mock-infected animals (data not shown). Cytocentrifuge smears were obtained from LWF of mock-infected and OPIV-3 infected animals and immunostained with rabbit anti OPIV-3 serum by indirect immunoalkaline phosphatase (IIAP). Again positive staining was
TABLE 5.2 Lymphocyte subsets in lung wash fluid (LWF) from control and PIV-3 infected animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CD4⁺</th>
<th>CD8⁺</th>
<th>γδTCR</th>
<th>B cells</th>
<th>CD4⁺/CD8⁺ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control  (n=5)</td>
<td>60.2±09.5¹</td>
<td>21.6±08.2</td>
<td>6.9±3.2</td>
<td>17.3±08.7</td>
<td>2.8±0.5</td>
</tr>
<tr>
<td>PIV-3 (n=5)</td>
<td>28.7±10.8³</td>
<td>31.3±09.3²</td>
<td>4.6±2.5</td>
<td>29.5±10.2</td>
<td>0.9±0.4³</td>
</tr>
</tbody>
</table>

1 Results are expressed as median of percentages ± SD of positively labelled cells in lymphocyte gate.
2 Significantly different from controls at p<0.05 (Mann-Whitney non-parametric rank test).
3 Significantly different from controls at p<0.001 (Mann-Whitney non-parametric rank test).
FIGURE 5.1 Frequency histograms showing the reactivity of OPIV-3 antisera (---) with AMΦs from 5 lambs (A-E) experimentally infected with OPIV-3 270-7. Lambs were obtained at birth and colostrum-deprived. They were inoculated with 5ml of tissue culture fluid containing the virus (OPIV-3 270-7); 2ml were given intranasally and 3ml transtracheally. The profiles show staining of AMΦs in the lung wash fluid (LWF) that was obtained from lambs euthanised and necropsied on post-inoculation (p.i.) day 7. Negative controls of staining (incubation with NRS) is shown for comparison (-----).
LW-AM/OPIV-3

Log10 Fluorescence Intensity

Cell Number
observed with Mφs but not with lymphocytes (Fig. 5.2). Mφs showed variable intensity of staining in cytoplasm. No staining of nuclei was detected.

5.2.4 Leucocytes distribution in lung lesions from normal and PIV-3 experimentally infected lambs.

The distribution of leucocytes in lungs from mock-infected animals is shown in Table 5.3. Mφs were observed around the bronchial or bronchiolar epithelium; in association with BALT; inside alveolar walls and free in alveolar space. In contrast, lymphocytes were located mainly in peribronchial and peribronchiolar areas as part of small BALT structures and less frequently migrating through the epithelium. They were only rarely seen in alveolar walls or in alveolar space. PMNs were occasionally seen inside blood vessels.

The distribution of leucocytes in lung sections from infected animals after 7 days p.i. with OPIV-3 270-7 is shown in Table 5.3. The numbers of lymphocytes increased considerably in peribronchial and peribronchiolar areas and heavily infiltrated the epithelium. They also appeared in association with oedema and in lymphatic vessels of the interlobular space. Lymphocyte cuffs were observed in many hyperemic blood vessels. Presence of lymphocytes in bronchiolar or bronchial lumen was sporadic. Mφs increased around the epithelium, interlobular space and subpleural area and inside bronchial and bronchiolar lumen, where they were occasionally seen in control sections. Their number increased inside alveolar walls and in bronchial BALT. Inside the alveolar space, Mφs did not show a noticeable increase, but some degenerative free, pneumocytes appeared. PMNs could sometimes be seen in bronchial and bronchiolar lumen and occasionally infiltrating the epithelium. More were observed inside hyperemic blood vessels.

5.2.5 Immunohistochemical detection of T lymphocyte subsets and macrophages in lung lesions from PIV-3 experimentally infected lambs

T lymphocyte subsets and Mφs were identified, by immunohistochemistry, in sections of colostrum deprived lungs showing lesions of interstitial pneumonia, after 7 days p.i. with OPIV-3 270-7. Serial frozen sections were cut and stained for CD4 and CD8
FIGURE 5. 2 OPIV-3 particles detection in LWF cells. Cytospin smear of LWF obtained from a lamb 7 days p.i. with OPIV-3 270-7. Immunostaining was carried out with OPIV-3 rabbit antisera. All four Mφs but none of the three lymphocytes show positive immunostaining (red colour) after incubation with OPIV-3 antisera. IIAP. The nuclei were counterstained with H/E. Magnification x400.
TABLE 5. 3 Leucocyte cell distribution in lung sections from control\(^1\) and PIV-3 infected\(^2\) lambs according to histological regions.

<table>
<thead>
<tr>
<th>Histological area</th>
<th>Lymphocytes</th>
<th>Mφs</th>
<th>PMNs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>PIV-3</td>
<td>C</td>
</tr>
<tr>
<td>Bronchi lumen</td>
<td>0.0(^3)</td>
<td>09.0±03.6</td>
<td>0.0</td>
</tr>
<tr>
<td>epithel</td>
<td>0.0</td>
<td>36.5±12.6</td>
<td>0.0</td>
</tr>
<tr>
<td>BALT</td>
<td>72.6±09.8</td>
<td>160.8±18.9</td>
<td>03.1±05.4</td>
</tr>
<tr>
<td>Bronchioles lumen</td>
<td>0.0</td>
<td>04.2±03.6</td>
<td>0.0</td>
</tr>
<tr>
<td>epithel</td>
<td>0.0</td>
<td>22.9±07.8</td>
<td>0.0</td>
</tr>
<tr>
<td>BALT</td>
<td>32.8±13.9</td>
<td>76.2±17.2</td>
<td>01.2±03.2</td>
</tr>
<tr>
<td>Alveolar space</td>
<td>0.0</td>
<td>02.8±01.7</td>
<td>06.3±04.9</td>
</tr>
<tr>
<td>Alveolar wall</td>
<td>20.4±00.9</td>
<td>75.6±18.8</td>
<td>05.6±06.3</td>
</tr>
<tr>
<td>Interlobular area</td>
<td>0.0</td>
<td>12.3±07.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Subpleural area</td>
<td>0.0</td>
<td>14.6±09.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Perivascular cuffs</td>
<td>0.0</td>
<td>26.9±08.6</td>
<td>0.0</td>
</tr>
</tbody>
</table>

1 formalin fixed paraffin embedded sections from five inoculated (7 days p.i.) PIV-3 positive lambs n= 25 (5 sections from each animal taken from affected lobes). Giemsa stain
2 formalin fixed paraffin embedded sections from five control PIV-3 negative lambs n= 25 (5 sections from each animal taken from anteroventral lobes). Giemsa stain
3 Results are expressed as the mean number(±SD) of cells found in each region considering 10 random observations per histological region in every section, magnification 600x
lymphocyte T cell markers. Mφs were detected by using monoclonal antibody VPM32. Corresponding areas were compared for staining for T cell and Mφ markers. The reactivity was detected by indirect alkaline phosphatase immunostaining. Table 5.4 shows the results with distribution of positive cells according to histological areas in lung tissue.

In control lung sections (mock-infected lambs) BALT structures were small and detected around a few bronchioles. Occasional peribronchiolar and peribronchial lymphocytes were observed. Most lymphocytes in BALT, around the epithelium and inside the alveolar walls were positive to CD4+ staining (Fig. 5.3a). Fewer cells were positive for CD8+ staining (Fig. 5.3b). This gave a CD4+/CD8+ ratio of 12.2±4.1 for bronchial BALT and of 8.8±3.4 for bronchiolar BALT (Table 5.4).

In contrast, immunostaining of lung sections from animals 7 days p.i. with PIV-3 270-7, showed significant differences in number and distribution of cells in interstitial pneumonic lesions (see Table 5.4). CD4+ lymphocytes were also detectable in BALT, around the epithelium and inside the alveolar walls in a similar number to those found in control lambs (Fig. 5.3c,d). In contrast the numbers of CD8+ had increased significantly (p<0.001) particularly in BALT (Table 5.4) where this accounted for most of the BALT hyperplasia. This markedly and significantly (p<0.001) changed the CD4+/CD8+ ratio of BALT, originally from 12.2±4.1 to 0.25±0.03 in bronchial BALT and from 8.8±3.4 to 0.30±0.10 in bronchiolar BALT (Fig. 5.4a-c). CD8+ lymphocytes were detected in the mantle and germinal centre areas of BALT nodes. Likewise in alveolar spaces there was a change in the CD4+/CD8+ from 2.4±0.5 to 0.97±0.01. In contrast, inside the alveolar walls the CD4+/CD8+ ratio did not change after infection. However, there was a significant (p<0.001) increase in absolute numbers of both CD4+ and CD8+ lymphocytes.

Immunostaining of sections from mock-infected animals, by monoclonal antibody VPM32 showed a few scattered Mφs located in the central areas of BALT, alveolar walls, alveolar space and around the bronchi/bronchiolar epithelium (Fig. 5.5a). In contrast, Mφ immunostaining of lung sections from animals 7 days p.i. with PIV-3 270-7, showed that these cells increased significantly (p<0.001) in all areas (Table 5.4). Mφs numbers increased particularly in interstitial tissue such as peribronchial and peribronchiolar areas, surrounding the epithelial wall and migrating towards the lumen (Fig. 5.5b,c). Also, Mφs with long cytoplasmic processes could be seen in central areas.
### TABLE 5

Distribution of lymphocyte subsets (CD4+ and CD8+) and macrophages in lung sections from control and PIV-3 infected lambs detected by immunohistochemistry.

<table>
<thead>
<tr>
<th>Region</th>
<th>Histological area</th>
<th>CD4+</th>
<th>CD8+</th>
<th>CD4+/CD8+</th>
<th>CD4+</th>
<th>CD8+</th>
<th>CD4+/CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchial BALT</td>
<td>S.3+±.5</td>
<td>4.4+1.9</td>
<td>3.8±1.2</td>
<td>1.16±0.59</td>
<td>5.3±1.1</td>
<td>2.3+±1.2</td>
<td>1.13±0.59</td>
</tr>
<tr>
<td>Bronchial BALT</td>
<td>S.3+±.5</td>
<td>4.6+1.2</td>
<td>1.86±1.1</td>
<td>2.56±1.6</td>
<td>4.6±1.2</td>
<td>1.86±1.1</td>
<td>2.56±1.6</td>
</tr>
<tr>
<td>BALT</td>
<td>S.3+±.5</td>
<td>4.6+1.2</td>
<td>1.86±1.1</td>
<td>2.56±1.6</td>
<td>4.6±1.2</td>
<td>1.86±1.1</td>
<td>2.56±1.6</td>
</tr>
</tbody>
</table>

*Results are expressed as the mean number (±SD) of cells found in each region considering 10 random observations per histological section.*

1 Frozen sections from five normal, PIV-3 negative lambs (7 sections from each animal, taken from anterior lobes).

2 Frozen sections from five inoculated (7 days p.i.) PIV-3 positive lambs (7 sections from each animal, taken from affected lobes). Indirect alkaline phosphatase immunostaining.

3 Significantly different from controls at p<0.05 (Mann-Whitney non-parametric rank test)

*Significantly different from controls at p<0.001 (Mann-Whitney non-parametric rank test)*

Region in every section measured in 600x magnification.

Lymphocytes detected by immunohistochemistry.
**FIGURE 5.3** Immunostaining of CD4+ and CD8+ T lymphocyte subsets in frozen lung sections of interstitial pneumonic areas from lambs after 7 days p.i. with OPIV-3 270-7. A series of frozen sections was cut and incubated with monoclonal antibodies SBU-T4 and SBU-T8 for CD4 and CD8 sheep lymphocyte T cell markers, respectively. The reactivity was detected by indirect immunoalkaline phosphatase (IIAP) staining.

a) Bronchiole surrounded by lymphocytes that showed positive immunostaining (red colour) for the CD4 marker. These cells are distributed around the bronchiolar epithelium where there is also a smaller number of non-stained lymphocytes (green nuclei). IIP with SBU-T4 mAb. The nuclei were counterstained by methyl green. Magnification x400.

b) Bronchiole surrounded by lymphocytes that showed positive immunostaining (red colour) for CD8 marker. These cells are distributed around the bronchiolar epithelium and the alveolar walls where there is also a larger number of non-stained lymphocytes (green nuclei). IIP with SBU-T8 mAb. The nuclei were counterstained with methyl green. Magnification x200.

c) A large BALT close to a bronchiole can be seen where there are some positive cells (red colour) for the CD4 marker. Most cells in this lymphoid structure remain negative (green nuclei). More scattered positive cells are observed in the alveolar walls. IIP with SBU-T4 mAb. The nuclei were counterstained with methyl green. Magnification x100.

d) Closer view of figure 5.5c. Detail of BALT node where most of the lymphocytes are negative for CD4 marker. SBU-T4 mAb. The nuclei were counterstained with methyl green. Magnification x400.
FIGURE 5.4 Immunostaining of CD8+ T lymphocytes in frozen lung sections of interstitial pneumonic areas from lambs after 7 days p.i. with OPIV-3 270-7. A series of frozen sections was cut and incubated with monoclonal antibody SBU-T8 for CD8 sheep lymphocytes T cell marker. The reactivity was detected by indirect immunoalkaline phosphatase (IAP) staining.

a) Two BALT nodes with marked hyperplasia can be seen. They show that most of the lymphocytes stained for the CD8 marker (red colour). A few cells remain negative in the periphery (green nuclei) of this lymphoid structure. IIP with SBU-T8 mAb. The nuclei were counterstained with methyl green. Magnification x100.

b) A closer view of the BALT located to the right in figure 5.6a. Most of lymphocytes in this lymphoid structure are positive (red colour) for lymphocyte marker CD8. IIP with SBU-T8 mAb. The nuclei were counterstained with methyl green. Magnification x400.

c) There is strong staining for the CD8 marker in lymphocytes located around the peribronchiolar area immediately below the epithelium. Staining of the cell membrane is noticeable. IIP with SBU-T8 mAb. The nuclei were counterstained with methyl green. Magnification x100.
FIGURE 5.5 Immunostaining of Mφs in frozen lung sections of interstitial pneumonic areas from lambs after 7 days p.i. with OPIV-3 270-7. A series of frozen sections was cut and incubated with monoclonal antibody VPM32 for ovine tissue Mφs. The reactivity was detected by indirect immunoalkaline phosphatase (IIAP) staining.

a) Scattered cells positive for VPM32 Mφ marker (red colour) can be observed around the bronchiolar epithelium and migrating towards the lumen. Also there are some positive cells in the alveolar walls which show increased thickness. IIP with VPM32 mAb. The nuclei were counterstained with methyl green. Magnification x200.

b) Overview of an atelectatic area around two bronchioles that show cell debris inside their lumens. Strong positive staining of many cells in the alveolar walls and the alveolar spaces. (green nuclei) can be seen. IIP with VPM32. The nuclei were counterstained with methyl green. Magnification x100.

c) Closer view of the peribronchiolar area from Fig. 5.7b. A large number of Mφs (red colour) can be observed either in small groups in the alveolar spaces or individually in the alveolar walls. IIP with VPM32 mAb. The nuclei were counterstained with methyl green. Magnification x400.

d) Close view of a hyperplastic BALT structure where there are some positive cells for Mφ marker VPM32. They are located in the centre of this lymphoid structure. Lymphocytes (green nuclei) remain negative. IIAP with VPM32 mAb. The nuclei were counterstained with methyl green. Magnification x400.
of BALT (Fig. 5.5d). The increment of AMΦs was less noticeable than that of the interstitial MΦs.

5.2.6 Immunohistochemical detection of PIV-3 infected macrophages and lymphocyte subsets in lung lesions from PIV-3 experimentally infected lambs by double immunostaining.

PIV-3 infected AMΦs or lymphocyte subsets CD4+ and CD8+ were detected by double immunostaining SA-HRP/IAP. First, sections were incubated with rabbit anti OPIV-3 sera, then with biotinylated goat anti-rabbit IgG and finally with HRP-conjugated avidin. The enzyme substrate was 4CN which gave blue product. Then sections were incubated with either mouse monoclonal antibody VPM32, SBU-T4 or SBU-T8 for MΦs, CD4+ or CD8+ T lymphocytes, respectively and then with AP-conjugated donkey anti-mouse IgG. The enzyme substrate was Naphtol AS-MX/Fast Red which gave a red product.

Lung sections showing interstitial pneumonia lesions after 7 days post experimental inoculation with OPIV-3 were double immunostained with rabbit antiserum and mAbs SUB-T4 and SBU-T8. They did not show positive staining for virus particles in cells staining for either CD4 or CD8 markers. In contrast sections double immunostained with rabbit anti OPIV-3 serum and mAb VPM32 showed that many cells which stained for the ovine tissue MΦ marker (VPM32) also stained for virus particles.

In double immunostained sections viral particle staining (blue colour) appeared not only associated with some MΦs (red colour) but also with some epithelial septal cells (pneumocytes) (Fig. 5.6a). In alveolar spaces large, round MΦs appeared intensely stained for MΦ marker VPM32. Some of these AMΦs also showed positive staining for virus particles (Fig. 5.6a,b). Interstitial MΦs also showed positive staining for virus particles. Large round cells, in alveolar spaces, which stained only for the virus (blue colour only) were more likely to correspond to detached degenerative epithelial cells. It was noted that in some areas there was patchy distribution of the virus particle staining following the lobular structure. Thus some lobules showed viral particle staining while the surrounding ones were negative (Fig.5.6c,d).
FIGURE 5. 6 PIV-3 infected AMφ detection by double immunostaining (SA-HRP/IAP) in frozen lung sections of interstitial pneumonic areas from lambs after 7 days p.i. with OPIV-3 270-7. Firstly, sections were incubated with rabbit anti OPIV-3 sera as primary antibody, then with secondary antibody biotinylated goat anti-rabbit IgG and finally with a third antibody HRP-conjugated avidin. The enzyme substrate was 4CN for a blue product. Then sections were incubated with mouse monoclonal antibody VPM32 as a primary antibody and then with AP-conjugated donkey anti-mouse IgG. The enzyme substrate was Naphtol AS-MX/Fast Red for a red product.

a) Many positive cells for VPM32 Mφ marker (red colour) can be seen in the alveolar walls or in the alveolar spaces. Positive staining for PIV-3 particles (blue colour) can be seen in some pneumocytes (small arrows) and also in some alveolar and interstitial Mφs (large arrow). SA-HRP/IAP with VPM32 mAb and OPIV-3 rabbit antisera. Nuclei were counterstained with methyl green. Magnification x400.

b) Small groups of Mφs in the alveolar spaces (arrows) are stained for both Mφ marker VPM32 (red colour) and OPIV-3 particles (blue colour). SA-HRP/IAP with VPM32 mAb and OPIV-3 rabbit antisera. The nuclei were counterstained with methyl green. Magnification x200.

c) Overview of an atelectatic area where the division between two lobules can be distinguished (large arrow). It is possible to observe OPIV-3 particle staining (blue colour) in the upper lobule, but not in the lower one. Positive cells for Mφ marker VPM32 (red colour) can be observed in both lobules. SA-HRP/IAP with VPM32 mAb and OPIV-3 rabbit antisera. The nuclei were counterstained with methyl green. Magnification x100.

d) Closer view of Fig. 5.8c. Detail of the division between two lobules where there is staining for OPIV-3 particles (blue colour) in the lobule to the left but not in the one to the right. SA-HRP/IAP with VPM32 mAb and OPIV-3 rabbit antisera. The nuclei were counterstained with methyl green. Magnification x400.
No PIV-3 particle staining was detectable in bronchial or bronchiolar epithelial cells. Likewise, no viral particles were detected by immunohistochemistry in BALT nodes. Neither lymphocytes nor any other type of leukocyte showed positive staining for the virus.
5.3 DISCUSSION

The results shown in this study demonstrate alterations in the normal composition of CD4⁺ and CD8⁺ lymphocyte subsets that could play a key role in the pathogenesis of OPIV-3 infection in the lung and could provide a better understanding of the pathogenic mechanisms of OPIV-3 in sheep pneumonia. This viral infection is associated with accumulation of lymphocytic cells, however the role for the different subsets remains unknown (Crouch, 1990).

An increased interstitial cellularity and a thickening of the alveolar septa due to Mφ and lymphocyte infiltration 7 days p.i. with OPIV-3 270-7 has been previously described in this study (section 4.2.5.2). These findings are similar to those from other reports on OPIV-3 experimental infection (Cutlip & Lehmkhul, 1982). Accordingly, an increased recovery of cells in lung wash fluid (LWF) could be expected. However, in this study the total LWF cell count in OPIV-3 infected animals, showed some increase, but did not differ significantly (p>0.01) from the controls. This could be due to the fact that in interstitial pneumonia most changes occur in the lung interstitial tissue rather than at the alveolar level. Moreover the lung pathology induced by OPIV-3 in the experimental lambs in this study were of focal distribution and restricted to anterior lobes. Furthermore, although the total cell count for LWF cell recovery after OPIV-3 infection was not significantly higher than in controls, there was a significant increase (p<0.001) in the proportion and also in absolute number of lymphocytes, when compared to controls. Also, lymphocytes were the main mononuclear cell type found in PIV-3 lung sections in this study. On the other hand, there was a decreased proportion of Mφs in LWF with a non-significant low increase in absolute number.

In contrast AMφs in LWF after 7 days p.i. with OPIV-3 showed a significant (p<0.05) decrease in frequency. Although their absolute number was increased this was not significant (p>0.01). AMφs although increased in pneumonic lung lesions their increment was less noticeable than the one of interstitial Mφs. This may reflect a cytolytic action of OPIV-3 for AMφs.

Similar changes in cellularity and both lymphocyte and Mφ recovery, from bronchoalveolar lavage (BAL), have been described after infection with MVV in sheep (Lujan et al., 1993). This virus not only induces interstitial pneumonic lesions
but also grows in AM\(\Phi\)s (Lee, 1994). In contrast, intratracheal inoculation of PIV-3 in guinea pigs (Folkerts et al., 1992) has been reported to induce airway hyper-responsiveness with significantly increased cellularity and recovery of AM\(\Phi\)s, monocytes, lymphocytes and even eosinophils after 4 days p.i. However, no data is available on the type and extent of lung pathology induced by OPIV-3 in these guinea pigs, showing airway hyper-responsiveness, for comparative purposes.

In addition to the increase in lymphocytes there was also an increase in PMNs in LWF from OPIV-3 infected animals. An increased secretion of Neutrophil Chemotactic Activity (CA) factor by alveolar cells from MVV pathologically affected animals has been reported (Cordier et al., 1990). It has also been described here (section 4.2.5.2.) and elsewhere (Cutlip & Lehmkhul, 1982) that necrosis is one of the histopathological findings in interstitial pneumonia induced by PIV-3. This could account for the increased recruitment of PMNs into the affected lungs. Also the release of granulocyte-macrophage colony-stimulating factor (GM-CSF) from AM\(\Phi\)s has been demonstrated to occur in vivo in the lung during HIV-1 infection (Agostini et al., 1992). This cytokine has well recognized effects on granulocyte and M\(\Phi\) growth and differentiation and plays some role in the mechanisms leading to the accumulation of M\(\Phi\)s and PMNs in patients with interstitial lung disease. These histopathological findings are concordant with many others from interstitial lung diseases in humans and animals (Martin et al., 1983). The local overproduction of GM-CSF is likely to play a role in the pathogenic events leading to the local accumulation of M\(\Phi\)s and recruitment of PMNs in PIV-3 infection.

The significant decrease (p<0.001) in the CD\(^4^+\)/CD\(^8^+\) ratio of lymphocytes from LWF of PIV-3 infected animals is due to both a significant increase of CD\(^8^+\) T lymphocytes and a significant decrease of CD\(^4^+\) T lymphocytes. This pattern of immune response is similar to that observed in murine lung lesions caused by the influenza virus (Allan et al., 1990) and also in inflammatory meningeal exudate after lymphocytic choriomeningitis (LCM) virus infection (Baezinger et al., 1988), where CD\(^8^+\) cells increase and largely surpass CD\(^4^+\) cells. In influenza CD\(^8^+\) cells promote viral clearance, monocyte release from bone marrow and induce severe inflammation in the absence of the CD\(^4^+\) T lymphocyte response (Allan et al., 1990).

The mechanism of protection against infection varies according to the etiological agent involved. In rats infected with the measles virus, CD\(^4^+\) cells are more important in protection than CD\(^8^+\) cells. In infections with LCM virus, CD\(^4^+\) cells are not
required when CD8+ cells are present, however, after CD8+ depletion, CD4+ cells can compensate this deficiency and play a protective role (Scott & Kaufmann, 1991).

CD8+ lymphocytes play an important role not only in viral diseases but they also are important in many diseases produced by bacteria and parasites such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Toxoplasma gondi*, *Plasmodium spp.*, *Theileria parvum* and *Tripanosoma cruzi* (Scott & Kaufmann, 1991).

The fact that CD8+ are induced to proliferate in response to PIV-3 infection as well as in response to other viral or non-viral infectious diseases is of considerable importance in the strategy and design of prophylactic schemes for these diseases. For example, vaccines against these pathogens could be designed to introduce antigens into MHC class I molecules, while recombinant bacteria could be used as carriers for vaccines against viral infections (Scott & Kaufmann, 1991).

In this study, after 7 days p.i with OPIV-3 virus particles were detected by histochemistry in association with pulmonary Mφs. In contrast, no virus was associated with CD4+ or CD8+ lymphocyte subsets. This seems to disagree with findings by Sieg et al., (1994) who have reported that HPIV-3 readily infects T cells *in vitro*; however, they also reported that T cell infection by PIV-3 may not be detectable, by immunostaining, before 1 or 2 weeks p.i. It is possible that the short period which had elapsed before taking the lung samples for this study (7 days p.i.) did not give time for infected lymphocytes to express viral proteins. Besides, no data is available on the time required to observe lymphocyte viral protein expression after *in vivo* infection with PIV-3. Furthermore, Basaraba et al. (1993) have reported, from *in vitro* infection of lymphocytes cultures with PIV-3, that despite the presence of lymphocyte-associated virus antigen, detected by direct immunofluorescence, no increase in PIV-3 titre above baseline was seen in infected lymphocytes, irrespective of mitogen stimulation. Likewise, lymphocytes did not contribute to the extracellular virus pool in lymphocyte-Mφ cultures as the increases in viral titre in supernatants were equal to levels released by Mφs alone. They conclude that the expression of viral antigen on lymphocytes stimulated in the presence of PIV-3-infected bovine AMφs suggests a non-productive or abortive infection of lymphocytes mediated through contact with infected Mφs. Similar findings were observed in this study and are described in the following chapter.
In lungs after 7 days p.i there was an increase of pulmonary lymphocytes both in the alveolar walls and the BALT nodes. Similarly, in the alveolar walls of MVV infected sheep lungs there was an increase of CD4+ and CD8+ lymphocytes with a CD4+/CD8+ ratio similar to control non infected animals. In regional lymph nodes of MVV infected animals there was also an increase of CD8+ T lymphocytes (Watt et al., 1992). This suggests that activated T lymphocytes, particularly CD8+ cells have an important role in the pathogenesis of lymph nodes and pulmonary lesions as part of the interstitial pneumonia induced by PIV-3 and MVV.

The accumulation of lymphocytes in the interstitial pulmonary tissue found in this study during PIV-3 infection can be the result of retention and proliferation of lymphocytes in response to cytokines, and/or the presence of viral antigens. In this study a clear increase in CD8+ T lymphocytes was observed as an inflammatory infiltrate distributed along the alveolar walls, around the bronchi and bronchioles, around the blood vessels and in particular as the main cell component of BALT hyperplasia. CD8+ lymphocytes were detected in the germinal centre and mantle areas of secondary BALT nodes. This is in agreement with observations in other viral induced interstitial pneumonia such as HIV in man and MVV in sheep (Watt et al., 1992). However, no OPIV-3 particles were detected by immunohistochemistry in these lymphoid structures. Similar findings has been reported in MVV infection by Watt et al. (1992). They suggest that the reaction in these lymphoid nodes can be driven by soluble antigens drained to this nodes or transported by cells from infected areas.

Generalized immunosuppression is not commonly associated with PIV-3 infection. Moreover, infected individuals often recover from the infection, if no secondary bacterial contamination occurs. This indicates that specific immune response is not totally obliterated. However, PIV-3 has the potential to both persist and reinfect in vivo (Basle et al., 1986). The mechanisms for these phenomena has not been described. HPIV-3 mediated immunoregulation of human T lymphocytes has been suggested to play an important role in the failure of the virus to induce lifelong immunity (Sieg et al., 1994; Ray et al., 1995).

It is likely that the equilibrium between the host and the viral parasite arises as a result of the complex interactions between the host immune system and the virus. It has been suggested that PIV-3 infection preferentially inhibits the establishment of specific
anti-PIV3 memory T cells (Sieg et al., 1994). So far, the role of specific T cells in the immune system to PIV-3 has not been thoroughly investigated. However, it seems likely that the virus has the potential for manipulating the immune response.

T cells have the capacity to lyse virally infected cells, help B lymphocytes produce immunoglobulins and establish long lived memory cells. Hence, the possibility that PIV-3 mediated immunoregulation of T lymphocytes plays a role in either viral persistence or reinfection should be clarified.

The increase in both alveolar and interstitial Mφs observed after 7 days of infection with OPIV-3 suggests a role for these cells in the host mechanisms of defence against infection and also in the mechanisms of lung pathology. Pulmonary interstitial Mφs, for instance, have a greater capacity to replicate and produce cytokines and growth factors that are essential for proliferation of fibroblasts (Shaw, 1991). This may contribute to the alveolar wall thickening and fibrosis observed in interstitial pneumonia. Furthermore, they are the most effective cells in stimulating T cell response to viral antigen (Holian & Scheule, 1990). Mφs can be activated during viral infection (Hearst et al., 1980) and this activation, during PIV-3 infection, is considered as an indirect effect possibly caused by the action of cytokines, in particular to γ-IFN (Babiuk et al., 1988).

A significant proportion of Mφs showed positive immunostaining for OPIV-3 suggesting that infection of these cells occurs in vivo. This agrees with evidence of PIV-3 replication in Mφs detected by electron microscopy (Bryson et al., 1983; Tsai, 1977). However, it was also observed in this study that many alveolar and interstitial pulmonary Mφs after 7 days p.i. showed no immunostaining for the virus. This could be the result of a lytic CPE effect of the virus combined with a rapid cell turnover. Also, it has been frequently observed in vitro (Moscona & Peluso, 1991a, 1992, 1993b) that HPIV-3 can induce a persistent viral infection with production of defective virus particles and no CPE. These particles may not be detectable by the monoclonal anti HPIV-3 protein HN used here to detect the virus. Therefore, it is necessary to carry out further studies in order to determine whether a proportion of the PIV-3 negative Mφs in PIV-3 infected animals is actually persistently infected with this virus.
CHAPTER SIX

IN VITRO INFECTION OF OVINE PERIPHERAL BLOOD DERIVED MACROPHAGES AND PULMONARY ALVEOLAR MACROPHAGES WITH PARAINFLUENZA TYPE 3 VIRUS

PART A: OPIV-3 INFECTION OF MONOCYTE/MACROPHAGE CELL CULTURES

PART B: PHENOTYPIC CHANGES
In the lung inhaled antigens can stimulate local and systemic primary immune responses. Many infectious organisms that invade the body via the lung are controlled by immune responses elicited locally. Furthermore, antigens inhaled by immune hosts are suspected of being responsible for triggering type I and type IV hypersensitivity reactions that can result in disease of the lung (Dubois, 1986). Most immune responses have been shown to require antigen presenting cells to stimulate the appropriate T lymphocytes (Godstein et al., 1973; Toews et al., 1984). It is likely that AM\(\phi\)s are important antigen-presenting cells and function in the initiation and secondary elicitation of immune responses within the lung. Likewise, it has been postulated that, in vivo, AM\(\phi\)s release cytokines critical for the activation of dendritic cells (Miller et al., 1992). This would take place during the afferent limb of the immune response. Alveolar M\(\phi\)s would respond to products of sensitized T-cells such as \(\gamma\)-IFN and IL-4 to interact with T-helper cells in an antigen-specific MHC-restricted manner during the efferent limb of the response (Miller et al., 1992). Their role, however, has not been completely investigated.

The monocyte/M\(\phi\) lineage originates from haemopoietic stem cells which subsequently develop into monoblasts or promonocytes in bone marrow (Godleski & Brain, 1972; Thomas et al., 1976). By complex cytokine stimulation and regulation, these cells differentiate into monocytes and are released into blood. Monocytes may remain in the blood for 36 to 104 hours and then migrate into tissues. Subsequently, monocytes differentiate and mature as resident M\(\phi\)s. These resident M\(\phi\)s have similar morphology and basic phenotype, but still display considerable functional heterogeneity in response to the resident environment (Beelen et al, 1989).

In pulmonary tissue, M\(\phi\)s may be found i) in the interstitial connective tissue of the alveolar wall ii) forming part of the lining epithelium of the alveolus and iii) free in the lumen of the alveolus. Some of the latter are true M\(\phi\)s and others are free defoliated large alveolar cells. The fine structure of free AM\(\phi\)s contains a moderate amount of smooth endoplasmic reticulum and relatively little rough endoplasmic reticulum. They have numerous processes and invaginations on their surfaces. Many
Inclusion bodies or lysosomes are present, often containing myelin figures or ferritin. Occasionally these structures are paracrystalline (Carr, 1973).

It is not difficult to establish a short-term mixed culture containing a preponderance of Mφs but much harder is to obtain a long term pure culture. Perhaps the best way to obtain a pure culture is by lavaging the serosal cavity, usually the peritoneal cavity or by lung washout. When cultured on a glass surface, other cells may be present. Polymorphonuclear cells degenerate rapidly and lymphocytes are non-adhesive. Only Mφs remain attached to glass after few hours of culture. The attached Mφs will change their shape and look very different from the relatively spherical cells obtained from lavaging out cavities but not very different from the cells that lie in the tissue spaces (Carr, 1973).

The structure of the Mφ in culture varies according to the length of time it has been in culture and with its source of origin. The phases in its culture include 1) adherence, 2) spreading and phagocytosis of debris, 3) mitosis and 4) extended culture. With the scanning electron microscope the shape of a peritoneal Mφ can be followed during the process of settling on a glass surface; it changes from a spherical to a flattened round form and then to a flat stellate form. Very fine protoplasmatic processes are extruded during this change. During prolonged culture the shape may be flattened and virtually circular, elongated or fully extended and may measure 15-80µm. Stellate forms may sometimes occur with curious end knobs. When the cell is moving a broad undulating flap-like membrane may act as the leading edge; sometimes two processes may be seen advancing in slightly different directions, each led by a small undulating membrane. Sometimes adjacent cells on a flat surface may apparently adhere to one another so closely as to form a membrane which will strip off the glass. Multinucleated giant cells may form (Carr, 1973).

The Mφ in culture has an oval or round nucleus which is often indented and resembles the nucleus of the blood monocyte. It contains dispersed chromatin granules but nucleoli are absent. The cytoplasm has a varied appearance which depends on the activity of the cell. They tend to show numerous ruffles and globules on the surface probably associated with pinocytosis (Stuart, 1970).

Interleukin 3 (IL-3) and granulocyte-Mφ-colony-stimulating factor (GM-CSF) have a broad specific activity on pluripotent stem cells leading to their differentiation and
proliferation (Schrader, 1986). IL-3 acts on early progenitors which develop into mature cells of multiple lineages (granulocytes, monocytes and megakaryocytes). Macrophage-colony-stimulating factor (M-CSF) and GM-CSF act at a late stage to stimulate progenitors to develop into monocytes and Mφs (Geissler et al., 1989; Eischen et al., 1991; Haig et al., 1990).

The leucocyte integrins (β2 integrins, CD11/CD18) are involved in mediating important leukocyte adhesion dependent interactions, including cell to cell communication thus imparting a significant role in host defence by mediating inflammatory and immune responses (Haines, 1992). The molecules of leucocyte integrins are exclusively expressed by leukocytes and are major cell surface molecules on monocyte/Mφs (Ross et al., 1989; Mackay & Imhof, 1993). The leucocyte integrin family has three structurally related glycoproteins, CD11a (LFA-1), CD11b (Mac-1, CR3) and CD11c (p150,95, CR4). Each of these three glycoproteins exist as an αβ complex in which a unique α subunit is associated with a common β subunit, the β2 chain (CD18) (Haines, 1992). These molecules are involved in stabilizing cell contact between Mφs and immune effector cells to allow antigen presentation to lymphocytes (Krensky et al., 1983; Denning et al., 1988). Intercellular adhesion molecules (ICAM) 1 and 2 act as ligands for CD11a. ICAM-1 is present on endothelium, leukocytes and many epithelial cell types. ICAM-2 is constitutively expressed by endothelial cells and leukocytes (Springer, 1990). Both CD11b and CD11c molecules are specific for the complement component, C3b, and are involved in phagocytosis (Myones et al., 1988; Owen et al., 1992).

Immunoglobulin Fc receptors (FcR’s) are involved in phagocytosis and endocytosis (Fridman, 1989, 1991). The expression of Fc receptors for IgG (FcγR) by Mφs enables them to phagocytose a variety of antigens in the presence of specific antibody thus enhancing the range of antigens ingested by phagocytes (Jungi & Hafner, 1986; Fanger et al., 1989; Sarmay, 1992). FcγRII (CDw32) and FcγRIII (CD16) have been identified on monocytes and Mφs and they bind complexed IgG only (Anderson, 1987; Ravetch & Kinet, 1991; Levy et al., 1991). All are members of the immunoglobulin superfamily with transmembrane and cytoplasmic regions and a variable number of extracellular domains (William & Barclay, 1988). Expression of these molecules on Mφs is enhanced by γ-IFN and IL-2 (Fanger et al., 1989; Heagy et al, 1984; Gonwa et al., 1986; Nash et al., 1992).
CD14 is a myeloid differentiation antigen expressed primarily on the cell surface of monocytes and Mφs. The CD14 is anchored to the cell surface via a glycosyl phosphatidyl inositol (GPI) linkage (Goyert et al., 1989). The antigen also exists in various soluble forms (Haziot et al., 1988; Durieux et al., 1994). Both the membrane and soluble forms of the antigen bind lipopolysaccharide (LPS) complexed to LPS binding protein (LBP), an acute phase protein present in serum. The binding of LPS-LBP complex to CD14 results in release of various inflammatory cytokines including TNF-α, leading to the induction of endotoxic shock (Ulevitch, 1993). CD14 is down-regulated by γ-IFN (Ziegler-Heitbrock & Ulevitch, 1993).

The direct effect on surface molecule expression of Mφs after infection with PIV-3 is not clear. Here, a study of the changes in the phenotype of sheep monocyte/Mφ cells including fresh monocytes and cultured both monocyte-derived Mφs (MDMφ) and alveolar Mφs (AMφ) was undertaken, before and after infection with OPIV-3.

There is often variation in monocyte/Mφ phenotype not only amongst different tissue types but also amongst different individuals. This heterogeneity may be related to variations in function (Lee, 1994) as well as in susceptibility to infection. Therefore, in this study, changes in monocyte/Mφ phenotype associated with culture and induced by OPIV-3 in vitro are analysed. The phenotype was characterised by detecting the expression of a number of molecules associated with differentiation and/or function, with monoclonal antibodies. They included CD4, CD8, VPM32 antigen, LFA-1, LFA-3 and CD14. Also, the susceptibility of different monocyte/Mφ populations and lymphocytes to infection by OPIV-3 (270-7 strain isolated in this study) is tested. These observations may help to explain functional changes observed both in vitro and in vivo functional studies.
6.2 RESULTS

6.2.1 PART A: OPIV-3 INFECTION OF MONOCYTE/MACROPHAGE CELL CULTURES

6.2.1.1 General observations in monocyte-derived macrophage (MDMϕ) cell cultures during maturation in vitro.

Monocytes were collected from peripheral blood (PBMC) and cultured in gelatin-coated flasks or plates, as previously described in section 2.2.3. The number of monocytes in the cultures was estimated by non-specific esterase staining (NSE). The presence of other leukocytes was detected by both Giemsa's stain and indirect immunofluorescence. The period of time the cells were allowed to attach, before the non-adherent cells were removed (by washing them off) seemed to affect both the cell number and composition of adherent cells, evaluated after 3 days in culture. Thus, when non-adherent cells were washed off after 1 or 2 hours incubation, a high proportion of adherent monocytes (over 80%) were obtained. However, they showed a low absolute number. Most of the contaminating cells were lymphocytes. It was difficult to obtain cell monolayers after 5 to 7 days with this method. In contrast, when non-adherent cells were washed off after overnight incubation (16 hrs.) the total number of cells, after 3 days in culture, was much higher but the proportion of monocytes was lower (70-80%), with more contaminating lymphocytes (data not shown). Many of the contaminating cells were trapped and attached to the flasks by strands of a material which was thought to be fibrin produced by MDMϕs. After 5 days of culture these aggregates of contaminating cells along with strands of fibrin became detached and were easily removed from the flasks or plates. The proportion of monocytes obtained with this method, after 5 days of culture, was much higher (near 95%), with a high number of cells in culture which form a continuous monolayer (Fig.6.1a). This method was chosen for providing MDMϕs, in the experiments described in this chapter. Monocytes cultured for 5 to 7 days markedly increased their size and cytoplasm/nucleus ratio and also showed very strong NSE staining compared to fresh monocytes (Fig.6.1b). Also, cytoplasmic vacuolization, binucleated cells and a few scattered syncytia were observed with nuclei disposition in
the central area of the cytoplasm (Fig. 6.1c). Some MDMφs from early cultures, that had come off the flask surface, could be passaged to 24 well plates where they became adherent and confluent. Contamination with fibroblasts, epithelioid and syncytial cells occurred in some long term MDMφ cultures, after 2-3 weeks. Therefore, MDMφs were infected after 5 days in culture and followed in most instances for no longer than 2 weeks.

6.2.1.2 General observations in alveolar macrophage (AMφ) cell cultures during maturation in vitro.

AMφs were collected from LWF and cultured in gelatin-coated flasks or plates, as previously described in section 2. The presence and number of AMφ in the cultures was estimated by non-specific esterases staining (NSE) and immunofluorescence with mAb VPM32. The presence of other leukocytes was observed by using Giemsa's stain. T lymphocytes were detected by immunofluorescence staining with mAbs SBU-T4 and SBU-T8. Large numbers of cells with a high proportion of Mφs (80 to 90%) were obtained from LWF. The period of time the cells were allowed to attach before the non-adherent cells were removed, did not affect either the cell number or composition of adherent cells, evaluated after 3 days in culture. Thus, even when non-adherent cells were washed off shortly after setting them up (1 or 2 hours incubation), the cultures showed high proportions of Mφs. Staining with mAb VPM32 showed always over 95% of positive cells (Fig. 6.2a). These cells formed monolayers, after a few hours in culture (12-24 hrs.). Staining with mAbs SBU-T4 and SBU-T8 showed that most of the scarce contaminating cells were predominantly T lymphocytes (Fig. 6.2b). After 3 to 5 days in culture AMφs markedly increased their size and cytoplasm/nucleus ratio with few scattered syncytia (larger than MDMφs) that showed nuclei located in the periphery of the cytoplasm (Fig. 6.2c). As well as in MDMφ cultures the production of a fibrin-like material was observed. Some Mφs, from early cultures, that had come off the flask surface, could be passaged to 24 well plates where they become adherent and confluent. In some long term AMφ cultures, after 2-3 weeks, fibroblast, epithelial and syncytial cell contamination also appeared. Therefore, cultures of AMφ, were infected after 2 or 3 days in culture and followed for no longer than two weeks.
FIGURE 6.1 Monocyte-derived macrophage (MDMφ) cell cultures. Peripheral blood monocytes (PBMC) were collected from clinically normal healthy sheep and cultured in gelatin-coated flasks or plates. Non-adherent cells were washed off after overnight incubation.

a) MDMφ after 3 days in culture are seen forming a continuous monolayer. H/E stain. Magnification x40.

b) MDMφ cultured for 5 days showed a marked increase in their size and cytoplasm/nucleus ratio. They also showed very strong NSE staining. H/E stain. Magnification x400.

c) MDMφ cultured for 7 days show some cytoplasmic vacuolization (v), binucleated cells (b) and cytoplasmic processes (p). H/E stain. Magnification x40.
FIGURE 6.2 Alveolar macrophage (AMφ) cell cultures. AMφ were collected from lung wash fluid (LWF) of clinically normal healthy sheep and cultured in gelatin-coated flasks or plates. Non-adherent cells were washed off after overnight incubation.

a) AMφ after 3 days in culture. It can be seen that the majority of cells are positive after immunostaining with ovine tissue Mφ marker VPM32. IIF with mAb VPM32. Magnification x400.

b) A 24 hour AMφ cell culture shows the presence of a few cells positive to the panlymphocyte marker. Low autofluorescence of Mφs is observed in the background. IIF with this mAb. Magnification x400.

c) AMφ after 5 days in culture show a marked increase in size and cytoplasm/nucleus ratio with a few scattered syncytia (arrows) that show nuclei located in the periphery of the cytoplasm. H/E stain. Magnification x40.
6.2.1.3 Cytopathic effect (CPE) of OPIV-3 in monocyte-derived macrophage (MDM\(\phi\)) cell cultures.

During OPIV-3 270-7 replication in MDM\(\phi\)s (infection on day 5 of culture), there were no remarkable morphological changes in the first day p.i. by light microscopic examination, compared with mock-infected controls. But after 2 days p.i. some infected cells became small and refractive and lysed in the culture, 3 to 5 days later. The presence of more numerous and larger syncytia than in mock-infected MDM\(\phi\)s was observed. These syncytia had nuclei located centrally (Fig. 6.3a). From day 3 p.i. presence of cytoplasmic, and less frequently nuclear, eosinophilic inclusion bodies (Fig. 6.3b) was observed in addition to more syncytia. Compared to mock-infected controls, 40-60% of cells were lost within the first 5 days of infection. By day 7 p.i. 80% to 90% of cells were detached from the cell monolayer.

6.2.1.4 Cytopathic effect (CPE) of OPIV-3 in alveolar macrophage (AM\(\phi\)) cell cultures.

In contrast, AM\(\phi\) infected with OPIV-3 270-7 (infection on day 3 of culture) showed CPE starting from day 1 p.i. with round refractive cells detaching from the flask surface, intense cytoplasmic vacuolation (Fig. 6.3c) and the presence of pleomorphic, eosinophilic inclusion bodies. By day 2 p.i. there were numerous cytoplasmic, and sometimes nuclear inclusion bodies. Also present were numerous large syncytia with nuclei located peripherally. They showed numerous inclusion bodies. After 5 days p.i. 40-50% of cells were lysed and detached from the surface. After 7 days 70-80% of cells were lost from the cell monolayer. Despite some cells showing syncytia and/or inclusion bodies the remaining cells were firmly attached to the cell surface and showed long cytoplasmic processes (Fig. 6.3d).

After infection with OPIV-3 270-7 syncytia were first seen from day 2 p.i. in MDM\(\phi\) and from day 1 p.i. in AM\(\phi\). These syncytia in MDM\(\phi\) (often 4-8 nuclei) were not as large as those seen in AM\(\phi\) (often more than 8 nuclei). Also, the nuclei arrangement was different for the two cell types as nuclei appeared located centrally in MDM\(\phi\)s and peripherally in AM\(\phi\)s.
FIGURE 6.3 Monocyte-derived macrophages (MDMφs) and alveolar macrophages (AMφs) cultured in gelatin-coated flasks or plates were inoculated with OPIV-3 270-7 (1 TICD₅₀/cell) after 5 days and 3 days in culture, respectively.

a) MDMφ cell culture after 2 days p.i. with OPIV-3 270-7. Some cells have become small and rounded (arrow). Large syncytia with nuclei centrally located are present. H/E stain. Magnification x200.

b) MDMφ cell culture after 3 days p.i. with OPIV-3 270-7. A large binucleated MDMφ showing presence of numerous pleomorphic cytoplasmic eosinophilic inclusion bodies is shown (c). Also there is an inclusion body located inside the nucleus (n) of this cell. H/E stain. Magnification x200.

c) AMφ cell culture after 1 day p.i. with OPIV-3 270-7. Some cells have become small and rounded (small arrows) and other show intense cytoplasmic vacuolation (large arrows). H/E stain. Magnification x400.

d) MDMφ cell culture after 7 days p.i. with OPIV-3 270-7. Most cells have become detached from the monolayer. A large multinucleated AMφ with long cytoplasmic processes and several pleomorphic cytoplasmic eosinophilic inclusion bodies is observed. H/E stain. Magnification x200.
There was a time-dependent loss of adherent AM\(\Phi\) in infected and mock-infected AM\(\Phi\) from 0 to 5 days p.i. (Table 6.1). There was no significant difference in adherent AM\(\Phi\) numbers between OPIV-3 infected and mock-infected cultures until 5 days p.i. (Fig. 6.4a). The viability of mock-infected AM\(\Phi\) remained constant in culture. In contrast OPIV-3 infected AM\(\Phi\) showed significantly lower (p<0.05) viability than mock-infected cells after 3 days p.i. From this day there was a sharp decrease in the viability of infected cells (Fig. 6.4b). There were always less adherent cells, with lower viability in comparison to mock-infected cultures (Fig. 6.4a,b).

6.2.1.5 Recovery of infectious virus

The time course of OPIV-3 270-7 replication in MDM\(\Phi\)s and AM\(\Phi\)s is shown in Fig. 6.5. No virus was detected in the supernatant of fresh MDM\(\Phi\)s infected with virus by day 1 p.i. However, virus production was seen by day 2 and peaked at day 5 p.i. In contrast, AM\(\Phi\)s infected with OPIV-3, showed \(10^3\) to \(10^5\) TCID\(_{50}\)/ml in the supernatant by day 1 p.i. and reached its peak at day 5 p.i. OPIV-3 replication in MDM\(\Phi\)s and AM\(\Phi\)s showed a steady increase in the virus titre in the supernatant with time. At the peak of virus production, approximately 50% of MDM\(\Phi\)s and AM\(\Phi\)s had degenerated and eventually lysed in culture. AM\(\Phi\) cultures released significantly more (p<0.001) infectious virus early after infection when compared to OPIV-3 infected MDM\(\Phi\). Equal numbers of MDM\(\Phi\)s failed to support viral replication to the levels of AM\(\Phi\)s throughout the length of the 9 day culture period (Fig. 6.5). Viral titres in AM\(\Phi\) cultures rapidly increased from time 0 to 48 hrs. p.i., and then increased steadily to become maximal at 5. p.i. The titre began to decrease from day 7 p.i.

Autologous lymphocyte cultures were obtained as described in section 2.9.3. and infected with OPIV-3. No virus replication was detected. A further experiment was carried out to assess the effect of mitogen stimulation on the susceptibility of lymphocytes to OPIV-3 infection. Mitogens have been shown to facilitate virus-cell surface interactions (Yamamoto et al., 1974). Cells that are not normally susceptible to paramyxovirus-mediated cytolysis have been made susceptible by treatment with concanavalin A (Con A) (Yamamoto et al., 1974). Also mitogen stimulation of lymphocytes has been reported to result in increased expression of PIV-3 antigen, detected by immunofluorescence (Adair et al., 1986; Basaraba et al., 1993). Cell
TABLE 6.1 Adherence and viability of OPIV-3 infected and non-infected AMϕ, A function of time in culture.

<table>
<thead>
<tr>
<th>Days Post inoculation</th>
<th>Mock-infected</th>
<th>OPIV-3 infected¹</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adherent($x10^4$)²</td>
<td>Viability³</td>
<td>Adherent($x10^4$)</td>
</tr>
<tr>
<td>0</td>
<td>6.8±1.2</td>
<td>92.1±1.6</td>
<td>6.5±0.6</td>
</tr>
<tr>
<td>1</td>
<td>5.8±0.7</td>
<td>95.2±6.3</td>
<td>5.7±0.4</td>
</tr>
<tr>
<td>2</td>
<td>5.0±0.6</td>
<td>94.7±5.9</td>
<td>4.6±1.5</td>
</tr>
<tr>
<td>3</td>
<td>4.3±0.8</td>
<td>94.1±2.6</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>4</td>
<td>4.0±1.5</td>
<td>93.4±2.9</td>
<td>2.9±1.4⁴</td>
</tr>
<tr>
<td>5</td>
<td>3.9±0.9</td>
<td>92.1±3.5</td>
<td>1.6±0.4⁵</td>
</tr>
</tbody>
</table>

¹ AMϕ cultures were infected with OPIV-3 270-7 (1 TCID₅₀/cell) strain after 3 days in culture.
² Mean adherence of alveolar Mϕs as determined by direct counting of plates; average of five separate experiments ±SD.
³ Viability expressed as mean percentage of non-adherent cells as determined by trypan blue dye exclusion; average of five separate experiments ±SD.
⁴ significantly different from non-infected AMϕ at p<0.05.
⁵ significantly different from non-infected AMϕ p<0.001 (Mann-Whitney non-parametric rank test).
A. Effect of OPIV-3 infection on alveolar macrophage (AM) adherence in cell culture.

B. Effect of OPIV-3 infection alveolar macrophage (AM) viability in cell culture.

FIGURE 6. 4 Time dependent effect on adherence (a) and viability (b) of OPIV-3 infected (●) and mock-infected (○) AMϕ, after infection with OPIV-3 -270. AMϕ were inoculated with OPIV-3 270-7 (1 TCID50/cell) after 3 days in culture. Data (Table 6.1) represents the average cell count ±SD from five different experiments.
Time dependent release of infectious virus from MDM and AM cultures infected with OPIV-3.

FIGURE 6.5 Time dependent release of infectious OPIV-3 from equal numbers (6.4x10^4 cells/well) of MDMs (●) and AMs (○). MDMs and AMs were inoculated with OPIV-3 270-7 (1 TCID_{50}/cell) after 5 and 3 days in culture, respectively. Data represent the average TCID_{50}/ml ±SD from five different experiments.
surface receptors for paramyxoviruses are ubiquitous and are found on many cell types including lymphocytes (Woodruff et al., 1974). Thus, crude Con A-supernatant generated from mesenteric lymph node lymphocytes was used for 3 days in order to stimulate cell cultures (0.5mg/well). Con A stimulated lymphocytes showed no increase in OPIV-3 titre above baseline (Fig. 6.6). Levels of infectious virus in medium decreased steadily over time and were at the limits of being detectable after 5 days p.i.

The rate of release of infectious OPIV-3 was also evaluated from Con A-stimulated AMϕs or non-Con A-stimulated AMϕs. Although the rate of virus release was always lower in Con A stimulated cultures, this was not statistically significant (p>0.05) (Fig. 6.7).

Direct contact of lymphocytes with AMϕs is enhanced during antigenic or mitogenic stimulation (Buono et al., 1989). These cellular interactions may be further strengthened by the functional activity of the PIV-3 fusion (F) glycoprotein which is expressed on the surface of virally infected cells (Moscona & Peluso, 1991a). Expression of fusion activity on the surface of PIV-3 infected cells allows the cell to fuse its outer membrane with that of adjacent uninfected cells resulting in intercellular spreading of the infection (Moscona & Peluso, 1992). Thus, the possibility that AMϕs, after becoming infected, could induce a productive infection of lymphocytes was also investigated. Autologous lymphocytes in 100μl of lymphocyte complete RPMI-1640 medium were added to AMϕs (Lymphocyte:AMϕ = 3:1). These co-cultures were stimulated or mock stimulated with Con A (0.5mg/well) or medium alone. Then they were infected with OPIV-3 270-7. The rate of release of infectious virus from AMϕs co-cultured with lymphocytes both Con A-stimulated and non-stimulated, showed no significant differences (Fig. 6.8). Furthermore, they paralleled the rate of infection of AMϕs alone. There was no significant difference in the release of infectious virus from AMϕs cultured alone or AMϕs co cultured with lymphocytes. Again the release of virus was always lower in Con A stimulated cultures.

Recovery of OPIV-3 from all Con A stimulated cultures was less than non-stimulated cells except at time 0. Yet in all cases the release of infectious virus into supernatants treated with Con A or the rate of virus inactivation in cell free cultures containing Con

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Kindly supplied by Dr. Barbara Blacklaws, Veterinary Pathology Department, University of Edinburgh.
Effect of Con A on time dependent release of infectious virus from lymphocyte cultures infected with OPIV-3.

FIGURE 6.6 Time dependent release of infectious OPIV-3 from lymphocytes cultures stimulated (•) and non-stimulated (○) with Con A (0.5mg/well). Data represent the average TCID$_{50}$/ml ±SEM from five different experiments.
Effect of Con A in time dependent release of infectious virus from AM cultures infected with OPIV-3

FIGURE 6.7 Time dependent release of infectious OPIV-3 from AMΦ cultures stimulated (•) and non-stimulated (o) with Con A (0.5mg/well). Data represent the average TCID<sub>50</sub>/ml ±SD from five different experiments.
Effect of ConA on time dependent release of infectious virus from lymphocyte-AM co-cultures infected with OPIV-3.

FIGURE 6.8 Time dependent release of infectious OPIV-3 from AMϕ (6.4 x10^4/well) cultures plus lymphocytes (2x10^5/well) stimulated (•) and non-stimulated (○) with Con A (0.5mg/well). Data represent the average TCID_{50}/ml ±SD from five different experiments.
A (data not shown), showed no statistically significant differences with non-stimulated cultures.

Flow cytometry with rabbit antisera showed that a similar proportion of virally infected AMφs were present in both mock-stimulated and Con A stimulated lymphocyte co-cultures. However, in non-stimulated cultures, few lymphocytes (15 to 22 %) expressed viral surface antigen after 3 days of co-culture with infected AMφs (Fig. 6.9a). In contrast, in Con A stimulated cultures more lymphocytes (30 to 63%) were viral antigen positive (Fig. 6.9b). In mock-infected cultures, neither mock-stimulated nor Con A-stimulated lymphocytes expressed viral antigens (data not shown).

In Con A stimulated and OPIV-3 infected co-cultures, AMφs were frequently covered and surrounded by aggregates of adherent autologous lymphocytes. These rosette forming lymphocytes were viral antigen positive by indirect immunofluorescence. There was a significant increase (p<0.05) in lymphocyte rosette formation in Con A stimulated cultures when compared with non-stimulated cultures (Table 6.2). No lymphocyte-AMφ rosettes were found in mock-infected co-cultures (mock-stimulated or Con A-stimulated) (data not shown). In order to investigate the distribution of T lymphocyte subsets in these rosettes, the cultures were immunostained for CD4 and CD8 lymphocyte markers and positive lymphocytes in rosettes were counted (Table 6.2). In rosettes of Con A stimulated AMφ/lymphocytes co-cultures there was a significantly higher (p<0.001) proportion of CD8+ than CD4+ lymphocytes. In mock-stimulated AMφ/lymphocytes co-cultures there was no significant difference in the proportion of CD4+ and CD8+ lymphocytes rosetting with Mφs, although there were slightly more CD8+ lymphocytes. Fig. 6.10a,b and c show immunostaining of T lymphocytes subsets CD4+ and CD8+ in rosettes with OPIV-3 270-7 infected AMφs.

6.2.1.6 Detection of viral haemagglutinin/neuraminidase (HN) in monocyte-derived macrophage (MDMφ) cell cultures infected with OPIV-3.

In order to study the relationship between phenotype alterations and viral antigen expression on MDMφs and AMφs, viral antigen HN expressed on the cell surface or within the cytoplasm was assayed using flow cytometry with mAb HN 4795. Viral
FIGURE 6. 9 Frequency histograms showing detection of OPIV-3 particles on lymphocyte and AMφ cell surfaces by flow cytometry. Autologous lymphocytes (2x10^6 cells/ml) in 100μl of lymphocyte complete RPMI-1640 were added to AMφ cultures. These co-cultures were either stimulated with Con A (0.5mg/well in lymphocyte complete RPMI-1640) or mock stimulated only with the medium. Then they were infected with OPIV-3 270-7. Cells were obtained after 1, 2, 3, 5 and 7 days p.i., immunostained with OPIV-3 rabbit antisera and then analyzed by flow cytometry. The profiles show the reactivity of OPIV-3 rabbit antisera (—) with both Con A stimulated and mock-stimulated infected co-cultures. Analysis by flow cytometry was carried out after setting a gate firstly in the lymphocyte area (A) and then another one in the Mφ area (B). The staining with NRS as negative control (......) is shown for comparison.
Lymphocytes/OPIV-3
1 Day p.i.

2 Days p.i.

3 Days p.i.

5 Days p.i.

7 Days p.i.

Log10 Fluorescence Intensity →

Mock-treated

Con A-treated
TABLE 6. 2 Effect of Con A stimulation in distribution of CD4+ and CD8+ lymphocyte subsets during specific rosetting of lymphocytes to AMφ cultures infected with OPIV-3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OPIV-3 infected AMφ/lymphocyte co-cultures</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SBU-T4 stained wells</td>
<td>SUB-T8 stained wells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%L/AMφ rosettes¹</td>
<td>%CD4+²</td>
<td>%L/AMφ rosettes</td>
<td>%CD8+³</td>
</tr>
<tr>
<td>Con A stimulated</td>
<td>74±4.6³</td>
<td>29±5.6</td>
<td>80±2.3³</td>
<td>71±3.1⁴</td>
</tr>
<tr>
<td>Non-stimulated</td>
<td>42±3.2</td>
<td>41±4.2</td>
<td>39±2.2</td>
<td>52±5.3</td>
</tr>
</tbody>
</table>

1 Specific rosetting of lymphocytes with alveolar Mφ= lymphocytes rosettes in OPIV-3 infected AMφ cultures minus rosetting in mock infected AMφ cultures. Results are expressed as median of percentage ±SD. Data represent the average from five different experiments.

2 Specific distribution of lymphocyte subsets= immunostained lymphocytes present in rosettes of virus infected AMφ cultures minus immunostained lymphocytes present in rosettes of mock infected AMφ cultures. Results are expressed as median of percentages ±SD. Data represent the average from five different experiments.

3 Significantly different from non-stimulated cultures at p<0.05 (Mann-Whitney non-parametric rank test)

4 Significantly different from % of CD4+ in Con A stimulated cultures at p<0.001 (Mann-Whitney non-parametric rank test)
FIGURE 6. 10  Immunostaining of T lymphocyte subsets CD4⁺ and CD8⁺ in lymphocyte rosettes with OPIV-3 270-7 infected AMΦs. Autologous lymphocytes (2x10⁶ cells/ml) were added to AMΦs (after 3 days p.i. with OPIV-3 270-7). Co-cultures were stimulated or mock stimulated with 0.5μg/well of a stock solution of Con A.

a) Immunostaining of T lymphocyte subset CD4⁺ in lymphocyte rosettes with OPIV-3 270-7 infected AMΦs. AMΦ/lymphocyte co-culture was stimulated with Con A. A few positive cells (red colour) for lymphocyte CD4 marker are in contact with MΦs (non-immunostained cells). IIAP with mAb SBU-T4. The nuclei were counterstained with H/E. Magnification x100.

b) Immunostaining of T lymphocyte subset CD8⁺ in lymphocyte rosettes with OPIV-3 270-7 infected AMΦs. AMΦ/lymphocyte co-culture was stimulated with Con A. Many positive cells (red colour) for lymphocyte CD8 marker surround and rosette with MΦs (non-immunostained cells). IIAP with mAb SBU-T8. The nuclei were counterstained with H/E. Magnification x100.

c) A closer view of Fig. 6.10b. An AMΦ is shown covered and surrounded by aggregates of adherent autologous lymphocytes. These rosette-forming lymphocytes are positive for T lymphocyte CD4 marker. IIAP with mAb SBU-T8 The nuclei were counterstained with H/E. Magnification x600.
antigen expressed on the cell surface was detected on unfixed cells as previously described (section 2.7.6). Detection of internal antigen was performed by fixing cells in 80% methanol (Lee, 1994). The time dependent reactivity after infection is shown in Table 6.3 and Fig. 6.11. HN antigen expression could not be detected in either the MDMφ cell membrane or cytoplasm until 2 days p.i. with OPIV-3 270-7 (ITCID$_{50}$/cell). By 2 days p.i. 17% of cells showed HN expression in the cytoplasm and 9% in the cell membrane. By 3 days p.i 38% of MDMφs were cytoplasmic antigen positive and 28% membrane antigen positive. By 5 days p.i. 62% of MDMφs were positive for HN antigen detected in cytoplasm and 53% of cells also expressed this antigen on the cell membrane. By 7 days p.i. 82% of MDMφs expressed antigen HN in the cytoplasm and 85% in the cell membrane. The expression of HN antigen in the cytoplasm was always higher than in the cell membrane. However, this was significantly higher (p<0.05) only until day 4 p.i.

6.2.1.7 Detection of viral haemagglutinin/neuraminidase (HN) in alveolar macrophage (AMφ) cell cultures infected with OPIV-3.

In contrast, OPIV-3 infection of AMφs with 1 TCID$_{50}$/cell resulted in the detection of OPIV-3 antigen HN within the first few hrs. p.i. The number of fluorescent positive AMφs increased linearly up to 5 days p.i. stabilizing between 5 and 7 days p.i. (Fig. 6.11). Cytoplasmic HN antigen was detected as early as 1 day p.i. in 27% of AMφs. Also 10% of AMφs showed HN expression on the cell membrane after 1 day p.i.. By 2 days p.i. 39% of AMφ were positive for HN antigen in the cytoplasm and 32% also showed antigen in the cell membrane. By 3 days p.i. 52% of AMφs were positive for HN antigen in the cytoplasm and 47% for HN in the cell membrane. By 5 days p.i. 91% of the cells were positive for cytoplasmic HN and 85% for HN on the cell membrane. The expression of HN antigen in cytoplasm was significantly higher (p<0.001) than in cell membrane only until day 1 p.i. After this although the cytoplasmic expression of HN was always higher no significant differences were detected. By 7 days p.i. the staining pattern was virtually the same for antigen HN expression, both at the cytoplasmic and cell membrane level. The percentage of cells expressing HN antigen both at the cytoplasmic and cell membrane level was significantly higher in AMφs than MDMφs until day 6 p.i. (p<0.05 until day 2 p.i.; p<0.001 from day 3 to 6 p.i.). Representative profiles of HN expression by both MDMφs and AMφs are shown in Figures 6.12A and 6.12B, respectively.
TABLE 6.3 Time dependent cytoplasmic and cell surface expression of OPIV-3 HN antigen in MDMφs and AMφs after *in vitro* infection.

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>OPIV-3 infected cultures</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDMφs</td>
<td>AMφs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>Cell membrane</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>1</td>
<td>0 1</td>
<td>0</td>
<td>27±1.9 3</td>
</tr>
<tr>
<td>2</td>
<td>17±1.6</td>
<td>09±1.2*</td>
<td>39±2.2 3</td>
</tr>
<tr>
<td>3</td>
<td>38±1.8</td>
<td>28±1.6*</td>
<td>52±1.5 2</td>
</tr>
<tr>
<td>4</td>
<td>45±2.2</td>
<td>32±1.8*</td>
<td>69±1.7 2</td>
</tr>
<tr>
<td>5</td>
<td>62±1.5</td>
<td>53±2.3</td>
<td>91±2.3 2</td>
</tr>
<tr>
<td>6</td>
<td>71±2.3</td>
<td>65±2.7</td>
<td>90±2.2 2</td>
</tr>
<tr>
<td>7</td>
<td>82±2.5</td>
<td>85±1.9</td>
<td>92±1.8</td>
</tr>
</tbody>
</table>

1. A 1% gate was set on the negatively stained control cells and cells staining positive above this are expressed as median of percentages of positively labelled cells (in Mφ gate) ±SD.
2. Data represent the average from five different experiments.
3. Significantly different from MDMφ same level (cytoplasm or membrane) at p<0.05 (Mann-Whitney non-parametric rank test).
4. Significantly different from MDMφ same level (cytoplasm or membrane) at p<0.001 (Mann-Whitney non-parametric rank test).
5. Significantly different from MDMφ cytoplasm at p<0.05 (Mann-Whitney non-parametric rank test).
6. Significantly different from AMφ cytoplasm at p<0.001 (Mann-Whitney non-parametric rank test).
Time dependent expression of protein HN in OPIV-3 infected MDM and AM by flow cytometry

FIGURE 6.11 Time dependent expression of HN antigen in OPIV-3 270 infected MDMs (•) and AMs (○) detected by flow cytometry. HN expressed on the cell surface or within the cytoplasm was detected with mAb 4795. Viral antigen expressed on the cell surface was detected on unfixed cells. Detection of internal antigen was performed by fixing cells in 80% methanol. Data represent the average ±SD from five different experiments.
FIGURE 6.12 Frequency histograms showing detection of the viral antigen HN expressed on the cell surface and in the cytoplasm, of MDMϕs (A) and AMϕs (B) infected with OPIV-3 270-7, by flow cytometry. The viral antigen expressed on the cell surface was detected on unfixed cells. Detection of internal antigen was performed by fixing cells in 80% methanol. MDMϕ and AMϕ cultures were infected and harvested on days 1, 2, 3, 4 and 5 p.i. HN antigen was detected by immunostaining with mAb HN4795 (—). Reactivity was analyzed by flow cytometry and incubation with NMS (…….) was used as the negative control of the staining.
6.2.1.8 Transmission electron microscopy (TEM) of monocyte-derived macrophage (MDMΦ) cell cultures infected with OPIV-3.

Ultrathin sections from monolayers of MDMΦ cell cultures harvested at 24, 48 and 72 hours post inoculation with OPIV-3 270-7 were observed by TEM. By 24 hrs. post infection, MDMΦs showed no major changes in cellular organelles, except for an increase in the amount of endoplasmic reticulum, vacuolation of the cytoplasm and swelling of mitochondria (Fig.6.13a). Noticeable morphological changes were seen in infected MDMΦs by 48 hrs. p.i. After 48 hrs. p.i. profound vacuolation of the cytoplasm and swelling of mitochondria could be seen. The nuclei contained dispersed heterochromatin margins and some infected MDMΦs lost their surface processes. Some small filamentous cytoplasmic inclusions, usually located in a perinuclear position were observed (Fig.6.13b). Inclusion material in the perinuclear region tended to be more tightly packed than that located in other parts of the cytoplasm. After 72 hrs. p.i., the filamentous cytoplasmic inclusions were much larger and more common (Fig.6.13c.). Further changes in the cytoplasm included an increased number of polyribosomes, swollen mitochondria and displacement of cytoplasmic organelles by the inclusions. Early virus budding was seen in cytoplasmic vesicle membranes and in cell membranes. Also virions accumulated inside cytoplasmic vesicles and in the intercellular spaces. During viral budding, an electron dense, crescent shaped nucleoid appeared. This fused with the vesicular or cytoplasmic membrane that appeared thickened and showed its outer aspect covered with a fringe. Both ends of the crescent eventually met, after which, virions were released from the membrane. They were roughly spherical pleomorphic viral particles completely surrounded by a similarly fringed membrane. These pleomorphic particles had an overall diameter of 150 to 320 nm. They were first observed inside the cytoplasm then budding through the membrane and finally detaching from the cell surface.
6.2.1.9 *Transmission electron microscopy (TEM) of alveolar macrophage (AM\(\Phi\)) cell cultures infected with OPIV-3.*

Similar experiments were performed with AM\(\Phi\) cell cultures. OPIV-3 replication in AM\(\Phi\)s was very similar to that found in MDM\(\Phi\)s, but changes associated with viral replication appeared much earlier in AM\(\Phi\) than in MDM\(\Phi\) cultures. There also seemed to be more virus particles and larger cytoplasmic inclusion bodies in infected AM\(\Phi\)s. In contrast to MDM\(\Phi\)s, by 24 hrs. p.i. AM\(\Phi\)s showed changes in cytoplasm with the presence of numerous, large vesicles, swelling of mitochondria and an increased amount of both endoplasmic reticulum and number of ribosomes. Some small cytoplasmic inclusions of filamentous material were seen. As with in MDM\(\Phi\)s these were usually located in a perinuclear position where they tended to be more tightly packed than those located in other parts of the cytoplasm. After 48 hrs. p.i. virus budding was also seen more frequently along vesicular membranes than in the cell membrane (Fig.6.13d,e.), with virus shedding inside these vesicles. Virus particles were first observed inside the cytoplasm then budding through the membrane and finally detaching from the cell surface (Fig.6.13f.). After 48 to 72 hrs. p.i. these changes increased in intensity and frequency, in particular virus budding and release of virus particles from the cell membrane.
FIGURE 6.13 Transmission electron microscopy (TEM) of MDMϕ and AMϕ cell cultures harvested at 24, 48 and 72 hours post inoculation with OPIV-3 270-7.

a) MDMϕ after 24 hrs. post infection with OPIV-3 270-7. MDMϕs showed no major changes in cellular organelles, except for an intense vacuolation of the cytoplasm. TEM. Magnification x4,600.

c) MDMϕs after 72 hrs. p.i. with OPIV-3 270-7. A large cytoplasmic inclusion containing filamentous material is seen in the cytoplasm (arrow). TEM. Magnification x4,600.

e) AMϕs after 48 hrs. p.i. with OPIV-3 270-7. Closer view of Fig. 6.13d. Virus budding in cytoplasmic vesicle membrane can be seen. Virions are accumulating inside this cytoplasmic vesicle. TEM. Magnification x215,000.

b) MDMϕs after 48 hrs. p.i. with OPIV-3 270-7. small filamentous cytoplasmic inclusions (arrow), located in a perinuclear position The inclusion material appears tightly packed. TEM. Magnification x16,500.

d) AMϕs after 48 hrs. p.i. with OPIV-3 270-7. There is displacement of cytoplasmic organelles by a large amount of filamentous inclusions (F). Also there is virus budding in the membrane of the cytoplasmic vesicles with an accumulation of virus particles inside these vesicles (arrow). TEM. Magnification x16,500.

f) AMϕs after 48 hrs. p.i. with OPIV-3 270-7. Viral budding on the cell membrane can be seen. The cytoplasmic membrane appears thickened and shows its outer aspect covered with a fringe (arrows). TEM. Magnification x215,000.
6.2.2 PART B: PHENOTYPIC CHANGES

6.2.2.1 Surface molecule expression of monocyte-derived (MDMφ) and alveolar (AMφ) cell cultures.

Cultured MDMφs and AMφs were variable in size, so they were gated on a large FSC and SSC area excluding lymphocytes and cell debris. They also increased in overall size with time in culture. Changes in AMφ size, observed during "live gating" are shown in Figure 6.14. Analysis of the cell staining from the FACScan often uses a 1% threshold set on negatively stained cells with the antibody of interest. The quantification of cellular constituents on AMφs using flow cytometry is complicated by two major factors. First, matching cell size and fluorescence is difficult due to the wide variation in cell size which is found among AMφs. The second complication involves the intense autofluorescence emitted by AMφs which interferes with the specific fluorescence of antibody-fluorophore complexes (Pankow et al., 1990). In this study it was observed that AMφ and MDMφ in culture showed an increase in size and in autofluorescence of the cells. Due to these factors, it was very difficult to separate positive and negative populations of MDMφ and AMφ, particularly when molecules were weakly expressed. Thus, using the percentage positive cells to express the phenotype of MDMφ may not reflect the actual situation, as reported by Pankow et al., (1990). For this reason the following data is expressed as the mean of fluorescence intensity (MF) of specific molecules, with negative control of fluorescence staining subtracted. The staining of negative controls was obtained by incubating with normal mouse serum (NMS, 1:400 dilution) as the primary antibody.

Fresh monocytes did not express either CD4 or CD8. After culture, they immediately and significantly (p<0.001) began to express both molecules on their surface after culture (Table 6.4) with maximal levels around day 7 (MF: CD4=21.5; CD8=35.6) which began to decrease after day 10. More CD8 was expressed than CD4. Fresh AMφ were virtually negative for CD4 or CD8 antigens and they remained so until day 10 in culture. Figure 6.15 shows the comparison between the expression of CD4 in either MDMφ or AMφ cell cultures. Figure 6.15 also shows the comparison between the expression of CD8 in either MDMφ or AMφ cell cultures.
FIGURE 6.14 Flow cytometry displays for "live gating" of AMφs using forward and side angle light scatter (FSC and SSC) profiles. The PMT voltage of both FSC and SSC was optimised for each population. A time dependent increase in both size and cytoplasm complexity is observed in culture. Cells were analyzed on days 0 (A), 3 (B), 5 (C), 7 (D) and 10 (E) of culture.
VPM32 is a monoclonal antibody which detects tissue Mφs, but not monocytes or T and B lymphocytes. The identity of the antigen of this mAb is not known, but is a single 55,000 MW polypeptide. It is thought to be specific for Mφ FCγRIII (González, 1989). Fresh monocytes (day 0 culture) showed very low expression of VPM32 antigen (MF=10.5); however with monocyte maturation in vitro, the expression of VPM32 antigen by MDMφ was greatly and significantly (p<0.001) increased from day 3 (MF=25.3) until day 7 (MF=45.3). (Table 6.4). Figures 6.16a and b compare the fluorescence staining with mAb VPM32 between fresh monocytes and 7 day cultured MDMφ. AMφ always expressed higher levels of VPM32 antigen than MDMφ (MF=35.4). However this was statistically significant (p<0.001) only on days 0, 3 and 5 of culture. This is because on day 1 of culture a noticeable decrease in VPM32 expression by AMφ was observed. MF on day 0 was 35.4 and on day 1 was 17.6. This change was transitory and then a rapid increase took place, returning to the initial levels by day 2. On day 5 (MF=49.4) they had reached maximum and significantly higher levels (p<0.05) than AMφ on day 0 of culture. Likewise, by this time the VPM32 expression of MDMφ had reached similar levels so no significant differences were detected between these two cell types (Fig. 6.15).

F10-150-39 (anti-LFA-1) and L180-1 (anti-LFA-3) monoclonal antibodies stained MDMφ and AMφ in a similar way. These molecules showed a tendency to increase after culture until day 7. However, this was variable and no significant differences were observed in the expression of these molecules during the culture of either MDMφ or AMφ (Table 6.4). The mean of fluorescence (MF) of LFA-1 and LFA-3 in MDMφ ranged between 46.2 to 54.4 and 50.3 to 63.5, respectively. This was slightly lower for AMφ with a MF ranging from 45.3 to 50.1 and 46.7 to 59.2 for LFA-1 and LFA-3, respectively. Figure 6.17 shows a representative profile of LFA-3 expression by MDMφ in culture.

Another monoclonal antibody, VPM65, reacts with ovine CD14, the cellular receptor for bacterial lipopolysaccharide (LPS) and LPS-binding protein (Gupta et al., 1993). Both fresh monocytes and cultured MDMφ showed a moderately high intensity (MF=51.4 on day 7) of staining for CD14 without much change during culture. AMφ also showed a moderately high intensity, decreasing or increasing during culture but with no significant changes (MF=45.8 on day 7) (Table 6.4).
### TABLE 6.4 Comparison of the phenotype of monocyte-derived macrophages (MDMφs) and alveolar macrophages (AMφs) during cell culture.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Molecules</th>
<th>Culture</th>
<th>Time in Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 days</td>
<td>1 day</td>
</tr>
<tr>
<td>CD4</td>
<td>MDMφ</td>
<td>0.5±0.7^{1,5}</td>
<td>5.7±1.8^{3,5}</td>
</tr>
<tr>
<td>AMφ</td>
<td></td>
<td>1.1±0.9</td>
<td>0.9±0.8</td>
</tr>
<tr>
<td>CD8</td>
<td>MDMφ</td>
<td>0.6±0.8^{3}</td>
<td>11.2±2.9^{3,5}</td>
</tr>
<tr>
<td>AMφ</td>
<td></td>
<td>1.5±1.0</td>
<td>0.7±0.6</td>
</tr>
<tr>
<td>VPM32</td>
<td>MDMφ</td>
<td>10.5±3.2^{3}</td>
<td>17.5±3.5^{4}</td>
</tr>
<tr>
<td>AMφ</td>
<td></td>
<td>17.6±4.5^{5}</td>
<td>32.1±4.2</td>
</tr>
<tr>
<td>LFA-1</td>
<td>MDMφ</td>
<td>46.2±3.4</td>
<td>50.1±4.7</td>
</tr>
<tr>
<td>AMφ</td>
<td></td>
<td>45.3±4.2</td>
<td>42.1±3.9</td>
</tr>
<tr>
<td>LFA-3</td>
<td>MDMφ</td>
<td>50.3±4.6</td>
<td>52.6±4.1</td>
</tr>
<tr>
<td>AMφ</td>
<td></td>
<td>46.7±4.8</td>
<td>49.3±5.2</td>
</tr>
<tr>
<td>CD14</td>
<td>MDMφ</td>
<td>43.5±5.2</td>
<td>45.7±4.9</td>
</tr>
<tr>
<td>AMφ</td>
<td></td>
<td>40.3±4.7</td>
<td>37.5±4.1</td>
</tr>
<tr>
<td>CD1</td>
<td>MDMφ</td>
<td>0.08±0.5^{3}</td>
<td>1.3±0.3^{3}</td>
</tr>
<tr>
<td>AMφ</td>
<td></td>
<td>26.6±2.9</td>
<td>20.7±3.7</td>
</tr>
</tbody>
</table>

1. Data (mean±SD) are expressed as mean fluorescence intensity (MF) where negative control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.
2. Significantly different from AMφ on the same day of culture at p<0.05 (Mann-Whitney non-parametric rank test).
3. Significantly different from AMφ on the same day of culture at p<0.001 (Mann-Whitney non-parametric rank test).
4. Significantly different from cells day 0 of culture at p<0.05 (Mann-Whitney non-parametric rank test).
5. Significantly different from at cells day 0 of culture p<0.001 (Mann-Whitney non-parametric rank test).
FIGURE 6. 15 Frequency histograms showing MDMϕ and AMϕ phenotypic changes in culture. MDMϕ cell cultures were obtained by collecting peripheral blood monocytes (PBMC). AMϕs were collected from the lung wash fluid (LWF). All cells were obtained from clinically normal healthy sheep. They were cultured in gelatin-coated flasks or plates. Non-adherent cells were washed off after overnight incubation. Cells were analyzed by flow cytometry for the expression of cell markers on days 0, 1, 3, 5, 7 and 10 of culture. These cell marker included CD4, CD8 and VPM32 (—). Staining with NMS as the negative control is shown for comparison (........).
Days in culture

0

1

3

5

10

Log10 Fluorescence Intensity →
Expression of ovine tissue MΦ marker by monocyte-derived macrophages (MDMΦs) in culture. Peripheral blood monocytes (PBMC) were collected from clinically normal healthy sheep and cultured in gelatin-coated flasks or plates. Cells were incubated with mAb VPM32 for ovine tissue MΦs. Reactivity was detected by indirect immunofluorescence (IIF) and cell were observed under UV microscope and analyzed by flow cytometry.

a) All MDMΦs after 12 hrs. in culture express a very low intensity of VPM32 macrophage marker. The insert shows a corresponding flow cytometry chart including the negative control of the staining with normal mouse serum (NMS). IIF with mAb VPM32. Magnification x200.

b) All MDMΦs after 7 days in culture express VPM32 MΦ marker with high intensity. Insert shows corresponding flow cytometry chart including negative control of staining with normal mouse serum (NMS). IIF with mAb VPM32. Magnification x200.
FIGURE 6. 17 Frequency histograms showing changes in LFA-3 expression (—) by MDM(°s in cell culture. MDM(°s were obtained by collecting peripheral blood monocytes (PBMC). They were cultured in gelatin-coated flasks or plates. Non-adherent cells were washed off after overnight incubation. Cells were analyzed by flow cytometry for the expression of cell markers on days 0, 1, 3, 5, 7 and 10 of culture. Staining with NMS as the negative control is shown for comparison (......).
Dendritic cells have been reported to be present in both lung sections (Holt et al., 1992) and LWF (Havenith et al., 1993) in the rat. Their presence in sheep LWF was investigated with monoclonal antibody VPM5 that reacts with ovine CD1b (Budjoso et al., 1989). It stains Langerhans cells, dendritic (veiled) cells in afferent lymph and dendritic cells within the paracortical areas of the lymph nodes. MDMΦ did not show specific staining for this marker either before or after culture (Table 6.4). In AMΦ suspensions there was always a small, variable, well defined subpopulation of cells expressing CD1 antigen that decreased noticeably after day 5 in culture and showed stronger intensity before culture than after (Fig. 6.18). The average percentage of cells in this population of AMΦ cell suspensions was 18% in fresh AMΦ (MF=26.55); 16% on day 1 of culture (MF=20.74); 13% on day 3 of culture (MF=15.2); 10% on day 5 of culture (MF=6.15); 9% on day 7 of culture (MF=4.04).

Crude Con A-supernatant generated from cultured mesenteric lymph nodes lymphocytes was used to activate cultures of both MDMΦ and AMΦ in concentration of 50%v/v for 3 days. Then cells were harvested for phenotypic analysis according to protocol described by Lee, (1994). Con A treatment did not have a significant effect (p>0.05) on the expression of CD8, CD4, LFA-1, LFA-2 or VPM32 antigens on these cells, in five different experiments (data not shown).

6.2.2.2 Effect of OPIV-3 infection on surface molecule expression.

MDMΦ and AMΦ were infected with OPIV-3, 1TCID₅₀ per cell, on day 5 and 3 of culture, respectively. Then virus infected and mock-infected (control) cells were harvested for phenotype analysis on days 1, 2, 3, 5, and 7 p.i. In infected MDMΦ cultures the expression of most surface molecules including CD4, CD8, LFA-1, LFA-3, CD14 and VPM32 was not significantly different from mock-infected cells up to 5 days post-infection (Table 6.5). After 5 days p.i., expression of most surface molecules was lower on the cell membrane of infected cells, however significant differences (p<0.05) were detected only for CD4, CD8 and VPM32 cell markers.

In infected AMΦ cultures (Table 6.6) the expression of CD4 and CD8 molecules was considered negative and it was similar to mock-infected controls. The expression of LFA-1, LFA-3 and CD14 was variable. Although it was either slightly higher or lower, no significant differences were detected after infection in comparison to mock-
FIGURE 6. 18 Frequency histograms showing changes in CD1 expression (---) by AMφs in cell culture. AMφs were collected from the lung wash fluid (LWF). They were cultured in gelatin-coated flasks or plates. Non-adherent cells were washed off after overnight incubation. Cells were analyzed by flow cytometry for the expression of cell markers on days 0, 1, 3, 5, 7 and 10 of culture. Staining with NMS as the negative control is shown for comparison (......).
AM

CD1

Days in culture

0

1

3

5

7

10

Cell Number

Log10 Fluorescence Intensity
infected controls. The expression of VPM32 antigen followed the same pattern in infected and mock-infected cells. Although it was slightly higher in mock-infected cells this was noticed from day 0 p.i. No difference was detected on day 7 as the control also showed decreased VPM32 expression in tissue culture by that time. CD1 expression decreased with time both in culture and after infection. However, on day 3 p.i. there was a significantly higher (p<0.05) expression in infected cultures when compared to mock-infected cells.
TABLE 6.5 Comparison of the phenotype of monocyte-derived macrophages (MDMφs) in culture in the presence or absence of infectious OPIV-3.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Group</th>
<th>Time Post Inoculation</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 day</td>
<td>1 days</td>
</tr>
<tr>
<td>CD4</td>
<td>Mock infected</td>
<td>15.0±1.2</td>
<td>17.4±4.9</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>14.9±3.1</td>
<td>18.9±3.1</td>
</tr>
<tr>
<td>CD8</td>
<td>Mock infected</td>
<td>32.4±6.1</td>
<td>28.7±7.9</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>29.7±6.9</td>
<td>25.5±5.8</td>
</tr>
<tr>
<td>VPM32</td>
<td>Mock infected</td>
<td>29.9±6.9</td>
<td>34.5±8.9</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>30.2±5.0</td>
<td>32.4±6.7</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Mock infected</td>
<td>52.0±6.9</td>
<td>50.9±6.2</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>47.4±9.3</td>
<td>49.5±5.9</td>
</tr>
<tr>
<td>LFA-3</td>
<td>Mock infected</td>
<td>54.5±8.6</td>
<td>55.5±5.1</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>52.4±6.0</td>
<td>53.8±8.3</td>
</tr>
<tr>
<td>CD14</td>
<td>Mock infected</td>
<td>53.5±7.3</td>
<td>52.1±6.2</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>50.4±6.9</td>
<td>53.6±7.4</td>
</tr>
</tbody>
</table>

1 Cultured MDMφ were infected on day 5 culture with OPIV-3 1TCID₅₀ per cell and cells were harvested on day 1, 2, 3, 5 and 7 p.i.

2 Data (mean±SD) are expressed as mean fluorescence intensity (MF) where negative control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.

3 Significantly different from mock-infected cells at p<0.05 (Mann-Whitney non-parametric rank test).

4 Significantly different from cells day 0 of culture at p<0.05 (Mann Whitney non parametric rank test).
TABLE 6. Comparison of the phenotype of alveolar macrophages (AM<sub>φ</sub>s) in culture in the presence or absence of infectious OPIV-3.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Group</th>
<th>Time Post Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0 day</td>
</tr>
<tr>
<td>Molecules</td>
<td>Mock infected</td>
<td>1.0±1.2&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4</td>
<td>OPIV-3 infected</td>
<td>1.9±0.7</td>
</tr>
<tr>
<td>CD8</td>
<td>Mock infected</td>
<td>2.4±0.5</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>2.7±0.9</td>
</tr>
<tr>
<td>VPM32</td>
<td>Mock infected</td>
<td>47.9±6.9</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>45.4±5.0</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Mock infected</td>
<td>47.0±6.9</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>47.4±9.3</td>
</tr>
<tr>
<td>LFA-3</td>
<td>Mock infected</td>
<td>57.5±8.6</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>61.4±6.0</td>
</tr>
<tr>
<td>CD14</td>
<td>Mock infected</td>
<td>51.7±8.9</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>52.4±7.7</td>
</tr>
<tr>
<td>CD1</td>
<td>Mock infected</td>
<td>5.8±3.4</td>
</tr>
<tr>
<td></td>
<td>OPIV-3 infected</td>
<td>6.0±3.6</td>
</tr>
</tbody>
</table>

<sup>1</sup> Cultured AM<sub>φ</sub> were infected on day 3 culture with OPIV-3 1TCID<sub>50</sub> per cell and cells were harvested on day 1,2,3,5 and 7 p.i.

<sup>2</sup> Data (mean±SD) are expressed as mean fluorescence intensity (MF) where negative control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.

<sup>3</sup> Significantly different from mock-infected cells at p<0.05 (Mann-Whitney non-parametric rank test).

<sup>4</sup> Significantly different from cells day 0 of culture at p<0.05 (Mann-Whitney non-parametric rank test).
Macrophages obtained from different anatomic locations differ in their ability to support viral replication (Stauber & Weston, 1984). In this study it has been shown that OPIV-3 infects ovine AMφ in vitro and in vivo. The in vitro infection is productive and characterised by the release of high titres of infectious virus. These findings agree with previous reports of PIV-3 infection of bovine AMφ (Tsai, 1977; Bryson et al., 1983; Hesse & Toth, 1983; Toth & Hesse, 1983; Basaraba et al., 1993). Infectious OPIV-3 virus titres increased in AMφ and AMφ/lymphocyte culture supernatants equally in a time dependent manner, a finding suggesting that extracellular virus was primarily Mφ derived. This was further supported by the inability to recover infectious virus, above base line levels, from supernatants of infected lymphocyte cultures.

Recently Sieg et al. (1994) suggested that BPIV-3 causes a productive infection in bovine lymphocytes in vitro. This productive infection seems to require T cell activation by using anti-CD3. However, BPIV-3 virus particles on lymphocyte cell surface were not detected by immunofluorescence before one week of infection. In this study some lymphocytes of OPIV-3 270-7 infected AMφ/lymphocyte co-cultures were found to show virus particles on cell surface after 3 days p.i. The presence of these particles on lymphocytes surface may be the result of the interaction of these cell with infected Mφs. In fact, a high proportion of lymphocyte rosette formation with infected AMφ was observed in AMφ/lymphocyte co-cultures. Basaraba et al., (1993) also failed in recovering infectious PIV-3 from both Con A stimulated and mock-stimulated bovine lymphocyte cultures. Furthermore, they suggested a possible interaction between Con A and PIV-3 as they found a significant decrease in the recovery of infectious virus from culture supernatant of AMφ, lymphocyte and AMφ/lymphocyte Con A stimulated cultures. In this study the recovery of OPIV-3 was lower from all Con A stimulated cultures. The reason for this observation is unclear. Binding of Con A to surfaces of virally infected cells may inhibit the release of virus through extensive mitogen cross linking similar to the action of antibody directed to cell associated viral antigens (Poste et al., 1974; Basaraba et al., 1993). Alternatively, Con A may be toxic to PIV-3 or it may cross link PIV-3 through recognition of mannose rich regions on the virus envelope, thus decreasing the
number of infectious viral particles when measured by infectivity (Basaraba et al., 1993).

In this study a non-productive or abortive type of infection was observed in lymphocyte cultures. This agrees with the absence of infected lymphocytes found in both LWF and in tissue sections obtained from pneumonic lungs after 7 days post experimental infection. PIV-3 infection is considered to be restricted primarily to epithelium and Mψs of the respiratory tract (Brysson et al., 1983; Chanock & McIntosh, 1990; Li & Castleman, 1991). However, more research is necessary to investigate the conditions both in vivo and in vitro which may lead to productive infection of lymphocytes. Also the susceptibility of the different lymphocytes subsets to PIV-3 infection should be clarified.

PIV-3 infections are often accompanied by depressed proliferation of mitogen stimulated peripheral blood lymphocytes (Ghram et al., 1989; Sieg et al., 1994), with prolonged viral shedding and incomplete immunity to viral proteins (Van Wyke-Coelingh et al., 1990). Reinfection by PIV-3 is common and like initial infection, virus is restricted primarily to the respiratory tract where both alveolar Mψs (AMψ) and respiratory epithelium are infected (Chanock & McIntosh, 1990). PIV-3 infection of AMψ results in altered Mψ function which is thought to contribute to the pathogenesis of this respiratory disease (Basabara et al., 1993). AMψ and lymphocytes are important cellular constituents of the pulmonary host defence system. As well as the direct effects virus interactions have on Mψs and lymphocytes, secondary lymphocyte dysfunction may be mediated through infected Mψs. Virally infected AMψ may be an important source of infectious virus or soluble mediators which can enhance or suppress lymphocyte functions. The mechanism of virus induced suppression of lymphocyte activation in the pathogenesis of PIV-3 infections and the role of the virally infected AMψ remain unknown (Basabara et al., 1993).

In this study, OPIV-3 in vitro replication in AMψ was found to be more rapidly lytic than in MDMψ. The differentiation and maturation state of Mψs may affect replication of PIV-3 with the virus being produced earlier and in higher levels in mature well differentiated AMψ than in less mature and differentiated MDMψ (5 days in culture before infection). These findings also support previous reports that AMψ is superior to peripheral blood monocytes (PBMC) and monocyte-derived-Mψs (MDMψ) in supporting PIV-3 growth in vitro (Rosenstreigh et al., 1976; Ghram et al., 1989;
This has also been previously shown for MVV (Gendelman et al., 1986; Narayan et al., 1983; Lee, 1994). A similar phenomenon has also been reported for HIV replication in fresh and mature monocytes (Potts et al., 1990). The results here suggest that MDM\(\phi\) do not support PIV-3 replication as productively as mature AM\(\phi\). However, these results may be variable depending on the viral isolate and type of M\(\phi\) culture used.

Low levels of expression of CD4 molecules by human monocytes and cultured MDM\(\phi\) have been reported (Collman et al., 1990). This molecule acts as a receptor for HIV particles allowing infection of M\(\phi\)s (Kazazi et al., 1989; Collman et al., 1990; Crowe et al., 1992). Expression of CD4 on sheep monocytes and cultured MDM\(\phi\) has not been studied in depth. The experiments reported here show that fresh sheep monocytes and AM\(\phi\)s did not express CD4 molecules on their surface, but low intensity staining was seen after short term culture of MDM\(\phi\)s. Perhaps the ovine CD4 expression is different to that seen in humans. Also ovine monocyte may contain internal CD4 and/or newly synthesized CD4 is expressed on the surface after culture. Moderate intensity staining for CD8 molecules was seen on MDM\(\phi\)s with similar kinetics of expression as CD4, but the biological significance of CD8 expression on MDM\(\phi\)s is not clear. A similar percentage (10%) of fresh human monocytes expressing CD8 has been reported (Moebious, 1989). An inverse expression of MHC class II and CD14 on AM\(\phi\) and peritoneal M\(\phi\)s has been reported (Andreesen et al., 1990). Some molecules such as Fc\(\gamma\)RIII (CD16), \(\alpha\)-chain of fibronectin (CD51), Max1 and Max3 are not expressed on fresh human monocytes, but are expressed on mature M\(\phi\)s (Eischen et al., 1991). This is similar to the pattern of expression of VPM32, CD4 and CD8 expression on cultured MDM\(\phi\) observed in this study.

Three day cultured monocytes underwent phenotypic changes and expressed CD4, CD8 and VPM32 antigen. They were, therefore, considered to be MDM\(\phi\)s and were used for infection with OPIV-3. OPIV-3 replication in these MDM\(\phi\)s was found to be slower and less productive than in AM\(\phi\)s. Furthermore, phenotypic changes seen after infection were variable and it was difficult to say whether they were caused by OPIV-3 infection, restriction of OPIV-3 infection or further differentiation of the cell with time in culture. Immature monocytes limit MVV replication and so monocyte cultures are not productively infected with MVV until they have differentiated (Narayan et al., 1983; Lee, 1994).
Seven day cultured MDM\(\phi\) had completely differentiated into M\(\phi\)s as defined by morphology, NSE staining and stable surface antigen expression. There was no significant difference in surface molecule expression on OPIV-3 infected and mock-infected MDM\(\phi\) up to 5 days of infection. However, in some cultures there was enhanced expression of surface antigen early after viral infection. MDM\(\phi\) may therefore increase their functional and biological activities in response to viral stimuli early in infection, but these changes were not further amplified by a cascade reaction due to the lack of lymphocytes in the culture. The interaction of infected M\(\phi\)s and lymphocytes may produce \(\gamma\)-IFN and enhance surface antigen expression as seen \textit{in vivo} (Lairmore \textit{et al.}, 1988a; Kennedy \textit{et al.}, 1985). A similar phenomenon of enhanced antigen expression has been seen in MDM\(\phi\) infected with MVV (Lee, 1994). There was no loss of surface antigen from MDM\(\phi\) or AM\(\phi\) up to 5 days post infection with OPIV-3; but since then, degenerative changes including a decrease in surface antigen expression were seen. This is probably due to productive replication of the virus as this is supported by the detection of viral antigen both in the cytoplasm and cell membrane (Fig.6.6). The kinetics of the changes also coincide with surface changes seen in OPIV-3 infected MDM\(\phi\) observed under TEM.

Fc receptors are important surface molecules for monocytes and M\(\phi\) function. These receptors participate in phagocytosis, antibody-dependent cell mediated cytotoxicity (ADCC) and superoxide production (Jungi \textit{et al.}, 1990; Sarmay, 1992). VPM32 which is thought to be specific for FcyRIII (González, 1989) showed no decrease in M\(\phi\) surface expression before 5 days p.i.
CHAPTER SEVEN

IN VITRO INFECTION OF MONOCYTE DERIVED MACROPHAGES AND
PULMONARY ALVEOLAR MACROPHAGES WITH PARAINFLUENZA
TYPE 3 VIRUS

PART A: EFFECT ON MAJOR HISTOCOMPATIBILITY COMPLEX
(MHC) MOLECULE EXPRESSION

PART B: EFFECT ON PHAGOCYTOSIS.
Macrophages function as the principal cellular, non-specific effector mechanism in the protection against microorganisms (Nathan, 1986). In addition they act to induce the immune response by presenting antigens to antigen-specific T cells (Harding et al., 1988; Weaver & Unanue, 1990; Unanue, 1992). Mφs exist throughout the body, but their functions are somewhat heterogeneous, depending on the microenvironment in which they are found (Brain, 1992).

Within the lungs the AMφ plays a fundamental role in both defence and homeostasis, particularly in inflammatory response and tissue repair. They take part in degradative processes through phagocytic function and in immunological surveillance through their capacity of antigen processing and presentation. Mφs are also involved in immune regulation and some non-specific effector mechanisms of cell mediated protection (Holian & Scheule, 1990).

AMφs contribute to primary non-specific defence mechanisms against invading pathogens in the lung. Many viral infections of the respiratory tract result in changes in the phagocytic and bactericidal activity of AMφs which may lead to concurrent bacterial infection (Fuentes & Piojan 1986; Boschert et al., 1988; Kang et al., 1992).

PIV-3 initiates infection in respiratory tract epithelial cells, but can also infect alveolar Mφs. This can lead to important pathological consequences during the pathogenesis of this respiratory tract viral disease (Stauber & Weston, 1984). The AMφ can be affected by different mechanisms, such as direct damage caused by viral replication or as a result of immune mediated cytotoxicity directed towards the virus infected Mφ. Not only can the number of Mφs be reduced by the viral infection, but also the function of the remaining cells can be altered. Some reports suggest that such Mφs have suppressed immunological and non-immunological activities. These activities include membrane receptor binding, phagocytic ingestion mediated by Fc receptor (FcR), non-receptor specific phagocytosis, phago-lysosome fusion, intracellular killing and bacterial degradation (Hesse & Toth, 1983; Davies et al., 1986; Slauson et al., 1987).
After infection of AMs by PIV-3, a reduction in receptor-mediated phagocytosis and antibody dependent cellular cytotoxicity (ADCC) can take place. PIV-3 can inhibit Mφ phagocytosis, mediated by Fc receptors, through the interaction with immunocomplexes (Jakab & Warr, 1981; Babiuk et al., 1988; Brown & Ananaba, 1988). Furthermore, the inhibited phagocytosis, intracellular degradation and intracellular killing induced by the virus, can alter the antigen processing and presenting functions of these cells (Horohov & Rouse, 1986).

Chemotactic, phagocytic and microbicidal activities are basic functions of monocytes and Mφs migrating into inflammatory areas against invading microorganisms. Receptors for immunoglobulin G (FcγRI, II and III) and for iC3b (CD11b/18) are the principal mediators of opsonized phagocytosis and phagocyte activation (Hed & Stendahl, 1982; Anderson, 1987; Fanger et al., 1989; Jungi et al., 1990; Sarmay, 1992). Monocytes and Mφs have been shown to express FcγRII (CDw32) and Fcγ RIII (CD16) (Anderson, 1987). Expression of these molecules on Mφs is enhanced by γ-IFN and IL-2 (Fanger et al., 1989; Heagy et al., 1984; Gonwa et al., 1986; Nash et al., 1992).

After antigen phagocytosis AMφs also activate T cells and induce the production of IL-2 (Elias et al., 1990). Thus, AMφs can modulate the tissue response during the inflammatory process. Therefore, analysis of Fc receptors and phagocytosis is commonly used to study the effector function of Mφs and their contribution to the pathogenesis of disease.

Other Mφ functions are related to the expression of major histocompatibility complex (MHC) class I and class II antigens. The MHC class II molecules play a central role in the induction of the immune response (Unanue & Allen, 1987). Among these functions the presentation of soluble antigens to T helper lymphocytes and the stimulation of alloreactivity are included. The induction of immune response towards antigenic proteins depends on recognition by T cell receptors associated to MHC class II molecules.

Over 90% of circulating human monocytes express HLA-DR, whereas, only 25-60% express HLA-DQ and DP (Gonwa et al., 1986). MHC class II on monocytes is closely linked to antigen presentation (Unanue & Cerottini, 1991). It has been reported that there is a decrease in HLA-DR expression in monocytes from HIV-
infected patients with AIDS (Heagy et al., 1984; Roy et al., 1987). In addition, AMφs from AIDS and AIDS related complex (ARC) patients did not express the normal levels of class II and LFA-1 antigen on their surface when compared to the normal controls (Roy et al., 1987; Bray et al., 1993). Documentation shows that there is a great reduction in MHC class II expression and function of dendritic cells and Langerhan’s cells in HIV-infected patients with ARC and AIDS (Eales et al., 1988; Belsito et al., 1984). In vitro, studies using the human promonocytic cell line U937 as a monocyte model have shown an increase in the expression of mature monocyte surface markers, except for HLA class II antigens, after infection with HIV (Pettit et al., 1987). It is thought that this contributes to the immunosuppression of HIV infection.

The expression of MHC class II molecules on Mφs is transitory. In vitro eventually all class II+ Mφs become class II−, and therefore lose their properties as APC (Unanue, 1984; Unanue & Allen, 1987). However, they can be rapidly reactivated to synthesize class II molecules after antigen uptake or stimulation with γ-IFN (Lucas & Epstein, 1985; Gonwa et al., 1986; Elias et al., 1990).

Viral infections can also induce the release of cell soluble factors. PIV-3 infection is an efficient γ-IFN inducer in peripheral blood mononuclear cells (Towsend et al., 1988). This virus is also involved in induction of other molecules of biological importance such as tumoral necrosis factor-alfa (TNF-α) (Vainionpaa et al., 1989; Bienhoff et al., 1992). Parainfluenza-3 virus/endotoxin combinations stimulated higher TNF-α release when compared with other virus/endotoxin combinations (Bienhoff et al., 1992).

The well-known inducers of γ-IFN are generally effective for inducing Mφ γ-IFN production. These inducers include viruses, endotoxin, synthetic polyribonucleotides, pyran and statolon (Lucas & Epstein, 1985). A variety of viruses are effective inducers of Mφ γ-IFN production including viruses which cause respiratory infections such as PIV-3.

γ-IFN has a wide range of antiviral effects such as decreased RNAm translation, the defective transmission of viral message and inhibition of the final events of the viral replicative cycle. In contrast to other classic patterns of action, the inhibition produced by γ-IFN in PIV-3 infection is not due to a decrease in viral RNA or viral
protein synthesis. On the contrary it is due to an interference with the process of virus maturation during late stages of viral replication. This can lead to defective virus assembly and virus budding, as well as release of virus particles with low infectivity due to a deficiency in glycoproteins (Unanue & Allen, 1987).

It has been pointed out that γ-IFN is the main inductor of MHC class II molecules (Watanabe & Jacob, 1991). γ-IFN is a biologically active molecule produced by T lymphocytes during antigen presentation. γ-IFN binds to Mφs inducing new expression of RNAm for MHC class II molecule synthesis (Unanue & Allen, 1987). γ-IFN-treated Mφs also exhibit cytocidal functions upon their interaction with other stimuli (called “second signals”) of which bacterial products like endotoxin are the most prominent (Adams & Hamilton, 1984).

On the other hand, TNF-α, acts as a potent stimulator of monocyte differentiation in myeloid leukemia cell lines, but TNF-α acts as an antagonist to γ-IFN to decrease MHC class II expression on mature monocytes (Watanabe & Jacob, 1991; Gonwa et al., 1986). This raises the question of whether MHC class II production by Mφs might be altered during PIV-3 infection and whether this has functional consequences.

The direct effect on surface MHC molecules of Mφs after PIV-3 infection in vitro is not clear. This can lead to alterations in the antigen presenting function of these cells. Therefore, a study of the changes of both phagocytic capacity and surface MHC molecule expression on cultured MDMφs and AMφs after OIV-3 infection was undertaken.
7.2 **Results.**

7.2.1 **PART A: EFFECT ON MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) MOLECULE EXPRESSION.**

7.2.1.1 **Kinetics of MHC molecule expression in monocyte-derived macrophage (MDMφs) cell cultures.**

Peripheral blood monocytes (PBMC) were collected and cultured to obtain MDMφ. AMφs were collected from lung wash fluid (LWF). All these cells were obtained from clinically normal healthy sheep and cultured in gelatin-coated flasks or plates. Non-adherent cells were washed off after overnight incubation. These cells were harvested every 24 hours and immunostained for MHC class I and II molecule expression on cell surface. The reactivity was analyzed by flow cytometry. Table 7.1 shows the results of MHC molecule expression by MDMφs before (day 0) and after cell culture (days 1 to 7).

VPM19 is a monoclonal antibody specific for a monomorphic region of MHC class I molecules that is expressed on most nucleated cells (Hopkins & Dutia, 1990). Fresh monocytes were found to express class I molecules with high intensity (MF=99.5) and they continued to express these molecules in culture during their *in vitro* differentiation towards MDMφs. However, the intensity of fluorescence staining, therefore density, of MHC class I on the surface started to decrease from day 1 of culture. It became significantly lower (p<0.05) on day 3 of culture in comparison to fresh monocytes (day 0 of culture). After 5 to 7 days of culture, although the expression was variable they stabilized in intermediate ranges of intensity of fluorescence (MF=68.5 and 67.2, respectively).

VPM 36 and VPM 38 are monoclonal antibodies specific for MHC class II DQ and DR α chains, respectively (Dutia *et al.*, 1990). The expression of MHC class II DQ antigen was of high intensity (MF=85.6) on fresh monocytes. However, after culture the expression of DQ started to decrease on day 1, being significantly (p<0.001) reduced by day 3 (MF=6.7). After day 5 the expression of DQ was almost at
background level (MF=2.3). The pattern of MHC class II DR expression was very similar to DQ (Table 7.1).

7.2.1.2 Kinetics of MHC molecule expression in alveolar macrophage (AMφ) cell cultures.

Table 7.2 shows the results obtained by flow cytometry of MHC molecule expression by AMφs before (day 0) and after cell culture (day 1 to 7). Fresh AMφs also expressed class I with high intensity (MF=95.3) and they also continued to express this molecule in culture. However, the intensity of fluorescence staining decreased significantly (p<0.001) and temporarily on day 1 after culture (MF=32.4). After day 2 of culture the level of MHC class I expression increased stabilizing to an intermediate level of expression.

Fresh AMφs were also positive for expression of MHC class II molecules DQ and DR, although this was somewhat lower than in fresh monocytes. After 1 day in culture the expression of MHC class II DQ molecule was significantly decreased (p<0.001) in comparison to fresh AMφs. The expression continued to decrease thereafter to minimal levels after days 5 to 7 (MF/DQ=2.7 and 3.6, respectively). No significant differences were observed between expression of MHC class II molecule DQ and MHC class II DR, either in fresh AMφs or in AMφs in culture (Table 7.2). MDMφs and AMφs were cultured on round coverslips set up in 24 well cell culture plates. Coverslips were removed from day 0 to 7 of cell culture and immunostained for MHC class II antigens DQ or DR by alkaline phosphatase (Fig. 7.1a). After 3 days of cell culture MHC class II staining in both MDMφs and AMφs was not detected by this technique. However in AMφ cultures the presence of a few scattered highly positive and pleomorphic cells, showing long cytoplasmic processes was observed on many occasions. They were in close contact with and surrounding AMφs (Fig. 7.1b,c). These cells remained highly positive for MHC class II antigens after the AMφs became negative (7-10 days). They are thought to be dendritic cells according to their morphology and MHC class II expression.
TABLE 7. 1 Time dependent expression of MHC molecules by monocyte derived macrophages (MDMφs) in cell culture.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Molecules</th>
<th>Time in cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
<td>1 days</td>
</tr>
<tr>
<td>MHC class I</td>
<td>99.5±5.31</td>
<td>87.2±4.9</td>
</tr>
<tr>
<td>MHC class II DQ</td>
<td>85.6±7.4</td>
<td>65.2±4.5</td>
</tr>
<tr>
<td>MHC class II DR</td>
<td>90.1±6.8</td>
<td>73.6±4.0</td>
</tr>
</tbody>
</table>

1. Data (mean±sd) is expressed as mean fluorescence intensity (MF) where non-specific control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.
2. Significantly different at p<0.05 (Mann-Whitney non-parametric rank test) from day 0 of culture (fresh cell).
3. Significantly different at p<0.001 (Mann-Whitney non-parametric rank test) from day 0 of culture (fresh cell).

TABLE 7. 2 Time dependent expression of MHC molecules by alveolar macrophages (AMφs) in cell culture.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Molecules</th>
<th>Time in cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
<td>1 days</td>
</tr>
<tr>
<td>MHC class I</td>
<td>95.3±6.3¹</td>
<td>32.4±7.1³</td>
</tr>
<tr>
<td>MHC class II DQ</td>
<td>68.5±6.9</td>
<td>11.5±4.5³</td>
</tr>
<tr>
<td>MHC class II DR</td>
<td>71.4±7.3</td>
<td>15.6±5.4³</td>
</tr>
</tbody>
</table>

1. Data (mean±sd) is expressed as mean fluorescence intensity (MF) where non-specific control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.
2. Significantly different at p<0.05 (Mann-Whitney non-parametric rank test) from day 0 of culture (fresh cell).
3. Significantly different at p<0.001 (Mann-Whitney non-parametric rank test) from day 0 of culture (fresh cell).
FIGURE 7. 1 Expression of MHC class II molecules in AMφs in tissue culture. AMφs were collected from the lung wash fluid (LWF) of clinically normal healthy sheep and cultured 24 well plates (gelatin-coated) with round coverslips. Non-adherent cells were washed off after 4 hours of culture. Coverslips were removed after 4, 24, 72 and 120 hours of culture. Cells were incubated with a 1:1 mixture of mAbs VPM36 and VPM38 for MHC class II molecules DQ and DR, respectively. Their reactivity was detected by indirect immunoalkaline phosphatase staining (IIAP).

a) AMφs after 4 hours in culture. Most cells show a strong positive staining (red colour) for MHC class II molecules. IIP with mAbs VPM36/38. Magnification x200.

b) AMφs after 120 hours in culture. All AMφs are negative for MHC class II molecule staining. A cell in close contact with an AMφ (arrow) shows a strong positive staining (red colour) for MHC class II molecules. This positive cell has long cytoplasmic processes and is contacting other AMφs with them. IIP with mAbs VPM36/38. Magnification x200.

c) AMφs after 120 hours in culture. All AMφs are negative for MHC class II molecule staining. Two pleomorphic cells are surrounding AMφs (arrows). These cells show strong positive staining (red colour) for MHC class II molecules. IIP with mAbs VPM36/38. Magnification x200.
7.2.1.3 \(\gamma\)-IFN induction of MHC class II expression

We were interested in looking at the effect of OPIV-3 infection on MHC class II expression by Mφs \textit{in vitro}. Hence, the loss of these cell surface molecules during culture was unfortunate. It was therefore decided to see if MHC class II expression by MDMφs and AMφs could be induced \textit{in vitro}. So, MDMφs and AMφs were cultured and then treated with \(\gamma\)-IFN (100U/ml) on day 3. The expression of MHC molecules on \(\gamma\)-IFN and mock treated MDMφs and AMφs was then analyzed from day 0 to day 7 of culture by flow cytometry.

Table 7.3 shows changes of MHC molecule expression in MDMφs cultures after 2 and 4 days of \(\gamma\)-IFN treatment. \(\gamma\)-IFN significantly enhanced (p<0.05) class I molecule expression from an intermediate intensity of expression (MF=62.8) to a high intensity after 2 days of treatment (MF=91.2). \(\gamma\)-IFN also significantly enhanced (p<0.001) MHC class II expression from minimal levels (MF/DQ=0.7; MF/DR=12.6) to a much higher expression after 2 days of treatment (MF/DQ=37.6; MF/DR=54.8). Representative profiles of two day \(\gamma\)-IFN treated MDMφs are shown in Fig. 7.2. Treatment of MDMφs with \(\gamma\)-IFN for 1 day only slightly enhanced MHC molecule expression. Treatment for 3 or 4 days did not greatly increased MHC expression.

\(\gamma\)-IFN treatment had very similar effects on AMφs (Table 7.4). Representative profiles of two day \(\gamma\)-IFN treated AMφs is shown in Fig. 7.3.

7.2.1.4 Effect of PIV-3 infection on major histocompatibility complex (MHC) molecule expression by monocyte-derived macrophages (MDMφs).

Individual cultures of MDMφs were infected with OPIV-3 270-7 (1 TCID\textsubscript{50}/cell) on days 3, 5, 7 and 9 of culture. MHC molecule expression on OPIV-3 and mock infected MDMφs was then analyzed on the same day (day 10 of culture). Only \(\gamma\)-IFN-treated Mφs expressed MHC class II molecules. Untreated cells were negative for class II expression.

MHC class I molecules were highly expressed on cultures of OPIV-3 infected MDMφs and this expression was not significantly altered during the 7 day p.i. period studied. Although some experiments showed a tendency for surface expression to
TABLE 7.3 Time dependent effect of γ-IFN treatment on MHC molecule expression by monocyte-derived macrophages (MDMφs) in cell culture.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Time in cell culture/γ-IFN treatment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecules</td>
<td>Treatment</td>
</tr>
<tr>
<td>MHC class I</td>
<td>Mock treated</td>
</tr>
<tr>
<td></td>
<td>γ-IFN</td>
</tr>
<tr>
<td>MHC class II DQ</td>
<td>Mock treated</td>
</tr>
<tr>
<td></td>
<td>γ-IFN</td>
</tr>
<tr>
<td>MHC class II DR</td>
<td>Mock treated</td>
</tr>
<tr>
<td></td>
<td>γ-IFN</td>
</tr>
</tbody>
</table>

1. γ-IFN treatment (100U/ml) was given on day 3 of cell culture.
2. Data (mean±SD) is expressed as mean fluorescence intensity (MF) where non-specific control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.
3. Significantly different at p<0.05 (Mann-Whitney non-parametric rank test) from mock-treated cells same day of culture.
4. Significantly different at p<0.001 (Mann-Whitney non-parametric rank test) from mock-treated cells same day of culture.

TABLE 7.4 Time dependent effect of γ-IFN treatment on MHC molecule expression by alveolar macrophages (AMφs) in cell culture.

<table>
<thead>
<tr>
<th>Surface</th>
<th>γ-IFN treatment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecules</td>
<td>Treatment(^1)</td>
</tr>
<tr>
<td>MHC class I</td>
<td>Mock treated</td>
</tr>
<tr>
<td></td>
<td>γ-IFN</td>
</tr>
<tr>
<td>MHC class II DQ</td>
<td>Mock treated</td>
</tr>
<tr>
<td></td>
<td>γ-IFN</td>
</tr>
<tr>
<td>MHC class II DR</td>
<td>Mock treated</td>
</tr>
<tr>
<td></td>
<td>γ-IFN</td>
</tr>
</tbody>
</table>

1. γ-IFN treatment (100U/ml) was given on day 3 of cell culture.
2. Data (mean±SD) is expressed as mean fluorescence intensity (MF) where non-specific control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.
3. Significantly different at p<0.05 (Mann-Whitney non-parametric rank test) from mock-treated cells same day of culture.
4. Significantly different at p<0.001 (Mann-Whitney non-parametric rank test) from mock-treated cells same day of culture.
FIGURE 7.2 Frequency histograms showing the effect after 2 days of γ-IFN treatment on the MHC molecule expression of MDMϕs in culture. Peripheral blood monocytes (PBMC) were collected and cultured in gelatin-coated flasks or plates to obtain MDMϕs. After 3 days of culture they were fed 100 U. of γ-IFN/ml. After 2 days γ-IFN treated and mock-treated cells were harvested and incubated with mAbs VPM19, VPM36 and VPM38 (——)in order to detect MHC class I, class II DQ and class II DR molecules on the cell surface by flow cytometry. Staining with NMS as the negative control (........) is shown for comparison.
FIGURE 7. 3 Frequency histograms showing the effect after 2 days of γ-IFN treatment on the MHC molecule expression of AMφs in culture. AMφs were collected from lung wash fluid (LWF) and cultured in gelatin-coated flasks or plates. After 3 days of culture they were fed 100 U. of γ-IFN/ml. After 2 days γ-IFN treated and mock-treated cells were harvested and incubated with mAbs VPM19, VPM36 and VPM38 (——) in order to detect MHC class I, class II DQ and class II DR molecules on the cell surface by flow cytometry. Staining with NMS as the negative control (........) is shown for comparison.
Mock-treated

IFN-treated

AM

MHC Class I

MHC Class I

Log_{10} Fluorescence Intensity →

Cell Number

MHC Class II/DQ

MHC Class II/DR

MHC Class II/DQ

MHC Class II/DR
increase slightly after 5 days p.i. (Table 7.5). After γ-IFN treatment for 2 days MHC class I molecule expression was significantly increased (p<0.05) in infected cells as well as in mock infected cells, in comparison to non-γ-IFN treated infected and mock infected cultures. These findings were similar to those observed in MDMϕ cultures of non-infected cells (Tables 7.1 and 7.3) and showed no effect on MDMϕ class I expression after OPIV-3 infection. Furthermore, γ-IFN MHC class I enhancement was not impaired (Table 7.5). A representative profile, of class I expression of MDMϕs after 5 days of OPIV-3 infection and two days γ-IFN treatment, is shown in Fig. 7.4. MHC class II molecule expression after infection remained almost undetectable and very similar in fashion to that observed during culture of non-infected MDMϕs (Table 7.1). Furthermore, after two days of γ-IFN treatment no significant increase in expression of either DQ or DR MHC class II molecules was observed in OPIV-3 infected cultures. In contrast mock infected control cultures showed a significant increase (p<0.001) in both DQ and DR MHC class II molecules, indicating a suppressive effect after OPIV-3 infection (Table 7.5). Representative profiles of class II expression of MDMϕs after 5 days of OPIV-3 infection, and two days γ-IFN treatment, are shown in Fig. 7.4.

7.2.1.5 Effect of PIV-3 infection on major histocompatibility complex (MHC) molecule expression by alveolar macrophages (AMϕs).

Two methods were used to examine the effect of OPIV-3 infection on AMϕ MHC phenotype. In the first experiment individual cultures of AMϕs were infected with OPIV-3 270-7 (1 TCID<sub>50</sub>/cell) on days 3, 5, 7 and 9 of culture. MHC molecule expression on OPIV-3 and mock infected AMϕs was then analyzed on the same day (day 10 of culture).

AMϕ cultures after OPIV-3 infection showed a similar pattern of MHC molecule expression to that observed in infected MDMϕ cultures and also a similar response to γ-IFN treatment for 2 days (Table 7.6). MHC class I molecules were highly expressed on cultures of OPIV-3 infected AMϕs and this expression was not significantly altered during the 7 days p.i. period studied. As well in MDMϕ cultures, here there was also a tendency for surface expression to increase slightly after 5 days p.i.; however no significant differences were detected due to variability in expression. After γ-IFN treatment for 2 days MHC class I molecule expression was significantly
TABLE 7. 5 Comparison of the MHC molecules expression by 10 day cultured monocyte-derived macrophages (MDMφs) after OPIV-3 infection with or without 2 days γ-IFN treatment.

<table>
<thead>
<tr>
<th>MHC molecules</th>
<th>Treatment</th>
<th>Time Post OPIV-3 Infection</th>
<th>Mock infected&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 day</td>
<td>3 days</td>
</tr>
<tr>
<td>Class I</td>
<td>mock-treated</td>
<td>64.2±6.5&lt;sup&gt;3&lt;/sup&gt;</td>
<td>63.1±4.8</td>
</tr>
<tr>
<td></td>
<td>γIFN&lt;sup&gt;4&lt;/sup&gt;</td>
<td>ND</td>
<td>99.3±5.2</td>
</tr>
<tr>
<td>Class II DQ</td>
<td>mock-treated</td>
<td>5.3±0.9</td>
<td>5.2±1.5</td>
</tr>
<tr>
<td></td>
<td>γIFN&lt;sup&gt;4&lt;/sup&gt;</td>
<td>ND</td>
<td>11.5±3.6</td>
</tr>
<tr>
<td>Class II DR</td>
<td>mock-treated</td>
<td>10.7±1.8</td>
<td>9.6±2.0</td>
</tr>
<tr>
<td></td>
<td>γ-IFN&lt;sup&gt;4&lt;/sup&gt;</td>
<td>ND</td>
<td>16.8±4.1</td>
</tr>
</tbody>
</table>

1. Cultured MDM were infected on days 3, 5, 7 and 9 of culture with OPIV-3 ITCID<sub>50</sub> per cell. Cells were harvested on day 10 of culture.
2. Mock infected cells on day 3 and harvested on day 10 of culture as controls.
3. Data (mean±SD) is expressed as mean fluorescence intensity (MF) where non-specific control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.
4. γ-IFN treatment (100U/ml) was given 2 days before harvesting the OPIV-3 infected or mock infected cells.
5. Significantly different at p<0.05 (Mann-Whitney non-parametric rank test) from non-γ-IFN treated cells.
6. Significantly different at p<0.001 (Mann-Whitney non-parametric rank test) from both non-γ-IFN treated cells and OPIV-3 infected-γ-IFN treated cells.
FIGURE 7.4 Frequency histograms showing the effect after 2 days of γ-IFN treatment on the MHC molecule expression of MDMφs infected for 5 days with OPIV-3. Peripheral blood monocytes (PBMC) were collected and cultured in gelatin-coated flasks or plates to obtain MDMφs. After 3 days of culture they were either infected with OPIV-3 270-7 at 1 TCID₅₀/cell or mock-infected only with medium. Three days later they were fed 100 U. of γ-IFN/ml. After 2 days γ-IFN treated and mock-treated cells were harvested and incubated with mAbs VPM19, VPM36 and VPM38 in order to detect the cell surface expression of MHC class I, class II DQ and class II DR molecules, respectively. Staining with NMS as the negative control is shown for comparison.
Mock-infected

IFN/MDM

MHC Class I

Log_{10} Fluorescence Intensity

OPIV-3 infected

MHC Class I

MHC Class II/DQ

MHC Class II/DR

Log_{10} Fluorescence Intensity
increased (p<0.05) in infected cells as well as in mock infected cells, in comparison to non-γ-IFN treated infected and mock-infected cultures (Table 7.2). These findings were similar to those observed in AMφ cultures of non-infected cells (Table 7.2 and 7.4). OPIV-3 infection showed no effect on either class I expression by AMφs or γ-IFN MHC class I enhancement (Table 7.6). A representative profile of class I expression of AMφs after 5 days of OPIV-3 infection and two days γ-IFN treatment, is shown in Fig. 7.5.

MHC class II molecule expression after infection by AMφs (Table 7.6) remained almost undetectable and very similar to that observed during culture of non-infected AMφs and infected MDMφs (Table 7.2). After two days γ-IFN treatment, no significant increase in expression of either DQ or DR MHC class II molecules was observed in OPIV-3 infected AMφ cultures. In contrast mock-infected control cultures showed a significant increase (p<0.001) in both DQ and DR MHC class II molecules, indicating a suppressive effect after OPIV-3 infection (Table 7.6.). A representative profile, of class II expression of AMφs after 5 days of OPIV-3 infection and two days γ-IFN treatment, is shown in Fig. 7.5.

In the second experiment, AMφs were infected with OPIV-3 1 TCID_{50} per cell, on day 3 of culture. Then after 2 days p.i. cells were either treated with γ-IFN (100U/ml) or mock-treated with medium. AMφs were harvested for phenotypic analysis on days 1, 3, 5 and 7 p.i. with OPIV-3 which were also days 0, 1, 3 and 5 post γ-IFN treatment, respectively (Table 7.7). Mock infected cells as well OPIV-3 infected cells showed high expression of class I molecules after day 3 post infection (day 1 post γ-IFN treatment). On days 5 and 7 p.i. which were also days 3 and 5 post γ-IFN treatment, respectively, there was a significant increase (p<0.05) in class I molecule expression in both infected and mock-infected cells. This illustrates that OPIV-3 infection does not affect MHC class I expression.

In contrast, OPIV-3 infection totally abrogates the effect of γ-IFN in its induction of both MHC class I IDR and DQ. Mock infected AMφs showed very low levels of both MHC class II DQ and DR molecules without γ-IFN treatment or after 1 day treatment, however, after 3 and 5 days of γ-IFN treatment they showed a significant increase (p<0.05) in both molecules. OPIV-3 infected AMφs also showed very low levels of both MHC class II DQ and DR molecules but in contrast to mock-infected cells they did not increase the expression of these molecules after γ-IFN treatment.
TABLE 7.6 Comparison of the MHC molecules expression by OPIV-3 infected alveolar macrophages (AMφs) in culture with or without 2 days of γ-IFN treatment.

<table>
<thead>
<tr>
<th>MHC molecules</th>
<th>Treatment</th>
<th>Time Post OPIV-3 Infection&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mock</th>
<th>infected&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 day</td>
<td>3 days</td>
<td>5 days</td>
</tr>
<tr>
<td>Class I</td>
<td>mock-treated</td>
<td>62.1±7.1&lt;sup&gt;3&lt;/sup&gt;</td>
<td>64.5±5.8</td>
<td>67.9±5.7</td>
</tr>
<tr>
<td></td>
<td>γIFN&lt;sup&gt;4&lt;/sup&gt;</td>
<td>ND</td>
<td>95.4±5.9</td>
<td>93.2±6.2&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Class II DQ</td>
<td>mock-treated</td>
<td>4.6±1.4</td>
<td>5.1±1.7</td>
<td>5.3±0.9</td>
</tr>
<tr>
<td></td>
<td>γIFN</td>
<td>ND</td>
<td>9.7±2.8</td>
<td>6.1±1.8</td>
</tr>
<tr>
<td>Class II DR</td>
<td>mock-treated</td>
<td>14.7±2.4</td>
<td>15.3±2.1</td>
<td>16.7±3.0</td>
</tr>
<tr>
<td></td>
<td>γ-IFN</td>
<td>ND</td>
<td>19.6±3.2</td>
<td>17.2±2.9</td>
</tr>
</tbody>
</table>

1. Cultured AM were infected on days 3, 5, 7 and 9 of culture with OPIV-3 1TCID<sub>50</sub> per cell. Cells were harvested on day 10 of culture.
2. Controls are mock infected cells on day 3 and harvested on day 10 of culture.
3. Data (mean±SD) is expressed as mean fluorescence intensity (MF) where nonspecific control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.
4. γ-IFN treatment (100U/ml) was given 2 days before harvesting the OPIV-3 infected or mock infected cells.
5. Significantly different at p<0.05 (Mann-Whitney non-parametric rank test) from non-γ-IFN treated cells.
6. Significantly different at p<0.001 (Mann-Whitney non-parametric rank test) from both non-γ-IFN treated cells and OPIV-3 infected-γ-IFN treated cells.
FIGURE 7. 5 Frequency histograms showing the effect after 2 days of γ-IFN treatment on the MHC molecule expression of AMφs infected for 5 days with OPIV-3. AMφs were collected from lung wash fluid (LWF) and cultured in gelatin-coated flasks or plates. After 3 days of culture they were either infected with OPIV-3 270-7 at 1 TCID₅₀/cell or mock-infected only with the medium. Three days later they were fed 100 U. of γ-IFN/ml. After 2 days, γ-IFN treated and mock-treated cells were harvested and incubated with mAbs VPM19, VPM36 and VPM38 (——) in order to detect the cell surface expression of MHC class I, class II DQ and class II DR molecules, respectively. Staining with NMS as the negative control (.........) is shown for comparison.
Log10 Fluorescence Intensity

Mock-infected

IFN/AM

MHC Class I

OPIV-3 infected

MHC Class I

MHC Class II/DQ

MHC Class II/DQ

MHC Class II/DR

MHC Class II/DR
TABLE 7. Comparison of the effect of γ-IFN treatment on MHC molecules expression by alveolar macrophages (AMφs) in culture, in the presence or absence of OPIV-3 infection.

<table>
<thead>
<tr>
<th>MHC molecules</th>
<th>Culture Treatment</th>
<th>Time Post Infection/γ-IFN treatment (days)</th>
<th>1/0</th>
<th>3/1</th>
<th>5/3</th>
<th>7/5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock infected</td>
<td>68.3±4.1(^2)</td>
<td>70.1±5.2</td>
<td>97.9±3.9(^3)</td>
<td>93.2±4.7(^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPIV-3 infected</td>
<td>59.8±5.7</td>
<td>65.1±4.3</td>
<td>98.2±5.1(^3)</td>
<td>95.4±3.9(^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class II DQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock infected</td>
<td>6.2±2.6</td>
<td>9.8±2.8</td>
<td>55.1±4.7</td>
<td>48.6±2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPIV-3 infected</td>
<td>7.1±2.8</td>
<td>6.5±2.5</td>
<td>5.1±2.2</td>
<td>5.4±3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class II DR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock infected</td>
<td>11.5±3.3</td>
<td>19.3±3.6</td>
<td>71.2±5.3(^4)</td>
<td>67.5±4.9(^4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPIV-3 infected</td>
<td>9.4±3.0</td>
<td>8.9±2.8</td>
<td>10.6±3.7</td>
<td>9.1±3.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Cultured AM were infected on day 3 of culture with OPIV-3 ITCID\(_{50}\) per cell. Cells were harvested on day 1,3,5, and 7 p.i. γ-IFN treatment (100U/ml) was given on day 2 p.i. with OPIV-3.

2. Data (mean±SD) is expressed as mean fluorescence intensity (MF) where nonspecific control fluorescence (NMS) has been subtracted. Data was collected from 5 separate experiments.

3. Significantly different at p<0.05 (Mann-Whitney non-parametric rank test) from non-γ-IFN treated cells.

4. Significantly different at p<0.001 (Mann-Whitney non-parametric rank test) from both non-γ-IFN treated cells and OPIV-3 infected-γ-IFN treated cells.
7.2.2 PART B: EFFECT ON PHAGOCYTOSIS.

In order to study functional alterations in MDM\(\phi\)s and AM\(\phi\)s after PIV-3 infection, these cells were cultured in 24 well plates and the erythrocyte rosetting and phagocytosis of opsonized sheep red blood cells (SRBC) were assayed by flow cytometry. Individual cultures of MDM\(\phi\)s and AM\(\phi\)s were infected with OPIV-3 270-7 (1 TCID\(_{50}\)/cell) on days 3, 5, 7 and 9 of culture. Erythrocyte rosetting and phagocytosis of opsonized SRBC were assayed on OPIV-3 and mock-infected MDM\(\phi\)s and AM\(\phi\)s on the same day (day 10 of culture).

7.2.2.1 Effect of OPIV-3 infection in SRBC rosetting

Table 7.8 shows that OPIV-3 infection of MDM\(\phi\)s had no significant effect on Fc rosetting. In mock-infected MDM\(\phi\) cultures 96.1±2.3% cells formed FcR rosettes. Following viral infection, the percentage of MDM\(\phi\)s forming FcR rosettes did not drastically differ from that of the controls. The lowest values were observed on day 7 of the virus infection, a time when 89.3±4.5 MDM\(\phi\)s demonstrated positive FcR rosettes. At all assay periods, incubations of normal M\(\phi\)s and virus infected ones with non-opsonized erythrocytes failed to demonstrate any non-immunologic binding. Thus, any interaction between M\(\phi\)s and opsonized erythrocytes was specific for the ligand and cell receptor.

FcR expression on the surface of OPIV-3 infected AM\(\phi\)s (Table 7.8), assayed by erythrocyte rosetting, showed a significant decrease (p<0.05) in immune complex FcR activity after 5 and 7 days p.i. when compared to mock-infected AM\(\phi\)s (Table 7.8). The lowest values were observed on day 5 of the virus infection, a time when 80.1±5.2 AM\(\phi\)s showed positive FcR rosettes. In mock-infected AM\(\phi\)s cultures 92.5±6.4% cells formed FcR rosettes.

7.2.2.2 In vitro phagocytic activity of OPIV-3 infected monocyte-derived macrophages (MDM\(\phi\)s) and alveolar macrophages (AM\(\phi\)s).

Using FITC-labelled SRBC as targets for phagocytosis, it is possible to use flow cytometry to analyse phagocytic activity. The time of incubation and the
TABLE 7.8 Effect of OPIV-3 infection on rosetting of cultured monocyte-derived macrophages (MDMφs) and alveolar macrophages (AMφs) with opsonized SRBC.

<table>
<thead>
<tr>
<th>Cells</th>
<th></th>
<th>Control</th>
<th>1 day</th>
<th>3 days</th>
<th>5 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDMφ</td>
<td>%</td>
<td>96.1±6.3</td>
<td>95.3±6.5</td>
<td>96.4±5.9</td>
<td>92.7±6.1</td>
<td>89.3±4.5</td>
</tr>
<tr>
<td>AMφ</td>
<td>%</td>
<td>92.5±6.4</td>
<td>90.8±5.7</td>
<td>88.5±6.3</td>
<td>80.6±5.9</td>
<td>80.1±5.2</td>
</tr>
</tbody>
</table>

1 Data is expressed as percentage of positive cells with 1% gate set on the negative control of the staining (incubation with NMS). Data was collected from 5 separate experiments.

2 Significantly different (p<0.05) from mock-treated cells same day of culture (Mann-Whitney non-parametric rank test).
concentration of antibody coated and FITC-labelled SRBC used in phagocytic assays were titrated in preliminary experiments (data not shown) according to the protocol described in section 2. Phagocytosis of SRBC is affected by opsonization as well as different concentrations of antiserum, and the fluorescence of the MDMϕs and AMϕs is related to the number of FITC-particles which are ingested by the cells (Oda & Maeda, 1986; Bjerknes et al., 1989; Buschmann & Winter, 1989; Lee, 1994).

Individual cultures of MDMϕs and AMϕs were infected with OPIV-3 270-7 (1 TCID_{50}/cell) on days 3, 5, 7 and 9 of culture. Phagocytosis of SRBC was assessed on both OPIV-3 and mock infected cells by flow cytometry on the same day (day 10 of culture). Previously, cells were fed with 50μL of opsonized FITC-SRBC and incubated for 1 hr. at 37°C.

In the early phase of viral infection (before 2 day p.i.), both OPIV-3 infected and mock-infected cells showed very high phagocytic activity, with 90.7% of cultured MDMϕs having high erythrocyte rosetting and phagocytic activity (MF=85.5) (Table 7.9). However, the phagocytic capacity of OPIV-3 infected MDMϕs was decreased to 67.2% (MF=65.3) by 3 days post infection. By day 5 p.i. phagocytic cells had further decrease to 47.2% (MF=58.5). There was a statistically significant difference between OPIV-3 infected and mock-infected MDMϕs at this time (p<0.05) (Table 7.9). By day 7 there was a recovery in both the percentage of phagocytic cells (67.4 %) and the fluorescence intensity (MF=65.5). Figure 7.6 shows representative profiles of these experiments.

The ability of AMϕs from virus infected cultures to ingest the surface bound IgG coated erythrocytes progressively decreased until day 5 p.i. In the early phase of viral infection (before 2 day p.i.), AMϕs showed a very high percentage of phagocytosis, with nearly 85.7% positive cells, although phagocytic activity (MF=76.9) was slightly lower in comparison to that of MDMϕs (Table 7.9). The phagocytic capacity of OPIV-3 infected AMϕs by 3 days post infection was decreased to 60.2% positive cells with not a decrease in phagocytic intensity (MF=63.3). By day 5 p.i. phagocytic cells had significantly decreased to 48.1% or less with a much lower phagocytic intensity (MF=58.2). There was a statistically significant difference between OPIV-3 infected and mock-infected MDMϕs at this point in time (p<0.001) (Table 7.9). By day 7, there was a recovery in both the percentage of phagocytic cells (67.4 %) and
the phagocytic intensity (MF=70.5) similar to that seen in the experiments with MDMφs. Figure 7.6 shows a representative profile of these experiments.

Treatment with γ-IFN did not result in significant changes in either rosetting or phagocytic capacity of MDMφs or AMφs between mock infected and OPIV-3 infected cells (data not shown).
<table>
<thead>
<tr>
<th>Cells</th>
<th>Control</th>
<th>1 day</th>
<th>3 days</th>
<th>5 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDMφ %</td>
<td>90.7±4.2</td>
<td>83.7±5.1</td>
<td>67.2±5.4</td>
<td>47.2±9.2&lt;sup&gt;3&lt;/sup&gt;</td>
<td>67.4±7.3</td>
</tr>
<tr>
<td>MF</td>
<td>85.5±6.3</td>
<td>75.5±6.1</td>
<td>65.3±5.2</td>
<td>58.5±4.4&lt;sup&gt;2&lt;/sup&gt;</td>
<td>65.5±4.7</td>
</tr>
<tr>
<td>AMφ %</td>
<td>85.7±6.3</td>
<td>82.9±5.9</td>
<td>60.2±4.1</td>
<td>48.1±3.9&lt;sup&gt;3&lt;/sup&gt;</td>
<td>67.4±4.6</td>
</tr>
<tr>
<td>MF</td>
<td>76.9±4.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td>77.6±5.3</td>
<td>63.3±4.9</td>
<td>58.2±4.2&lt;sup&gt;2&lt;/sup&gt;</td>
<td>70.5±5.1</td>
</tr>
</tbody>
</table>

-1 Data is expressed as specific mean fluorescence intensity (which is MF with FITC SRBC minus MF without FITC SRBC) and as percentage of positive cells. Data was collected from 5 separate experiments.

2. Significantly different (p<0.05) from mock-treated cells same day of culture (Mann-Whitney non-parametric rank test).

3. Significantly different (p<0.001) from mock-treated cells same day of culture (Mann-Whitney non-parametric rank test).
Individual cultures of MDMφs and AMφs were infected with OPV-3 270-7 (1 TCID$_{50}$/cell) in a series on days 3, 5, 7 and 9 of culture. Phagocytosis of SRBC was assessed on both OPV-3 and mock infected cells by flow cytometry on the same day (day 10 of culture). Previously, cells were fed with 50μl of opsonized FITC-SRBC (----) and incubated for 1 hr. at 37°C. Data was obtained by flow cytometry. Staining with NMS as the negative control (........) is shown for comparison.
SRBC/Phagocytosis
Mock-infected

1 Day p.i.

3 Days p.i.

5 Days p.i.

7 Days p.i.

Log10 Fluorescence Intensity →
7.3 DISCUSSION

The migration of monocytes into tissues results in the generation of organ specific populations of resident Mφs which show different phenotypes and functions (Andreesen et al., 1990). For example, AMφs express high MHC class II levels, but low levels of CD14, whilst peritoneal Mφs show low MHC class II levels but high CD14 expression (Andreesen et al., 1990). The factors attributing to these differences in vivo may be intrinsic differentiation pathways of monocytes influenced by organ specific microenvironments. In vitro, it is possible to alter culture conditions (by the addition or absence of serum, growth factors or cytokines) to grow monocytes in different differentiation states and showing different levels of surface antigen expression and function (Geissler et al., 1989; Eischen et al., 1991; Kreutz et al., 1992). The in vitro states are therefore comparable to different organ specific Mφs seen in vivo.

Serum contains many factors important for monocyte differntiation in vitro. In fact monocytes cultured in the absence of serum cannot mature and die rapidly in culture. They can however, be rescued by the addition of growth factor (Geissler et al., 1989; Eischen et al., 1991; Kreutz et al., 1992). Indeed, GM-CSF will increase surface antigen expression of CR3 and FcyllR, phagocytic ability, cytotoxicity and TNF-α secretion of Mφs (Eischen et al, 1991). GM-CSF treated human MDMφs exhibit low grade proliferation (Elliot et al., 1989), but GM-CSF stimulates mouse MDMφ proliferation (Chen et al., 1988).

Other factors which are also present in serum are cytokines. γ-IFN is a very important cytokine which can activate Mφs and increase class II DR, MHC class I and LFA-1 surface expression (Gonwa et al., 1986; Watanabe et al., 1991; Nash et al., 1992), but it restricts monocyte maturation (Andreesen et al., 1990). Another cytokine, TNF-α, acts as a potent stimulator of monocyte differentiation in myeloid leukemia cell lines, but TNF-α acts as an antagonist to γ-IFN to decrease MHC class II expression on mature monocytes (Watanabe et al., 1991; Gonwa et al., 1986).

In the experiments shown, monocytes were grown in high serum concentrations (10%FCS and 10%NLS) to allow the growth and the differentiation of the monocytes in vitro. However, these serum concentrations were not enough to overcome the loss
of MHC class II expression in vitro. A similar result in cultured human monocytes has been reported (Sztein et al, 1984). However, cultured human monocytes in teflon bags (suspension) in the presence of GM-CSF persistently expressed MHC class II DR for 2 weeks (Eischen et al., 1991), suggesting that adherence to plastic may down regulate MHC class II expression. In vitro activated T-helper cells secrete IL-2 which is thought to be the primary stimulus causing γ-IFN release from immune T-cells (Torres et al., 1982). γ-IFN could then act on monocytes to allow MHC class II expression. In vitro, cultured MDMφs do not come into contact with activated lymphocytes. In this experiment γ-IFN enhanced the expression of MHC class II (both DR and DQ) molecules. This has also been observed with human monocytes where γ-IFN causes an induction of specific mRNA (Nash et al, 1992).

AMφs lost MHC class II molecules in culture as assessed by both by flow cytometry and immunohistochemistry. However, in the same cultures there were a few scattered highly MHC class II positive cells found in close contact with Mφs. These cells are thought to be dendritic cells (DC) according to morphology and class II molecule expression. Furthermore, staining of AMφ cultures with anti-CD1b mAb VPM5 showed a positive population that decreased in proportion from 18% on fresh cells to 9% after 7 days in culture. This mAb not only reacts with afferent lymph dendritic cells but also with dendritic cells in the skin and paracortical T cell areas of lymph nodes (Budjoso et al., 1989). Immunohistochemical analysis of frozen sections of rat lung tissue (Holt et al., 1992) identified a widely distributed population of highly pleiomorphic Ia+ cells in alveolar septal walls, which are negative for the pan-Mφ marker ED4 and the related markers ED1, ED2 and ED9. They were also identified as DC. Furthermore, flow cytometric parameters, density fractionation, and a series of ED monoclonal antibodies raised against rat Mφ antigens showed that both AMφs and DC cells are present in LWF and that they are diverse populations (Havenith et al., 1993).

This small population of CD1b+ cells was detected in AMφ but not in MDMφ cultures. CD1 proteins are generally not expressed on circulating monocytes however, they can be induced in vitro using GM-CSF (Porcelli et al., 1992) or IL-3 (Porcelli et al., 1993). Apparently this involves transcriptional activation of CD1 and new protein synthesis. This in vitro cytokine induction is inhibited in the presence of human serum or plasma and the CD1+ monocyte-derived do not appear obviously dendritic in morphology (Porcelli et al., 1995). It should be pointed out that there is evidence for
expression of human CD1 proteins on a subset of tissue Mϕs. However, these Mϕs are found in inflammatory or infectious lesions in vivo (Modlin et al., 1983). Furthermore, these molecules have by no means been found on all tissue macrophages in situ. For example, one study reported no detection of CD1a on a variety of different tissue macrophages in normal and diseased tissues (Wood et al., 1983). The existence of CD1+ macrophages in chronic infectious and inflammatory lesions may be a consequence of in situ generation of cytokines that could induce CD1 during the process of monocyte migration and differentiation in tissues. This may reflect the complex regulation of CD1 on monocyte-derived cells that could restrict the expression to a limited subset of macrophages in a restricted spectrum of pathologic states (reviewed by Porcelli et al., 1995).

In HIV-infected MDMϕs, HLA-DR increased, HLA-DQ decreased and HLA-DP was unchanged (Mann et al., 1990a). A different study has shown that simian immunodeficiency virus (SIV) infected H9 cells had increased MHC class II antigen expression. This was caused directly by viral infection and not mediated by γ-IFN activity (Kannagi et al., 1987). In MVV infection, an increase in MHC class II molecule expression by AMϕs has been reported to occur in vivo (Lujan et al., 1993; Lee, 1994) and in vitro (Lee, 1994). In contrast, in this study the in vitro infection of both MDMϕs and AMϕs with OPIV-3 showed a suppressive effect on MHC class II molecule expression. The difference between results seen here with OPIV-3 and those with HIV, MVV and SIF may be due to the chronic (non-lytic) infection seen with these lentiviruses, whilst PIV-3 causes a lytic infection of Mϕs. Furthermore, in MVV infection γ-IFN is elevated whereas TNF-α is unaffected (Ellis et al., 1991). In contrast in PIV-3 infection both γ-IFN and TNF-α are also elevated (Towsend et al., 1988). PIV-3 virus/endotoxin combinations have been shown to stimulate higher TNF-α release when compared with other virus/endotoxin combinations (Bienhoff et al., 1992).

Further research should be directed to elucidate the potential association between suppressed Mϕ MHC class II molecule expression and impaired lymphocyte proliferation after PIV-3 infection. Experiments have been carried out with lymphocytes stimulated with concanavalin A (Con A) in the presence of bovine parainfluenza virus type 3 (PIV 3) infected bovine alveolar Mϕs (BAM) or monocytes (Basaraba et al., 1993). Lymphocytes showed depressed [3H]thymidine incorporation. This failure of lymphocytes to proliferate occurred only in the presence
of live virus. It was also time dependent and most pronounced when BAMφs were infected 48 hr. before the addition of lymphocytes. The rate of infection of AMφs and the release of infectious virus into culture supernatants paralleled suppression of lymphocyte mitogenesis by PIV-3. However, the peak titre of exogenous, live or inactivated virus was not suppressive when added to lymphocyte Mφ cultures just prior to Con A stimulation. Neither the loss of viable AMφs nor a shift in antigen or mitogen dose response in virally infected cultures could account for the deficit in [3H]thymidine incorporation by these lymphocytes. In this study despite the presence of lymphocyte-associated virus antigen detected by flow cytometry, no increase in PIV-3 titre above baseline was seen from infected lymphocytes, irrespective of mitogen stimulation. Likewise, lymphocytes did not contribute to the extracellular virus pool in lymphocyte-Mφ cultures as the increases in viral titre above basal levels in supernatants were equal to levels released by Mφs alone. The expression of viral antigen on lymphocytes stimulated in the presence of PIV-3-infected BAMφs suggests a non-productive or abortive infection of lymphocytes mediated through contact with infected Mφs. PIV-3 has been reported to cause impairment in lymphocyte proliferation. This is thought to be associated with PIV-3 induced Mφ malfunction (Basaraba et al., 1993).

Different approaches to determining phagocytosis and Fc receptor expression have been developed. In general, the assay most frequently used to quantify phagocytosis directly counts the number of cells containing ingested particles under a microscope (Boschert et al., 1988; Washburn et al., 1985). However, this procedure is very tedious and time consuming, and is subject to operator error due to personal perception and the accuracy of counting large numbers of cells by eye. More automated assays include measurement of ingested RBC by a colorimetric assay using haemoglobin as the substrate (Jungi et al., 1990); the total fluorescence intensity of ingested FITC-labelled particles in lysates of the phagocytic cells is detected by spectrofluorometry (Oda et., 1986; Ragsdale et al., 1989); and radioactive counting of 51 Chromium-labelled particles instead of FITC-labelled particles are all employed in phagocytic assays (Salmon et al., 1984). However, these methods have some limitations which include: equivalence of the phagocytic cell number in parallel wells, harvesting errors and labeling levels with radiations as well as operator safety. Flow cytometry (Bjerknes et al., 1989; Buschmann et al., 1989, Tuijman et al., 1990) is the best and simplest approach for the phagocytic assays. It can not only quantitatively analyze the number of cells with phagocytic activity, but can also qualitatively analyze
the number of ingested RBC in each phagocyte by fluorescence intensity. Moreover, it can count more than 5,000 cells within specific parameters in a couple of minutes. Therefore, it was an affective and convenient method to approach the phagocytic assays in this study.

Compared to yeast and bacteria, RBC are the best targets in phagocytic assays, as external RBC can easily be lysed with distilled water. The assay then only measures those cells which have internalized RBC. Bacteria and yeast cannot be removed from the surface by this method and so some labelled particles still bind to the cell surface. Therefore, results with yeast and bacteria are the sum of surface binding and internalization (Lee, 1994). When crystal violet was used to quench external fluorescence (Hed & Stendahl, 1982), it was found that all fluorescence was interfered with and so this method could not be used in flow cytometry. A method for stripping the external particles from the cell surface is therefore necessary (e.g. protein digestion) before surface binding and internalization of yeast and bacteria can be separated. However, treatment with trypsin to remove externally bound Pasteurella haemolytica has been reported as being unsuccessful (Lee, 1994).

Both Fcγ and C3b receptors are very important surface molecules which mediate phagocytosis on Mφs and PMNs (Sarmay, 1992; Jungi et al., 1990; Anderson, 1987; Hed & Stendahl, 1982). The C3b receptor primarily promotes attachment so that the particle is bound onto the membrane, whereas the FcR mediates ingestion (Hed & Stendahl, 1982). The ingested microorganism in phagosomes may be killed by the peroxidase-hydrogen peroxidase system (Locksley et al., 1987). Internalized particles may also be partially degraded and peptides derived can become associated with MHC class II molecules. Antigen breakdown and re-expression on the surface of Mφs play a major role in initiating an immune response (Brodsky & Guagliardi, 1991).

During this study, FcγR expression on AMφs and their phagocytic activity were decreased, after OPIV-3 infection. This agrees with experiments reported previously (Davies et al., 1986; Slausson et al., 1987); furthermore, modulation of the FcγR associated with a functional phagocytic impairment has been reported (Fleit et al., 1982). The percentage of MDMφs with immune complex FcR activity was not greatly changed during the course of the infection. However, they had a significantly reduced capacity of SRBC phagocytic activity on days 3 (p<0.05) and 5 (p<0.001) after infection. In contrast AMφs showed both a significantly lower (p<0.05) proportion of
immune complex FcR activity on day 3 and 5 p.i. and a significantly reduced capacity of SRBC phagocytic activity on days 3 (p<0.05) and 5 (p<0.001) after infection. Not only less MDMφs and AMφs ingested particles attached to Fc receptors, but those Mφs that were still phagocytic ingested fewer particles as suggested by the mean of fluorescence intensity. This virus-associated defect was more marked on day 3 to 5 of the infection and correlates with the increased susceptibility of the host to secondary bacterial pneumonia (Jakab, 1982; Jakab & Warr, 1983). After day seven the FC-mediated phagocytic activity of Mφs started to recover slowly.

The results here suggest that direct infection of Mφs by OPIV-3 leads to the suppression of both phagocytic activity and MHC class II molecule expression. However, the mechanisms of suppression in both cases remain to be elucidated. Viral infections interrupt the functions of infected cells and generally produce cell disruption and death (Jakab, 1982). Also, the incorporation of OPIV-3 particles into the cell membrane may alter membrane fluidity and permeability. This may be of importance after the infection has been established and when a large amount of viral proteins are present in the cell membrane.
CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS
8.1 **Role of PIV-3 infection in ovine respiratory disease**

Viral involvement is important in ovine respiratory disease, however the relative contributions of different viruses as causative agents are unclear. Ovine parainfluenza type 3 (PIV-3), a paramyxovirus, was first associated with ovine respiratory disease in the UK in 1969 (Hore & Stevenson, 1969). It has been isolated from sheep with respiratory disease in many countries (Ditchfield, 1966; St.George, 1969; Carter & Hunter, 1970; Epstein, B. 1974; Lehmkhul & Cutlip, 1982). In this study, three OPIV-3 isolates (270-7, 392-10 and 430-7) were obtained from three field cases of sheep pneumonia in the South of Chile (Berríos et al., 1991). In two of these cases, *Pasteurella haemolytica* was isolated. No previous isolations of this virus from field cases of sheep pneumonia had been reported before. However, according to serological data, the frequency of infection by PIV-3 virus in sheep from this area had been reported to be very high. Furthermore, this was thought to be the cause of severe cases of pneumonia in association with *Pasteurella haemolytica* (Berríos et al., 1987; Riedemann et al., 1990).

The precise relationship of this virus to ovine respiratory disease has been considered as unclear (Sharp et al., 1978). The association of bovine and ovine PIV-3 virus with respiratory disease has been carried out with a number of laboratory tests. The most commonly reported technique is the demonstration of the induction of serum antibodies to PIV-3 antigens (Adair, 1986). This test is often inconclusive because seroconversion to PIV-3 may be associated with clinical disease in some animals (Bryson et al., 1979) and may be asymptomatic in others (Allan et al., 1978). Another means of implication has been the isolation of the virus from tissues or body fluids of animals with respiratory disease. However, virus has been isolated from healthy animals (Dawson & Cruikshank, 1963) as well as from respiratory disease cases.

The uncertainty concerning the role of PIV-3 in cases of ovine respiratory disease may result, in part, from the failure to detect and evaluate accurately the location of the virus in tissues of affected animals. A more recent technique for associating viruses with disease is direct microscopic demonstration of virus in infected tissues using immunohistochemistry. Immunoenzyme stains are permanent preparations visible with ordinary light microscopy and may be simultaneously counterstained for optimal demonstration of the histologic lesions, which may assist in establishing the
pathogenicity of the agent. This advantage may be particularly important in assessing the role of the PIV-3 virus to the pathology of ovine respiratory disease.

In this study OPIV-3 virus was not only isolated from clinical cases of ovine respiratory disease and characterised by traditional methods but was also detected by immunohistochemistry in lesions of natural occurring viral interstitial pneumonia. On the other hand, the isolated PIV-3 ovine strain, 270-7, was successfully used in reproducing clinical and pathological signs of interstitial pneumonia in colostrum deprived lambs. The virus was recovered, seroconversion was detected and the virus was demonstrated in association with lung lesions of interstitial pneumonia. Moreover, the virus induced phagocytic and immunological alterations in AM after infection in vitro. This demonstrates the pathogenicity of this OPIV-3 isolate and points out a role for OPIV-3 in predisposing sheep to acquiring secondary bacterial pneumonia. It should be considered that although under some circumstances no clinical signs are apparent after infection with PIV-3 the virus may interfere with AM functions, allowing concurrent or subsequent infections by other viruses and bacteria.

8.2 Antigenic differences between OPIV-3 and BPIV-3 or HPIV-3.

Little was known about the antigenic relationship between OPIV-3 and BPIV-3 or HPIV-3. Studies had been carried out so far to determine antigenic variations only between BPIV-3 and HPIV-3 (Ray & Compans, 1986; Klippmark et al., 1990). Three human and six bovine PIV-3 strains were examined by the use of 60 mAbs. Fifty-three mAbs to the human C243 strain (Rydbeck et al., 1986) were directed against six, four, nine and seven epitopes of the haemagglutinin-neuraminidase (HN), fusion (F), nucleocapsid (N) and matrix proteins, respectively. Seven mAbs to the bovine strain were directed against three epitopes of the HN protein and three epitopes of the F protein. Each strain was characterized in ELISA and immunofluorescence tests with all mAbs and in a haemagglutination inhibition assay with the anti-HN mAbs. There were marked differences between human and bovine viruses, primarily in the HN protein where five epitopes differed. One epitope of the F and one of the N protein also differed. BPIV-3 was found to be a homogeneous subtype and distinct from HPIV-3 (Klippmark et al., 1990).
From this panel of 53 mAbs, against C243 strain of HPIV-3 (Rydbeck et al., 1986), 11 which showed cross reactivity with BPIV-3 were selected to be used in this study. They would help in establishing the antigenic relationship between OPIV-3 and both BPIV-3 and HPIV-3. The reactivity of these mAbs showed that the three Chilean OPIV-3 strains isolated in this study together with the British OPIV-3 reference strain can be distinguished from HPIV-3 and BPIV-3. They also showed that only limited variation occurs among OPIV-3 strains recovered at different geographical locations, in a similar fashion to that observed with BPIV-3 (Klippmark et al., 1990). With the reagents employed here, epitopes of viral proteins common to HPIV3 and BPIV-3 were found to be absent or to be expressed in a lower proportion in OPIV-3 strains. Antigenic differences were found predominantly in the F protein and to a lesser extent in the M protein. F protein seems to have a conserved character among OPIV-3 strains. This has also been observed in HPIV-3 and BPIV-3 strains (Rydbeck et al., 1986; Klippmark et al., 1990). In this case three distinct epitopes of protein F that are found in HPIV-3 and BPIV-3 strains were absent in OPIV-3.

These differences should be considered when using vaccines produced with PIV-3 from other species. Antigenic differences suggest that a bovine strain may well induce important neutralization antibodies in sheep but a sheep strain may induce a wider spectrum of neutralizing antibodies against OPIV-3.

8.3 Interaction of macrophages and PIV-3

The research undertaken here looked at the interaction of OPIV-3 and macrophages with 3 main aspects: OPIV-3 replication in macrophages; the effect of OPIV-3 infection on the phenotype of immune related receptors and effect on functional capacities (phagocytic and MHC class II expression for antigen presentation) of macrophages.

In this study it has been shown that AM are infected by OPIV-3 in vivo and in vitro. The virus induces extensive CPE and finally cell lysis in vitro. This led to defects in the phagocytic capacity of AM. Likewise, PIV-3 has been associated with defects in the bactericidal capacity of macrophages, chemotaxis and phagocytosis in cattle (Brown & Ananaba, 1988) and humans (Henricks et al., 1994) in vitro. Loss of macrophage functions may contribute to infection by opportunistic pathogens. Indeed,
concurrent bacterial infections are often found in OPIV-3 infected sheep with pneumonia. This was also observed in two cases of sheep pneumonia, reported during this study, from where OPIV-3 isolates were obtained (3.2.3.3). Other examples from field cases (Lehmkuhl & Cutlip, 1982) and experimental cases (Sharp et al., 1978) have been previously reported.

In this study it has been shown that in vitro infected AM not only show loss of phagocytic functions but also ability to express MHC class II molecules upon stimulation with γ-IFN. The expression of class II molecules is directly related to AM immunological functions. When macrophages are maintained in standard culture media both class II molecule expression and accessory cell function are lost. The accessory cell function can be recovered upon stimulation with γ-IFN and is related quantitatively to the level of class II molecule expression (Belles, 1984).

Anger & Ross (1992) have pointed out the criteria which must be fulfilled for the macrophage to be capable of presenting antigen to T-cells for the initiation of an immune response. The macrophage must be able to:

1) Internalize antigen by phagocytosis, to allow processing to occur;
2) process antigen by proteolysis, the primary mode of antigen degradation;
3) transcribe the products of MHC class II (and/or Class I) genes and express these at the cell surface in sufficient quantity;
4) associate the processed peptide fragments, several amino acids long, with the MHC molecules and express these on the cell surface; and
5) provide the necessary regulatory signals in the form of cytokines to responding cells.

According to this at least two steps, which are necessary for the AM to be capable of presenting antigen to T-cells, are impaired after infection with OPIV-3. They are steps 1 and 3 associated with phagocytosis (for antigen internalization) and expression of class II MHC antigens (at the cell surface in sufficient quantity), respectively.

This shows that some viruses can down-regulate MHC antigens. There is also evidence that the infection of cells with hepatitis virus inhibits IFN-induction of MHC class II antigens (Onji et al., 1987). Such effects may be more widespread than has hitherto been recognized, leading to the subversion of immune responses by viruses.
T cells recognize proteolytic fragments of antigenic proteins that are presented at the cell surface by either major histocompatibility complex (MHC) class I or class II molecules. CD4+ T cells recognize peptides presented by MHC class II molecules (Grey et al., 1989). This implies that CD4+ T cells which help in eliciting an antibody response may not be appropriately stimulated during PIV-3 infection. This may help explain why humoral immunity against PIV-3 seems to be limited. It has been reported and demonstrated that infection recurs within short time intervals (Well et al., 1976; Lehmkuh & Cutlip, 1983; Morein et al., 1983; Ray et al., 1985). It appears that infection with PIV-3 does not result in the establishment of a state of long-lasting protective immunity (Sieg et al., 1994).

CD8+ T cells recognize peptides presented by MHC class I molecules (Grey et al., 1989). The expression of these molecules on AM surfaces was not altered after OPIV-3 infection. This agrees with the noticeable accumulation of lymphocytes in both lesions of interstitial pneumonia and LWF obtained from animals after 7 days p.i. with OPIV-3. In these lesions as well as in LWF there was a predominance of CD8+ T cells.

Mammals have both non-specific (NK cells, PMNs and macrophages) and specific (cellular and humoral immunity) defences against invading pathogens. During the early phase of primary infection viruses rapidly replicate in target cells and cause lesions with a large progeny being produced and spread. It is at this first stage that non-specific effector mechanisms are important in limiting the amount of virus produced. Once sufficient inflammation and antigen have been produced the host develops specific immunity which is important in eliminating these pathogens and allowing recovery from infection. However, for patients with defects in immunity, infection may result in death. This basic model is normally seen in most viral infections (Sissons & Oldstone, 1980).

8.4 Cellular immune response

The complexity of the role of CD8+ (cytotoxic, Tc) and CD4+ (helper, Th) T-cell subsets continues to emerge as more virus systems are examined. Adoptive transfer studies using cloned lines of CD8+ class I MHC-restricted Tc have demonstrated the beneficial role of these cells in certain virus infections, such as influenza, where Tc are
able to protect against lethal infection and to clear the virus more rapidly from the lung (Mackenzie et al., 1989). In other viral infections, such as lymphocytic choriomeningitis virus (LCMV), the role of Tc depends on the type and site of infection. Following intracerebral infection Tc are detrimental to the host and increase pathology, for example by their direct effect on the infected cells in brain tissues (Baezinger et al., 1988). These Tc cells can cause damage without the need for recruited inflammatory cells. Studies with respiratory syncytial virus (RSV) specific Tc lines or clones have shown that viral clearance is accompanied by an enhanced pathology, unless low Tc cell numbers (>10⁶) are transferred (Canon et al., 1988).

Proliferation of CD8⁺ T lymphocytes is a common feature in many viral infections (McMichael et al., 1983) and PIV-3 is not an exception. A great increase in CD8⁺ lymphocytes in lung wash as well as in BALT was seen after infection during this study (section 5.2.2). An increase in CD8⁺ lymphocytes is also seen in bronchoalveolar lavage, CSF or synovial fluid from MVV infected sheep, particularly those sheep which have developed lesions (Harkiss et al., 1991; Torsteinsdottir et al., 1992; Lujan et al., 1993). There is no significant alteration in lymphocyte phenotypes in PBMC and good antibody and cellular mediated immune (CMI) responses appear to be present in MVV infected sheep. However, there are significant changes in CD4⁺:CD8⁺ lymphocyte ratios and lymphocyte and macrophage phenotypes in lung, synovium and CSF (Cordier et al., 1992; Torsteinsdottir et al., 1992; Lujan et al., 1993), suggesting that the situation in blood may not really reflect the situation in organs targeted by MVV infection. No information is available on changes of distribution of lymphocytes subsets in peripheral blood during PIV-3. However, it has been reported that no major changes in differential leucocyte counts are observed in peripheral blood during PIV-3 infection (Stauber & Weston, 1984). Also, PIV-3 infection is restricted primarily to the respiratory tract (Chanock & McIntosh, 1990).

Concurrent infection with pasteurellosis is a common feature in PIV-3 infection, especially in field cases (Davies et al., 1977). Pulmonary defence against infection is very complex and consists of local humoral (IgG and IgA) and cellular immunity, complement, ciliary activity, mucus production and phagocytosis (Burrels, 1985; Christensen & Mousing, 1992). The major immunoglobulin generated in the respiratory tract particularly in the upper respiratory tract is secretory IgA which can efficiently agglutinate invading bacteria; this may enhance mucociliary clearance of pathogens and inhibit bacterial colonization of the mucosa. However, significant
amounts of IgG and complement components are also found in the lower respiratory tract (Burrels, 1985). These plasma components mainly enter the respiratory system by transduction, although small amounts may be produced locally (Kaltreider, 1976). IgG and complement components are an important source of opsonins in the pulmonary environment therefore act together in destroying invading pathogens (Kaltreider, 1976). In PIV-3 lungs lesions from naturally occurring and experimental disease the alveolar walls are greatly thickened which may decrease the transduction of plasma into the lung, as has also been suggested in MVV infection in sheep (Lee, 1994). This would reduce the amount of opsonins in the respiratory system available for action with AM, allowing the replication of gram negative bacteria (which require opsonization for phagocytosis) entering alveolar areas. These together with macrophage functional alterations in phagocytosis and antigen presentation would lead to bacterial pneumonia.

8.5 Immune mechanisms of Pathology

It has been reported that proliferation of bacteria occurs more frequently, and hence pneumonia develops more often, when animals are inoculated with *P. haemolytica* 6 days after PIV-3 than when they were inoculated earlier in the course of the virus infection or when they are inoculated with *P. haemolytica* alone (Davies et al., 1977; Sharp et al., 1978; Davies et al., 1986). This maximum impairment of pulmonary clearance coincided with declining virus titres in the lung and the first detectable evidence of the host’s immune response to the virus observed in this study. A similar impairment of pulmonary clearance at the time that the host’s immune response is first detected has been reported previously (Davies et al., 1982; Jakab & Warr, 1983). This impairment of clearance can be prevented by immunosuppression with cyclophosphamide (Jakab & Warr, 1981a) or anti-lymphocyte serum (Jakab, 1982). Thus the defect is likely to be immunologically mediated and is not due to the direct effect of the virus replication on host cells. Similar impairment of phagocytosis was produced by exposing virus-infected AM to antiviral antibody and complement or sensitised lymphocytes *in vitro* (Jakab & Warr, 1981b).

It was observed during this study (Chapter 4) that the presence of a large number of lymphocytes, particularly on days 5 and 7 p.i., combined with a minimal to moderate cytolysis in antigen bearing cells. This, suggests that PIV-3 induced pulmonary
disease may have an important immunopathological component. Lymphocytes were first seen at 3 days p.i. and reached a maximum 7 days p.i., an observation consistent with a specific T-cell response to the virus (Porter et al., 1991).

Pathology accumulated during PIV-3 infection seems to be mediated by immune responses. It is not known however, if this is through PIV-3 antigens or responses to autoimmune antigens. Persistent, small amounts of viral antigen being produced in target organs which continuously stimulate immune responses and cytokine production (low levels) have been suggested to be the cause of chronic inflammation in MVV interstitial pneumonia (Nathanson et al., 1976).

During interactions between PIV-3 infected alveolar macrophages and lymphocytes, activation of some lung T-cell populations takes place (Basaraba et al., 1993; Sieg et al., 1994). As a consequence, it is likely that several cytokines with immunoregulatory activities are simultaneously produced. These cytokines could play a major role in pathogenesis of PIV-3 infection, probably being the main component of the inflammatory nature of the viral lesions. Some of those cytokines, such as TNF-α, IL-1, IL-6 or GM-CSF, could induce the accumulation of immunocompetent cells (including T lymphocytes) in the lung interstitium (potentiating lung tissue damage) and migration of these cells into the bronchoalveolar milieu. Whether or not cytokines have any indirect effects that lead to proliferation of CD8⁺ cytotoxic T cells and inhibition of CD4⁺ helper T cells is not known (Narayan et al., 1985). Increased levels of cytokines in PIV-3 infection have been reported. PIV-3 virus/endotoxin combinations had been shown to stimulate higher TNF-α release when compared with other virus/endotoxin combinations (Bienhoff et al, 1992). IFN-γ is also elevated in alveolar macrophages following infection with PIV-3 (Towsend et al, 1988). γ-IFN also elevated in MVV that is also associated with interstitial pneumonia (Narayan et al, 1985); however, TNF-α release is not (Ellis et al., 1991).

In vitro studies (Kennedy et al., 1985; Narayan et al., 1985) have demonstrated the contribution of an ovine lentivirus-induced IFN (LV-IFN) to the pathogenesis of MVV infection. LV-IFN induces MHC class II antigen expression on alveolar macrophages and as a result, the infected cell remains in a state of continuous or chronic antigen presentation. LV-IFN inhibits maturation of infected monocytes to macrophages, and thus, indirectly restricts completion of the virus life cycle. It also
causes reduction in the number of transcripts of viral RNA in infected macrophages, giving rise to the phenomenon of “slow replication”.

Likewise, PIV-3 infection is associated with γ-IFN production (Towsend et al., 1988). However, the role of γ-IFN in the pathogenesis of this respiratory infection remains to be investigated. In vivo, the lung is exposed to complex mixtures rather to individual cytokines, and cytokines in combination have quantitatively different effects than they have individually (Elias et al., 1990; Kelley, 1990).

Macrophages were productively infected with OPIV-3 in vitro (Section 6.2.1). The studies on phagocytosis and MHC class II expression in these cells have shown functional defects (Chapter 7). In vivo there are low percentages of productively infected macrophages (Section 5.2.3). However, a decrease in phagocytosis and killing of bacteria has been reported to occur in vivo (Brown & Ananaba, 1988). Cytokines produced from lymphocytes interacting with these activated macrophages (TNF-α and IL-1) may also enhance expression from the viral LTR and increase viral antigen products as it has been suggested in MVV (Clements et al., 1979). If this occurs, free viral antigen may interfere with uninfected cell functions. Free virus antigen may also add to the general immune activation, if macrophages take it up and present it to lymphocytes. In MVV the gag p25 antigen has been seen in efferent lymph plasma (Bird et al., 1993). So free viral antigens may be present in the body fluids of MVV infected sheep. Cells infected with paramyxoviruses release soluble material such as viral envelope glycoproteins (Vainionpaa et al., 1989). However, little is known about the pathogenic effect of individual paramyxovirus proteins.

Further studies are necessary to determine whether there is any connection between infected pulmonary macrophages and level of expression of MHC class II molecules in vivo. Also, it is necessary to investigate the possibility of persistent infection of pulmonary macrophages as the activation of these cells, chronically infected, could trigger the transition from latency to active viral replication. Infection of pulmonary macrophages apart from leading to the production of cytokines, might contribute to perturbation of the cellular pulmonary immune response by some other mechanisms. In this study it has been shown that cells of the macrophage lineage can be major host cells for replication of PIV-3 in vivo and in vitro. If the cells involved in the non-specific cellular defence system of the host via viral presentation, are at the same time the source of viral replication and are possibly damaged, this could result in
disruption of the immune system and incapability of responding efficiently to the infecting virus. Additionally, infected macrophages, by remaining in this state of continuous antigen presentation may mediate immunopathologic disease.

In summary the pulmonary macrophage acts as a major reservoir for PIV-3. Pulmonary lesions rather than the result of the direct effect of the virus per se will result from the response of diverse elements of the immune response to viral infection. During natural and experimental PIV-3 infection the pulmonary immune response is characterised by proliferative response of interacting macrophages and lymphocytes which should lead to production of cytokines. This interaction involves a series of immunological phenomena associated with lung tissue damage and immune dysfunction which, in some instances, may lay the ground for bacterial colonization, pneumonia and eventually death. In other cases, lesions are confined to restricted anteroventral lung areas where in the presence of highly specialised effector and immune surveillance mechanisms, the progress of the disease will remain under control.

Research has focused on the suppressive effect of AM, via nitric oxide, on pulmonary immunity (Mills, 1991; Holt, 1986). Nitric oxide is functional against microorganisms and tumour cells (Liew and Cox, 1991), but also suppresses pulmonary DC function and lymphocyte responses (Holt et al., 1986,1993). One hypothesis suggests that the suppressive effect of AM is necessary for the control of the non-specific activity of AM. AM frequently come into contact with foreign antigens from air or the upper respiratory tract which may non-specifically trigger AM to release accessory cell factors leading to immunopathological lesions. The major physiological function of AM is thought to be the release of chemotactic factors, ingestion and degradation of antigen and microbicidal activity (Brain, 1992) rather than an antigen presenting function for lymphocytes. Therefore, the increase in the number of lymphocytes and AM and their activation in PIV-3 infection is not only responsible for the induction of the active chronic inflammation seen, but also may cause a suppressive state in local immunity. This may contribute to the concurrent infections seen in PIV-3 infected lungs in field cases.
In this study three isolates (270-7, 392-10 and 430-7) of OPIV-3 were obtained from field cases of sheep pneumonia in Chile. The reactivity of a monoclonal antibody panel cross-reacting with HPIV-3 and BPIV-3 viral proteins showed that these OPIV-3 strains can be distinguished from HPIV-3 and BPIV-3 strains. They also showed that only limited variation occurs among OPIV-3 strains recovered at different geographical locations. With the reagents employed here, epitopes of viral proteins common to HPIV3 and BPIV-3 were found to be absent in the OPIV-3 strains. Antigenic differences were found predominantly in the F protein and to a lesser extent in the M protein. Three distinct epitopes of F protein and one of M protein, that are found in HPIV-3 and BPIV-3 strains, were absent in OPIV-3.

The present study confirmed the pathogenicity of ovine PIV-3 strain 270-7 and showed that this virus is able to induce clinical and pathological symptoms corresponding to interstitial pneumonia. One of the most striking histopathological findings in lambs inoculated with OPIV-3 270-7 strain was an intense proliferation of lymphocytes. This was most noticeable in the resulting BALT hyperplasia. This could be associated with the immune response elicited against OPIV-3.

The results shown in this study demonstrate alterations in the normal composition of CD4+ and CD8+ lymphocyte subsets in the lungs of animals experimentally infected with OPIV-3. This could play a key role in the pathogenesis of the interstitial pneumonia associated with this infection. There was a significant decrease (p<0.001) in the CD4+/CD8+ ratio of lymphocytes due to both a significant increase of CD8+ T lymphocytes and a significant decrease of CD4+ T lymphocytes. In this study a non-productive or abortive type of infection was observed in lymphocyte cultures. This agrees with the absence of infected lymphocytes found in both LWF and in tissue sections obtained from pneumonic lungs after 7 days post experimental infection. However, more research is necessary to investigate the conditions, both in vivo and in vitro which may lead to productive infection of lymphocytes. Also the susceptibility of the different lymphocytes subsets to PIV-3 infection should be clarified.
The increase in both alveolar and interstitial MΦs observed after 7 days of infection with OPIV-3 suggests a role for these cells in the host mechanisms of defence against infection and also in the mechanisms of lung pathology. A significant proportion of MΦs showed positive immunostaining for OPIV-3 proving that infection of these cells occurs in vivo. It has also been shown that OPIV-3 infects ovine AMΦ in vitro. The in vitro infection is productive and characterised by the release of high titres of infectious virus.

In this study, OPIV-3 in vitro replication in AMΦ was found to be more rapidly lytic than in MDMΦ. The differentiation and maturation state of MΦs may affect replication of PIV-3 with the virus being produced earlier and in higher levels in mature well differentiated AMΦ than in less mature and differentiated MDMΦ (5 days in culture before infection).

The culture of sheep monocytes from blood induced the expression of some cell markers, such as CD4, CD8 and VPM32 antigen which were not expressed on fresh monocytes.

Seven day cultured MDMΦ had completely differentiated into MΦs as defined by morphology, NSE staining and stable surface antigen expression. There was no significant difference in surface molecule expression on OPIV-3 infected and mock-infected MDMΦ up to 5 days p.i.. There was no loss of surface molecules (such as CD8, VPM32, or CD14) from MDMΦ or AMΦ up to 5 days p.i.; but after day 5 p.i., degenerative changes including a decrease in surface expression of these molecules were seen. This is probably due to productive replication of the virus as this is supported by the detection of viral antigen in both the cytoplasm and cell membrane. The kinetics of these changes also coincides with surface changes seen in OPIV-3 infected MDMΦ observed under TEM.

In the experiments shown, monocytes were grown in high serum concentrations (10%FCS and 10%NLS) to allow the growth and the differentiation of these cells in vitro. However, these serum concentrations were not enough to overcome the loss of MHC class II expression in vitro. γ-IFN treatment restored the expression of MHC class II (both DR and DQ) molecules although, at a lower level in comparison to the expression in fresh cells.
AMφs also lost MHC class II molecules in culture as assessed by both flow cytometry and immunohistochemistry. However, in the same cultures there were a few scattered highly MHC class II positive cells found in close contact with Mφs. These cells were thought to be dendritic cells (DC) according to morphology and class II molecule expression. Also, staining of AMφ cultures with anti-CD1 mAb VPM5 showed a positive population that decreased in proportion from 18% on fresh cells to 9% after 7 days in culture.

In this study the in vitro infection of both MDMφs and AMφs with OPIV-3 showed a suppressive effect on γ-IFN induction of MHC class II molecule expression.

During this study, FcγR expression on AMφs and their phagocytic activity were decreased after in vitro OPIV-3 infection. The percentage of MDMφs with immune complex FcR activity was not greatly changed during the course of the infection. However, they had a significantly reduced capacity of SRBC phagocytic activity on days 3 (p<0.05) and 5 (p<0.001) after infection. In contrast AMφs showed both a significantly lower (p<0.05) proportion of immune complex FcR activity on day 3 and 5 p.i. and a significantly reduced capacity of SRBC phagocytic activity on days 3 (p<0.05) and 5 (p<0.001) after infection. Not only less MDMφs and AMφs ingested particles attached to Fc receptors, but those Mφs that were still phagocytic ingested fewer particles as suggested by the mean of fluorescence intensity. This virus-associated defect was more marked from day 3 to day 5 of the infection. After day seven the Fc-mediated phagocytic activity of Mφs started to recover slowly.

The results here show that direct infection of Mφs by OPIV-3 leads to the suppression of both phagocytic activity and MHC class II molecule expression. However, the mechanisms of suppression in both cases remain to be elucidated.

Infected macrophages are targets for immunosurveillance (CTL and NK/LAK cells) and the low levels of infected cells and antigen seen in vivo may be, in part, due to clearance from the host. Also mechanisms for evasion of the immune response may exist and include latency or restricted replication in immature macrophages. Future work should focus on the production of sheep monocyte/macrophage cell lines or the definition of cell culture conditions which will allow the study of persistent PIV-3 infection of macrophages.
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