OBSERVATIONS ON THE PATHOGENESIS
OF BLUETONGUE VIRUS INFECTIONS IN SHEEP

By


A Thesis submitted in part fulfilment for the degree of Doctor of Philosophy

March, 1979

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Animal Virus Research Institute
Pirbright, Surrey
BLUETONGUE VIRUS

DOMESTIC HOST SPECIES

VECTOR—CULICOIDES SPECIES
ACKNOWLEDGEMENTS

I am extremely grateful for the encouragement and advice given to me by Dr. E.P.J. Gibbs and Dr. R.F. Sellers of the Animal Virus Research Institute, and Dr. M. Butler of the University of Surrey during the course of this work and the preparation of this thesis. I would also like to thank Dr. J.B. Brooksby, the Director of the Institute, for allowing me to undertake this study. I am indebted to many other members of the Institute staff for helpful discussions and criticisms; in particular I wish to thank Dr. R.C. Wardley. I would like to thank Miss Jenny Bryant for valuable laboratory assistance, Dr. Fiona Hamilton for her patience and time-consuming work in producing the thin section electron micrographs, Miss Jenny Ryder for the photography, and the Librarians Mrs. Penny Scott and Mrs. Irene Gregory, for their always cheerful and willing help.

Finally I would like to acknowledge the constant encouragement and support given to me by my wife, and her superb effort in the preparation of this typescript.
SUMMARY

This study was instigated to provide basic information on the pathogenesis of bluetongue virus infection in sheep (and, where considered appropriate, to include cattle and goats) in support of other studies at the Animal Virus Research Institute, Pirbright, on the epidemiology and control of bluetongue infections. The investigation was conducted in four parts: 1) distribution of virus in infected sheep (a sequential slaughter study), 2) haematology of infected sheep, goats and cattle, 3) replication of virus in organ culture and 4) the growth of virus in cells derived from the haemopoietic system of sheep, goats and cattle.

From the study on virus distribution in sheep it was evident that the lymphoreticular system was involved in the primary location and replication of virus. Once a viraemia was detected, virus was isolated from a large number of tissues and organs.

The major haematological feature of bluetongue virus infection in sheep, goats and cattle was a transient pan-leucopenia; maximum leucopenia preceded peak viraemia and peak pyrexia. Studies on the type of the viraemia revealed it to be cell associated with both the erythrocyte and leucocyte fraction. For technical reasons, attempts to ascertain the identity of the cell types involved in the leucocyte fraction were inconclusive since the techniques for cell separation were not sufficiently specific. This meant that although the preparations were relatively pure the number of infected cells in each cell population was below that of the contaminating cells; therefore it was not possible to say which of the cell populations were infected.

Organ culture studies indicated that most tissues and organs were capable of supporting virus replication, in particular lymph nodes and
other tissues and organs of the haemopoietic system with the exception of the liver.

Prior to studies conducted on virus replication in various cell types (especially those isolated from the haemopoietic system) methods for the production, separation and purification of these cells were assessed. Where possible a basic technique was established for a particular cell type from any of the species of animal examined. Of importance during these preliminary experiments was the successful production of established macrophage cell lines derived from mononuclear cells from various sources: alveolar, bone marrow, spleen, peripheral blood and mammary gland. These cell lines originated from sheep, goats and cattle. (See Appendix I).

In studying the susceptibility of the various populations of cells to the virus it was found that endothelial cells from the vascular systems of sheep, goats and cattle supported the growth of bluetongue at high and low multiplicities of infection and exhibited a cytopathic effect. Monocytes and macrophages from these three species of animal also supported growth of virus at high and low multiplicities of virus, and exhibited a cytopathic effect. An important difference was observed in macrophages of goat and cattle at low multiplicities; whilst these cells were susceptible there is evidence that they become persistently infected with low levels of virus being released without cytopathic effect occurring for at least 12 days post infection. The importance of this finding is discussed in relationship to over-wintering mechanisms for bluetongue in goats and cattle in certain parts of the world.

The following conclusions were made from the study in this thesis.

1. Bluetongue replicates in the haemopoietic system namely

   a) primary replication occurs in the lymph nodes and other tissues of the lymphoreticular system.
b) the cells involved in the primary replication are probably
the avidly phagocytic cells of the mononuclear phagocytic system
which includes monocytes and macrophages.

c) endothelial cells of blood vessels are involved in replication
of the virus and

d) neutrophils may be involved in virus replication.

2. Monocytes and macrophages are probably involved in the mechanism
of persistent infections in cattle and goats.

On the basis of this study areas of future studies are discussed
and these fall into three categories: 1) mechanisms of clinical
disease 2) mechanisms of persistent infection and 3) vaccination.
**CONTENTS**

| ACKNOWLEDGEMENTS                      | i                  |
| SUMMAR Y                              | ii                 |

**CHAPTER I**
Review - Pathogenesis of viruses of the bluetongue group

1. INTRODUCTION
2. HISTORY AND GEOGRAPHICAL DISTRIBUTION
3. PHYSICAL AND CHEMICAL CHARACTERISTICS
4. EPIDEMIOLOGY
5. CLINICAL DISEASE AND SPECIES AFFECTED
   I Horizontal transmission
   II Vertical transmission
6. PATHOLOGY AND PATHOGENESIS

**AIMS OF THESIS**

**TERMINOLOGY OF SYSTEMS**

**CHAPTER II**
Observations on the pathogenesis of bluetongue virus infection in sheep: - *In vivo* pathogenesis

INTRODUCTION
MATERIALS AND METHODS
RESULTS
DISCUSSION
Tables and Figures

**CHAPTER III**
Observations on the pathogenesis of bluetongue virus infection in sheep: - *Viraemia* and haematology - *In vivo*

INTRODUCTION
MATERIALS AND METHODS
RESULTS
DISCUSSION
Tables and Figures
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>Observations on the pathogenesis of bluetongue virus infection in sheep: Replication of bluetongue virus in organ culture</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td><strong>INTRODUCTION</strong></td>
<td>78</td>
</tr>
<tr>
<td></td>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>78</td>
</tr>
<tr>
<td></td>
<td><strong>RESULTS</strong></td>
<td>81</td>
</tr>
<tr>
<td></td>
<td><strong>DISCUSSION</strong></td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Tables and Figures</td>
<td>84</td>
</tr>
<tr>
<td>V</td>
<td>Observations on the pathogenesis of bluetongue virus infection in sheep: The growth of bluetongue virus in cells derived from the haemopoietic system of sheep, goats and cattle</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><strong>INTRODUCTION</strong></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td><strong>RESULTS</strong></td>
<td>102</td>
</tr>
<tr>
<td></td>
<td><strong>DISCUSSION</strong></td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>Tables and Figures</td>
<td>116</td>
</tr>
<tr>
<td>VI</td>
<td>Conclusions and areas for further study</td>
<td>157</td>
</tr>
<tr>
<td>I</td>
<td>Purification and culture of cells from the haemopoietic system of sheep, cattle and goats</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td><strong>INTRODUCTION</strong></td>
<td>174</td>
</tr>
<tr>
<td></td>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>174</td>
</tr>
<tr>
<td></td>
<td><strong>RESULTS</strong></td>
<td>185</td>
</tr>
<tr>
<td></td>
<td><strong>DISCUSSION</strong></td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>Tables and Figures</td>
<td>196</td>
</tr>
<tr>
<td>II</td>
<td>Media, materials and methods</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td>Media</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>Methods</td>
<td>214</td>
</tr>
</tbody>
</table>
TABLES AND FIGURES - CONTENTS

Tables

CHAPTER I

1.1 Classification of Orbiviruses 3

1.2 Geographical distribution of bluetongue, epizootic haemorrhagic disease of deer, African horse sickness and Ibaraki virus as compared by virus isolation from infected vertebrates and/or vectors 6

1.3 Physical and Chemical characteristics of the bluetongue virus group 12

CHAPTER II

2.1 Tissues and organs collected post mortem and examined for virus recovery 38

2.2 Viraemia (whole blood) in 30 Dorset horn sheep after intradermal inoculation of 2.0 log_{10} TCID_{50} of bluetongue virus Type 4 45

2.3 Isolation of bluetongue virus Type 4 from tissues and organs removed from bluetongue infected sheep, killed at daily intervals - Haemopoietic and lymphoreticular tissues and organs 46

2.4 Isolation of bluetongue virus Type 4 from tissues and organs removed from bluetongue infected sheep, killed at daily intervals - Vascular tissues and organs 47

2.5 Isolation of bluetongue virus Type 4 from tissues and organs removed from bluetongue infected sheep killed at daily intervals - Glandular tissues and organs 48

2.6 Isolation of bluetongue virus Type 4 from tissues and organs removed from bluetongue infected sheep killed at daily intervals - Miscellaneous tissues and organs 49

Figures

2.1 Temperature response of four sheep to infection with bluetongue virus Type 4 50

2.2 a Isolation of bluetongue virus, Type 4, from the blood of infected Dorset horn sheep 51

2.2 b Serum neutralising antibody response of Dorset horn sheep to infection with bluetongue virus, Type 4 51
CHAPTER III

Mean percentage purity and range in cell populations derived from sheep blood

Whole blood viraemia in sheep, goats and cattle after intravenous inoculation of $2.0 \log_{10} \text{TCID}_{50}$ of bluetongue virus Type 4

Association of bluetongue virus with cells removed from infected sheep blood

Schematic representation of virus isolation from tissues of bluetongue infected sheep killed at daily intervals

CHAPTER IV

Tissues and organs examined by organ culture

Thermal inactivation of bluetongue virus in various media containing protein

Multiplication of bluetongue virus Type 4 in organ cultures from 1 week old Dorset horn lambs
#### Tables

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Growth of bluetongue virus in lymphocytes from sheep (Dorset horn)</td>
<td>116</td>
</tr>
<tr>
<td>5.2</td>
<td>Percentage fluorescence of bluetongue infected macrophages from sheep and goat peripheral blood and cattle mammary gland macrophages</td>
<td>117</td>
</tr>
<tr>
<td>5.3</td>
<td>Growth of bluetongue virus in lymphocytes from goats</td>
<td>118</td>
</tr>
<tr>
<td>5.4</td>
<td>Growth of bluetongue virus in lymphocytes from cattle</td>
<td>119</td>
</tr>
</tbody>
</table>

#### Figures

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Growth of bluetongue virus Type 4 in lymph node suspension cultures from an adult Dorset horn sheep</td>
<td>120</td>
</tr>
<tr>
<td>5.2</td>
<td>Growth of bluetongue virus Type 4 in bone marrow suspension cultures from a Dorset horn lamb</td>
<td>121</td>
</tr>
<tr>
<td>5.3</td>
<td>Growth of bluetongue virus Type 4 in spleen suspension cultures from a Dorset horn lamb</td>
<td>122</td>
</tr>
<tr>
<td>5.4</td>
<td>Growth of bluetongue virus Type 4 in theuffy coat cells (leucocytes) from peripheral blood of a Dorset horn sheep</td>
<td>123</td>
</tr>
<tr>
<td>5.5</td>
<td>Growth of bluetongue virus Type 4 in peripheral blood monocytes from a Dorset horn sheep</td>
<td>124</td>
</tr>
<tr>
<td>5.6</td>
<td>Growth of bluetongue virus Type 4 in established cultures of macrophages derived from peripheral blood monocytes of a Dorset horn sheep</td>
<td>125</td>
</tr>
<tr>
<td>5.7</td>
<td>Thin section electron micrograph of established sheep peripheral blood macrophages infected with bluetongue virus Type 4 (24 hours post inoculation) (x50,000)</td>
<td>126</td>
</tr>
<tr>
<td>5.8</td>
<td>Thin section electron micrograph of established sheep peripheral blood macrophages infected with bluetongue virus Type 4 (24 hours post inoculation) (x61,000)</td>
<td>127</td>
</tr>
<tr>
<td>5.9</td>
<td>Growth of bluetongue virus Type 4 in neutrophils stimulated in the mammary gland of a Dorset horn ewe</td>
<td>128</td>
</tr>
<tr>
<td>5.10</td>
<td>Growth of bluetongue virus Type 4 in established macrophages derived from monocytes stimulated in the mammary gland of a Dorset horn ewe</td>
<td>129</td>
</tr>
<tr>
<td>5.11</td>
<td>Growth of bluetongue virus Type 4 in alveolar macrophages on primary isolation, from the lung of a Dorset horn lamb</td>
<td>130</td>
</tr>
</tbody>
</table>
Growth of bluetongue virus Type 4 in established alveolar macrophages derived from primary cultures of macrophages from the lung of a Dorset horn lamb

Growth of bluetongue virus Type 4 in established macrophages from bone marrow of a Dorset horn lamb

Growth of bluetongue virus Type 4 in established macrophages from the spleen of a Dorset horn lamb

Growth of bluetongue virus Type 4 in thymocytes (T cells enriched) from a Dorset horn lamb

Growth of bluetongue virus Type 4 in fibroblastic cells derived from the thymus of a Dorset horn lamb

Growth of bluetongue virus Type 4 in cultured endothelial cells isolated from the vena cava of a Dorset horn lamb

Growth of bluetongue virus Type 4 in cultured endothelial cells from the aorta of a Dorset horn lamb

Growth of bluetongue virus Type 4 in cultured endothelial cells from the ventricles of the heart of a Dorset horn lamb

Growth of bluetongue virus Type 4 in peripheral blood monocytes from a goat

Growth of bluetongue virus Type 4 in peripheral blood monocytes from a heifer

Growth of bluetongue virus Type 4 in established cultures of macrophages derived from peripheral blood monocytes of a goat

Growth of bluetongue virus Type 4 in established macrophages derived from monocytes stimulated in the mammary gland of a nanny goat

Growth of bluetongue virus Type 4 in established macrophages derived from monocytes stimulated in the mammary gland of a virgin heifer

Growth of bluetongue virus Type 4 in alveolar macrophages on primary isolation from the lung of a goat kid

Growth of bluetongue virus Type 4 in alveolar macrophages on primary isolation from the lung of a calf
Growth of bluetongue virus Type 4 in established alveolar macrophages derived from primary cultures of macrophages from the lung of a goat kid

Growth of bluetongue virus Type 4 in established alveolar macrophages derived from primary cultures of macrophages from the lung of a calf

Growth of bluetongue virus Type 4 in neutrophils stimulated in the mammary gland of a nanny goat

Growth of bluetongue virus Type 4 in neutrophils stimulated in the mammary gland of a virgin heifer

Growth of bluetongue virus Type 4 in cultured endothelial cells from the carotid artery of a goat kid

Growth of bluetongue virus Type 4 in cultured endothelial cells from the carotid artery of a heifer

CHAPTER VI

Pathogenesis of bluetongue virus infection in sheep (Hypothesis)

APPENDIX I

Recovery rates of various sheep cell populations and percentage contamination in the populations at different ficoll/hypaque densities

Published methods for the separation and purification of haemopoietic cell populations from various species

Methods employed in this study and the cell populations separated

Percentage survival of haemopoietic cells in vitro culture

Sheep peripheral blood monocyte culture 24-48 hours post culture (x800)

Sheep peripheral blood monocyte culture 72-96 hours post culture (x700)

Sheep peripheral blood monocytes 5-7 days post culture (x700)

Sheep peripheral blood monocytes 7-10 days post culture (x1,400)
AI. 2 e Confluent monolayer of established macrophages derived from sheep peripheral blood 12-14 days post culture (x1,600) 206

AI. 2 f Established macrophage cell line derived from sheep peripheral blood (x2,400). 206

AI. 3 Cytospin preparation of established sheep peripheral blood macrophages. 207

AI. 4 Thin section electron micrograph of established sheep peripheral blood macrophages (x41,000) 208

AI. 5 Cell production in sheep mammary glands inoculated with E. coli lipopolysaccharide 209

AI. 6 Cell production in cattle mammary glands inoculated with E. coli lipopolysaccharide 210

AI. 7 Cell production in goat mammary gland inoculated with E. coli lipopolysaccharide 211

Table

APPENDIX II

AII. 1 Fixation procedure of cells for thin section electron microscopy 215

Figures

AII. 1 Shandon Elliott cytospin 218

AII. 2 Centrifuge buckets for Shandon Elliott cytospin 219

AII. 3 Cell deposit (stained with giemsa) after centrifugation in cytospin centrifuge 220
CHAPTER I

Review - Pathogenesis of viruses of the bluetongue virus group
The double stranded RNA arboviruses that showed a resistance to lipid solvents (ether, chloroform and sodium deoxycholate), were pH labile and lacked any antigenic relationship to the lipid containing Toga viridae and Bunyawiridae, were separated and named Orbiviruses (Borden et al., 1971; Fenner, 1976) a genus within the family Reoviridae.

Bluetongue virus is the type species for the genus Orbivirus. Within this genus are included the following: epizootic haemorrhagic disease of deer virus, African horse sickness virus, Ibaraki virus, equine encephalosis virus, Wallal sub group, Warrego sub group, Palyam sub group, Eubenangee sub group, Corriparta sub group, Colorado tick fever virus, Changuinola sub group, Kemerov sub group, Lebombo virus, Abadina virus, Orungo virus, Japanaut virus, Umatilla virus and UgMP359 virus (Table 1.1).

The term arbovirus was introduced to describe viruses that are maintained biologically in nature by a transmission cycle involving a vertebrate host and a haematophagus insect vector (WHO, 1967; Porterfield, 1975). The criteria for this classification are based on epidemiological and ecological conditions and do not correspond to those criteria, such as morphology and physico/chemical structure, that are used by the International Committee on Taxonomy of Viruses. The term arboviruses which describes a 'biological' property of a virus is a very broad term encompassing viruses from many distinct and unrelated groups. Arboviruses are both RNA and DNA viruses (Fig. 1.1).

In this review, only the viruses of the bluetongue group, namely African horse sickness, epizootic haemorrhagic disease of deer and Ibaraki will be discussed because little is known about the pathogenesis of the other viruses placed in this genus.
### Table 1.1 Classification of Orbiviruses

(Verwoerd et al., 1979)

**FAMILY**

REOVIRIDAE

**GENUS**

ORBIVIRUS

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of types</th>
<th>Vector</th>
<th>Vertebrate host</th>
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<td>Sheep, cattle, goats &amp; wild ungulates</td>
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<td>2</td>
<td>Culicoides</td>
<td>White tailed deer</td>
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<tr>
<td>Ibaraki virus</td>
<td>1</td>
<td>Culicoides</td>
<td>Cattle</td>
</tr>
<tr>
<td>African horse sickness virus</td>
<td>9</td>
<td>Culicoides</td>
<td>Horse, donkey &amp; mule</td>
</tr>
<tr>
<td>Equine encephalosis virus</td>
<td>5</td>
<td>-*</td>
<td>Horses</td>
</tr>
<tr>
<td>Colorado tick fever virus</td>
<td>2</td>
<td>Ticks</td>
<td>Humans, rodents</td>
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<td>Eubenangee sub group</td>
<td>3</td>
<td>Mosquitoes</td>
<td>Marsupials</td>
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<td>Changuinola sub group</td>
<td>8</td>
<td>Phlebotomines</td>
<td>-</td>
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<tr>
<td>Corriparta sub group</td>
<td>2</td>
<td>Mosquitoes</td>
<td>Ungulates</td>
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<td>Kemerovo sub group</td>
<td>18</td>
<td>Ticks</td>
<td>Humans</td>
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<td>-</td>
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</tr>
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<td>Warrego sub group</td>
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<td>Japanaut virus</td>
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<td>-</td>
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<tr>
<td>UgMP359</td>
<td>-</td>
<td>Mosquitoes</td>
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*-* = not known
Fig. 1.1 Genera and families containing arboviruses (Based on Fenner, 1976)

*Those genera containing arboviruses are shown in boxes*
2. HISTORY AND GEOGRAPHICAL DISTRIBUTION

The geographical distribution of the diseases caused by orbiviruses is wide, involving the major continents (table 1.2). Most of the countries where the viruses have been isolated lie in the area between 40° North and South of the equator.

**Bluetongue virus**

The disease called "epizootic catarrh" was first reported in 1881 by Hutcheon but it was not until 1902 that Hutcheon gave a complete description of the clinical disease then named "Malarial catarrhal fever of sheep". He also described the epidemiology of the infection. The viral nature of the causative agent was not established until 1906 by Theiler who observed that the infectious agent was found to be closely associated with the blood from infected animals. Biological transmission of the virus via haematophagous insects particularly Culicoides species was not conclusively demonstrated until the work of Foster et al., 1963; Jochim and Jones, 1966; Luedke et al., 1967; Foster et al., 1968; and at the present time the many isolates of the virus are designated types 1 to 20 on the basis of the serum neutralisation test.

The disease was originally confined to the African continent. However, in 1943 the disease was reported in Cyprus (Gambles, 1949) and then in the Middle East (Komarov and Goldsmit, 1951). Hardy and Price (1952) reported an outbreak in Texas which occurred in 1948 and there are now 4 types recognised in the U.S.A. (Barber and Jochim, 1973). In 1956/57 an epidemic occurred in the Iberian peninsular (Manso-Ribeiro et al., 1957; Campano, 1957; Manso-Ribeiro, 1958; Lopez and Botija, 1958). Since then the disease has been reported in India (Sapre, 1964) and West Pakistan (Sarwar, 1962). There is serological evidence that bluetongue virus has occurred in cattle in British Colombia. In 1977 the virus was isolated from Culicoides species
Table 1.2. Geographical distribution of bluetongue, epizootic haemorrhagic disease of deer, African horse sickness and Ibaraki virus as compared by virus isolation from infected vertebrates and/or vectors

<table>
<thead>
<tr>
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<th>Current Type(s) Recognised</th>
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<td></td>
<td></td>
<td></td>
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<td>West Pakistan</td>
<td>1959</td>
<td>16, untyped</td>
<td>Howell (1960); Sarwar (1962)</td>
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<td>India</td>
<td>1968</td>
<td>1, 10, 16 untyped</td>
<td>Sapre (1964); Bhambani and Singh (1968)</td>
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<td></td>
<td>Cyprus</td>
<td>1943</td>
<td>4, 3</td>
<td>Gambles (1949); Howell (1960)</td>
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<td>Middle East</td>
<td>1943</td>
<td>1, 2, 4, 6, 10, 12, 16</td>
<td>Komarov and Goldsmit (1951); Dafni (1967); Ayoub and Singh (1970); Sellers, personal communication</td>
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<td></td>
<td>NORTH AMERICA</td>
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<td>U.S.A.</td>
<td>1948</td>
<td>10, 11, 13, 17</td>
<td>Hardy and Price (1952); McGowan (1953); Bushenell (1977)</td>
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<td></td>
<td>Canada</td>
<td>1975</td>
<td>(serological evidence only)</td>
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<td></td>
<td>AFRICA</td>
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<td>South Africa</td>
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<td>1-15, 18, 19</td>
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</tr>
</tbody>
</table>
Table 1.2 cont'd

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<td>Placidi (1957)</td>
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<td><strong>EUROPE</strong></td>
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<td>1956/57</td>
<td>10</td>
<td>Howell (1960); Manso-Ribeiro (1958)</td>
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<td></td>
<td>Spain</td>
<td>1956/57</td>
<td>10</td>
<td>Manso-Ribeiro et al. (1957); Campano (1957); Lopez and Botija (1958)</td>
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<tr>
<td></td>
<td><strong>AUSTRALIA</strong></td>
<td>1977</td>
<td>20</td>
<td>St. George et al. (1978)</td>
</tr>
<tr>
<td>Epizootic Haemorrhagic Disease</td>
<td><strong>NORTH AMERICA</strong></td>
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<td></td>
<td>U.S.A.</td>
<td>1955</td>
<td>New Jersey</td>
<td>Shope et al. (1955)</td>
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<td></td>
<td>Canada</td>
<td>1960</td>
<td>Alberta</td>
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<td><strong>AFRICA</strong></td>
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<td></td>
<td>West Africa</td>
<td>1974</td>
<td>untyped</td>
<td>Moore and Lee (1972)</td>
</tr>
<tr>
<td>Virus</td>
<td>Country</td>
<td>First Report</td>
<td>Current Type(s) Recognised</td>
<td>Reference</td>
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<td>African Horse Sickness</td>
<td>AFRICA</td>
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<td>South Africa</td>
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<td>1960</td>
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<td>FAO/OIE (1960); OIE(1960)</td>
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<td>Cyprus</td>
<td>1960</td>
<td>9</td>
<td>Orhan (1961)</td>
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<td></td>
<td>Middle East</td>
<td>1959/60</td>
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<td>Rafyi (1961)</td>
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<td>EUROPE</td>
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<tr>
<td></td>
<td>Spain</td>
<td>1966</td>
<td>9</td>
<td>Montilla and Marti (1967, 1968)</td>
</tr>
<tr>
<td></td>
<td>Ibaraki</td>
<td>1959/60</td>
<td>1</td>
<td>Omori (1960)</td>
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</table>
caught in the Northern Territories of Australia (St. George et al., 1978).

**Epizootic haemorrhagic disease of deer virus**

This disease of deer was first reported by Shope et al., (1955) from New Jersey U.S.A. They also showed the causative agent to be of viral aetiology and this was later confirmed by Fay et al., (1956). As with bluetongue virus, the virus is found in blood from infected animals (Gibbs and Lawman, 1977), and has been shown to replicate in Culicoides species (Boorman and Gibbs, 1973). The virus produces a severe and often fatal disease in white tailed deer (Odocoileus virginianus).

Since the initial outbreak of epizootic haemorrhagic disease of deer in New Jersey in 1955 (Shope et al., 1955) the disease is still confined to the North American continent. In 1960 the disease was reported in Canada (Alberta) (Chalmers et al., 1964; Ditchfield et al., 1964). Confirmed epidemics amongst deer have been recorded in South Dakota, North Dakota, Wyoming, Missouri, Washington, British Columbia, Nebraska, Michigan and Iowa. Viruses related to the virus of epizootic haemorrhagic disease have been found in West Africa (Moore and Lee, 1972) and South Africa (Verwoerd et al., 1979).

**Ibaraki virus**

An epidemic of an acute infectious disease occurred amongst cattle of southern Japan in 1959/60 (Omori, 1960, 1966). The virus, whilst showing morphological and some physical/chemical characteristics of bluetongue, was shown to be serologically distinct. The virus was given the name of the district in which it was observed (Ibaraki) (Omori, 1966) and has not been isolated outside Japan.

**African horse sickness virus**

The disease has been known since the turn of the century and was first diagnosed in Southern Africa. In 1900 M'Fadyean showed that the
causal agent was of viral aetiology and later Howell (1962) reported the existence of more than one serotype. Nine serotypes are now recognised. The disease is endemic in most of the African continent. Epidemics have occurred however in the Middle East and India during 1959 and 1960 (O.I.E., 1960; Rafyi, 1961) and in North Africa and Spain in 1965 and 1966 (Montilla and Marti, 1967, 1968; Laaberki, 1969).

3. PHYSICAL AND CHEMICAL CHARACTERISTICS

The physical and chemical characteristics and morphology of the Orbiviruses are similar and have been thoroughly reviewed (Verwoerd et al., 1979). A comparison of the physical and chemical characteristics of these viruses is shown in table 1.3.

4. EPIDEMIOLOGY

The most prominent features of these diseases are the seasonal incidence and the geographical location of the epidemics which suggest a close correlation with climatic conditions and therefore the possibility of an insect vector being involved. The role of an insect vector in the epidemiology of these diseases has been proven both experimentally and naturally for bluetongue virus (Du Toit, 1944; Foster et al., 1963) and African horse sickness virus (Du Toit, 1944; Mellor et al., 1975 and Boorman et al., 1975). The insect vector incriminated in these reports were the Culicoides species namely Culicoides variipennis and Culicoides nubeculosus. Culicoides variipennis, nubeculosus and riethi have also been shown to support the replication of epizootic haemorrhagic disease of deer virus after experimental inoculation into the thorax or oral ingestion of infected blood meals (Boorman and Gibbs, 1973). Furthermore the disease has been transmitted experimentally by Culicoides variipennis to white tailed deer (Foster et al., 1977) and it was suggested by Jones et al., (1977) that Culicoides variipennis was the probable vector of an
Epidemic of epizootic haemorrhagic disease which occurred in Kentucky in 1971. These observations were based on epidemiological data and virus isolation from parous female midges. Despite the seasonal incidence and geographical distribution of Ibaraki virus no direct evidence has been obtained to implicate Culicoides or any other insect species as a vector. Apart from Culicoides species, limited evidence exists that other species of insects may transmit bluetongue, epizootic haemorrhagic disease of deer and African horse sickness virus. For instance Gray and Bannister (1961) implicated the sheep ked (Melophagus ovinus) in the mechanical transmission of bluetongue virus, by finding that sheep keds from infected sheep harbour virus. They were unable to demonstrate natural transmission of the virus with infected keds. Ozawa et al., (1966 and 1972) transmitted African horse sickness to horses by permitting artificially infected Aedes aegypti, Culex pipiens and Anopheles stephensi to feed on uninfected horses. However Wetzel et al., (1970) were unable to infect either mosquito or Culicoides species with African horse sickness virus.

The distribution of Orbiviruses will be governed largely by ecological factors favouring the life cycle of the insect vectors, for instance temperature, humidity, rainfall, altitude and wind will be important factors. The optimum conditions for Culicoides, which have a short life span, are temperatures of about 13-35°C and humidity. In endemic areas disease is only seen in animals introduced from elsewhere. The numbers of Culicoides are associated with the rainy season, peaks being found at the beginning and end. Most infection is also associated with these times. In sub tropical areas as in Israel and Cyprus, insect numbers will generally start to increase in Spring reaching a peak in late Summer or early Autumn and decline with the onset of Winter. The incidence of disease will generally follow
Table 1.3 Physical and Chemical characteristics of the bluetongue virus group (based on Verwoerd et al., 1979)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Bluetongue virus</th>
<th>Epizootic haemorrhagic disease of deer virus</th>
<th>Ibaraki virus</th>
<th>African horse sickness virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Morphology of virion</td>
<td>Icosahedral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Nucleic acid</td>
<td>Double strand RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Molecular wt. of nucleic acid x 10^6</td>
<td>12</td>
<td>12-15</td>
<td>11-12</td>
<td>?</td>
</tr>
<tr>
<td>4. Number of segments in genome</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Capsid symmetry</td>
<td>Cubic (naked icosohedron)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Number of capsomers</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Size of virion (nm)</td>
<td>50-60</td>
<td>60-80</td>
<td>50</td>
<td>50-80</td>
</tr>
<tr>
<td>8. pH sensitivity</td>
<td>Labile</td>
<td></td>
<td>Resistant</td>
<td></td>
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<tr>
<td>9. Reaction to lipid solvents</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Thermo-stability</td>
<td>stable (except at -20°C)</td>
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</tbody>
</table>
the same pattern, therefore giving it an epidemic nature.

Wind probably plays a particularly important part in the distribution of epidemics due to its profound influence over the distribution of the vectors, which do not have the power or ability to move themselves far. Sellers et al., (1977 and 1978) analysed outbreaks of African horse sickness and bluetongue and showed that the most likely cause of the spread of disease was the movement of infected Culicoides over great distances on prevailing winds.

Transmission of these orbiviruses, other than by insects, has not been recorded.

5. CLINICAL DISEASE AND SPECIES AFFECTED

I. Horizontal transmission

a) Bluetongue

All breeds of sheep are susceptible to bluetongue to varying degrees but the greatest susceptibility occurs in those that are exotic to enzootic areas. In general, age does not seem an important factor in their susceptibility although Moulton (1961) claims that sheep about 1 year of age are more susceptible than older sheep.

The response to infection can be either inapparent or give rise to acute fulminating disease. The first sign of disease in sheep is usually a febrile response, lasting an average 6-7 days. Infected sheep however, that do not have a febrile reaction may still exhibit the other clinical signs.

The incubation period before clinical signs develop varies according to the method of infection and may be as little as 2 days or may be as long as 15 days post inoculation (Moulton, 1961; Howell, 1963; Erasmus, 1975). The incubation after natural infection is generally considered to be about 7 days, but the length depends on which clinical signs are used as the measure (Howell, 1963; Erasmus, 1975). For instance, leucopenia develops 3-5 days after infection.
whereas lesions in the mouth occur from 1-7 days after infection (Luedke et al., 1964).

Peak viraemia in infected sheep lies between 6-7 days post infection with virus being recovered as early as 3 days post inoculation and lasting up to 10 days (Luedke, 1969). The duration of viraemia and concentration of virus per ml of blood in sheep infected with epizootic haemorrhagic disease of deer virus is comparable (Gibbs and Lawman, 1977).

Following this febrile response the tissue of the buccal cavity and nasal mucosa of the sheep becomes hyperaemic and this is accompanied by an increase in salivation and frothing from the mouth; a nasal discharge, initially serous but becoming mucopurulent and speckled with blood is common. The nasal discharge often dries forming a crusty layer on the muzzle. Hyperaemia and lacrimation are also observed. Lips, tongue and intermolar spaces become oedematous with petechial haemorrhages developing on the mucous membrane. There is a loss of condition associated with severe oral lesions and anorexia (Bowne, 1971) and the main cause of death is thought to be aspiration pneumonia (Luedke et al., 1964; Luedke and Jochim, 1968; Erasmus, 1975).

The coronary band of the feet exhibit reddening, and pain is evident when the feet are subjected to pressure and foot lesions are present. Animals exhibiting foot lesions and coronary band involvement are usually reluctant to walk and tend to be recumbent.

The oedema may be very severe and extensive and is often confused with photosensitization (Luedke, 1969; Erasmus, 1975). Hyperaemia may be visible in the skin, involving the whole animal. Wool break may occur some weeks later. Muscular degeneration sometimes occurs.

Goats are susceptible to bluetongue but are not as sensitive as sheep for the disease may be mild or inapparent. Infected goats usually exhibit a febrile response with slight hyperaemia of conjunc-
tiva and mucous membranes. Luedke and Anakwenze (1972) detected bluetongue up to 21 days post infection. Peak viraemia occurred between 6-9 days (Luedke and Anakwenze, 1972) and may last as long as 19 days (Barzilai and Tadmor, 1971). As with sheep, virus has also been detected in the blood when high levels of serum neutralising antibody were also present (Haig, 1959). Similar observations concerning viraemia have been made on sheep (Luedke, 1969).

Bluetongue infection in cattle may be inapparent, acute or chronic. Experimentally infected animals, despite becoming viraemic, show minimal clinical response but naturally infected cattle may show stiffness in all four legs with involvement of the laminae. Oedema and lesions are present on the dental pad, tongue, nose and muzzle and excessive salivation with nasal discharge, which becomes mucopurulent, occurs. As with sheep, the nasal discharge forms a crusty layer on the muzzle. Occasionally, lesions are evident on the teat and udder of infected cows (Bowne et al., 1968; Hourrigan and Klingsporn, 1975).

Natural bluetongue virus infection in the North American species of white tailed deer has been fully reported (Robinson et al., 1967; Stair et al., 1968; Thomas and Trainer, 1970 a, b; Hoff et al., 1974). The disease can be either acute, showing a febrile response with depression, viraemia, oedema, hyperaemia of the lips and conjunctiva, ulceration and coronitis or it can be inapparent despite a viraemia being present. Experimental infection of white tailed deer induces severe clinical disease resembling bluetongue infection in sheep (Trainer et al., 1967; Vosdingh et al., 1968). Viraemia was also detected from 2 days to 10 days post inoculation.

In Israel, Barzilai et al., (1971) have also recorded natural bluetongue infection of the mountain gazelle (Gazella gazella), a
species of antelope. There was no clinical sign of infection but virus was recovered, however, from the blood of these animals and neutralising antibody developed even in others from which virus was not isolated.

No clinical disease was observed in mountain gazelles experimentally inoculated with the virus (Barzilai and Tadmor, 1972). Virus was isolated, however, from the blood from day 1 to 31 days post inoculation. Peak viraemia \(10^{4.0} \text{ TCID}_{50}/\text{ml}\) occurred from day 6 to 10 days post inoculation.

The blesbuck \((\text{Damaliscus alibifrons})\) was also susceptible to infection (Neitz, 1933), but there was no clinical disease, although virus was recovered from the blood on the 8th, 10th, 14th and 17th day post infection. A disease reported to resemble bluetongue was observed in Topi \((\text{Damaliscus korrigum ugandae})\), a species closely related to the blesbuck of South Africa. However, no virus or antibody was isolated. The clinical signs observed were anorexia, lameness and ulceration of the coronary band and mouth (Wells, 1962).

In North America clinical disease has been recorded in pronghorn antelope \((\text{Antilocapra americana})\) (Hoff and Trainer, 1972). Low level viraemia developed and the animals were anorexic, showed central nervous system involvement and dyspnoea. Haemorrhage was also evident. The North American elk \((\text{Cervus canadensis})\) was susceptible to infection but clinical disease was either inapparent or mild; that is, there was a slight increase in temperature, conjunctivitis and viraemia (Murray and Trainer, 1970).

In 1970/71 at the San Diego Zoo U.S.A., an acute and fatal disease characterised by haemorrhaging and oedema was recorded in several species of cervidae and bovidae but the virus was only isolated from two of the species concerned - the kudu \((\text{Trigelaphus})\)
b) Ibaraki in cattle

The clinical disease resembles that of bluetongue. In experimental infection the incubation is usually 4–7 days but can extend to 12 days post infection. The onset of disease is abrupt with a pyrexia of 40°C. Other signs are anorexia, lacrimation, depression and salivation. The disease is also characterised by congestion and oedema of the conjunctival mucous membranes of the oral and nasal cavities and muzzle. Erosions and ulcerations sometimes occur in the nose, on the gingivae, hard palate and coronets. The disease however is usually mild and frequently inapparent. Lesions occur in the oesophagus, larynx, pharynx and tongue and infected cattle may exhibit difficulty in swallowing and rumenating. Aspiration pneumonia is consequently a major cause of death (Omori et al., 1969; Omori, 1970).

c) Epizootic haemorrhagic disease of deer

Epizootic haemorrhagic disease of deer which is a severe and often fatal disease was first recorded in 1955 in white tailed deer in the U.S.A. (Shope et al., 1955). Since then further outbreaks have been recorded in the same species of deer in various parts of the U.S.A. (Fay and Boyce, 1955; Fay et al., 1956; Guenther, 1956; Shope et al., 1960; Pirtle and Layton, 1961; Parikh et al., 1967; Hoff et al., 1973; Prestwood et al., 1974; Frank and Willis, 1975; Roughton, 1975). The clinical disease reported is similar to bluetongue infections of the white tailed deer and is characteristically an acute and fatal disease in deer. Initial signs of the disease are anorexia with the animal rapidly becoming weaker. Salivation is evident, the animal exhibits tachycardia and dyspnoea, finally becoming comatose, the resulting death being caused by profound shock. Gross haemorrhage of mucous
membranes is prominent with progression of disease and the deer exhibit the characteristic "blue tongue" as is seen with bluetongue disease in sheep.

Other wild ungulates have been reported to be susceptible, responding with similar acute clinical manifestations as are found in the white tailed deer (Chalmers et al., 1964). For example the prong-horn antelope (Antilocapra americana) and the mule deer (Odocoileus hemionus). Of the latter species 1 out of 3 died from infection, which implied that this species was relatively refractile to the disease (Pirtle and Layton, 1961). The North American elk (Cervus canadensis) was studied to determine its susceptibility to the virus (Hoff and Trainer, 1973) but no clinical disease was apparent, although both animals exhibited a viraemia, one lasting 30 days. The moose (Alces alces) was not apparently susceptible (Karstad - personal communication; Hoff and Trainer, 1973).

Infection of British species of deer, red (Cervus elaphus), fallow (Dama dama) and roe (Capreolus capreolus) was successful in so far as the development of viraemia was concerned, but no clinical disease occurred (Gibbs and Lawman, 1977).

Species other than deer may be susceptible. The virus replicates in sheep but without causing clinical disease; however a viraemia is detected (Gibbs and Lawman, 1977). Cattle support a viraemia but no clinical signs have been recorded experimentally (Gibbs and Lawman, 1977) but clinical signs in naturally infected cattle have been observed (Metcalf, 1977).

d) African horse sickness

African horse sickness has been described on many occasions (Theiler, 1921; Henning, 1956; Rafyi, 1961; Maurer and McCully, 1963; Erasmus, 1972; Mirchasmy and Hazrati, 1973). Horses, donkeys and mules
have all been found to be susceptible to varying degrees although horses are considered the most susceptible, having high morbidity and mortality rates. There is also a relatively high morbidity rate in mules but the mortality rate is low; donkeys are the least sensitive, with only a mild febrile response and no recorded mortality.

The clinical disease has been described under 4 distinct types (Mornet and Gilbert, 1968). 1) Horse sickness fever, 2) pulmonary disease, 3) oedematous/cardiac disease and 4) combinations of 1-3.

1) Fever: pyrexia reaches 40-41°C and lasts approximately 10-15 days, after which the animal appears healthy. Occasionally other signs such as dyspnoea, depression, anorexia and hyperaemia of the conjunctiva develop.

2) Pulmonary disease: this is the most serious form of the infection in the horse. It is characterised by an increase in temperature and the development of acute anorexia, hyperaemia of conjunctiva and mucous membranes with dyspnoea. Coughing spasms become frequent and a serous fluid is discharged via the nares, which is sometimes frothy. The animal becomes weak, and on pulmonary involvement, death follows rapidly. Just prior to death the mucous membranes are congested and the conjunctiva are ecchymotic. Apart from the nasal serous discharge the clinical signs are similar to those observed for bluetongue in sheep and cattle, and epizootic haemorrhagic disease of deer virus in deer.

3) The oedematous or cardiac disease: this form of the disease has a longer incubation period than the other two. There is a pyrexia and the animal becomes anorexic but as the temperature begins to fall, oedemas become visible in the sub orbital region. Eventually, the oedema spreads to the mandibular spaces, nostrils and larynx. The eyes become irritated by the oedema and swell giving the animal a 'fixed gaze'. From the head region the oedema spreads to the
withers, then the breast region and finally the upper limbs, and oedema has been recorded affecting the hind quarters. The cardiac involvement starts when the oedema becomes apparent. The animal suffers dyspnoea and the cardiac rate increases but there is no serous exudate or cough as is seen in the pulmonary form of the disease. The oedematous areas are not painful to touch or pressure. Death usually follows once the oedema generalizes and death is considered to be due to cardiac arrest.

4) Mixed types: Montilla and Marti, (1967) found that the pulmonary form was evident first, with subsequent development of the cardiac/oedematous form. Borkovec (1966) however, found cases of widespread oedema as a primary sign, with coughing and serous discharge developing subsequent to oedema. Rafyi (1961) maintains that the co-existence of the two types is only distinguishable during or after autopsy.

All the characteristic lesions of bluetongue, epizootic haemorrhagic disease of deer virus and Ibaraki are exhibited by one or all of the four disease types of African horse sickness. It should be mentioned, however, that apart from the equine species in which African horse sickness virus causes severe clinical reactions, clinical disease has been documented in experimentally infected dogs (Theiler, 1921) in which the clinical signs are similar to those in horses and mules. Clinical disease has also been reported in dogs that had fed upon infected horse meat (Bevan, 1911; Piercy, 1951).

II Vertical transmission - (Teratogenic involvement of orbiviruses in congenital abnormalities of the foetus)

To produce congenital defects in the foetus the virus teratogen exerts its effect in three ways:- a) the inhibition or distortion of normal cellular development by interfering with proliferation,
differentiation and maturation at the cellular and sub-cellular level; b) cellular destruction by the replication of virus in developing tissues, or c) by the destruction of infected tissues by the host immune mechanism (Done, 1979).

Recognition that bluetongue virus was capable of producing foetal abnormalities was first reported by Schultz and De Lay (1955). They observed that ewes vaccinated with a live attenuated bluetongue vaccine produced spastic lambs, mummified foetuses and still born lambs, some with congenital defects.

Congenital abnormalities have also been recorded after natural infection of sheep and cattle with bluetongue virus but only on the basis of serology and histopathology (Griner et al., 1964; McKercher et al., 1970; Richards et al., 1971; Schmidt and Panciera, 1973). The abnormalities recorded by the above authors were mainly concerned with cerebral damage - hydranencephaly, porencephaly, cerebral mineralisation and encephalitides.

Experimental studies of the teratogenic effect of the virus in sheep and cattle have confirmed these field observations (Luedke et al., 1970; Osburn et al., 1970; Silverstein et al., 1971; Osburn et al., 1971 a, b; Osburn and Silverstein, 1972; Enright and Osburn, 1974; Barnard and Pienaar, 1976). Osburn and Silverstein (1972) also suggested that the susceptibility of the foetus to the virus was dependent on the gestational period at the time of foetal infection - severe encephalopathy was seen in lambs infected, in utero, at 50-58 days of gestation, whilst among those inoculated after 100 days, apart from mild focal encephalitis, no gross abnormalities were observed.

The pathology of bluetongue-caused congenital abnormalities has also proved of interest, as an animal model, to those investigating the congenital abnormalities, in man, of unknown aetiology (Osburn and
In most of the above studies, sheep and cattle foetuses have been inoculated experimentally, in utero, directly and unfortunately therefore, there is little information on the mechanisms of infection of the foetus by the dam.

Recent work by Gibbs et al., (1979) in which the foetus was infected transplacentally, has indicated that the virus may also be an important cause of growth retardation in foetal lambs without obvious pathology. Furthermore, this study has also revealed that such apparently normal lambs are often viraemic at birth and remain so for up to 2 months after birth and this may, therefore, represent an overwintering mechanism for virus perpetuation in certain areas of the world. A similar observation was made by Osburn et al., (1975) when they stated that, at birth, infected lambs were capable of becoming persistently viraemic. Similar observations have also been made by Luedke et al., (1977a, b) where calves were born viraemic, one of which remained so up to 3 years.

Vertical transmission of bluetongue virus has also been recorded in white tailed deer (Thomas and Trainer, 1970a).

Very little, if anything, is known about the teratogenic effect of epizootic haemorrhagic disease of deer virus, Ibaraki virus and African horse sickness have on the developing foetus.

6. PATHOLOGY AND PATHOGENESIS

Pathology

The pathology of natural and experimental cases of bluetongue, African horse sickness, epizootic haemorrhagic disease of deer and Ibaraki disease has been reviewed by several authors (Moulton, 1961; Luedke et al., 1964; Stair et al., 1968; Howell and Verwoerd, 1970; Erasmus, 1975; Rafyi, 1961; Maurer and McCully, 1963; Karstad et al.,
auu i v c i x a c c i a, lyo/* Ishitani, 1967; Omori et al., 1969;
Omori, 1970) and an outline of these findings is given below.

The predominant feature of these infections is haemorrhage involving most of the organs and tissues of the body but particularly those of the cardiovascular and haemopoietic system.

a) Bluetongue

(i) Cardiovascular system: involvement of the cardiovascular system during disease is marked by widespread petechial and ecchymotic haemorrhage, oedema and hyperaemia. Cardiovascular lesions are evident in all the species of animals infected by this disease.

(ii) Haemopoietic system: lymph nodes become enlarged, hyperaemic and oedematous. In these hyperaemic lymph nodes, due to the oedema, there is separation of cellular elements, deposition of haemosiderin and infiltration of neutrophils and monocytes. The spleens show splenomagaly, hyperaemia and perifolicular neutrophil invasion. The lymph nodes and spleen also show petechial and ecchymotic haemorrhages and the liver tends to be swollen and congested. Haematological studies show that this disease produces a pan-leucopenia in infected animals.

(iii) Respiratory and oral systems: the nasal mucosa becomes haemorrhagic and congested and the lung hyperaemic and oedematous with infiltration of neutrophils into the alveolar. Erosions may develop on the lips and dental pad and these erosions are often haemorrhagic. Erosions are also found on the tongue, and the upper and lower gums become hyperaemic with the tops of the papilla often eroded.

(iv) Skeletal musculature: apart from haemorrhage the most important pathological change is the damage to the skeletal musculature: the most frequently affected are the muscles of the thigh, shoulder, back and neck. Both ecchymotic and petechial haemorrhage into the
musculature with advance hyalin degeneration have been recorded. Infiltration of monocytes and lymphocytes have also been described.

b) Ibaraki

Ibaraki virus, the other important orbivirus affecting cattle, causes pathological changes similar to those ascribed to bluetongue in sheep. Congestion and ulceration of the mucous membranes occur, in particular in the gingival, hard palate, abomasum and nose; there is ulceration of the coronet and muzzle and to a lesser extent, ulceration of the oesophagus and rumen. Musculature is extensively affected and most lesions appear in the striated muscle of the larynx, pharynx and tongue. There is loss of striation, hyalin degeneration, infiltration of neutrophils and lymphocytes. Perimysial cells are swollen and there is hyperplasia of fibroblasts and histocytes. Haemorrhage and oedema are found in the lesions of the muscle. Striated muscle of the breast and limbs is also affected. The musculature lesions described are similar to those found in bluetongue infected sheep.

c) Epizootic haemorrhagic disease of deer

In epizootic haemorrhagic disease of deer, the pathology is also similar. Haemorrhages are found in the heart, aorta, myocardium and blood vessels of the small intestine. Lymphatic organs are oedematous and haemorrhagic, the lungs are congested and haemorrhagic as are the kidneys, liver, adrenal gland and pancreas. The spleen is swollen and engorged with erythrocytes. The spleen is swollen and engorged with erythrocytes. A major pathological change is found in the musculature: degenerative hyalin, loss of striation, infiltration of leucocytes and haemorrhages.

d) African horse sickness virus

i) Pulmonary disease

The primary lesions are petecchial haemorrhages of the mucous
membranes of the nasal cavity, pharynx, trachea and bronchial tubes and lung, and the bronchial tubes and lung are filled with a white serous exudate. On palpation, the pulmonary tissues have lost all elasticity. Petechial haemorrhages are also present on the heart. Haemorrhages and oedema are evident in the intestine and stomach. The kidneys show slight congestion but the spleen is often normal. The oedema fluid contains a high proportion of eosinophils.

ii) Cardiac disease

The main lesions are found in the subcutaneous conjunctiva and cardiovascular system. The cardiac lesions consist of haemorrhage and oedema. The failure of the heart denotes pulmonary congestion. The pericardial sac is distended with fluid (up to 2 litres). Haemorrhages are present in the valves, papillary muscles and myocardium. There is oedema of subcutaneous conjunctiva and intramuscular tissues. The oedema involves the head and neck, sometimes extending as far as the head and shoulders. The lesions of the intestine and stomach are similar to the pulmonary form. The spleen is haemorrhagic and hyperplasic. Muscular fibres lose their striations and some of the destroyed muscle fibres are replaced by small zones of fibro-vascular tissue.

iii) Mixed form of disease

Lesions of either the pulmonary or cardiac form are found.

Pathogenesis

In contrast to pathology, there are relatively few reports on the pathogenesis of bluetongue, African horse sickness, epizootic haemorrhagic disease of deer and Ibaraki disease. In 1968 Stair studied the pathogenesis of bluetongue by observing the clinical reactions and studying the progress of disease by immunofluorescence and histopathology on infected sheep slaughtered daily up to 16 days. His findings were that bluetongue viral antigen had an affinity for
the endothelial and periendothelial cells of the vascular system. The greatest concentration of virus assessed by immunofluorescence was in the capillaries underlying stratified squamous epithelium, especially that of the oral mucous membrane, skin and coronet of the hoof. The highest concentration of antigen occurred between day 3 to 11 days post inoculation. The fluorescent antigen was also found in the reticuloendothelial cells of the lymph nodes but none was detected in the epithelial cells.

Histopathological examination of the endothelial cells revealed pyknotic cells which exhibited karyorhexia, cytoplasmic vesiculation, nuclear and cytoplasmic swelling and hypertrophy. The infected endothelial cells became enlarged and necrotic which resulted in vascular occlusion, stasis and exudation. The consequential inflammatory response resulted in hypoxia of the overlying epithelium.

In a sequential slaughter study conducted by Pini (1976), organs from sheep which had been inoculated subcutaneously with virus were sampled over a period of 11 days and assayed for viral infectivity. Virus was recovered as early as 4 days post inoculation from the lymphoreticular system, especially the lymph nodes, followed after 2 days, by viraemia. From the sixth day, virus was, not surprisingly, widespread and Pini postulated that the primary site of localisation of bluetongue virus was the lymph nodes. He assumed that after penetration into the host, the virus was probably first associated with the macrophage or other lymphoid cells from which dissemination to the regional lymph nodes occurred; spread was then via the lymphatics or blood stream to other lymphoreticular tissues, where further replication took place.

Erasmus (1972) studied the pathogenesis of African horse sickness virus in 6 infected horses serially slaughtered over a 6 day period. Two days after inoculation, virus was recovered at relatively high
titre from the spleen, lung, caecum, pharynx, choroid plexus and in 12 out of 20 lymph nodes but no viraemia was detected. In tissues and organs removed from other horses that had died from both experimental and natural infections, high titres of infectious virus were recovered from the lung, spleen and lymph nodes (Erasmus, 1972). Erasmus also suggested that the different clinical forms of the disease could be explained if the field strains of virus were composed of a population of virus particles heterogeneous with regard to tissue tropism, that is, some particles would selectively replicate in the endothelium of pulmonary capillaries, others in the lymphoreticular system or in the endothelium of blood and lymph vessels.

Tsai and Karstad (1973) studied the pathogenesis of epizootic haemorrhagic disease of deer virus in deer by electron microscopy of selected tissues. Virus particles were seen in the cytoplasm of endothelial cells of small blood vessels and they suggested that injury to the endothelial cell would give rise to the intravascular thrombosis and that the consequent haemorrhage was a reflection of virus-cell interaction.

The pathogenesis of Ibaraki virus has not been studied.

Leucopenia has been recorded for bluetongue and epizootic haemorrhagic disease of deer (Luedke et al., 1964; Wilhelm and Trainer, 1969; Debbie and Abelseth, 1971; Luedke and Anakwenze, 1972) but it has not been resolved whether the cells were sequestered to other areas in an infected animal (Moulton, 1961; Karstad and Trainer, 1967) or whether they were directly destroyed by virus replication, or whether they were destroyed as a result of virus replication in organs such as thymus and bone marrow where the cell types constituting the leucocyte population are formed.
As early as 1906 bluetongue virus was reported to be closely associated with the cellular fraction of blood (Theiler, 1906) and since then others (Pini et al., 1966; Luedke, 1969; 1970) have also shown the virus to be associated with cells in the blood. Pini et al. (1966) found it in the buffy coat whilst Luedke recorded 10 to 100 times more virus to be associated with washed erythrocytes than with the leucocytes. The virus of epizootic haemorrhagic disease has also been isolated from the buffy coat and erythrocyte fractions of infected blood (Hoff and Trainer, 1974; Gibbs and Lawman, 1977). Ozawa et al., (1972) concluded too that the virus of African horse sickness was also very closely associated with the erythrocyte and buffy coat fraction. Frequently, any one of these viruses could be recovered from blood containing high levels of specific neutralising antibody and it has been suggested that these viruses are all afforded protection from the neutralising antibody by residence within reticulo-endothelial cells. The actual location and nature of association these viruses have with the erythrocyte - whether they are intracellular or absorbed to the erythrocyte membrane - has not been elucidated. However, Colorado tick fever virus (a rodent orbivirus which also infects humans) has been shown to be within the erythrocyte by thin section electron microscopy (Emmons et al., 1972).

Karstad et al., (1961) concluded that the virus of epizootic haemorrhagic disease of deer caused extensive haemorrhaging by a derangement of the clotting mechanism in conjunction with degenerative changes in the blood vessel lining. Karstad and Trainer (1967), Trainer and Karstad (1970) and Hoff and Hoff (1976) later state that the prime pathogenetic mechanism seemed to be disseminated intravascular coagulation. The mechanism for disseminated intravascular coagulation has not been determined but they postulated that the virus causes damage to the endothelial cells of the vascular system which
lead to platelet aggregation and breakdown and release of thromboplastin. Certainly a decrease in the platelet count of peripheral blood was recorded by Fletch and Karstad (1967), which correlated with the appearance of platelet and fibrin thrombi in small blood vessels. A thrombocytopenia has also been recorded by Markowitz (1963) in Colorado tick fever infection. Debbie and Abelseth (1971) in their studies on blood coagulation during infection with epizootic haemorrhagic disease virus infection found a decrease in fibrinogen and activating of factor VIII and related this decrease to their utilization in clotting and there was also a reduction of the number of platelets. They suggested that a combination of damage to the vascular wall and abnormalities of platelets were involved in the pathogenesis of epizootic haemorrhagic disease. Tsai and Karstad (1973) suggested that the clinical manifestation of epizootic haemorrhagic disease could be the result of a disturbance of integrity of the vascular system by virus replication in endothelial cells as well as disturbance in the homeostasis of the clotting mechanisms. The presence of fibrin in the capillary lumen, intermingled with platelets which appeared to be intact or completely degranulated was taken as evidence for this.
AIMS OF THESIS

From the above review it is evident that gaps exist in our knowledge of bluetongue. At present we have an incomplete understanding of the mechanisms of clinical disease and persistent infection on which to base control measures for bluetongue especially by vaccination.

Basic information on the pathogenesis of bluetongue is required for full investigation of these problems and this study was instigated in an attempt to provide such information. At the outset it was realised that only certain aspects of the pathogenesis of bluetongue could be investigated within the time available. It was decided to restrict the study to sheep, only extending it to include cattle and goats when considered appropriate. The thesis is presented in 4 Chapters:-

i) Distribution of bluetongue virus in infected sheep

ii) Haematology of bluetongue virus infection of sheep

iii) Replication of bluetongue virus in organ cultures

iv) Growth of bluetongue virus in cells derived from the haemopoietic system of sheep, goats and cattle
It is pertinent at this stage to define the terminology used in this and subsequent sections for cells and cell systems within the haemopoietic system. In this thesis the haemopoietic system is used to include both the lymphoreticular and reticuloendothelial system (table 1.4).

The lymphoreticular system is normally regarded to be composed of lymph nodes, thymus, spleen, bone marrow and liver (Jubb and Kennedy, 1970). Lymph nodules are also part of the lymphoreticular system. They occur in mucous membranes of urinogenital and respiratory tract, intestine (Payers patches). Lymph nodules are distinct from lymph nodes as they do not possess any afferent lymphatics.

The terminology reticuloendothelial system is less easily defined. The first attempt to define the system was in 1892 by Metchnikoff. He was the first to classify the cells as 'macrophages' and 'microphages' and to demonstrate their phagocytic activity. He also observed the relationship between the phagocytic cells of the spleen, lymph nodes and bone marrow and the macrophages existing 'outside' these organs. Metchnikoff termed the cell system the 'Macrophage System'. Aschoff (1924) introduced the term reticuloendothelial system. This was composed of several groups of cells which were placed in order of increasing phagocytic activity. The system excluded endothelial cells because of their low phagocytic activity. Aschoff's criterion for inclusion into the reticuloendothelial system was the uptake of a vital dye by phagocytosis.

The reticulo-histiocyte system was first suggested by Volterra (1927) and preferred by Thomas (1949). This classification included not only histiocytes but other cells which could acquire this 'histiocytic state' at certain times. The criteria used by Thomas were not
only phagocytosis and mobility but also an enhanced metabolic activity. Whilst the term reticuloendothelial system of Aschoff is still in general use today, Van Furth et al. (1972) recommended that phagocytic mononuclear cells be classified under the mononuclear phagocyte system. The criteria for this system are that phagocytic cells share the same morphological characteristics and that they originate from the same precursor cell type derived from bone marrow. The cells included in this system are shown in table 1.5 (Van Furth et al. (1972).

This system, however, omits endothelial cells. It is considered, however, that these cells, even though their phagocytic activity is low, should also be in the reticuloendothelial system, albeit not within the proposed mononuclear phagocyte system. For the purpose of this study the endothelial cells are included in the term reticuloendothelial system.

Table 1.5 The mononuclear phagocyte system (Van Furth et al. (1972)

<table>
<thead>
<tr>
<th>Cell</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor cell</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Promonocytes</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Bone marrow and blood</td>
</tr>
<tr>
<td>Macrophage</td>
<td>Connective tissue (histiocytes)</td>
</tr>
<tr>
<td></td>
<td>Liver (Kupffer cells)</td>
</tr>
<tr>
<td></td>
<td>Lung (alveolar macrophages)</td>
</tr>
<tr>
<td></td>
<td>Spleen (free and fixed macrophages)</td>
</tr>
<tr>
<td></td>
<td>Lymph node (free and fixed macrophages)</td>
</tr>
<tr>
<td></td>
<td>Bone marrow (macrophages)</td>
</tr>
<tr>
<td></td>
<td>Serous cavity (pleural and peritoneal macrophages)</td>
</tr>
<tr>
<td></td>
<td>Bone tissue (osteoclasts)</td>
</tr>
<tr>
<td></td>
<td>Nervous system (Microglial cells)</td>
</tr>
</tbody>
</table>
Table 1.4 The various organs, tissues and cell types grouped by their systems and the relationship of the various systems to each other

<table>
<thead>
<tr>
<th>Organ/tissue/cell type</th>
<th>System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph nodes</td>
<td>Lymphoreticular system</td>
</tr>
<tr>
<td>Lymph nodules</td>
<td>(non phagocytic cells)</td>
</tr>
<tr>
<td>Haemolymph nodes</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>Bone marrow derived monocytes and macrophages of Liver</td>
<td>Haemopoietic system</td>
</tr>
<tr>
<td></td>
<td>(all blood cells and including stem cells)</td>
</tr>
<tr>
<td>Connective tissue</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
</tr>
<tr>
<td>Serous cavity</td>
<td></td>
</tr>
<tr>
<td>Bone tissue</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td></td>
<td>(avidly phagocytic)</td>
</tr>
<tr>
<td>Nervous system</td>
<td>Reticulo-endothelial system</td>
</tr>
<tr>
<td></td>
<td>(all phagocytic cells)</td>
</tr>
<tr>
<td>Polymorphonuclear cells (neutrophils)</td>
<td></td>
</tr>
<tr>
<td>Species of epithelial cells</td>
<td>partially phagocytic</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td></td>
</tr>
</tbody>
</table>
In conclusion, therefore, the terms as used in this thesis are defined as:

1. **Lymphoreticular system**: contains those cells within the haemopoietic system which are non phagocytic - namely lymphocytes and also contains the organs and tissues in which these cells are produced or sequestered to.

2. **Mononuclear phagocyte system**: contains the avidly phagocytic mononuclear cells which are derived from the bone marrow.

3. **Reticuloendothelial system**: encompasses all the phagocytic cells. This includes the cells of the mononuclear system as well as those cells which are not avidly phagocytic e.g. neutrophils and endothelial cells.

4. **Haemopoietic system**: includes all blood cells and stem cells of the lymphoreticular and reticuloendothelial system.
CHAPTER II

Observations on the pathogenesis
of bluetongue virus infection in sheep

In vivo pathogenesis
INTRODUCTION

The use of sequential slaughter as a method for the study of early infection, dissemination and growth of virus in the body was first reported by Fenner (1948) in his classic studies on the pathogenesis of mousepox. Since then it has been used for studies on other virus diseases including myxomatisis (Fenner and Woodroffe, 1953), rabbit pox (Bedson and Duckworth, 1963), African swine fever (Heuschele, 1967; Plowright et al., 1968; Colgrove et al., 1969; Greig, 1972), rinderpest virus (Plowright, 1968), canine herpes virus (Appel et al., 1969) and bluetongue virus (Stair, 1968; Pini, 1976).

MATERIALS AND METHODS

Virus

Type 4 (Cyprus strain) from the 1969 outbreak in Cyprus (Parker et al., 1975). The virus was isolated by intravenous inoculation of fertile eggs and subsequently passaged 7 times in BHK/21 monolayers (Macpherson and Stoker, 1962). This virus was inoculated intravenously into a sheep, and blood 7 days post inoculation was used to infect a further 3 sheep intravenously. Blood taken after 7 days, at a titre of $4.5 \log_{10} \text{TCID}_{50}/\text{ml}$, was stored and used as the stock virus for all animal studies.

Sheep

Thirty Dorset horn sheep, recommended as the species most susceptible to bluetongue virus (Howell and Verwoerd, 1970), were housed in an isolation unit (Brooksby, 1965).

Experimental Design

Virus (1 ml; 100 tissue culture infective doses $\text{TCID}_{50}/\text{ml}$) was inoculated intradermally into the left ear. The sheep
were slaughtered using a humane killer in pairs at 12 hours post inoculation and at 6, 8, 10 and 14 days post inoculation, and in groups of 4 at 1, 2, 3, 4 and 5 days post inoculation. All surviving sheep were examined daily for clinical disease, pyrexia and viraemia. Post mortem sheep were examined for gross pathological lesions, and tissues were collected for histological examination from both the left and right side of the carcase. The selected tissues and organs were categorised as haemopoietic, vascular, glandular or miscellaneous (Table 2:1).

The nature and the intradermal site of inoculum was chosen to mimic the natural route of inoculation by Culicoides species. The concentration of virus in the inoculum was also chosen so as to reflect the dose likely to be transmitted by the vector. Infected Culicoides were not used to infect the sheep because when this work started there were restrictions on their use with animals.

Collection of samples

The samples were collected to ensure that they were of equal size, from the same area and there was no cross contamination of samples with infected instruments. Blood was obtained immediately after the animal was killed by severing the jugular vein; all the blood was collected to minimise the contamination of subsequently excised samples with virus from the blood. Sterile instruments were used for collection of each sample. Tissue samples (approximately 1 gram), for isolation of virus, were removed aseptically and washed in phosphate buffered saline supplemented with 3% antibiotic solution (Appendix II) before being placed separately in labelled universal bottles containing 10 ml phosphate buffered saline held at 4°C so as to minimise thermal inactivation of virus.
Table 2.1  Tissues and organs collected post mortem and examined for virus recovery

<table>
<thead>
<tr>
<th>HAEMOPOIETIC TISSUES OR ORGANS</th>
<th>VASCULAR TISSUES OR ORGANS</th>
<th>GLANDULAR TISSUES OR ORGANS</th>
<th>MISCELLANEOUS TISSUES OR ORGANS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prescapular (Left)</td>
<td>Heart muscle</td>
<td>Thyroid</td>
<td>Lung</td>
</tr>
<tr>
<td>Mandibular</td>
<td>Tricuspid valve</td>
<td>Adrenal</td>
<td>Liver</td>
</tr>
<tr>
<td>Parotid</td>
<td>Bicuspid valve</td>
<td>Pancreas</td>
<td>Kidney (left and right)</td>
</tr>
<tr>
<td>Retropharyngeal</td>
<td>Vena cava</td>
<td>Parotid salivary gland (left)</td>
<td>Small intestine</td>
</tr>
<tr>
<td>Bronchial</td>
<td>Carotid artery</td>
<td>Parotid salivary gland (right)</td>
<td>Muscle (Biceps femoris)</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>Aorta upper 1/3</td>
<td>Mandibular salivary gland (left)</td>
<td>Trachea</td>
</tr>
<tr>
<td>Inguinal</td>
<td>Aorta middle 1/3</td>
<td>Mandibular salivary gland (right)</td>
<td>Tongue</td>
</tr>
<tr>
<td>Precrural</td>
<td>Aorta lower 1/3</td>
<td></td>
<td>Glossopharynx</td>
</tr>
<tr>
<td>Iliac</td>
<td>Pulmonary artery</td>
<td></td>
<td>Dental pad</td>
</tr>
<tr>
<td>Popliteal</td>
<td>Iliac artery</td>
<td></td>
<td>Normal skin</td>
</tr>
<tr>
<td>Prescapular (Right)</td>
<td>Aorta scraping</td>
<td></td>
<td>Skin-inoculation site</td>
</tr>
<tr>
<td>Mandibular</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parotid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retropharyngeal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Popliteal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemolymph nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tonsil (left and right)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Virus isolation and identification

The sample in the collection fluid was ground in a pestle and mortar. The homogenate was centrifuged (1000g, 15 minutes at 4°C) and the supernatant was decanted and retained at 4°C. Ten fold dilutions of the suspensions were prepared in phosphate buffered saline containing 1% bovine albumin and 0.2 ml volumes were inoculated per tube cultures of BHK/21 cell monolayers, using 5 tubes per dilution. The inoculum was left to adsorb for 1 hour at room temperature after which the tube cultures were washed twice with phosphate buffered saline (free of calcium and magnesium ions) and 2 ml maintenance medium (Appendix II) containing 1% foetal calf serum was added. The monolayers in the tubes were examined daily for 7 days for the development of cytopathic effects. The virus titres were calculated using the Kärber equation (1931) and were expressed as log_{10} tissue culture infective doses 50% per gram of tissue (log_{10} TCID_{50}/gm of tissue). Selected virus isolates from the samples were identified by neutralisation against antiserum to type 4 bluetongue virus by the microtitre method (Herniman, unpublished data).

Serology

The antibody titres were calculated by the method of Kärber (1931) and expressed as the reciprocal of the serum dilution at the 50% end point.

Viraemia

The method for isolation of virus from heparinised blood removed from the jugular vein of the experimental animals (final concentration of heparin = 10 i.u./ml), was as described by Luedke (1970) for bluetongue virus and Gibbs and Lawman (1977) for epizootic haemorrhagic disease of deer virus.
RESULTS

Clinical disease, viraemia, serology, gross and microscopic pathology

None of the sheep inoculated showed severe disease. The only clinical signs observed, and these were not observed in all the sheep, were hyperaemia of the gums and dental pad and a transient pyrexia (Fig. 2.1). The transient temperature increase occurred between 6 and 10 days post inoculation.

No viraemia was detected between 12 hours and 4 days post inoculation but thereafter until the conclusion of the experiment at 14 days all sheep were viraemic. The maximum titre of viraemia was detected at 8 days post inoculation (Table 2.2 and Fig. 2.2 a). The virus content of the cellular fraction of the blood was greater than that of the plasma and a greater concentration of the virus was associated with the erythrocyte fraction than with the leucocyte fraction.

Serum neutralising antibody was first detected in the sheep on day 10 with reciprocal titres of 40 rising to 120 on day 14 (the last day of the sequential slaughter programme. Fig. 2.2 b).

No gross pathological abnormalities attributable to bluetongue infection were seen apart from haemorrhage at the base of the pulmonary artery. The histological examination of the tissues was also unremarkable and revealed a lack of histopathological change (A. Rowlands, personal communication).

Virus assay in tissues and organs

In general, virus was first recovered 24 hours post inoculation from the lymph nodes of the facial and neck area of the side (left) that was inoculated (Table 2.3 and Fig. 2.3); virus was not recovered from lymph nodes on the right hand side until 2 days later and by this time the majority of the lymph nodes sampled on the left were positive.
By the 5th day all the lymph nodes were positive, but a viraemia was now detectable. Virus was recovered from the other lymphoreticular tissues such as the spleen, bone marrow and thymus from the 3rd day onwards and the infectivity titres per gram between the 6th and 8th day were comparable to those in the blood at this time as expressed per ml. In most lymph nodes, titres higher than the blood virus titre were recorded on the 5th day post inoculation. By 1½ days post inoculation, 7 out of 15 lymph nodes were still positive despite the fact that only traces of virus were present in the blood.

In the vascular tissues, consistent virus recovery was only successful once the animals exhibited a detectable viraemia, that is from day 5 to day 10 (Table 2.4; Fig. 2.3) and the majority of tissues sampled were negative by the 14th day.

Similar results were obtained for the glandular and miscellaneous tissues (Table 2.5 and 2.6; Fig. 2.3). One area sampled, the glossopharynx, remained negative throughout the experiment (Table 2.6). Very little virus was recovered from the skin, yet virus at the inoculation site remained high in 14 out of the 30 animals including 1 animal at 14 days post inoculation.

DISCUSSION

The results of these experiments are in general agreement with results published both recently and after the completion of this study (Pini, 1976). In studying the pathogenesis of African horse sickness virus, Erasmus (1972) also used a sequential slaughter programme and found high titres of virus in lymph nodes and other lymphoid tissues in the absence of a detectable viraemia. The virus was first isolated in lymphoid tissues within 2½ hours post inoculation and was then found in the lymph nodes of the neck and facial regions.
This is what might be expected due to the pattern of lymph drainage in the area, since initial experiments were conducted using Indian ink marker mixed with the virus inoculum. The Indian ink was found, at 12 and 24 hours post inoculation, to have travelled to the facial and prescapular lymph nodes on the side of inoculation. The association of bluetongue virus is predominantly with lymphoid tissue in particular the lymph nodes. The subsequent detection of virus at 72 hours post infection in lymph nodes in other parts of the body indicates that there was an undetectable viraemia present before 72 hours. Virus isolation from other tissues throughout the body generally coincided with detectable viraemia at 5 days post inoculation. This is 96 hours after virus was first isolated in the lymph nodes.

Despite work reported by Stair (1968) where bluetongue antigen was shown to be present by the fluorescent antibody test in the endothelial cells of blood vessels in the capillary beds, no virus was isolated from vascular tissue until generalised spread of the virus occurred. However, Stair (1968) also reported early fluorescence in the lymphoid tissues of the neck region which is in accordance with the results described here and also with those described by Pini (1976). Pini (1976) also recorded that the liver and kidney were two organs not showing consistent virus isolation and virus was eliminated at an early stage compared to other tissues. In these present experiments, virus in the liver and kidney was only isolated during the days when viraemia was detected which suggests that these tissues were contaminated and not sites of virus replication. The absence of virus in the glossopharynx was surprising since virus was recovered from the tonsilar crypts. However, the pH of the glossopharynx is reported to be alkaline (pH 8.0 - 9.0) and the known lability of bluetongue virus may explain its absence.
Another area showing inconsistent isolation of virus was the normal skin even though virus could obviously survive there as is evident from the isolation of virus at the site of inoculation.

The lack of clinical disease exhibited by the experimental sheep which has been reported by other workers (Luedke, personal communication) cannot be explained, although it is very likely that the excellent health of the experimental sheep and the husbandry was such as to minimise the consequences of infection. Inability to induce clinical signs is a common phenomenon of infectivity studies.

Viraemia was still detectable at day 10 post inoculation despite the presence of neutralising antibody. The viraemia, however, was declining and by day 14 there was only trace virus present.

One of the difficulties of interpreting results of a sequential slaughter study when such a limited number of animals is available is that the data comes from different animals and individual variation could distort the overall interpretation of the results. Furthermore, such experiments are a relatively crude method for determining the distribution and levels of virus because they give little direct information on the cell types which may be involved in the replication of the virus. An assumption is also made that any virus present within tissues or organs at a time when a viraemia is not detected indicates primary virus replication within that tissue or organ. Allowance however, should be made for the fact that an undetected primary viraemia may have occurred and therefore be responsible for virus dissemination and that the virus assayed within these tissues may not be due to virus replication there.

However, in conclusion, it is possible to claim that the primary replication sites are lymph nodes which drain the site of inoculation. It is likely that the virus first replicates in the phagocytic cells
which occur in high concentration in the lymph node and are the primary cellular defence mechanism against invasion by a pathogen; it is not known how the virus overcomes the potential antivirus activities of the phagocytes. The phagocyte cells of importance are the monocytes, macrophages and the neutrophils since these infected cells have access via the lymph and blood, to lymph nodes and other target organs.
CHAPTER II

Tables and Figures
Table 2.2 Viraemia (whole blood) in 30 Dorset horn sheep after intradermal inoculation of $2.0 \log_{10} TCID_{50}$ of bluetongue virus Type 4

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* No virus isolated ($<0.3 \log_{10} TCID_{50}/ml$)

+ Heparinized blood. Titre expressed as $\log_{10} TCID_{50}/ml$ of blood
Table 2.3 Isolation of bluetongue virus Type 4 from tissues and organs removed from bluetongue infected sheep, killed at daily intervals - Haemopoietic and lymphoreticular tissues and organs.

* virus not isolated  
+ log$_{10}$ TCID$_{50}$/gm of tissue/organ  
δ log$_{10}$ TCID$_{50}$/ml of blood
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Table 2.4 Isolation of bluetongue virus Type 4 from tissues and organs removed from bluetongue infected sheep, killed at daily intervals - Vascular tissues and organs.

* virus not isolated

+ log_{10} \text{TCID}_{50}/gm of tissue/organ

<log_{10} \text{TCID}_{50}/ml of blood
## Vascular Tissues and Organs

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Table 2.5  Isolation of bluetongue virus Type 4 from tissues and organs removed from bluetongue infected sheep, killed at daily intervals - Glandular tissues and organs.

* virus not isolated

+ $\log_{10} \text{TCID}_{50}/\text{gm of tissue/organ}$

$\div \log_{10} \text{TCID}_{50}/\text{ml of blood}$
### Clandular Tissues and Organs

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## Miscellaneous Tissues and Organs

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<td>2.2</td>
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<td>Tongue</td>
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<td>2.0</td>
<td>2.2</td>
<td>3.7</td>
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<td>Glossopharynx</td>
<td>-</td>
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<td>-</td>
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<td>Dental Pad</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.8</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Normal Skin</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>1.5</td>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td>1.7</td>
</tr>
</tbody>
</table>
Figure 2.1 Temperature response of four sheep to infection with bluetongue virus Type 4
Temperature in °C:

Days post inoculation
Figure 2.2a  Isolation of bluetongue virus, Type 4, from the blood of infected Dorset horn sheep.

(• • •) whole blood  
(-----) erythrocytes fraction  
(——) buffy coat (leucocyte) fraction  
(★★★) plasma  
(<→) input virus titre

Figure 2.2b  Serum neutralising antibody response of Dorset horn sheep to infection with bluetongue virus, Type 4.
Figure 2.3 Schematic representation of virus isolation from tissues of bluetongue infected sheep killed at daily intervals.
CHAPTER III

Observations on the pathogenesis of bluetongue virus infection in sheep:

Viraemia and haematology - in vivo
INTRODUCTION

During viraemia, bluetongue virus is closely associated with the cellular fraction of blood (Pini et al., 1966; Luedke, 1970). Other members of the bluetongue group e.g. epizootic haemorrhagic disease of deer virus and African horse sickness virus have also been shown to be associated with the cellular components of blood during a viraemia. (Ozawa et al., 1972; Hoff and Trainer, 1974; Gibbs and Lawman, 1977). Bluetongue virus has been reported to be associated with both the erythrocyte (Luedke, 1970) and leucocyte fractions (Pini et al., 1966). No evidence is available to show whether the association with the erythrocyte is intracellular – as is the case with Colorado tick fever virus, a related virus (Emmons et al., 1972) or which type of leucocyte is associated with bluetongue virus during viraemia.

Information on this feature of the pathogenesis of bluetongue is required to support investigations on the mechanism of virus persistence in ruminants and infection of the arthropod vector.

MATERIALS AND METHODS

Animals and experimental design

Four pedigree Dorset horn sheep, 1 year old and female, were inoculated intradermally into the left ear with approximately 100 TCID_{50} of bluetongue virus type 4 (Cyprus 1969). The passage history of the virus was:- isolation in the chick embryo, 7 passages in BHK/21 and 2 passages in sheep. The sheep were examined daily for clinical disease.

For comparative studies, 4 goats and 2 Friesian heifers were inoculated with virus as above. The sheep, goats and cattle were housed separately. Blood samples (40 ml) were removed from the jugular vein at daily intervals.
Fractionation of blood

Forty ml of heparinised blood were withdrawn from the jugular vein into syringes. Ten ml of blood were removed and used for routine assay of viral infectivity of the erythrocyte and buffy coat fractions using a simple procedure as described by Gibbs and Lawman (1977) for epizootic haemorrhagic disease of deer virus.

The remaining 30 ml of blood were centrifuged (200 g, 30 mins at 4°C) and the leucocyte fraction (buffy coat) was removed. The buffy coat sample was diluted with 5 ml of Hanks medium containing yeast and lactalbumin hydrolysate (LYH), layered onto a 3 ml gradient of ficoll/hypaque (at a density of 1.0774 g/cm^3 at 25°C) (see Appendix I) and centrifuged (200 g, 20 mins at 4°C). The cells at the interface of the gradient and LYH medium were removed and contaminating erythrocytes were lysed with sterile distilled water (Fig. 3.1). The resulting leucocyte suspension was washed 3 times with LYH medium and finally resuspended in Rose/Park Memorial Institute (RPMI) 1640 growth medium (Appendix II).

The cells were incubated in glass petri dishes for 2 hours in an incubator gassed with CO_2 (5% in 95% air) after which the non-adherent cells were removed by 3 washes of LYH medium, centrifuged and resuspended in RPMI 1640 growth medium. These cells were termed lymphocytes. The adherent cells were incubated with versene trypsin for 20 minutes, then removed using a rubber scraper from the glass surface. These cells were termed monocytes.

The cells at the base of the gradient were removed, the erythrocytes lysed and the resulting leucocytes — termed neutrophils — were washed 3 times in LYH medium and finally resuspended in RPMI 1640 growth medium as above.

The purity of each of the sub population was checked by staining a cytospin smear with Giemsa (Appendix II).
Purified populations of erythrocytes were obtained by washing the erythrocytes in PBS 3 times, discarding the top 1 ml after each wash.

**Assay of viral infectivity**

Infected buffy coat cells were collected for assay on days 2, 4, 6 and 8 post infection and fractionated as above.

The cell concentrations were estimated by the method of dye exclusion in a Fuchs-Rosenthal counting chamber; each of the suspensions was then diluted to give $1.0 \times 10^6$ cells/ml and subjected to serial 10 fold dilution in phosphate buffered saline before assay for viral infectivity. The virus titre in the cell suspensions was assayed in microplates, inoculating 0.1 ml per well and using 4 wells per dilution. BHK/21 cells were added in suspension at a concentration of $5 \times 10^5$/cells. The microplates were read daily for 5 days for cytopathic effect and the titres expressed as the minimum number of cells with which virus was associated or were calculated by Kärber (1931) and expressed as $\log_{10}$ tissue culture infective doses at the 50% end point per ml ($\log_{10} \text{TCID}_{50}/\text{ml}$).

**Thin section electron microscopy**

Washed erythrocytes and the various leucocyte populations were fixed in 1% glutaraldehyde and prepared for thin section electron microscopy by the method of Oshiro et al., (1978) (Appendix II). Sections were cut and stained using saturated uranyl acetate and lead citrate and were examined using a Philips 301 electron microscope.

**Haematology**

Total and differential leucocyte counts were made each day by conventional techniques for each experimental animal from 7 days before inoculation of virus until 10 days after inoculation.
RESULTS

Sheep

Clinical disease and haematology

No clinical disease was observed although pyrexia was detected between 6 and 8 days after virus inoculation. The predominant haematological feature was a pan-leucopenia that was at its maximum between 4 and 7 days after inoculation. Data for one sheep are shown in Fig. 3.2 a.

Virus isolation

The techniques used to separate the buffy coat into the different cell types within the leucocyte population gave consistent results irrespective of the stage of infection, and the purity of the cell populations obtained is shown in Table 3.1.

The titre of the maximum viraemia in the sheep was $4.5 \log_{10} \text{TCID}_{50}/\text{ml}$ (Fig. 3.2 b and Table 3.2) with peak pyrexia occurring on day 7. The virus was associated with the cellular fraction of the blood (Fig. 3.2 b). Ninety per cent of virus in whole blood was associated with the erythrocyte fraction whereas plasma only contained trace amounts.

Data on the number of cells within the leucocyte series that was infected with bluetongue virus are given in Table 3.3. The numbers of infected leucocytes were low within each cell population and there was no significant difference in virus content of the different cell types.

Electron microscopy

No virus was observed by thin section electron microscopy in any of the leucocyte cell populations or erythrocytes examined.

Cattle and goats

Clinical disease and pyrexia were not observed in the cattle or
goats, but a viraemia and a pan-leucopenia were detected (Table 3.2 and Figs. 3.3 a,b and 3.4 a, b). It is interesting to note that the maximum leucopenia in goats and cattle occurs between 5 and 7 days post inoculation (similar to the sheep). The viraemia, however, differs; in goats, maximum viraemia occurs 8 days post inoculation whilst in cattle, by day 10, the viraemia is still increasing. As had been observed with the infected sheep, the virus was principally associated with the cellular fraction of the blood but the number of infected cells within the sub-populations of the leucocyte series was low.

DISCUSSION

The affinity of bluetongue virus with the cells present in the blood of infected sheep as shown in these experiments is in agreement with previous reports (Pini et al., 1966; Luedke, 1970). There was no significant difference between sheep, cattle and goats in the distribution of bluetongue virus between the erythrocyte and buffy coat fractions. Most of the virus was associated with the erythrocytes. The association of another Orbivirus - Colorado tick fever virus - with the erythrocytes of infected rodents and man has been recognised for several years (Emmons et al., 1972; Hughes et al., 1974). Emmons and his colleagues (1972) demonstrated that Colorado tick fever virus was intraerythrocytic, in viraemic animals, and Oshiro et al., (1978) published evidence that the virus replicates with erythropoietic cells. When virus replication is concurrent with differentiation of the infected erythroblast, infected erythrocytes are released into circulation.

Various attempts in this study by electron microscopy and immuno-fluorescent techniques to demonstrate a similar pathogenesis for bluetongue virus failed; but Emmons and his colleagues stressed the
difficulty they experienced in demonstrating the presence of virus in erythrocytes and their stem cells. So this failure does not invalidate the proposal that bluetongue virus, as with Colorado tick fever virus, replicates within the haemopoietic system.

In addition to the erythrocyte fraction the association of bluetongue virus with the buffy coat of blood of infected sheep has been previously reported (Pini et al., 1966) but no attempt was made to identify whether any particular cell type was important. The fractionation of the infected leucocyte population into neutrophils, monocytes and lymphocytes in an attempt to identify which cell type, if any, was associated with bluetongue virus lead to no firm conclusions. It was disappointing that the techniques for cell separation were not sufficiently specific which meant that although the preparations were relatively pure, the number of infected cells recorded for each cell population was below that of the contaminating cells so that it was not possible to say which of the cell populations were infected. Had the titre of viraemia been higher as shown by Wardley and Wilkinson (1977 b) in a parallel study with African swine fever virus — where titres in blood reached 9.0 log_{10} TCID_{50}/ml — then there would probably have been a greater number of infected cells and the identity of which type of leucocyte supported growth of bluetongue virus would have been simplified.

The demonstration of a pan-leucopenia which is a consistent feature of infection with bluetongue virus in cattle, sheep, goats and wild ruminants even in the absence of clinical disease (Luedke et al., 1964; Vosdingh et al., 1968; Gleiser et al., 1969; Murray and Trainer, 1970; Barzilai and Tadmor, 1972; Luedke and Anakwenze, 1972) supports the proposal that bluetongue virus replicates not only in the stem cells of the erythrocyte series but also in leucocytes or in their stem cells.
During a virus infection, a leucopenia may be caused either by
1) sequestration of leucocytes in tissues where the virus is
replicating or 2) by replication and cytolytic infection of either
the leucocytes present in the circulation or in the stem cells
present in bone marrow and elsewhere.

When clinical bluetongue disease is present some sequestration
of leucocytes does occur (Moulton, 1961), but no histological
evidence (A. Rowlands, 1978 unpublished observation) of sequestration
of leucocytes exists for sub-clinical disease. It is possible that
bluetongue virus produces the leucopenia by replicating in the
leucocytes and/or in the stem cells within the haemopoietic system.
CHAPTER III

Tables and Figures
Table 3.1  Mean percentage purity and range in cell populations derived from sheep blood

<table>
<thead>
<tr>
<th>% purity</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
<th>Erythrocytes</th>
</tr>
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<tbody>
<tr>
<td>mean</td>
<td>93.5</td>
<td>97.7</td>
<td>98.0</td>
<td>100.00</td>
</tr>
<tr>
<td>range</td>
<td>(89.0-96.0)</td>
<td>(97.0-99.0)</td>
<td>(96.0-99.0)</td>
<td>(100.00)</td>
</tr>
<tr>
<td>predominant</td>
<td>Monocyte</td>
<td>Neutrophil &amp; Lymphocyte</td>
<td>Monocyte</td>
<td>-</td>
</tr>
<tr>
<td>contaminant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2 Whole blood viraemia in sheep, goats and cattle after intravenous inoculation of $2.0 \log_{10} TCID_{50}$ of bluetongue virus type 4

<table>
<thead>
<tr>
<th>Animal species/no.</th>
<th>Days post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Sheep No. 1</td>
<td>0.4*</td>
</tr>
<tr>
<td>Sheep No. 2</td>
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</tr>
<tr>
<td>Sheep No. 3</td>
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</tr>
<tr>
<td>Sheep No. 4</td>
<td>0.5</td>
</tr>
<tr>
<td>Goat No. 1</td>
<td>+</td>
</tr>
<tr>
<td>Goat No. 2</td>
<td>-</td>
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<tr>
<td>Goat No. 3</td>
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<tr>
<td>Goat No. 4</td>
<td>-</td>
</tr>
<tr>
<td>Cattle No. 1</td>
<td>-</td>
</tr>
<tr>
<td>Cattle No. 2</td>
<td>-</td>
</tr>
</tbody>
</table>

* $\log_{10} TCID_{50}$/ml of blood

+ $\leq 10^{0.3}$ TCID$_{50}$/ml of blood
Table 3.3 Association of bluetongue virus with cells removed from infected sheep blood

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Days post inoculation</th>
<th>Whole blood titre</th>
<th>Number of infected cells per $10^6$ cells assayed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neutrophils</td>
</tr>
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<td>6</td>
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<td>1.0</td>
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<td>3.2</td>
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<td>3.4</td>
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<td>&lt;1.0</td>
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<td>3.8</td>
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<td>0.75</td>
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<tr>
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<td>8</td>
<td>4.2</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* $\log_{10} \text{TCID}_{50}/\text{ml of blood}$
+ $\log_{10}$
Figure 3.1 Schematic diagram of ficoll/hypaque separation of leucocytes.
FICOLL/HYPAQUE SEPARATION OF LEUCOCYTES

LEUCOCYTE SUSPENSION

FICOLL/HYPAQUE (1.0774 g/cm³ at 25°C)

CENTRIFUGE
200g, 20mins
4°C

MONOCYTE LAYER
LYMPHOCYTE LAYER
NEUTROPHIL LAYER
ERYTHROCYTE LAYER
Figure 3.2 a Alteration in blood cell dynamics of one sheep following infection with bluetongue virus Type 4.

Cells studied were total leucocyte population, lymphocytes, monocytes, neutrophils and eosinophils.
Total cell count ($\times 10^6$/ml) vs. Days post inoculation

LEUCOCYTE
Total cell count ($10^6$/ml) vs Days post inoculation for LYMPHOCYTE and MONOCYTE.
Total cell count (x10^6/ml)

NEUTROPHIL

Total cell count (x10^6/ml)

EOSINOPHIL

Days post inoculation
Figure 3.2b  The distribution of virus in the blood, and temperature response of one sheep infected with bluetongue virus Type 4.

(●—●) whole blood
(☆—☆) erythrocyte fraction
(○—○) buffy coat (leucocyte) fraction
(☆—☆) plasma
Figure 3.3 - Alteration in blood cell dynamics of one goat following infection with bluetongue virus Type 4.
Cells studied were total leucocyte population, lymphocytes, monocytes, neutrophils and eosinophils.
LYMPHOCYTE

Total cell count (x10^6/ml)

Days post inoculation

MONOCYTE

Total cell count (x10^6/ml)

Days post inoculation
**NEUTROPHIL**

Total cell count ($\times 10^6$/ml)

Days post inoculation

**EOSINOPHIL**

Total cell count ($\times 10^6$/ml)

Days post inoculation
Figure 3.3 b  The distribution of virus in the blood, and temperature response of one goat infected with bluetongue virus Type 4.

(●—●) whole blood
(●—●) erythrocyte fraction
(○—○) buffy coat (leucocyte) fraction
(★—★) plasma
Figure 3.4a Alteration in blood cell dynamics of one heifer following infection with bluetongue virus Type 4. Cells studied were total leucocyte population, lymphocytes, monocytes, neutrophils and eosinophils.
Total cell count ($\times 10^6$/ml)

Days post inoculation

LEUCOCYTE
LYMPHOCYTE

MONOCYTE

Days post inoculation

Total cell count (x10^6/ml)
Figure 3.4 b The distribution of virus in the blood, and
temperature response of one heifer infected with
bluetongue virus Type 4.

(●——●) whole blood
(★——★) erythrocyte fraction
(○——○) buffy coat (leucocyte) fraction
(★——★) plasma
CHAPTER IV

Observations on the pathogenesis of bluetongue virus infection in sheep:

Replication of bluetongue virus in organ culture
INTRODUCTION

Organ culture is the maintenance in vitro of either embryonic rudiments or small portions of organs or tissues during which their fundamental structure remains intact and they retain their natural susceptibility to infection. The techniques of organ culture and its use in virology as both a method of growing viruses and as a tool for studying pathogenesis of infection has been extensively reviewed by Hoorn and Tyrrell (1969).

Other authors have used organ cultures as methods for studying growth and behaviour of viruses in tissues and organs - human respiratory viruses (Hoorn and Tyrrell, 1965); bovine rhinotracheitis virus (Shroyer and Easterday, 1968); human enteroviruses (Rubinstein and Tyrrell, 1970); influenza virus (Basarabs and Smith, 1970); porcine enteroviruses (Darbyshire and Collins, 1971); foot and mouth disease virus (Williams and Burrows, 1972); swine vesicular disease virus (Mann, unpublished observations).

The study of the growth of bluetongue virus in organ cultures from sheep was undertaken to give an indication as to which tissues or organs bluetongue virus would replicate in and therefore give an idea as to the probable replication sites in early stages of infection.

MATERIALS AND METHODS

Virus

Bluetongue virus type 4 isolate from Cyprus 1969. Passage history was 2 egg passages and 7 passages through BHK/21 cells.

Tissues and organs removed

The tissues and organs taken from 9 healthy Dorset horn lambs aged 1 week old for study in organ culture are shown in Table 4.1.
<table>
<thead>
<tr>
<th>Haemopoietic tissues and organs</th>
<th>Vascular tissues and organs</th>
<th>Other tissues and organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parotid</td>
<td>Heart muscle</td>
<td>Muscle</td>
</tr>
<tr>
<td>Retropharyngeal</td>
<td>Tricuspid valve</td>
<td>Skin</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>Bicuspid valve</td>
<td>Tongue</td>
</tr>
<tr>
<td>Prescapular</td>
<td>Iliac</td>
<td>Thyroid</td>
</tr>
<tr>
<td>Bronchial</td>
<td>Aorta</td>
<td>Trachea</td>
</tr>
<tr>
<td>Inguinal</td>
<td>Carotid artery</td>
<td>Adrenal</td>
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<td>Popliteal</td>
<td>Vena cava</td>
<td>Kidney</td>
</tr>
<tr>
<td>Mandibular</td>
<td></td>
<td>Pancreas</td>
</tr>
<tr>
<td>Precrural</td>
<td></td>
<td>Glossopharynx</td>
</tr>
<tr>
<td>Post cervical</td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Tonsil</td>
<td></td>
<td>Intestine</td>
</tr>
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<td>Spleen</td>
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<td>Dental pad</td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td>Ovary</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td>Uterus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mandibular salivary gland</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Parotid salivary gland</td>
</tr>
</tbody>
</table>

**Organ culture method**

Small portions of the tissues and organs were aseptically removed from a freshly slaughtered lamb and placed in phosphate buffered saline containing 1% (final concentration) antibiotic solution. Each tissue segment (approximately 0.1gm) was cut into 9 mm square pieces and sets of 3 were placed into McCartney bottles which contained 10 ml of Eagle's maintenance medium. To allow for
temperature equilibrium, cultures were incubated at 37°C for 2 hours before inoculation with bluetongue virus.

**Inoculation procedure**

The organ cultures were removed from 37°C, the medium decanted and 1 ml of virus suspension (4.0 log_{10} TCID_50/ml) was added (5.0 log_{10} TCID_50/ml for ovary and uterus). The organ cultures were replaced at 37°C for an adsorption period of 1 hour. After 1 hour the inoculum and extracellular virus was removed either by 1) the addition of 5 ml of an acid buffer (pH 6.0) for 30 minutes (which would inactivate extracellular virus) followed by 3 rinses with 10 ml of a neutral buffer, so as to neutralise the acid buffer; or 2) the explants were simply rinsed in phosphate buffered saline (pH 7.2) 5 successive times. Finally, 10 ml of Eagles maintenance medium was added to each of the organ explants which were then incubated at 37°C.

**Growth curves**

At daily intervals, the medium from each of the 3 bottle cultures was removed and pooled. Fresh medium (10 ml) was added and the cultures were reincubated. The sampling was maintained for 9 days post infection. The pooled samples for each day were diluted in log_{10} dilution steps and 0.1 ml of the dilutions inoculated onto BHK/21 cell monolayers grown in microplates. The plates were examined daily for 6 days for development of cytopathic effects. The titres were calculated using Karber's (1931) method and expressed as log_{10} tissue culture infective doses at the 50% end point per gram (log_{10} TCID_50/0.1 gm).

**Survival of virus in support media**

Thermal inactivation of virus at 37°C over the period of the growth curve in organ culture was tested in 5 types of protein containing medium. Two of the controls contained 0.1 g of cell
debris from BHK/21 cells or from lamb liver powder. Two further controls contained foetal calf serum and BHK/21 lysate and were used at a concentration of 10%. The final control used was Eagles medium without supplements as this was the medium used in all the organ culture experiments.

**Histological procedure**

The explants were fixed with 10% formol saline, sectioned by conventional techniques and stained with haematoxylin and eosin and were examined for the retention of the overall architecture and the viability of the cells within the explants.

**RESULTS**

a) **Histology**

In most explants, even though some dead cells were present, the basic architecture of the organ was retained for at least 3-4 days post culture except for lung, liver, trachea, which altered within 2-3 days post culture. Despite some changes in the architecture however, a high proportion of the cells remained viable for up to 6 days post culture in the majority of tissues. There was leaching of cells into the maintenance medium with some explants particularly the lymph nodes, bone marrow, liver and spleen.

b) **Growth curves**

Bluetongue virus replicated in most tissues, (the results shown in Figs. 4.1 - 40 represent the mean of nine experiments) with peak titres of $4.0 \log_{10} \text{TCID}_{50}$/0.1 gm occurring between day 4 to 7 post inoculation. Very little virus, however, was recovered from skin, liver, glossopharynx and heart muscle between 2-6 days post culture. The intestine only produced significant virus on day 7 post culture. All the tissues of the haemopoietic system supported the growth of bluetongue virus in particular the various lymph nodes. In the
control, containing maintenance medium only, most virus was lost by the third day; only trace virus (0.5 log$_{10}$ TCID$_{50}$/ml) was found on day 6 and no virus was detected from 7-9 days post culture (Table 4.2). No virus was detected after day 4 in the control containing the BHK/21 lysate. Two logs of virus, however, were recovered up to and including day 8 in the control containing cell debris. Reduced amounts of virus were recovered in the controls containing liver powder and foetal calf serum. Trace virus was detectable in these controls on day 8.

**DISCUSSION**

The rationale for not using serum supplement in the maintenance medium was to prevent selective survival and outgrowth of fibroblastic cell types and thus alter the structure of the explant. Williams and Burrows (1972) found no improvement in the survival of explants in cultures, either maintained or those with additions of various supplements including serum. When cells in the explant adapt to the environment there is usually a loss of specialisation and non differentiated cell types predominate under these conditions; therefore the relationship between organ culture and the tissues of the animal become tenuous (Williams and Burrows, 1972). Hoorn and Tyrrell (1965) considered that the virus specificity of organ cultures is more akin to that of in vivo than monolayer cultures of that particular organ. The susceptibility of organs to virus infections may be influenced by inducers such as hormones, interferon and immune mechanisms and therefore must be considered in the interpretation of the in vitro responses of organ culture to virus infection.

Although some cells within most of the explants in this study died, some at a faster rate than others, many organs or tissues apparently supported bluetongue virus replication. The relatively low virus growth in various organs could be interpreted as virus
persistence, virus elution from cells or the release of aggregates of virus from cellular material. The results do, however, reflect the low virus titres usually recorded in the blood from infected animals and also the low virus concentration isolated during sequential slaughter from tissues and organs.

The thermal inactivation of bluetongue virus at 37°C over the same period showed the virus to be significantly inactivated over the 9 day period. Most of the virus inoculum was lost by day 3 and the virus was not detectable by day 7-8. There are also tissues and organs which showed no significant increase in virus titre. This suggests that in those tissue explants where an increase in virus titre was recorded, the virus replicated. It is worth noting that all 10 ml of the medium was harvested at each time interval and was replaced with 10 ml of fresh maintenance medium. The amount of virus recorded over the 9 days was far in excess of the input titres.

In conclusion, the above results and those from Chapters 2 and 3 support the proposal that bluetongue virus replicates in lymphoreticular tissues and those containing an endothelial layer, and once infection is systemic, other tissues and organs are capable of supporting the replication of bluetongue virus.
CHAPTER IV

Tables and Figures
Table 4.2 Thermal inactivation of bluetongue virus in various media containing protein

<table>
<thead>
<tr>
<th>Days post inoculum</th>
<th>Lamb liver powder*</th>
<th>BHK lysate+</th>
<th>BHK cell debris</th>
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<th>Foetal calf serum*</th>
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</table>

*liver powder dried in acetone 0.1 gm in 10 ml media

+ supernatant only - cell debris removed. Used at 10%

*0.1 gm of cell debris from above

*Eagles basal medium no supplements

*used at 10%

**log_{10} TCID_{50}/ml

++ < 10^{-3} TCID_{50}/ml
Figure 4.1 - 4.40 Multiplication of bluetongue virus Type 4 in organ cultures obtained from 1 week old Dorset horn lambs.

(Note) LN = lymph node
Days post inoculation

PAROTID LN

Log_{10} TCID_{50} / 0.1 gm.

RETROPHARYNGEAL LN

MESENTERIC LN

Days post inoculation
THYMUS

BONE MARROW

HEART MUSCLE

Days post inoculation

Log\textsubscript{10} TCID\textsubscript{50} / 0.1 gm.
LUNG

Log TCID\textsubscript{50} /0.1gm.

Days post inoculation

LIVER

INTESTINE
Days post inoculation

Log$_{10}$ TCID$_{50}$ / 0.1gm.

AORTA (UPPER)

AORTA (MIDDLE)

AORTA (LOWER)
Days post inoculation
Days post inoculation

OVARY

UTERUS

Log_{10} TCID_{50}/0.1gm.
CHAPTER V

Observation on the pathogenesis of bluetongue virus infection in sheep:-

The growth of bluetongue virus in cells derived from the haemopoietic system of sheep, goats and cattle
In previous sections the pathogenesis of bluetongue virus infection in sheep was studied by sequential slaughter, viraemia and haematology and organ culture. The results indicate that the primary site of the replication of bluetongue virus is in the haemopoietic system.

In this section an in vitro investigation of the replication of bluetongue virus within cells of the haemopoietic system derived from sheep, goats and cattle is reported.

MATERIALS AND METHODS

Virus

The virus used was bluetongue virus type 4 isolated from Cyprus (1969). The passage history was 2 passes, intravenously, in fertile eggs with 7 subsequent passages through BHK/21 cell monolayers.

Cell cultures

The cell cultures and the methods for preparation are described in Appendix I.

Growth curves - virus assay

Growth curves of bluetongue virus were set up at both high and low multiplicities of infection. Low multiplicity was tested because some of these cells were phagocytic and therefore might be able to eliminate the virus inoculum at a low concentration. High multiplicity was at a ratio of \( \frac{1 \text{ TCID}_{50}}{\text{cell}} \) and low multiplicity was at a ratio of \( \frac{1 \text{ TCID}_{50}}{10^4 \text{ cells}} \). All cell cultures were seeded at a final cell concentration of \( 1 \times 10^6 \) cells in both bottle cultures and microplate cultures. Inoculated cells were incubated for a 1 hour adsorption period at 37°C. Following this the cultures were washed 5 times with 20 ml phosphate buffered saline to remove the inoculum, after which fresh RPMI 1640 maintenance medium (15 ml) was added. All the growth
curves were carried out at 37°C. The medium was replaced with fresh medium daily. The spent medium was used for tests of viral infectivity. All cultures, where possible, were examined for cytopathic effect or for increase of non-viable cells present in the culture. The viability of cells in suspension was tested by dye exclusion. Samples were taken at daily intervals, before fresh medium was added to the cultures, and the virus infectivity was assayed. Samples were diluted in log_{10} dilution steps and 4 tubes or 4 wells (microplates) were used per dilution. The titrations were read daily for 7 days and the titres calculated by Karber's (1931) method and expressed as log_{10} tissue culture infective doses at the 50% end point per ml (log_{10} TCID_{50}/ml).

A minimum of 4 experiments was performed for each cell type. All graphs, of virus growth curves in the various cell populations, depict results typical of each group of experiments.

**Growth curves - thin section electron microscopy**

Monocytes, macrophages, neutrophils and lymphocytes were prepared for examination by thin section electron microscopy immediately before infection and at 5, 15 and 24 hours post infection. The samples were fixed in 1% glutaraldehyde and prepared for thin section electron microscopy as described by Oshiro et al. (1978). All thin section samples were examined using a Philips 301 electron microscope, after staining with saturated uranyl acetate and lead citrate.

**Growth curves - fluorescent antibody technique**

The growth of virus in the haemopoietic cells was also monitored by the indirect fluorescent antibody technique. Growth curves were set up at high and low multiplicity of infection. The cells were, if possible, grown in cover slips; otherwise infected cells were centrifuged onto glass slides using a haematocrit centrifuge. (Appendix II). Samples were taken at 0, 3, 5, 15, 20 and 24 hours and then daily
for 13 days. The cells were fixed in acetone at -20°C, incubated with hyperimmune bluetongue antisera and stained with antispecies conjugate, using the method described by Pini et al. (1968). Any fluorescence observed was scored as a percentage of the total number of cells in any one field observed.

RESULTS

I SHEEP CELLS

A. MIXED POPULATION OF CELLS

1. Lymph node suspension

The cell suspension obtained from the lymph node (prescapular) appeared to support the growth of bluetongue virus (Fig. 5.1 A and B) whether inoculated with high or low multiplicities of infection.

2. Bone marrow and spleen cultures

Virus replicated to high titres in both bone marrow and spleen cultures at both high and low multiplicities of infection (Fig. 5.2 A and B and Fig. 5.3 A and B). Virus was also detected to high titre in bone marrow and spleen cultures that had been previously subjected to rapid lysis to remove the erythrocytes. The results obtained were similar to those shown for the whole bone marrow and spleen cultures.

B. SPECIFIC CELL POPULATIONS

1. Peripheral blood

No growth of virus was recorded in whole blood or erythrocyte cultures. Some growth of virus in buffy coat cells was recorded over a period of 6 days (Fig. 5.4 A and B).

(i) Lymphocytes

The sensitivity of the sheep lymphocytes to bluetongue virus infection was difficult to assess by use of virus assay, fluorescent antibody and thin section electron microscopy. In the growth curve
study (Table 5.1) there was no significant difference between the viability of infected lymphocytes and the non-infected controls since there was a rapid decline in the number of viable cells in both the cultures and only small increases in virus titres were observed in the infected lymphocytes. Furthermore, no virus was observed in cells under thin section electron microscopy nor was fluorescence observed in infected lymphocytes.

(ii) Monocytes

Virus grew to high titre in peripheral blood monocytes inoculated at high and low multiplicities of infection (Fig. 5.5 A and B) and caused a cytopathic effect. This reached its maximum cytopathic effect 48-72 hours post inoculation at high multiplicity but not until after 96 hours at low multiplicity.

(iii) Macrophages

Macrophages formed from peripheral blood monocytes in in vitro culture were also sensitive to bluetongue virus at both high and low multiplicities of infection (Fig. 5.6 A and B) and showed full cytopathic effect as early as 48 hours post inoculation. The growth of virus in peripheral blood macrophages was examined by thin section electron microscopy. Evidence of virus growth in macrophages was seen (Fig. 5.7, 5.8). Virus factories were observed in the cytoplasm close to the nuclear membrane.

Studies on the growth of bluetongue virus in macrophages by the indirect fluorescent antibody test gave further evidence of virus replication in these cells. Perinuclear inclusions were observed and the percentage fluorescence is shown in Table 5.2.

2. Mammary gland

(i) Neutrophils

Neutrophils supported the growth of bluetongue virus at both high and low multiplicities of infection (Fig. 5.9 A and B), but
the presence of virus within these cells could not be demonstrated by thin section electron microscopy. In comparison with control cells, however, infected cells showed marked degeneration of cytoplasmic and nuclear structure. No virus inclusions were observed in infected neutrophils when examined by indirect immunofluorescence.

(ii) Macrophages

Macrophages infected with virus gave similar results to those obtained with established peripheral blood macrophages (Fig. 5.10 A and B).

3. Lung

(i) Alveolar macrophages

Alveolar macrophages (primary isolation) inoculated with virus at high multiplicity of infection showed similar growth patterns and cytopathic effect (Fig. 5.11 A and B).

In established alveolar macrophages similar growth patterns were recorded to those of the established macrophages of the peripheral blood (Fig. 5.12 A and B).

4. Bone marrow and spleen

Macrophages isolated and grown in vitro from bone marrow and spleen were shown to support the replication of virus at high and low multiplicities of infection and also produced cytopathic effects (Fig. 5.13 A and B, and 5.14 A and B).

5. Thymus

At high multiplicity (Fig. 5.15 A) virus grew in suspension of thymocytes, maximum virus infectivity titre occurring 48 hours post inoculation, but at low multiplicity (Fig. 5.15 B) maximum titre did not occur until 96 hours post inoculation and was low.

The fibroblastic cells grown from trypsinised thymus, however, gave relatively high yields of bluetongue virus at both high and low
multiplicity of infection (Fig. 5.16 A and B). Maximum titres at high multiplicity occurred 48 hours post infection and at low multiplicity, 96 hours post infection.

6. Cardio-vascular system

Isolated endothelial cells from blood vessels and heart supported the growth of virus at both high and low multiplicity (Fig. 5.17 A and B, 5.18 A and B, 5.19 A and B), and cytopathic effect was observed. There appeared to be no significant difference in the sensitivity of endothelial cells isolated from the various blood vessels or from the heart.

II GOAT AND CATTLE CELLS

(Note: only specific cell populations were examined)

1. Peripheral blood

(i) Lymphocytes

The growth curve studies can be seen in tables 5.3 and 5.4. The results obtained in the goat and cattle lymphocytes were similar to those shown for sheep lymphocytes (Table 5.1).

(ii) Monocytes and macrophages

Virus grew to a high titre in monocytes when inoculated at high multiplicity, and caused a cytopathic effect which reached maximum effect 6 days post infection (Fig. 5.20 A and 5.21 A). At low multiplicity, virus was isolated at a lower level than observed in the sheep monocytes (Fig. 5.20 B and 5.21 B). In cattle monocytes, only trace cytopathic effect was observed and the virus titre, over 6 days, never exceeded $1.5 \log_{10} \text{TCID}_{50}/\text{ml}$. Virus titres in goats were also low (maximum $2.5 \log_{10} \text{TCID}_{50}/\text{ml}$) but a greater amount of cytopathic effect was seen.

Established macrophages from goat peripheral blood monocytes infected at high multiplicity gave similar results to those obtained
with sheep peripheral blood macrophages (Fig. 5.22 A). At low
multiplicity however, the titres of virus were lower, and more
significantly, maximum cytopathic effect was not evident until 16
days post inoculation; between 1 and 9 days post inoculation only
trace cytopathic effect was evident (Fig. 5.22 B). At the time of
this study, established macrophages from cattle peripheral blood
were not obtained. This was probably due to cultural conditions.
The growth of virus in the blood macrophages of goats was examined
by thin section electron microscopy. No virus factories or virions
were observed. These cells showed, however, marked degeneration of
the cytoplasm and nucleus compared to uninfected control cultures.

Studies on the growth of virus, using the indirect fluorescent
antibody test, in macrophages of goat and cattle showed that at
high multiplicity, perinuclear inclusions were present and a high
percentage of the cells were involved (Table 5.2). At low multi-
PLICITY however, no discrete inclusions were observed in goat and
cattle macrophages; the fluorescence observed was usually in single
cells or in groups of 2 to 3 cells with diffuse fluorescence through-
out the cytoplasm.

2. Mammary gland

(i) Macrophages

A similar picture of virus growth was obtained using goat
macrophages isolated from the mammary gland (Fig. 5.23 A). At low
multiplicity only trace cytopathic effect was evident for 11 days
post inoculation with \( 4.0 \log_{10} \text{TCID}_{50}/\text{ml} \) of virus being recovered
daily up to 18 days post inoculation (Fig. 5.23 B). Bovine
macrophages isolated from the mammary gland of virgin heifers
inoculated at high multiplicity, were susceptible to infection
(Fig. 5.24 A) and maximum cytopathic effect occurred 72 hours post
inoculation. After low multiplicity of infection similar growth patterns to those found with goat macrophages were seen in these macrophages; that is very little cytopathic effect, with a maximum cytopathic effect occurring as late as 23 days post inoculation but with $4.0 \log_{10} \text{TCID}_{50}/\text{ml}$ of virus being released daily for 29 days post inoculation (Fig. 5.24 B).

(ii) Neutrophils

Growth of virus occurred in goats and cattle neutrophils at both high and low multiplicity (Fig. 5.29 A and B, and Fig. 5.30 A and B). Furthermore, examination by thin section electron microscopy could not demonstrate the presence of virus in either cattle or goat neutrophils nor was antigen demonstrated by immunofluorescence.

3. Lung

Alveolar macrophages (primary isolation) prepared from goats and cattle inoculated at high multiplicity of infection showed similar growth patterns with production of cytopathic effect (Fig. 5.25 A and Fig. 5.26 A).

Differences in the growth pattern at low multiplicity of infection with virus occurred, however, when compared to that of sheep. In the sheep alveolar macrophages growth at low multiplicity was similar to that of high multiplicity; whilst at low multiplicity, the growth of virus in goat and cattle alveolar macrophages was to a lower level of virus and reduced cytopathic effect (Fig. 5.25 B and 5.26 B).

In established alveolar macrophages similar growth patterns to those of the peripheral blood macrophages were achieved (Fig. 5.27 A and B and 5.28 A and B); that is at high multiplicity the growth of virus in the goats and cattle was similar with high titres of virus and rapid cytopathic effect being attained, whilst at low
multiplicity, the established macrophages showed a much more prolonged cytopathic effect with significantly lower titres of virus.

4. Cardio-vascular system

Isolated endothelial cells from the blood vessels of goats and cattle supported the growth of virus at both high and low multiplicity (Fig. 5.31 A and B and Fig. 5.32 A and B) and the virus was cytopathic.

DISCUSSION

An important aspect of viral pathogenesis is the host resistance to virus infections. Host resistance is a complex phenomenon and may involve both humoral and cellular responses.

The outcome of virus-leucocyte relationship is important in determining the course of infection. Once the virus is intracellular, within the leucocyte, the final outcome of the infection could be 1) failure to replicate within the leucocyte, the infection becoming abortive, 2) replication of the virus within the cell, thereby permitting the infection to progress to an acute stage or 3) the leucocyte, after failing to inactivate the virus, may carry the virus passively to more distant sites; thus further infection is initiated which may ultimately progress to clinical disease.

It was difficult to assess whether bluetongue virus was capable of infecting and replicating in unstimulated lymphocyte (and thymocyte) cultures because only low titres of virus were found and no virus was observed by electron microscopy studies. The apparent lack of virus replication or low level virus replication within the lymphocytes may account for the good antibody response elicited during bluetongue infections in vivo. It was interesting to note,
however, that despite the low level of virus produced by the thymocytes, the fibroblastic cell type from the thymus was highly susceptible to virus.

Unlike in the lymphocytes, high titres of virus with cytopathic effect were produced in monocytes and macrophages derived from peripheral blood. A significant difference between sheep monocytes and macrophages and the monocytes and macrophages of goats and cattle was observed at low multiplicity of infection. In sheep cells, high titres of virus and rapid cytopathic effect were produced; however, at low multiplicity of infection in goat and cattle cells, virus was recovered at low titres and there was little or no cytopathic effect. This was also found with alveolar and mammary gland macrophages of goats and cattle.

Infected macrophages were maintained for up to 23 days post infection before total cytopathic effect was observed. The interpretation of this result is difficult. It could be considered that resistant clones of cells were present; however, on superinfection of these cultures 100% cytopathic effect was observed within 24-36 hours post superinfection (Lawman, unpublished observations). Perhaps therefore these cells became persistently infected at a low multiplicity and the gradual increase in cytopathic effect was due to the fact that it is impossible to remove all the infectious virus produced when replacing the medium daily; thus a gradual increase in virus concentration occurred resulting in the cells becoming in effect superinfected. If it were possible to remove the extracellular virus it might have been possible to maintain persistently infected cultures of macrophages. Further evidence for persistent infection came from immunofluorescent studies of goat and cattle cells infected with low multiplicity.
Only a small percentage of cells showed fluorescence and these did not produce an intra-cytoplasmic inclusion peripheral to the nucleus, which was the normal fluorescence observed in cells which were highly susceptible to bluetongue virus. Goats in the Middle East and cattle in the United States of America have been implicated in the overwintering cycle of bluetongue virus in those areas (Luedke et al., 1977; Luedke et al., 1977 a and Luedke et al., 1977 b). Although the overwintering mechanism has not been elucidated the results shown here may be an indication of this.

Another interesting observation was the recovery of virus from peripheral blood macrophages of goats 50 days post infection (Lawman, unpublished data). The virus was recovered on two occasions from the macrophages of these goats, and this correlates with the fact that virus has been recovered from the blood of cattle 1783 days after infection (Luedke et al., 1977). Persistence of infection in macrophages was also recorded for bluetongue virus in bovine peripheral blood macrophages (Rossi and Kiesal, 1977).

The growth of bluetongue virus in the bone marrow, spleen and lymph node cultures is probably a reflection of the ability of the virus to grow readily in the sheep monocyte and macrophage isolated from various organs including the lung, bone marrow and spleen. One organ, however, was conspicuous by its absence in the replication of bluetongue (Chapter III and IV) and that was the liver. This was an unexpected result since the liver contains a high proportion of macrophages, namely von Kupffer cells, and during clinical disease is reported to be involved pathologically. During the study of sequential slaughter (Chapter II), bluetongue virus was in general only isolated from the liver once there was a detectable viraemia and, in vitro, liver explants (Chapter IV) did not support the growth of bluetongue virus. Mims (1967) states that the liver
macrophages "are quantitatively the most important cells responsible for the removal of intravenously ingested particles both viral and non-viral". Allison (1974) points out the importance of the liver as a barrier for those viruses that are introduced into the blood stream or those that have a viraemia stage during their replication within the host. This is of particular importance with the arboviruses since they both enter the host via the blood and also involve a viraemic stage.

The failure of a virus to replicate within the liver is usually attributed to the efficiency of the macrophage to act as a barrier to infection of the liver. This is important epidemiologically for arboviruses, since if the liver fails to remove such viruses, the likelihood is that the viruses would be available as infective blood meals for the biting vectors. The failure of the virus to replicate within the liver Kupffer cell, however, does not necessarily mean that the virus is not capable of replication within the hepatocytes of the liver. It has been shown that the CL strain of vaccinia virus, even though it is taken up by the Kupffer cell, is destroyed by the macrophage therefore failing to initiate a replication cycle within these cells; if the CL strain of vaccinia is inoculated however into the bile duct, high concentrations of antigen can be found 10-18 hours post inoculation, in the periportal hepatic cells (Mims, 1964). This has also been shown for influenza virus and myxoma virus in the mouse and ectromelia virus in the rat (Mims, 1964). Mims, during these studies, concluded therefore, that viruses sometimes apparently fail to replicate within the liver hepatocytes because the virus has not had the opportunity to come into contact with these cells since the viruses are rapidly removed by the phagocytic Kupffer cell. This is apparently not the case with bluetongue virus as replication in organ culture was not detected.
Some viruses, for example influenza in mice, are capable of crossing the "macrophage barrier" and infecting hepatocytes without a replication cycle occurring within the macrophage (Mims, 1960). The dose of influenza virus was critical— a sub toxic dose, intravenously, was dealt with by the macrophage, but the Kupffer cells were unable to cope with a larger toxic dose, with virus being able to infect the hepatocyte. The virus infecting the hepatocytes could however come from a) the egestion of intact virus by the Kupffer cell or b) by virus passing through spaces between the Kupffer cell. Rift valley fever virus also behaves in a similar manner to influenza virus (Mims, 1957).

Finally, some viruses on entering the Kupffer cell are capable of replication; this has been reported for ectromelia virus (Mims, 1959). In those studies with ectromelia virus, even with large doses, only small numbers of infective foci in the Kupffer cell were observed. Mims stated that only one in every four Kupffer cells which took up virus became infected; therefore not all liver macrophages are infected with equal readiness and they probably differ in phagocytic activity. The lack of bluetongue antigen and replication in the liver could be attributed to the efficiency of the Kupffer cell in inactivating or destroying bluetongue virus. It would prove interesting to study the liver Kupffer cells and hepatocyte relationship to bluetongue virus both in vitro and in vivo and observe if bluetongue virus does fail to replicate in these cells and this might explain the failure to isolate bluetongue virus routinely from the liver. The in vitro studies would be possible since the methodology for long term cultivation of hepatocytes has been recorded from human liver (Tisquaye et al., 1978) and the growth of Kupffer cells in culture has also been observed (Lawman, unpublished data).
It does not necessarily follow that because one virus from a particular virus group replicates in macrophages the rest of the group will also replicate in these cells. It has been reported that not all herpes viruses for instance will replicate in macrophages; herpes simplex virus growth is restricted in macrophages (Stephens and Cook, 1971) whereas infectious bovine rhinotracheitis virus will replicate in macrophages (Rouse and Babiuk, 1978). A similar finding was reported to the poxvirus group; mousepox fails to replicate in murine macrophages (Blandon, 1971) while cowpox and pseudocowpox will replicate in these cells (Rossi and Keisel, 1977).

The resistance of an animal to infection with a virus has been related to the macrophage, that is macrophages from susceptible animals are themselves susceptible to infection with the agent. However, macrophages removed from a resistant animal are themselves resistant to infection. This phenomenon has been reported for mouse hepatitis virus (Bang and Warwick, 1960), some flaviviruses (Goodman and Kaprowski, 1962) and ectromelia virus (Mims, 1964). Mims and Gould (1978) also reported that the efficiency of murine cytomegalovirus infection in macrophages could be affected depending on whether the macrophages had been previously stimulated or not. The stimulated macrophages were more resistant to cytomegalovirus infection than unstimulated macrophages. In this thesis however, no significant difference was observed in the susceptibility to bluetongue virus in unstimulated macrophages isolated from peripheral blood and those macrophages stimulated with E. coli lipopolysaccharide, during isolation from the udder of sheep, goats and cattle.

Another important consideration in viral pathogenesis is the suggestion that the virulence of a virus strain relates to its ability to grow in macrophages (Allison and Malluci, 1965); that is,
virulent strains are capable of infecting macrophages more readily than attenuated avirulent strains. This has been shown for ectromelia virus (Roberts, 1964) and mouse hepatitis virus (Allison and Malluci, 1965). Mims and Gould (1978) however, report the converse - tissue cultured passaged cytomegalovirus (with a decreased pathogenicity) had an increased ability to infect macrophages when compared to virus from infected salivary glands. A further complication was observed (Lawman, unpublished observations) when bluetongue type 4 attenuated vaccine strain was found to replicate in sheep macrophages as readily as the virulent type 4 bluetongue reported in this thesis.

Mims and Gould (1978) suggest, therefore, that the virulence factor of viruses relates to the ability of the macrophage to cope with the invading virus; that is whether the macrophage will be permissive or non-permissive to the virus replication.

Despite the recorded growth of virus in cultures of neutrophils of the 3 species the results are somewhat difficult to explain. Virus titres in neutrophils appear to be lower than those shown for macrophages. However, it must be remembered that despite the purification procedure for the preparation of the neutrophil population, the purity achieved was only between 98-100%. Therefore in every $10^6$ cells of the purified neutrophil population $10^{4.3}$ cells could possibly be monocyte contaminants. The growth of virus observed in these neutrophil cultures could therefore be interpreted not as growth of bluetongue virus in neutrophils but more probably as growth in contaminating monocytes. Studies by electron microscopy also failed to show evidence of virus present in neutrophil cultures. The growth of virus in buffy coat cells of sheep, goats and cattle is probably due to the presence of monocytes and macrophages in the cultures.
The lack of growth of bluetongue virus in erythrocytes is probably due to the absence of nucleated cells and the lack of virus replication in whole blood may be due to the low number of susceptible cells per ml of whole blood. (The whole blood experiments were conducted with 1/100 diluted blood).

Virus was shown to replicate in endothelial cells causing a cytopathic effect and the release of infectious virus. There appeared to be no difference in susceptibility between the three species of animal. The growth of virus in endothelial cells in vitro might have relevance to the vascular lesions observed in sheep, cattle and goats suffering from acute bluetongue disease; that is the haemorrhage and oedema may be due to the direct replication of virus in the endothelial cells of arterioles and capillaries. This study is in agreement with Stair (1968) who observed bluetongue antigen, by fluorescence, in capillary endothelial cells. Another orbivirus has also been observed to replicate in endothelial cells - epizootic haemorrhagic disease of deer virus was found to be present, by electron microscopy, in endothelial cells of capillaries from infected deer (Tsai and Karstad, 1973).
CHAPTER V

Tables and Figures
Table 5.1 Growth of bluetongue virus in lymphocytes from sheep (Dorset horn)

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>Viability of control lymphocytes as a.%</th>
<th>Viability of infected lymphocytes as a %</th>
<th>Virus titre *SV 6.0</th>
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<td>5</td>
<td>+†</td>
<td>+½</td>
<td>3.0</td>
</tr>
</tbody>
</table>

*SV = starting virus titre
† - no viable cells recorded
Table 5.2 Percentage fluorescence of bluetongue infected macrophages from sheep and goat peripheral blood and cattle mammary gland macrophages

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>Sheep</th>
<th>Goat</th>
<th>Cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>high</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>80*</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>5/40</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>10</td>
<td>10/15</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>10</td>
<td>10/15</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>20/30</td>
<td>20/30</td>
<td>20/25</td>
</tr>
</tbody>
</table>

* percentage fluorescence
<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>Viability of control lymphocyte as a %</th>
<th>Viability of infected lymphocyte as a %</th>
<th>Virus titre *SV 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>100</td>
<td>2.5</td>
</tr>
<tr>
<td>1</td>
<td>55</td>
<td>35</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
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<tr>
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<td>5</td>
<td>-</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*SV = starting virus titre

+ - no viable cells recorded
Table 5.4 Growth of bluetongue virus in lymphocytes from cattle

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>Viability of control lymphocyte as a %</th>
<th>Viability of infected lymphocyte as a %</th>
<th>Virus titre SV 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>2.0</td>
</tr>
<tr>
<td>1</td>
<td>45</td>
<td>50</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
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<td>3.95</td>
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<tr>
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<td>10</td>
<td>≤5</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*SV = starting virus titre

+ - no viable cells recorded
Figure 5.1 Growth of bluetongue virus Type 4 in lymph node suspension cultures from an adult Dorset horn sheep

A - high multiplicity of infection
B - low multiplicity of infection

(---) extracellular virus

Note: cells were in suspension cultures, therefore no cytopathic effect was recorded.
Figure 5.2 Growth of bluetongue virus Type 4 in bone marrow suspension cultures from a Dorset horn lamb

A - high multiplicity of infection
B - low multiplicity of infection

(---) extracellular virus

Note: cells were in suspension culture, therefore no cytopathic effect was recorded.
Figure 5.3  Growth of bluetongue virus Type 4 in spleen suspension cultures from a Dorset horn lamb

A - high multiplicity of infection

B - low multiplicity of infection

(——) extracellular virus

Note: cells were in suspension culture, therefore no cytopathic effect was recorded.
Figure 5.4 Growth of bluetongue virus Type 4 in the buffy coat cells (leucocytes) from peripheral blood of a Dorset horn sheep

A - high multiplicity of infection

B - low multiplicity of infection

extracellular virus

Note: cells were in suspension culture, therefore no cytopathic effect was recorded.
Figure 5.5  Growth of bluetongue virus Type 4 in peripheral blood monocytes from a Dorset horn sheep

A - high multiplicity of infection
B - low multiplicity of infection

(-----) extracellular virus
(----) % cytopathic effect
Figure 5.6  Growth of bluetongue virus Type 4 in established cultures of macrophages derived from peripheral blood monocytes of a Dorset horn sheep

A = high multiplicity of infection
B = low multiplicity of infection

extracellular virus

% cytopathic effect
Figure 5.7 Thin section electron micrograph of established sheep peripheral blood macrophages infected with bluetongue virus Type 4 (2½ hours post inoculation). (VF) virus factory (N) nucleus. (x16,500)
Figure 5.8 Thin section electron micrograph of established sheep peripheral blood macrophages infected with bluetongue virus Type 4 (24 hours post inoculation). (VF) virus factory (N) nucleus. (x50,000)
Figure 5.9  Growth of bluetongue virus Type 4 in neutrophils stimulated in the mammary gland of a Dorset horn ewe.

A - high multiplicity of infection
B - low multiplicity of infection

(—) extracellular virus

Note: cells were in suspension cultures, therefore no cytopathic effect was recorded.
A

Days post inoculation

B

Days post inoculation
Figure 5.10  Growth of bluetongue virus Type 4 in established macrophages derived from monocytes stimulated in the mammary gland of a Dorset horn ewe

A - high multiplicity of infection
B - low multiplicity of infection

(— — —) extracellular virus

(← — —) % cytopathic effect
Figure 5.11 Growth of bluetongue virus Type 4 in alveolar macrophages on primary isolation, from the lung of a Dorset horn lamb

A - high multiplicity of infection
B - low multiplicity of infection

(---) extracellular virus
(----) % cytopathic effect
A

Log$_{10}$ TCID$_{50}$/ml

Days post inoculation

1 2 3 4 5 6 7

B

Log$_{10}$ TCID$_{50}$/ml

Days post inoculation

1 2 3 4 5 6 7

% CPE
Figure 5.12 Growth of bluetongue virus Type 4 in established alveolar macrophages derived from primary cultures of macrophages from the lung of a Dorset horn lamb

A - high multiplicity of infection
B - low multiplicity of infection

(---) extracellular virus
(----) % cytopathic effect
Figure 5.13 Growth of bluetongue virus Type 4 in established macrophages from bone marrow of a Dorset horn lamb

A - high multiplicity of infection
B - low multiplicity of infection

(---) extracellular virus

(-----) % cytopathic effect
Figure 5.14  Growth of bluetongue virus Type 4 in established macrophages from the spleen of a Dorset horn lamb

A  high multiplicity of infection
B  low multiplicity of infection

extracellular virus

% cytopathic effect
Figure 5.15  Growth of bluetongue virus Type 4 in thymocytes  
(T cells enriched) from a Dorset horn lamb  
A - high multiplicity of infection  
B - low multiplicity of infection  
(---) extracellular virus  
Note: cells were in suspension culture, therefore  
no cytopathic effect was recorded.
A

Log_{10} TCID_{50}/ml

Days post inoculation

B

Log_{10} TCID_{50}/ml

Days post inoculation
Figure 5.16 Growth of bluetongue virus Type 4 in fibroblastic cells derived from the thymus of a Dorset horn lamb

A - high multiplicity of infection

B - low multiplicity of infection

(-----) extracellular virus

(---) % cytopathic effect
Figure 5.17  Growth of bluetongue virus Type 4 in cultured endothelial cells isolated from the vena cava of a Dorset horn lamb

A - high multiplicity of infection
B - low multiplicity of infection
(---) extracellular virus
(-----) % cytopathic effect
Figure 5.18 Growth of bluetongue virus type 4 in cultured endothelial cells from the aorta of a Dorset horn lamb

A - high multiplicity of infection

B - low multiplicity of infection

(—) extracellular virus

(−−−) % cytopathic effect
A

Days post inoculation

Log_{10} TCID_{50} / ml

B

Days post inoculation

Log_{10} TCID_{50} / ml
Figure 5.19 Growth of bluetongue virus Type 4 in cultured endothelial cells from the ventricles of the heart of a Dorset horn lamb

A - high multiplicity of infection
B - low multiplicity of infection

(- - -) extracellular virus
(- - -) % cytopathic effect
A

B

Days post inoculation

Log$_{10}$ TCID$_{50}$/ml

Days post inoculation

Log$_{10}$ TCID$_{50}$/ml

% CPE

% CPE
Figure 5.20 Growth of bluetongue virus Type 4 in peripheral blood monocytes from a goat.

A - high multiplicity of infection
B - low multiplicity of infection

(-----) extracellular virus

(-----) % cytopathic effect
Figure 5.21 Growth of bluetongue virus Type 4 in peripheral blood monocytes from a heifer

A - high multiplicity of infection
B - low multiplicity of infection

(—) extracellular virus
(——) % cytopathic effect
Figure 5.22 Growth of bluetongue virus Type 4 in established cultures of macrophages derived from peripheral blood monocytes of a goat.

A - high multiplicity of infection

(——) extracellular virus

(——) % cytopathic effect
Figure 5.22 Growth of bluetongue virus Type 4 in established cultures of macrophages derived from peripheral blood monocytes of a goat.

- low multiplicity of infection

(----) extracellular virus

(-----) % cytopathic effect
Figure 5.23  Growth of bluetongue virus Type 4 in established
macrophages derived from monocytes stimulated in
the mammary gland of a nanny goat
A - high multiplicity of infection
(---) extracellular virus
(-------) % cytopathic effect
Days post inoculation

Log_{10} TCID_{50} / ml

o/o CPE
figure 5.23  Growth of bluetongue virus Type 4 in established macrophages derived from monocytes stimulated in the mammary gland of a nanny goat

$\delta$ - low multiplicity of infection

(---) extracellular virus

(----) % cytopathic effect
Figure 5.24  Growth of bluetongue virus Type 4 in established macrophages derived from monocytes stimulated in the mammary gland of a virgin heifer

A - high multiplicity of infection

(---) extracellular virus

(----) % cytopathic effect
Figure 5.24  Growth of bluetongue virus Type 4 in established macrophages derived from monocytes stimulated in the mammary gland of a virgin heifer

B - low multiplicity of infection

---------- extracellular virus

---------- % cytopathic effect
Figure 5.25  Growth of bluetongue virus Type 4 in alveolar macrophages on primary isolation from the lung of a goat kid.

A - high multiplicity of infection
B - low multiplicity of infection

(—) extracellular virus
(—-—) % cytopathic effect
Figure 5.26  Growth of bluetongue virus Type 4 in alveolar macrophages, on primary isolation, from the lung of a calf

A - high multiplicity of infection
B - low multiplicity of infection

(—) extracellular virus
(→→) % cytopathic effect
Figure 5.27 Growth of bluetongue virus Type 4 in established alveolar macrophages derived from primary cultures of macrophages from the lung of a goat kid

A - high multiplicity of infection

(-----) extracellular virus

(----) % cytopathic effect
Log$_{10}$ TCID$_{50}$/ml vs Days post inoculation

Days post inoculation

0/0 CPE
Figure 5.27  Growth of bluetongue virus Type 4 in established alveolar macrophages derived from primary cultures of macrophages from the lung of a goat kid

B - low multiplicity of infection

(-----) extracellular virus

(-----) % cytopathic effect
Figure 5.28 Growth of bluetongue virus Type 4 in established alveolar macrophages derived from primary cultures of macrophages from the lung of a calf.

A - high multiplicity of infection

(-----) extracellular virus

(••••) % cytopathic effect
Figure 5.28 Growth of bluetongue virus Type 4 in established alveolar macrophages derived from primary cultures of macrophages from the lung of a calf

B - low multiplicity of infection

(—) extracellular virus

(——–) % cytopathic effect
Figure 5.29  Growth of bluetongue virus Type 4 in neutrophils stimulated in the mammary gland of a nanny goat

A - high multiplicity of infection

B - low multiplicity of infection

(——-) extracellular virus

Note: cells were in suspension cultures, therefore no cytopathic effect was recorded.
Figure 5.30  Growth of bluetongue virus Type 4 in neutrophils stimulated in the mammary gland of a virgin heifer

A - high multiplicity of infection
B - low multiplicity of infection

(——) extracellular virus

Note: cells were in suspension cultures, therefore no cytopathic effect was recorded.
Figure 5.31 Growth of bluetongue virus Type 4 in cultured endothelial cells from the carotid artery of a goat kid

A - high multiplicity of infection

B - low multiplicity of infection

(—) extracellular virus

(----) % cytopathic effect
Figure 5.32  Growth of bluetongue virus Type 4 in cultured endothelial cells from the carotid artery of a heifer

A - high multiplicity of infection
B - low multiplicity of infection
(---) extracellular virus
(- - -) % cytopathic effect
CHAPTER VI

Conclusions and areas for further study
CONCLUSIONS AND AREAS FOR FURTHER STUDY

The following observations arise from the study presented in this thesis:-

1. Bluetongue virus replicates in the haemopoietic system:
   a) primary replication occurs in the lymph nodes and other tissues of the lymphoreticular system.
   b) the cells involved in the primary replication are probably the avidly phagocytic cells of the mononuclear phagocytic system, which include the monocytes and macrophages.
   c) endothelial cells of blood vessels and possibly lymph vessels are involved in virus replication.
   d) the less avidly phagocytic cells, the neutrophils, may be involved in the replication of the virus.

2. Monocytes and macrophages are probably involved in the mechanism of persistent infection (carrier state) in cattle and goats.

   From this and previous studies (Stair, 1968; Pini, 1976) it is proposed that the pathogenesis of bluetongue virus in sheep is as indicated in Fig. 6.1.

   Bluetongue virus on penetration via the bites of Culicoides species is probably taken up by phagocytic cells of the mononuclear system and by polymorphonuclear cells. These infected cells either enter a regional drainage lymph node or are carried into the blood circulatory system. The deposition of these infected cells in the lymph nodes initiates a primary viraemia which disseminates the virus to further target organs and also sets up infection in the endothelium of the blood vessels. The replication of bluetongue virus in these target organs and cells would disseminate the virus via a secondary and detectable viraemia to other organs and tissues thereby culminating in a generalised infection with (in some cases)
Figure 6.1 Pathogenesis of bluetongue virus infection in sheep
(Hypothesis)
PATHOGENESIS OF BLUETONGUE VIRUS INFECTION IN SHEEP
(HYPOTHESIS)

DAYS POST INOCULATION

In Vivo

Observation

Cellular

SITE OF INOCULATION

REGIONAL DRAINAGE LYMPH NODE

Lymphatic System

Blood Vascular System

MONOCYTE MACROPHAGE (NEUTROPHIL)

LYMPHATIC SYSTEM

BLOOD VASCULAR SYSTEM

MONOCYTE MACROPHAGES
LYMPHATIC ENDOTHELIAL ERYTHROBLASTS

VASCULAR ENDOTHELIAL CELLS

OTHER TARGET CELLS

ASSOCIATED WITH
ERYTHROCYTE, MONOCYTE MACROPHAGE, NEUTROPHIL

Virus Dissemination

SITE OF INOCULATION

PRIMARY REPLICATION

PRIMARY VIRAEMIA

SECONDARY REPLICATION

SECONDARY VIRAEMIA

(0 - 1)

(1 - 3)

(3 - 4)

(5 - 14)

(5 - 10)
In the 'Aims of the Thesis' the comment was made that basic information on the pathogenesis of bluetongue virus would be useful (if not a pre-requisite) for investigation of the major gaps in our knowledge of bluetongue virus infections.

These major gaps exist within the limits of clinical disease, persistent infection and vaccination. Although discussed previously it is appropriate to reiterate them briefly.

The major problem with bluetongue infection is an explanation of the "expression of clinical disease". In many areas of the world, bluetongue is endemic, remaining sub-clinical or mild whilst in other areas the virus can cause severe clinical disease. A good example of this was the 1956/57 bluetongue type 10 epidemic in the Iberian Peninsular during which 175,000 sheep died and yet type 10 exists in North America without causing great economic loss or concern.

For many years bluetongue was believed to be principally a disease of sheep with cattle and goats showing disease on rare occasions. It has now been recognised that cattle and other ruminants may become infected and become carriers, but without ever showing signs of clinical disease. The persistence of viraemia is of prime importance when considering the epidemiology of the disease. In cattle, viraemia has been recorded to persist for as long as 5 years (Luedke et al., 1977 c), but the mechanism of this persistence is unknown.

Commercial vaccines for bluetongue are based on live attenuated vaccine strains of the antigenic types. Relatively little is known of the pathogenesis of these vaccine strains, their effectiveness and whether these vaccines are capable of setting up persistent infection in reservoir hosts.
The question must now be asked: in which way(s) will the information gained in this study direct future work on these problems?

Probably the two most important observations in this study were the recognition that cells of the mononuclear phagocytic system - i.e. monocytes and macrophage - support the growth of the virus and that endothelial cells are also capable of supporting the growth of the virus.

Recognition of the fundamental importance of the macrophage in immunity and pathogenesis of virus diseases is comparatively recent. (Mims, 1964; Gresser and Lang, 1969; Glasgow, 1970; Mims, 1977). The following notes on these topics, reviewing recent advances, preface comments on how bluetongue virus replicates in the monocyte-macrophage and how this information may influence future research on the above mentioned problems of bluetongue. It is also pertinent at this stage to offer a few comments on the function of endothelial cells as these cells also have relevance and importance in the guidance of future studies of bluetongue infection.

The following notes are compiled from a recent symposium held on the importance of macrophages in immunity (Friedman, 1977; Beller et al., 1977; Pierce and Kapp, 1977; Rosenthal et al., 1977; Unanue, 1977), and from a recent review by Cline and Golde (1979) on haemopoiesis. Macrophages are an important member of a triad of specific cells involved in humoral and cell mediated responses. In contrast to the other two cell types (T cells and B cells) macrophage function is not antigen specific. Macrophages, apart from their phagocytic activity, play a more important role in a variety of immunological responses. They are a heterogeneous population of cells sharing different biochemical, morphological and functional characteristics depending upon their location in the body. In relation to immunity there are three functions of importance. Phagocytosis is considered a primary function of the macrophage for a) the localization and degradation of antigen or b) processing and presentation of antigen to lymphocytes. Macrophages have a critical role to play in the presentation of antigen to the responding T cell and B cell in a highly immunogenic form that will initiate an antibody response. These cells are involved in the presentation of both T cell dependent and independent antigens for the development of antibody
secretory B cells. The macrophage interaction with lymphocytes appears to act either, indirectly, via the secretion of antigen complexed to soluble macrophage products which are cytphilic for lymphocytes or by direct cell interaction via the clustering of lymphocytes around the antigen primed B lymphocyte in contact with an antigen bearing macrophage resulting in cell proliferation and immune response (Neilson et al., 1974; Werdelin et al., 1974). The molecular events during presentation and the subsequent stimulation of lymphocytes is still not clear. Finally, macrophages make and release large numbers of different molecules. The secretory function of these cells is complex, varying with the state of differentiation and activation. The secretory products are enzymes (lysosomes, elastases, collagenases and esterases), molecules involved in primary defence mechanisms (interferon (Glasgow and Habel, 1963; Glasgow, 1965) and complement factors C2, C4, C6 and C7) and a variety of factors that effect lymphocyte function - a) non specific factors that inhibit lymphocyte proliferation b) enhance lymphocyte proliferation and c) facilitates the maturation of thymocytes into T cells.

It can therefore be appreciated that macrophages constitute a diverse population of cells which are involved in many important specific and non specific roles in immunity and hence pathogenesis.

Endothelial cells constitute a single layer of cells lining the lumen of the blood vessel or lymph vessel. In the finer branches of the circulatory system, these cells represent the total structure of these vessels. The endothelial lining covers a vast surface area and because of its position in the host it is in direct contact with the blood and lymph and therefore is probably vulnerable to possible immune and haemostatic mechanisms and disease processes.

The role of the endothelial cell in the prevention of thrombosis is crucial. Moncarda et al. (1976) reported that endothelial cells release an enzyme which is a powerful inhibitor of platelet aggregation and also dilates blood vessels. Thus the endothelial cell possesses a primary function which prevents platelets from adhering to its surface and also sustains the flow of blood by dilation of the vessel. Endothelial cells are capable of taking up foreign particles. The actual process of particle uptake is difficult to define; it has been referred to as phagocytosis or pinocytosis. Since the terminology is not clear it may be suitable to term it endocytosis as this encompasses both types of ingestion. Endocytosis by these cells may be important in the prevention of haemorrhage in thrombocytopenic animals. It has been shown that platelets can be assimilated or even incorporated into endothelial cells to restore vascular integrity (Johnson, 1971; Kitchens and Weiss, 1975). Lymphocytes have also been shown to pass through endothelial cells to gain access to sites of
delayed hypersensitivity (Astrom et al., 1968).

Whilst it may be stated that the functions of the endothelial cells are not fully understood, it is known that these cells, apart from assisting with the integrity of the vascular systems (blood and lymph), play an important role in preventing thrombosis.

CLINICAL DISEASE

Haemorrhage and oedema characterise the clinical disease and pathology of bluetongue, but an adequate explanation of why this should occur is lacking. It is logical to conclude that this could be caused by destruction of the endothelium by virus replication in the endothelial cells. As shown, endothelial cells are susceptible to bluetongue virus. Replication of viruses in endothelial cells is not uncommon and has been recorded for varicella virus (Tyzzer, 1905), hog cholera virus (Seifried and Cain, 1932), infectious canine hepatitis virus (Coffin et al., 1953), parvovirus (Margolis and Kilham, 1970), Sendai virus (Mims and Murphy, 1972), epizootic haemorrhagic disease of deer virus (Tsai and Karstad, 1973), African swine fever virus (Wilkinson and Wardley, 1978), dengue virus (Andrews et al., 1978) and Junin virus (Andrews et al., 1978).

Despite the evidence for bluetongue virus replication in isolated and cultured endothelial cells it must be remembered that this may not reflect the situation present in an in vivo infection or that this represents the major cause of vascular injury. The problem is compounded because we do not understand why severe clinical disease is rarely seen in experimental animals or for that matter in many epidemics. Why this should occur is not known. It may be argued that this is purely a virulence factor, in that the virus type used in experimental studies has often been isolated
in tissue culture and subsequently passaged in this system prior to use in animals; however, work carried out using clinical material from a confirmed bluetongue outbreak in Cyprus, 1977, inoculated experimentally into sheep without any tissue culture passage did not produce clinical disease, nor did using Culicoides, infected with this isolate to infect sheep produce disease (Sellers, Herniman, unpublished data, 1977). This suggests that there are environmental or physiological factors involved in the enhancement of clinical disease. Naturally infected animals may exhibit clinical disease because of extremes of weather, diet or concomitant infection with other ecto or endo-parasites. It was suggested by Stair (1968) that there might be a correlation between the distribution of lesions and temperature gradients within the host. The most severe lesions occurred in tissues exposed to the environment. It has been observed that bluetongue disease is more severe in sheep that had been recently shorn (Neitz and Riemerschmid, 1944).

The replication of viruses such as bluetongue in endothelial cells could be important epidemiologically since this virus relies on insect vector transmission. For such transmission to occur, the viraemia must be of a certain level and duration. Also viruses that have a long intravascular half life have a greater chance of infecting endothelial cells compared to those viruses that are readily cleared from the circulatory system (Mims, 1964). The localisation of antigen in the endothelium may be influenced in two ways. Firstly, the vascular endothelium in certain parts of the body may only be involved. The capillary endothelium of various organs differs in either the number of antigen receptors it has or in the virus' ability to replicate within it. This may be due to localised conditions such as a local inflammatory response or the affinity of endothelium for virus in various regions of the body may differ.
It has been shown that the antigenicity of the organ extract lies in the vascular bed (Pressman and Sherman, 1951). Secondly, the lack of endothelial involvement may be due to efficient clearance of antigen by the reticulo-endothelial system. Benacerraf et al. (1959) showed that carbon would localise in endothelial cells when the reticulo-endothelial system was impaired experimentally.

The involvement of the monocyte-macrophage cells in the pathogenesis of virus disease has been well reviewed by Mims (1964) and (1977), and as these cells are central to many immunological responses, replication in them may be an important factor in the susceptibility of the host and contribute to the pathogenesis of that virus infection. It has been recorded that some viruses will replicate in monocytes and macrophages both in vitro and in vivo:— yellow fever and West Nile virus (Goodman and KOprowski, 1962), ectromelia virus (Roberts, 1964), lymphocytic choriomeningitis virus (Mims and Subrahmanyan, 1966), Aleutian disease agent (Porter et al., 1969), lactate dehydrogenase-elevating virus (Evans, 1970), Germiston virus (Olson et al., 1975), measles virus (Joseph et al., 1975), Semliki forest virus (Van der Groen, 1976), African swine fever virus (Wardley and Wilkinson, 1977 a), parainfluenza-3 (Probert et al., 1977), dengue virus (Halstead et al., 1977; Halstead and O'Rourke, 1977), pox viruses, herpes viruses and enteroviruses (Rossi and Kiesel, 1977), murine cytomegalovirus (Mims and Gould, 1978), rinderpest and peste des petits ruminants virus (Lawman, unpublished data).

Since bluetongue virus grows in the monocytes and macrophages the question arises, in which way does this finding influence clinical disease. At this time there is a lack of published information on the involvement of monocytes and macrophages in clinical disease of bluetongue, and therefore, this area is worth further investigation. The functions of the reticulo-endothelial
system may be suppressed by virus replication within the monocytes and macrophages and this could reduce the efficiency of this system in preventing virus replication and spread and this, therefore, would increase the chance of endothelial cells becoming infected and destroyed. Similarly macrophage secretory products may suffer because of virus replication and hence, complement factors, interferon and factors influencing lymphocyte function could all be reduced. The above hypotheses are based on the assumption that the haemorrhage and oedema associated with bluetongue are caused principally by mechanical defects in the integrity of the vascular system. This hypothesis is probably too simple and the interaction of monocytes and antibody may also be important in influencing clinical disease. It has been shown that antibody will enhance the in vitro infection and growth of dengue virus in mononuclear cells (Halstead et al., 1973; Halstead and O'Rourke, 1977). Monocytes, from non-immune humans and monkeys, in culture are non permissive for dengue virus replication. Enhancement was produced by multi-heterotypic anti-dengue antibody and only by low concentration of monotypic antibody. These authors suggested that this antibody enhancement of infection in cultured mononuclear cells for dengue virus provides a mechanism for the dengue shock syndrome seen in dengue 'sensitized' patients. In areas of endemic bluetongue, there is serological evidence that more than one bluetongue type exists and therefore it may be possible that this heterotypic antibody may be important in the expression of clinical disease. Virus/antibody complexes may be formed, with the possibility of complexes becoming deposited on the endothelium causing autoimmune disease. It has been shown that endothelial cells, in culture, can be damaged by antibody in presence of complement (De Bono, 1974; De Bono et al., 1977) or by antibody dependent leucocyte mediated cytotoxicity (Hirschberg et al., 1975; De Bono et al., 1977).
There is another important mechanism which may play a part in the expression of clinical disease and that is disseminated intravascular coagulation. This is a complex phenomenon and not easily defined. This subject is well reviewed by McKay and Margaretta (1967) and Sharp (1977). There are complex changes in the blood coagulation and fibrinolytic systems; these changes may lead to alteration of clotting factors and platelets. The changes result in abnormal bleeding, minor blood vessel occlusion by fibrin and/or platelets, tissue necrosis and organ dysfunction. The mechanism of disseminated intravascular coagulation is not a disease process in itself, but is a process associated with many different conditions. This mechanism has been offered as a model for those diseases characterised by oedema, vascular haemorrhage and thrombosis and may be important in such diseases as bluetongue (Wigton et al., 1976; Hoff and Hoff, 1976). The destruction of endothelial cells would also result in the inability of these cells to produce the enzyme involved in the inhibition of platelet aggregation and vascular dilation (Moncarda et al., 1976). The absence of this enzyme would increase the probability of microthrombi being deposited in damaged blood vessels.

PERSISTENT INFECTION

Mechanisms for survival of arboviruses (overwintering) during adverse conditions have been reviewed by Reeves (1974). Overwintering may be defined as the "endemic persistence of any arbovirus through adverse periods, by various mechanisms so as to allow for the reappearance of the arbovirus under favourable conditions". Overwintering can occur by:-

1. Virus persistence in the arthropod vector
   a) long lived blood feeding stages of the primary vector surviving unfavourable periods for blood feeding but remaining infective.
b) transovarian transmission

c) undetected arthropod vectors acting as an alternative vector to the primary vector.

(No evidence has been provided to prove that virus persistence in the vector occurs for bluetongue).

2. Virus persistence in the vertebrate host

a) chronic and latent infection

b) vertical transmission

Persisting virus in the vertebrate host would obviously have to evade the host defence mechanisms. In the case of bluetongue the virus is a good immunogen eliciting a high antibody response and thus a satisfactory method of avoiding this antibody would be needed. During viraemia bluetongue is associated with the cellular fraction of blood. Evidence reported in this thesis suggests that the virus is probably associated with the monocytes, macrophages, erythrocytes and possibly the neutrophils.

The association of bluetongue virus with the erythrocyte is difficult to explain. No evidence exists to show whether bluetongue virus is intra-erythrocytic or extra-erythrocytic. Many viruses have been shown to adsorb to the surface of erythrocytes but very few viruses have been shown to be intra-erythrocytic - Colorado tick fever virus (Emmons et al., 1972), Friend and Rauscher virus (Reilly and Schloss, 1971), mammary tumor virus (Nandi and Haslam, 1971), feline C-type virus (Oshiro et al., 1972) and an unclassified membrane encircled virus of Rana pipiens (Bernard et al., 1968). In this study thin section electron microscopy failed to show whether bluetongue virus was 'inside' or 'outside' the erythrocyte. This failure was probably due to the low level of virus in the erythrocyte.
fraction, during viraemia, the probability therefore of finding infected erythrocytes was low. Experiments were conducted to attempt to adsorb bluetongue virus to erythrocytes in vitro but without success (Lawman, unpublished data).

The intra-erythrocytic or intra-macrophage location of arboviruses (including bluetongue if it is proved to be the case) is an interesting epidemiological phenomenon. The intra-cellular location affords the virus protection from circulating antibody and cellular immune mechanisms and also the virus is in a very convenient location for biological transmission via a blood meal to the insect vector (and with Colorado tick fever, persistence in erythrocytes has been recorded, Hughes et al., 1974).

Since the erythrocyte lacks a nucleus it is unlikely that the virus replicates within the mature erythrocytes. Viruses may gain entry by pinicytosis but more probably, if bluetongue proves to be intra-erythrocytic, the mode of entry is at the stem cell-erythroblast stage in the bone marrow or haemopoietic liver. Studies by thin section electron microscopy on the haemopoietic liver infected with Colorado tick fever virus have shown the presence of viral replication within the erythroblast cell (Oshiro et al., 1978).

The possibility of infection after degeneration of the nucleus cannot be ignored since it has been shown that polio virus, echovirus, Semliki Forest virus, reovirus, respiratory syncytial virus, vesicular stomatitis virus, Klamath and Mokola virus are produced in enucleated cells (Crocker et al., 1966b; Follett et al., 1975). The intra-cellular content of erythrocytes, however, is somewhat different to those of enucleated cells especially in its polyribosome content (Marks et al., 1963) and therefore virus replication in mature erythrocytes would be unlikely.

Growth in macrophages or in other cells of the immune system
may also affect the efficiency of the immune response and/or provide a reservoir of infection and thus increase the probability of virus persistence. Virus persistence in monocytes and macrophages has been shown for other viruses; Aleutian mink disease virus (Porter et al., 1969), equine infectious anaemia (McGuire et al., 1971; Coggins, 1975), lactate-dehydrogenase elevating virus (Evans, 1970) and lymphocytic choriomeningitis virus (Mims and Subrahmanyan, 1966).

The evidence reported in this thesis of virus persistence in macrophages is of course only from in vitro experimentation and may be a laboratory artifact. However, this persistence in the monocyte and macrophage of goats and cattle may offer a method of virus overwintering during periods of adverse conditions and low insect activity.

Vertical transmission in cattle, sheep and goats has been suggested as an alternative method for the overwintering of blue-tongue virus in the vertebrate host (Luedke et al., 1977; Gibbs et al., 1979). Depending on the stage of development of the foetus at the time of infection, virus replication within the foetus is tolerated and the offspring are born viraemic but without any obvious abnormalities and remain viraemic for varying periods thereafter, some developing latent infection (Luedke et al., 1977). In cattle, it has been suggested that insect bites 'activate' latent virus (Luedke et al., 1977). The initial bites of Culicoides stimulate a viraemia which enhances the chance of other Culicoides becoming infected.

In none of the vertebrate hosts are the mechanisms or sites of persistence of virus known.

VACCINATION

One of the main reasons for studying the pathogenesis of a disease is to increase our understanding of the disease process with a view to introducing better control in the field. Vaccination is one such
control measure which could utilise such information. Commercial vaccines for bluetongue are based on live attenuated vaccine strains of the various antigenic types. Such vaccines however, pose problems. The pathogenesis of these vaccine strains is not known; for instance do these live attenuated strains replicate in the same organs and tissues as the wild type virus, or more importantly, are monocytes and macrophages involved in the replication of the vaccine viruses and if used in cattle and goats, will the vaccine strains persist in these cells? In persistently infected goats and cattle it is not known whether these vaccines will eradicate the wild type virus infection or complicate the picture. Will the vaccine strain replicate in the insect vector and if so, is reversion to virulence a possibility? In endemic countries where more than one type exists, cross-hybridisation could possibly occur after vaccination causing more virulent strains to arise (Gorman et al., 1978).

If vaccination is feasible in the eradication of bluetongue, it may be more pertinent to direct studies to inactivated vaccines. Some work has been carried out on this subject (Parker et al., 1975) and preliminary results would indicate that this may be a possibility. The main problem is that monovalent vaccines are specific and therefore do not give adequate protection against the many other strains that may be present. Another area of interest would be to use temperature sensitive mutant vaccine strains. It might be possible to isolate temperature sensitive mutants that would only replicate at the permissive host body temperature but not at the non permissive temperature of the insect vector; therefore the cycle of host-vector-host would be broken. However, the problem still may arise of reversion to virulence at some time during its replication in the host.
CONCLUDING STATEMENT

The many lines of future work discussed may require lengthy study. However, further detailed study of the macrophage and endothelial cell may bring long term rewards.
APPENDIX I:

Purification and culture of cells from the haemopoietic system of sheep, cattle and goats
INTRODUCTION

To investigate the role played by the haemopoietic system in the pathogenesis of virus infections, it is important to obtain purified populations of the various cell types within the system. Various methods have been developed to produce purified populations of the different cell types.

The object of the work reported here was to develop and standardise these methods for use with cattle, sheep and goats as the initial stage of an investigation of the pathogenesis of bluetongue virus.

MATERIALS AND METHODS

A. MIXED POPULATION OF CELLS

1. Lymph node suspension

The prescapular lymph nodes from lambs were removed, finely minced and trypsinised at 37°C with continual agitation for 1 hour. The resulting cell suspension was pelleted by centrifugation and the cells resuspended in RPMI 1640 growth medium (Appendix II). The cell suspension was seeded into 6 x 4 (1.5 cm diameter) welled microplates and placed in an incubator gassed with 5% CO₂, 95% air and maintained at 37°C.

2. Bone marrow suspensions

The method of preparing bone marrow cultures was based on that described for pigs by Plowright and Parker (1967) and Plowright et al., (1968). The long bones from 7 day old lambs were aseptically removed and all muscle and cartilage cut away to expose the bone shaft. The bones were broken open and the fragments of bone plus exposed bone marrow were placed in a conical flask containing
phosphate buffered saline supplemented with 2% antibiotic solution, and agitated at room temperature for 30 minutes. The resulting supernatant was separated from the bone fragments by decanting the fluid into a sterile container. The cell suspension was centrifuged and the cell deposit resuspended in RPMI 1640 growth medium. The cell suspensions were seeded into glass tissue culture flasks and incubated at 37°C. In some cultures the erythrocytes were removed by rapid lysis (Naylor and Little, 1975) prior to incubation at 37°C.

3. Spleen suspension culture.

The spleens from 7 day old lambs were removed and the capsules removed. The red pulp was scraped into a flask containing phosphate buffered saline plus 2% antibiotic solution and agitated for 30 minutes at room temperature. The resulting cell suspension was centrifuged and the cell pellet resuspended into RPMI 1640 growth medium. The spleen cell suspension was seeded into tissue culture flasks and incubated at 37°C. As with the bone marrow cultures, the erythrocytes in some of the cultures were removed by rapid lysis (Naylor and Little, 1975).

B. SELECTION OF SPECIFIC CELL POPULATIONS

1. Peripheral blood cultures

(i) Whole blood cultures

Heparinised, peripheral whole blood (20 ml) was obtained from the carotid artery of adult sheep using a 50 ml syringe. The 20 ml of whole blood were put into flasks containing 80 ml of RPMI 1640 medium and stirred continuously while incubated at 37°C.

(ii) Erythrocyte cultures

The erythrocytes were purified by centrifugation (200 g, 15 mins, 4°C) of the whole blood. The buffy coat (leucocyte) layer was removed and the erythrocytes were resuspended in 20 ml of phosphate
buffered saline and centrifuged again and the top 1.0 ml removed and discarded. This procedure was repeated a further 2 times. The erythrocytes were diluted to a cell concentration of $1 \times 10^6$ cells per ml in 20 ml of RPMI 1640 medium supplemented with 20% foetal calf serum. The erythrocyte suspension (100 ml) was kept at $37^\circ{C}$ and stirred continuously.

(iii) Preparation of total leucocyte population

Forty ml of peripheral blood was withdrawn from the jugular vein into a 50 ml disposable syringe containing 10 international units of heparin. The blood was transferred into a cooled (4°C) glass universal container. All the following procedures were carried out at 4°C. The blood was centrifuged (200 g, 10 mins) and the buffy coat layer (leucocyte fraction) was withdrawn, using a pasteur pipette, from the interface of the plasma and erythrocyte layers and resuspended in 5 ml of Hank's medium (containing yeast extract and lactalbumin hydrolysate) (LYH). Contaminating erythrocytes were removed either by rapid lysis with sterile distilled water (Naylor and Little, 1975) or with ammonium chloride/tris solution (ACT) (Symons and Binns, 1975) (Appendix II). The leucocytes were then washed 3 times in RPMI 1640 medium and finally resuspended in RPMI 1640 growth medium. The cell suspension diluted to a total cell concentration of $1 \times 10^6$ cells, was dispensed into either 50 mm diameter plastic petri dishes (50 ml of cell suspension) or microplates (1.5 ml of cell suspension) (15 mm diameter well with 24 wells per plate).

2. Isolation and preparation of monocytes, macrophages, neutrophils and lymphocyte cultures

The above cells were mostly prepared from 3 sources, peripheral blood, mammary gland or lung.
Peripheral blood

(i) Preparation of monocyte cultures.

Forty ml of heparinised blood was centrifuged (as above) and the leucocyte layer removed. The different leucocytes were then separated by methods similar to those described by Bough (1964, 1968) and Wardley and Wilkinson (1977 a, b). The leucocytes were resuspended in 5 ml of LYH and then layered onto a 3 ml *ficoll/hypaque* gradient at varying densities. The gradients were centrifuged (200 g, 20 mins, 4°C) and the cells remaining at the interface of the LYH and ficoll/hypaque gradient were removed. Any remaining contaminating erythrocytes were removed by a single cycle of rapid distilled water lysis (as previously described). The cells were washed 3 times in phosphate buffered saline and finally resuspended in RPMI 1640 growth medium. The monocyte concentration was counted in the presence of neutral red (1%) (monocytes will rapidly phagocytose the neutral red granules). The cell suspension was diluted to 1 x 10^6 total monocytes suspension. The cell suspension was seeded into 50 mm diameter petri dishes (5.0 of cell suspension) or microplates (1.5 ml of cell suspension) and incubated at 37°C. After a 2 hour incubation period the supernatant was discarded with non adherent cells (lymphocytes and neutrophils) and the adherent cells were washed once in phosphate buffered saline and the growth medium replaced. The adherent cells were incubated for a further 48 hours with daily replacement of medium to remove all non-adherent cells. At 48 hours, cultured cells were tested for phagocytic activity and their morphology examined and compared to monocytes. (See page 183).

sodium hypaque: Winthrop Laboratories, Surbiton-on-Thames, Surrey

+Ficoll 400: Pharmacia Fine Chemicals A.B., Uppsala, Sweden
(ii) Preparation of macrophage cultures

The monocytes, obtained by the above procedure were maintained in culture with frequent change of medium (every 12-24 hours) for approximately 7 days. After this, small clones of cells were observed which rapidly multiplied to form complete monolayers. These cells were tested for phagocytic activity and other characteristics of macrophages (page 183).

(iii) Preparation of lymphocyte cultures

Forty ml of heparinised blood was centrifuged and the leucocyte layer removed (as described previously). The leucocyte suspension was layered onto a 3 ml gradient of ficoll/hypaque at various densities and centrifuged (200 g, 20 mins, 4°C) and the cell band at the interface was removed and any contaminating erythrocytes removed by rapid distilled water lysis. This layer contained a high percentage of lymphocytes but also some contaminating monocytes and neutrophils. These contaminant cells were removed by the addition of carbonyl iron at a concentration of 3 x 10^6/cells ml to 4 mg/ml followed by incubation at 37°C for 30 to 45 minutes with occasional agitation to ensure mixing. After incubation the phagocytic monocytes and neutrophils, which had taken up carbonyl iron, were removed by drawing a magnet across the outside surface of the container thus pulling down the excess carbonyl iron and those cells containing carbonyl iron from the supernatant. The supernatant was removed and the procedure repeated twice. The cells remaining in the supernatant were centrifuged and any extraneous carbonyl iron was removed by magnetic attraction. The lymphocyte population was checked for purity.
Mammary gland

(i) Preparation of neutrophil and monocyte cultures

The preparation of purified populations of neutrophils and monocytes from the mammary gland of cattle has been reported (Wardley et al., 1976), sheep (Outteridge et al., 1971) and pigs (Wardley and Wilkinson, personal communication). The method used here was based on those mentioned above. Two ml of 5 \mu g/ml of lipopolysaccharide* (extracted from Escherichia coli) was inoculated via the teat duct into the mammary gland. This technique can only be used on animals with teats having single ducts and not on those with multi ducts. Samples were taken at 6 hours and then at daily intervals for 6 days. At each sample time, 10 ml of prewarmed saline were infused into the gland, via the teat duct, the gland was gently massaged and the saline expressed into a sterile container. The cells in the saline were centrifuged (200 g, 10 mins, 4°C) and washed twice in phosphate buffered saline supplemented with antibiotic solution. The cells were finally resuspended in LYH medium and differential cell counts made.

To purify the neutrophil population (taken at height of neutrophil production - see results) the cells were washed and placed in 5 ml of LYH and layered onto a ficoll/hypaque gradient; the gradient was centrifuged (200 g, 20 mins, 4°C). The neutrophils were deposited at the base of the gradient and most of the contaminating monocytes and lymphocytes remained at the interface. The neutrophil deposit was resuspended and used accordingly.

Purification of monocytes (taken at height of monocyte production - see results) was by the above method for neutrophil separation with

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the exception that the cells are collected from the interface of the ficoll/hypaque gradient and the LYH medium. Contaminating lymphocytes and neutrophils were removed as previously described for peripheral monocytes.

(ii) Preparation of established macrophage cultures

The monocytes from the udder were maintained in culture with frequent media changing as described for the peripheral blood monocytes. Clones of cells were observed 7-14 days post culture which multiplied to form confluent monolayers. These cells were also tested for the characteristics of macrophages.

Lung

The method used for the production of pulmonary macrophages was similar to that described by Fox (1973). The lungs from one week old lambs, one week old kids and three month calves were removed and washed in phosphate buffered saline plus 2% antibiotics. Approximately 500 ml of prewarmed saline was infused into the alveolar sacs via the trachea and bronchus using a 50 ml syringe. The lung was very gently massaged and the infused saline was then expressed into a sterile container. Care was taken not to contaminate the saline wash with blood. The saline was centrifuged (200 g, 10 mins, 4°C) to deposit the alveolar macrophages. The cells were washed 3 times in phosphate buffered saline and finally resuspended in RPMI 1640 growth medium. The cell suspension (10 x 10^6/ml of suspension) was seeded into 4 oz tissue culture bottles and incubated at 37°C. The medium in the bottles was changed every day. Established macrophage cultures were also obtained 7-14 days post culture using the same method as that described for peripheral blood and the mammary gland.
Macrophages from other sources

Macrophage cultures were produced from both the spleen and bone marrow. Both cell populations were isolated from the cell suspensions made from the spleen red pulp and the 'red marrow'. Contaminating erythrocytes were removed by rapid lysis and the remaining cells pelleted and finally resuspended in RPMI 1640 growth medium. The cells were incubated at $37^\circ C$ in 4 oz tissue culture bottles. The medium overlying the adherent cells was changed daily and observed for any gross morphological change. Any resulting monolayers of cells were tested for phagocytic activity and other monocyte or macrophage characteristics.

3. Thymocytes and thymic fibroblast cultures

The thymus was removed from 7 day old lambs, finely minced using a scalpel, and then trypsinised (0.5% trypsin) at $37^\circ C$ with continual agitation for 20 minutes. The resulting cell suspension was pelleted by centrifugation (200 g, 20 mins, $4^\circ C$), resuspended in RPMI 1640 growth medium and dispensed, 20 ml of cell suspension per tissue culture flask. The cultures were allowed to remain stationary at $37^\circ C$ for 2 hours to allow fibroblastic and other supporting tissue cells to settle and attach to the glass. The unattached cells were removed with the medium and pelleted and resuspended in fresh RPMI 1640 growth medium. The cell suspension was dispensed (0.1 ml) into microplates at a cell concentration of $5 \times 10^6$ cells/ml and incubated at $37^\circ C$ in a gassed incubator (5% CO$_2$, 95% air). The adherent cells were maintained and allowed to form monolayer cultures. Confluent monolayers of these cells were dispersed with versene trypsin, centrifuged (200 g, 5 mins) and resuspended in fresh growth medium and finally dispensed into fresh culture bottles.
4. Endothelial cell cultures

(i) Preparation of heart endothelial cell cultures

The heart from a 1 week old lamb was removed and placed in a beaker containing calcium and magnesium free phosphate buffered saline plus antibiotics. From the heart the atria and attached blood vessels were removed exposing the ventricles. The ventricles were washed out with the calcium/magnesium free phosphate buffered saline to remove blood clots. The ventricles were minced and then placed in a trypsinising flask containing 200 ml of calcium and magnesium free phosphate buffered saline and left at room temperature for 5 minutes before the phosphate buffered saline was discarded. Fresh trypsin was added and trypsinisation proceeded at 37°C and each 50 ml trypsin digest was removed every 10 minutes for 30 minutes. The cells were deposited by centrifugation (200 g, 5 mins) and were resuspended in LYH plus 10% foetal calf serum and 1% antibiotic solution. The cells from each digest were kept separately and resuspended in growth medium at a cell concentration of 10 x 10^6 cells/ml and were dispensed in 15 ml amounts into tissue culture bottles. Clones of endothelial cells were observed in only the first and occasionally the second trypsin digest. These cells were maintained until confluency and then dispersed using versene trypsin (0.5%), resuspended in growth medium and reseeded in clean bottles at a ratio of 1 bottle of confluent cells to 3 clean bottles.

(ii) Preparation of blood vessel endothelial cell cultures

A suitable section of blood vessel (dorsal aorta, carotid artery or vena cava) was freed from the connective tissue and removed from the carcase of 7 day old lambs, 3 month old calves and 7 day old kids. The lumen of the blood vessel was rinsed 3 times in phosphate buffered saline plus antibiotics to ensure the lumen was free of contaminating blood cells. The open ends of the blood vessel were
ligated using artery forceps. The outer wall of the blood vessel was wrapped in parafilm* to stop leakage of the introduced trypsin (0.5%) through any severed minor vessel. The trypsin was injected into the lumen of the blood vessel via the vessel wall. The blood vessel was placed in an empty sterile beaker and incubated at 37°C for 20 minutes. The trypsin was removed and kept at 4°C. Fresh trypsin was added and gently flushed in and out of the blood vessel lumen using a syringe. The first and second trypsin washes were pooled and centrifuged (500 g, 2 mins) and the cell deposit was resuspended in LYH plus 10% foetal calf serum and 1% antibiotics and seeded into tissue culture flasks. Cell counts were made but counts of only $1 \times 10^2 - 1 \times 10^3$ were achieved. The medium on the endothelial cells was changed daily until the cells became established and a monolayer was formed. At confluency the monolayers were passaged as for the heart endothelial cells described above.

C. TEST PROCEDURES FOR IDENTIFYING POSSIBLE MONOCYTES AND MACROPHAGES

The following criteria were used to identify cells isolated as possible monocytes or macrophages. They are based on those used by Van Furth et al., (1972).

Cells were thought to be monocytes or macrophages on the basis of the following characteristics:

a) avid adherence to glass or plastic

b) phagocytosis of particulate matter (Cohn and Weiner, 1963; Sanderson et al., 1975).

c) presence of cytoplasmic vacuoles and granules

d) development of pseudopodia

e) presence of non-specific esterase activity (Yam et al., 1971)
f) presence of cytoplasmic phagosomes in cells examined by thin section electron microscopy

Phagocytic activity of these cell types was tested as follows by:

a) the uptake of neutral red stain using a method similar to that described by Cohn and Weiner (1963). The neutral red stain was prepared at a final concentration of 0.005% and added to the cell suspensions. The mixture was incubated at 37°C for 10 minutes, the cells washed in phosphate buffered saline to remove excess stain and then finally examined by microscopy to ascertain the presence of dye within the cells.

b) the uptake of carbonyl iron, similar to that described by Sanderson et al., (1975). Carbonyl iron (20 mg/ml) was pretreated with growth medium (RPMI 1640), sonicated and then incubated at 37°C for 1 hour. The carbonyl iron was mixed with the cell suspension (cell concentration of 1 x 10^7 cells/ml) to give a final concentration of carbonyl iron of 4 mg/ml. The mixture was incubated at 37°C for 45 minutes, after which time the cells were removed and then examined for the uptake of carbonyl iron.

The cell morphology was determined using cytospin preparations of cell suspensions (5 x 10^5/ml) made using a Shandon Elliott Cytospin (Appendix II). The cell preparations were finally stained with Wrights stain (0.2% W/V in methanol). The cells were examined for the presence of vacuolation, granules and pseudopodia.

Cytochemical evidence was obtained using the method for identifying non-specific esterase activity as described by Yam et al. (1971).

The presence of phagosomes in the cytoplasm of these cells was sought by thin section electron microscopy.
RESULTS

A. MIXED POPULATION OF CELLS

Suspensions of lymph node, bone marrow and spleen cells were adequately prepared by these methods but no cell differentiation or viability studies were undertaken.

B. SPECIFIC POPULATION OF CELLS

Cells derived from peripheral blood

Ficoll/hypaque gradients were found to give adequate separation of the cellular components of blood. Gradients of various densities were tried in attempts to find optimum purification and separation of the various cell types. The results of the sheep peripheral blood leucocyte separation on the various gradients are shown in Table AI.1. It should be pointed out that Table AI.1 shows monocytes and lymphocyte recoveries in separate columns. However, on ficoll/hypaque gradients, monocytes and lymphocytes are recovered from the same (interface) layer. The monocytes and lymphocytes were finally separated after further treatments, therefore the rate of contamination shown in the table for these two populations only refers to neutrophils and erythrocytes found at the interface of the gradient. The separation, recovery and purity of the cell populations of cattle and goats gave similar results to those of sheep. Further experiments on cell separation using ficoll/hypaque gradients were carried out using a gradient of 1.0774 g/cm³ at 25°C (6.35 ficoll and 10 g sodium hypaque in 100 ml distilled water) since this gave the highest yield of cells with the best purity attained. Neutrophil populations, however, from blood were not used since the rate of recovery was low or the rate of contamination too high.

The removal of erythrocytes by either ACT treatment or rapid lysis with distilled water had no deleterious effects on the leucocytes or any of the purified populations. All the procedures needed to be
carried out at 4°C so as to prevent clumping of leucocytes and inhibit the adherence of the monocytes to the surface of the glass containers. The separation of the lymphocytes from monocytes using carbonyl iron was found to be satisfactory and the purification was very high (98%).

The behaviour of the enriched populations in culture varied considerably. Lymphocyte cultures did not adapt well to in vitro culture (Fig. AI.1A). It was found consistently that 50% of the lymphocyte population was lost during the first 24 hours culture. No viable cells remained in the culture following a further 72 hours culture. The total leucocytes were less sensitive to culture (Fig. AI.1F). However, a large percentage (40-45%) of the leucocytes were lost during the first 24 hours. Approximately 25-30% of the leucocytes remained viable in culture for up to 6 days post culture.

The cells which adapted best to in vitro culture were monocytes (Fig. AI.1C). Whilst there was a continual loss of viable cells over 7 days in culture there was only a 40% reduction in the number of viable monocytes during this culture period. In the culture of monocytes from sheep and goats, established cells were found (7-10 days post culture) which readily divided to form a continuous cell line (Fig. AI.2a-f). These established cells were tested for phagocytic activity and for other criteria of macrophages. They were found to be highly phagocytic and cytospin preparations showed them to be 20 - 50 nm in diameter, highly vacuolated and to display many pseudopodia (Fig. AI.3). Thin section electron microscopy revealed phagosomes in the cytoplasm of these cells (Fig. AI.4). It was felt justified to assume that these larger established cells were macrophage cell lines of the mononuclear-phagocytic series of Van Furth et al., (1972) and had been established from blood monocytes.
Cells from mammary gland

The production of neutrophils and monocytes in the mammary gland has been shown by other authors to be a convenient source of enriched cell populations. In this study, it was found that in sheep, goats and cattle the cellular content of the udder, prior to inoculation and stimulation with lipopolysaccharide, was very low \((10^4.0 - 10^6.0 \text{ cells/ml})\). Six hours post stimulation there was a marked increase in the total cell population in all 3 species \((10^8.0 - 10^9.0 \text{ total cell concentration})\) (Figs. A1.5, A1.6, A1.7). There was a gradual decline in the cell numbers over a period of 6 days. In studying the differential cell counts of the various cell populations in each species, it can be seen that at prestimulation, lymphocytes were the predominant cell accounting for 70% whilst neutrophils and monocytes were low in number \((10 - 20\%)\). Six hours post stimulation, the neutrophil population was dramatically increased \((80 - 90\% \text{ of the cell population})\) while that of the lymphocytes dropped and the monocytes remained low. The neutrophil population was observed to remain high for 24 to 48 hours post stimulation but then declined slowly over 6 days.

There was a marked increase in the number of monocytes 48 - 72 hours post stimulation. At 96 hours, 50 - 60% of the total cell population were monocytes whilst the neutrophils and lymphocytes accounted for 20 - 30% of the remaining population. There appeared to be no significant difference between cell species if the mammary gland was the source of enriched cell populations.

Further purification of the neutrophil population was achieved by removing lymphocytes and monocytes on a ficoll/hypaque gradient and then allowing residual monocytes to adhere to glass or plastic as described above for purification of cells from peripheral blood.
The purity of the resulting neutrophil population was between 98 - 100%. The survival of the neutrophil population in vitro was low (Fig. AI.1B). Most of the cells (90 - 95%) were nonviable by 72 hours post culture. The mammary gland monocytes, however, were maintained in culture, losing only 40% of viable cells over 7 days post culture (Fig. AI.1D). This correlated well with the survival of peripheral blood monocytes in culture. As with peripheral blood monocytes, 7 - 10 days post culture, established cells were observed in the culture. These established cells also showed the same characteristics as did those derived from the peripheral blood monocytes and therefore it was assumed that they were also established macrophage cell lines of the mononuclear phagocytic series.

Cells derived from the lung

High yields of alveolar macrophages were obtained. The viability of the cells in culture was good over 7 days, only 30 - 35% of the cells were nonviable over this period (Fig. AI.1E). When the cells were maintained with repeated washing and replenishing of medium, clones of established cells were observed and these cells eventually formed complete monolayers. These cells were highly phagocytic and also exhibited other characteristics of macrophages.

The macrophage cell lines, of peripheral blood, mammary gland and alveolar derived grew well in vitro culture and were passaged 20 to 30 times. The cells recovered well after storage in 10% dimethyl sulphoxide (DMSO) at -70°C and even after 6 months showed 90% viability on recovery. The cell lines were checked routinely for their phagocytic activity and were still found to be phagocytic at the 20 - 30 pass level.

Established cell monolayers derived from spleen and bone marrow were also found to be phagocytic and possess other characteristics of
macrophages. It was possible to assume that these cells were established macrophages of the mononuclear phagocytic series.

These cells were also passaged to the same level (20 - 30) as the other macrophage cell lines and were still found at the late passage levels to be highly phagocytic.

Cells derived from the cardio-vascular system

The preparation of endothelial cells from the heart was successful. Previously described methods of endothelial cell isolation (Pomerat and Slick, 1963; Spiers and Turner, 1966; Tautsumi and Gore, 1969; Jaff et al., 1973; De Bono, 1974) were attempted and met with varied success. No muscle cells were ever cultured. Endothelial cells from blood vessels of sheep, goats and cattle were difficult to isolate. However, once cells were isolated they readily grew to confluency. These endothelial cells revived well from storage at -70°C.

DISCUSSION

Many methods have been developed for the separation of leucocytes from peripheral blood and for the separation of various cell populations from the leucocyte population as well as the production of enriched populations of cells (Table AI.2). The methods described by the various authors are based upon the method first described by Boyum (1964) and the majority of these methods utilized centrifugation and an erythrocyte aggregating agent (Dextran, methyl cellulose) for the separation of leucocytes from peripheral blood. For larger quantities of cells particularly those of the mononuclear phagocytic series and polymorphonuclear cells, other methods have been used such as the stimulation of the mammary gland or washing out of the lung. It was felt important to evaluate some of these methods despite their publication by other authors, so as to be aware of the difficulties
involved and to be able to have a standard approach for all the procedures used. Most of the published techniques were developed using human blood but some have been adapted for use with blood from various animal species. It was decided that, if possible, a single method was to be employed to isolate a particular cell type from all 3 species since this would remove criticism of varied treatments for the isolation of a particular cell type which might alter or influence their survival or growth in \textit{in vitro} culture. The use of these cells for studies on the pathogenesis of bluetongue virus infection could then be satisfactorily assessed. Three basic methods were employed and were based upon the cell types required (Table A1.3). Ficoll/hypaque at a density of $1.0774 \text{ g/cm}^3$ at $25^\circ \text{C}$ gave the best separation of the leucocyte population from all 3 species because at this density between 80-100\% of the lymphocytes and monocytes were recovered with a low percentage contamination (5 - 10\%). The neutrophil population, however, was always heavily contaminated and was therefore discarded and neutrophils from a different source were obtained. The rapid loss of lymphocytes was a problem in as far as the growth curve studies of bluetongue virus were concerned, since low multiplicity studies required 3 - 4 days to complete. Growth curve studies in monocytes would represent no problem at either high or low multiplicities of infection due to their better survival rates in \textit{in vitro} culture. Production of neutrophils and monocytes in the mammary gland was found to be a useful technique for all 3 species. This in part reproduces the work of Wardley \textit{et al.}, (1976) in the bovine and of Outeridge \textit{et al.}, (1971) in the sheep. The results of the goat have not been published before and are in agreement with Outeridge \textit{et al.}, (1971) and in general agreement with Wardley \textit{et al.}, (1976) since the results reported here for cell production in the goat mammary gland show a
higher percentage production (60%) of monocytes than does the work reported by Wardley et al., (1976) where they report 10 - 20% of monocytes were produced. From the results it was shown that to obtain a high yield of neutrophils a sample taken at 6 hours post stimulation would yield a cell population of $1 \times 10^9$ total cell count, of which neutrophils accounted for 90%. To further purify the neutrophil population the cells were subjected to a ficoll/hypaque gradient centrifugation and residual monocytes removed by adherence to glass. To obtain a high percentage yield of monocytes samples were taken at 4 days post stimulation when 60% of the population represented monocytes. These were further purified by adherence of the monocytes to a glass surface.

It was presumed that these cells, both mononuclear monocytes and the polymorphonuclear cells, were recruited from the peripheral blood on stimulation with lipopolysaccharide. The monocytes were, however, kept as a distinct cell population from the peripheral blood isolated monocytes since these cells have been 'stimulated' and therefore have probably undergone morphological changes during the period of stimulation in the mammary gland. Like the lymphocyte population the neutrophils did not adapt well to culture and therefore these cells pose the same problem concerning low multiplicity of bluetongue virus infection in neutrophils as do the lymphocytes. The monocytes were well adapted to culture as were the peripheral blood monocytes.

It has been shown that the alveolar macrophage differs in some physiological aspects and are probably of different origin (Oren et al., 1963; Nelson, 1969) to peripheral blood macrophages. It was concluded worthwhile, therefore, to compare their behaviour to infection with bluetongue virus in in vitro culture. A disadvantage observed during isolation and culture of alveolar macrophages in the
above manner was the high risk of contamination with fungi and bacteria. A further difficulty encountered when removing alveolar macrophages was the contamination of the lung lavage with blood, since peripheral blood macrophages could be isolated and grown as a contaminant cell in the alveolar macrophage cultures.

All the established cell types derived from the various sources of monocytes, demonstrated many morphological and cytochemical characteristics of macrophages and therefore in this thesis, these cells are presumed to be and are referred to as macrophages whether derived from peripheral blood, mammary gland, spleen and bone marrow or from the lung. The literature contains reports of apparent human (Paul, 1958) and horse (Moore et al., 1970) macrophage cell lines.

In conclusion, to obtain purified populations of various leucocytes for further experimentation, the following methods were selected:

1. **Leucocytes**

Obtained from heparinised peripheral blood. The blood was centrifuged (200 g, 10 mins, 4°C) and the buffy coat layer (leucocyte fraction) was withdrawn and resuspended in 5 ml of LYH. Contaminating erythrocytes were removed by either rapid lysis with distilled water or ammonium chloride/tris solution. Leucocytes were washed 3 times in RPMI 1640 medium, finally resuspended in RPMI 1640 growth medium.

2. **Lymphocytes**

Obtained from heparinised peripheral blood. The leucocyte layer was removed, as above, layered onto a 3 ml ficoll/hypaque gradient (6.35 ficoll and 10 g sodium hypaque in 100 ml distilled water, 1.0774 g/cm³ at 25°C) and centrifuged (200 g, 20 mins, 4°C). The cell band at the interface was removed, any contaminating erythrocytes were removed by rapid distilled water lysis. Contaminating monocytes and neutrophils were removed by the addition of carbonyl iron and their subsequent removal was by using a magnet to draw down excess
carbonyl iron and cells which had phagocytosed carbonyl iron. The supernatant plus lymphocytes were removed, washed and resuspended in RPMI 1640 growth medium.

3. Monocytes

a) Obtained from heparinised peripheral blood. The leucocyte layer was removed, resuspended in 5 ml of LYH and then layered onto a 3 ml ficoll/hypaque gradient (1.0774 g/cm³ at 25°C) and centrifuged (200 g, 20 mins, 4°C). The cells remaining at the interface of LYH and ficoll/hypaque were removed, and any contaminating erythrocytes were lysed with distilled water. The cells were washed and then resuspended in RPMI 1640 growth medium. To purify the monocyte population the cells were seeded into glass or plastic tissue culture flasks and incubated at 37°C. After 2 hours incubation the supernatant was discarded with the non adherent cells and the adherent cells were washed 3 times in phosphate buffered saline and the growth medium replaced. The adherent cells were incubated for a further 48 hours with daily replacement of growth medium to remove all non adherent cells. Monocytes were used after 48 hours culture.

b) Obtained from mammary gland. Two ml of 5 μg/ml of lipopolysaccharide (E. Coli) was inoculated via the teat duct into the mammary gland. Twenty ml of prewarmed saline was introduced in the mammary gland 4 - 5 days post inoculation, massaged, then expressed into a breaker (50 - 60% of cells expressed on this day were monocytes). The purification and culture of these monocytes is as described for the peripheral blood monocytes.

4. Established macrophages cell lines

a) Obtained from peripheral blood monocytes. The peripheral blood monocytes were maintained in culture with frequent media changing (every 12 - 24 hours for 7 - 14 days post culture). Clones of established macrophages appear 7 - 14 days post culture. These
clones were allowed to form monolayers after which they were passaged on as and when necessary. These established cells could be stored at -70°C in 10% dimethyl sulphoxide (DMSO) preservative.

b) Mammary gland - as for peripheral blood.

c) Bone marrow and spleen - as for peripheral blood.

5. Neutrophils

Obtained from the mammary gland. Two ml of lipopolysaccharide were inoculated via the teat duct into the mammary gland. Twenty ml of prewarmed saline were introduced in the mammary gland 6 - 24 hours post inoculation, massaged, then expressed into a beaker (80 - 90% of the cells expressed were neutrophils). The cells were washed with phosphate buffered saline and then placed in 5 ml of LYH and layered onto a ficoll/hypaque gradient (1.0774 g/cm³ at 25°C) and centrifuged (200 g, 20 mins, 4°C). The neutrophils were deposited at the base of the gradient. The neutrophil deposit was resuspended in RPMI 1640 growth medium and used accordingly.

6. Alveolar macrophages (primary)

Lungs were removed and approximately 500 ml of prewarmed saline was infused into the alveolar air sacs via the trachea and bronchus using a 50 ml syringe. The lung was gently massaged and the infused saline expressed into a sterile beaker. Care must be taken to prevent blood from contaminating the 'lung wash'. The saline cell suspension was centrifuged (500 g, 10 mins, 4°C) to deposit the cells. The cells were washed 3 times in phosphate buffered saline and finally resuspended in RPMI 1640 growth medium. The cells were then seeded at appropriate rates into sterile tissue culture flasks and incubated at 37°C and the cultures used accordingly.
7. Established alveolar macrophages

Cultured 'primary' alveolar macrophages were maintained with frequent media changes - every 24 hours for 7 - 14 days post culture. Clones of established macrophages appeared 7 - 14 days post culture. These clones were allowed to reach confluency after which they were passaged as and when necessary. These alveolar macrophages could be stored at -70°C in 10% DMSO as a preservative.
APPENDIX I

Tables and Figures
Table AI.1  Recovery rates of various sheep cell populations and percentage contamination in the populations at different ficoll/hypaque densities

<table>
<thead>
<tr>
<th>Ficoll/hypaque densities</th>
<th>Monocytes Rec.%</th>
<th>%Cont.</th>
<th>Lymphocytes Rec.%</th>
<th>%Cont.</th>
<th>Neutrophils Rec.%</th>
<th>%Cont.</th>
<th>Erythrocytes Rec.%</th>
<th>%Cont.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.060*</td>
<td>60</td>
<td>5-10</td>
<td>50-60</td>
<td>5-10</td>
<td>80-90</td>
<td>30</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1.0774</td>
<td>80-100</td>
<td>5-10</td>
<td>80-100</td>
<td>5-10</td>
<td>70-10-80</td>
<td>20</td>
<td>90-100</td>
<td></td>
</tr>
<tr>
<td>1.088</td>
<td>80-100</td>
<td>20-30</td>
<td>80-100</td>
<td>20-30</td>
<td>60-15</td>
<td></td>
<td>90-100</td>
<td></td>
</tr>
<tr>
<td>1.095</td>
<td>90-100</td>
<td>20-30</td>
<td>80-100</td>
<td>20-30</td>
<td>60-15</td>
<td></td>
<td>90-100</td>
<td></td>
</tr>
</tbody>
</table>

* density x g/cm³ @ 25°C  
+ percentage recovery  
× percentage contamination
Table AI.2 Published methods for the separation and purification of haemopoietic cell populations from various species

<table>
<thead>
<tr>
<th>Primary method</th>
<th>Secondary method</th>
<th>Species</th>
<th>Source</th>
<th>Cell population</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. SEDIMENTATION</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(no centrifugation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>glass adherence</td>
<td>?</td>
<td>Blood</td>
<td>Lymphocytes</td>
<td>McFarland &amp; Heilman (1965)</td>
</tr>
<tr>
<td></td>
<td>Methyl cellulose/sodium metrizoate</td>
<td>Man, Rat, Mouse, Rabbit, Guinea pig, Sheep</td>
<td>Blood</td>
<td>Leucocytes</td>
<td>Hulliger &amp; Blazkovec (1967)</td>
</tr>
<tr>
<td></td>
<td>Dextran &amp; glass adherence</td>
<td>Man</td>
<td>Blood</td>
<td>Monocytes &amp; lymphocytes</td>
<td>Hersh &amp; Harris, (1968)</td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>Guinea pig</td>
<td>Blood</td>
<td>Lymphocytes</td>
<td>Oppenheim et al., (1967)</td>
</tr>
<tr>
<td></td>
<td>Nylon fibre/glass bead column</td>
<td>Man</td>
<td>Blood</td>
<td>Lymphocytes</td>
<td>Oppenheim et al., (1968)</td>
</tr>
<tr>
<td>2. CENTrifUGATION</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine, Man, Rabbit, Deer, Sheep, Cattle</td>
<td>Blood</td>
<td>Leucocytes</td>
<td>Aalund et al., (1970); Buening (1973)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lymphocytes</td>
<td>Shah &amp; Dickson (1974)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gibbs &amp; Lawman (1977)</td>
</tr>
<tr>
<td>Primary method</td>
<td>Secondary method</td>
<td>Species</td>
<td>Source</td>
<td>Cell population</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------</td>
<td>---------</td>
<td>--------</td>
<td>-----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>b) Density gradient</td>
<td>Isopaque/methyl cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isopaque/Dextran</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isopaque/Ficoll</td>
<td>Man</td>
<td>Blood</td>
<td>Leucocytes</td>
<td>Boyum (1964)</td>
</tr>
<tr>
<td></td>
<td>Ficoll/EDTA</td>
<td>Horse</td>
<td>Thymus</td>
<td>Leucocytes, thymocytes</td>
<td>Boone et al., (1968)</td>
</tr>
<tr>
<td></td>
<td>EDTA/Dextran</td>
<td>Rabbit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Goat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>?</td>
<td>Blood</td>
<td>Lymphocytes</td>
<td>Carson et al., (1975)</td>
</tr>
<tr>
<td>+ adherence</td>
<td>Pig</td>
<td></td>
<td></td>
<td>Monocytes</td>
<td>Owen et al., (1975)</td>
</tr>
<tr>
<td>+ carbonyl iron</td>
<td>Pig</td>
<td></td>
<td></td>
<td>Lymphocytes</td>
<td>Wardley et al., (1977)</td>
</tr>
<tr>
<td>Ficoll/Triosill</td>
<td>?</td>
<td>Blood</td>
<td>Lymphocytes</td>
<td>Ting &amp; Morris (1971)</td>
<td></td>
</tr>
<tr>
<td>Liquid silicone</td>
<td>Cattle</td>
<td>Blood &amp; lung</td>
<td>Monocytes</td>
<td>Stott et al., (1975)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td>Blood</td>
<td>Lymphocytes</td>
<td>Joel et al., (1969)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran &amp; glass wool</td>
<td>Rat</td>
<td>Bone marrow</td>
<td>Lymphocytes</td>
<td>Morrison (1967)</td>
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</table>
Table AI.2 cont'd

<table>
<thead>
<tr>
<th>Primary method</th>
<th>Secondary method</th>
<th>Species</th>
<th>Source</th>
<th>Cell population</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c) Isopycnic/sedimentation</td>
<td>Rapid lysis</td>
<td>Cattle</td>
<td>Blood</td>
<td>Lymphocytes &amp; Granulocytes</td>
<td>Naylor &amp; Little (1975)</td>
</tr>
<tr>
<td></td>
<td>Ficoll/hypaque</td>
<td>Goat</td>
<td>Blood</td>
<td>Lymphocytes</td>
<td>Perper et al., (1968)</td>
</tr>
<tr>
<td>3. ELECTROPHORESIS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Beckman Instruments Bull. 7096-B</td>
</tr>
<tr>
<td>4. LIPOPOLYSACCHARIDE STIMULATION</td>
<td>Ficoll/hypaque</td>
<td>Cattle</td>
<td>Mammary gland</td>
<td>Neutrophils &amp; Monocytes</td>
<td>Wardley et al., (1976)</td>
</tr>
<tr>
<td></td>
<td>Ficoll/hypaque</td>
<td>Sheep</td>
<td>Mammary gland</td>
<td></td>
<td>Outeridge et al., (1971)</td>
</tr>
<tr>
<td>5. LAVAGE</td>
<td></td>
<td>Cattle</td>
<td>Lung</td>
<td>Macrophages</td>
<td>Fox (1973); Stott et al., (1975)</td>
</tr>
</tbody>
</table>
Table AI.3  Methods employed in this study and the cell populations separated

<table>
<thead>
<tr>
<th>Method</th>
<th>Cell population</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ficoll/hypaque</td>
<td>1. Leucocytes</td>
</tr>
<tr>
<td>Density centrifugation of</td>
<td>2. Lymphocytes</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>3. Monocytes/macrophages</td>
</tr>
<tr>
<td>2. Lipopolysaccharide treatment of mammary gland</td>
<td>1. Neutrophils</td>
</tr>
<tr>
<td></td>
<td>2. Monocytes/macrophages</td>
</tr>
<tr>
<td>3. Lung lavage</td>
<td>1. Alveolar macrophage</td>
</tr>
</tbody>
</table>
Figure A1.1 Percentage survival of haemopoietic cells in \textit{in vitro} culture

A) lymphocyte

B) neutrophils

\begin{itemize}
  \item \textit{\begin{tabular}{c}
  \text{\hspace{0.5cm}}
  \end{tabular}}\hspace{0.5cm} sheep
  \item \textit{\begin{tabular}{c}
  \text{\hspace{0.5cm}}
  \end{tabular}}\hspace{0.5cm} goat
  \item \textit{\begin{tabular}{c}
  \text{\hspace{0.5cm}}
  \end{tabular}}\hspace{0.5cm} cattle
\end{itemize}
Figure AI.1 Percentage survival of haemopoietic cells in in vitro culture

C) monocytes
D) mammary gland monocytes

(---) sheep
(---) goat
(---) cattle
Figure AI.1 Percentage survival of haemopoietic cells in

in vitro culture

E) alveolar macrophages

F) leucocytes

(—···) sheep

(—···) goat

(—···) cattle
Figure AI. 2a Sheep peripheral blood monocyte culture 24 - 48 hours post culture (x800). Cells stained with methylene blue.

Figure AI. 2b Sheep peripheral blood monocyte culture 72 - 96 hours post culture (x700). Cells stained with methylene blue. Note appearance of macrophage cells.
Figure AI. 2c  Sheep peripheral blood monocytes 5 - 7 days post culture (x700). Cells stained with methylene blue.

Figure AI. 2d  Sheep peripheral blood monocytes 7 - 10 days post culture (x1,400). Island of established macrophage cells. Cells stained with methylene blue.
Figure AI. 2e Confluent monolayer of established macrophages derived from sheep peripheral blood 12 - 14 days post culture (x1600). Cells stained with methylene blue.

Figure AI. 2f Established macrophage cell line derived from sheep peripheral blood (x2400). Cells stained with methylene blue.
Figure AI.3  Cytospin preparation of established sheep peripheral blood macrophages. Note the vacuolated cytoplasm and the many pseudopodia. (x2,400)
Figure A1.4 Thin section electron micrograph of established sheep peripheral blood macrophages. (x41,000)

(P) phagosomes

(MF) microfilaments

(Mt) mitochondria

(N) nucleus
Figure AI.5  Cell production in sheep mammary glands inoculated with *E.coli* lipopolysaccharide

A) cell production

B) percentage of each cell type

(—) total count

(—■) neutrophils

(—■■) monocytes

(—■■■) lymphocytes
Figure AI.6  Cell production in cattle mammary gland inoculated with *E. coli* lipopolysaccharide

A) cell production

B) percentage of each cell type

(---) total count
(---) neutrophils
(---) monocytes
(---) lymphocytes
Figure AI. 7  Cell production in goat mammary gland inoculated
with \textit{E. coli} lipopolysaccharide

A) cell production

B) percentage of each cell type

(- - - ) total count

(- - - ) neutrophils

(- - - ) monocytes

(- - - - ) lymphocytes
APPENDIX II

Media, materials and methods
Media

1. **Antibiotic solution:**
   A stock solution contained 2,500 international units (i.u.) of
   *mycostatin/ml, 5,000 i.u. of Neomycin sulphate/ml, 2,500 i.u.
   Crystapen (benzyl penicillin (sodium) B.P.) and 500 i.u. Aerosporine
   (sulfate de Polymyxine B). The antibiotic solution was used at
   either 1% or 2% final concentration.

2. **BHK/21 Growth medium:**
   Eagles basal medium was supplemented with 10% tryptose broth,
   10% foetal calf serum and 1% antibiotic solution.

3. **BHK/21 Maintenance medium:**
   Eagles basal medium was supplemented with 10% tryptose phosphate
   broth, 2% foetal calf serum and 1% antibiotic solution.

4. **Leucocyte Growth medium:**
   Rose/Park Memorial Institute 1640 medium (RPMI 1640) (Moore
   *et al.,* 1967) was supplemented with glutamine (300 mg per litre),
   10% foetal calf serum and 1% antibiotic solution.

5. **Leucocyte Maintenance medium:**
   RPMI 1640 medium supplemented with glutamine and 2% foetal calf
   serum and 1% antibiotic solution.

6. **Foetal calf serum:**
   As supplied by Flow Laboratories, Irvine, Ayrshire, Scotland.

* E.R. Squibb and Sons Ltd., Twickenham, Middlesex
* Upjohn Ltd., Fleming Way, Crawley, Sussex
* Glaxo Ltd., Greenford
* Wellcome Foundation and Co. London
Methods

1. Preparation and fixation of cells for thin section electron microscopy

The cells were washed first in phosphate buffered saline and then fixed in 1% glutaraldehyde for 10 to 15 minutes, preferably in situ, and the cells were then washed 3 times in phosphate buffered saline. The cells were transferred to a glass test tube by detaching them into the second or third saline wash using a glass rod. The cells were centrifuged and the supernatant removed. One drop of 1% osmium tetroxide was then placed on the pellet. After 30 minutes the cells were washed 3 or more times with distilled water until the smell of osmium was removed and all the washes were discarded into saturated sodium hydroxide. If there was only a small number of cells, these were transferred into 0.4 ml polythene minicentrifuge tubes. Two tubes were used for each sample. The cells were then pelleted using the mini-centrifuge (400 g, 5 mins) and then left in a pellet for the rest of the process. Larger quantities of cells were left in glass conical test tubes and the cells were agitated after each stage and re-centrifuged. At the Epon stage of the process, the cells were transferred to beam capsules using 2 capsules per sample.

After washing with distilled water the process was as shown in Table A.II. 1.

The capsules or tubes were then placed in a 60°C oven for 48 hours to facilitate Epon hardening after which the sections could be cut using a Reichert OMU 3 microtome.
### Table A.II 1 Fixation procedure of cells for thin section electron microscopy

<table>
<thead>
<tr>
<th>Stage</th>
<th>Small Nos. of cells</th>
<th>Large Nos of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% alcohol</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
<tr>
<td>50% alcohol</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
<tr>
<td>70% alcohol</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
</tbody>
</table>

After the 70% alcohol stage the processing must continue to the end. After removing the supernatant the replacing liquid must be put on immediately to prevent the cells drying out.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Small Nos. of cells</th>
<th>Large Nos of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>90% alcohol</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
<tr>
<td>95% alcohol</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
<tr>
<td>Absolute</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
<tr>
<td>Absolute</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
<tr>
<td>50:50 (v/v) abs. alcohol/propylene oxide</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
<tr>
<td>Propylene oxide</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
<tr>
<td>Propylene oxide</td>
<td>10 minutes</td>
<td>10 minutes and mix</td>
</tr>
<tr>
<td>50:50 prop. oxide/epon</td>
<td>1 hour</td>
<td>1 hour</td>
</tr>
<tr>
<td>Epon (stored)</td>
<td>2 hours</td>
<td>2 hours</td>
</tr>
<tr>
<td>Epon (stored)</td>
<td>overnight</td>
<td>transfer to beam capsules. overnight</td>
</tr>
<tr>
<td>Epon (fresh)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2. Stains for thin section electron microscopy

a) Lead citrate stain - pH 12.0

Into a 50 ml volumetric flask were added 1.33 g of lead nitrate (Pb (NO$_3$)$_2$), 1.76 g sodium citrate (Na$_3$ (C$_6$H$_5$O$_7$)). 2H$_2$O and 30 ml of distilled water. The flask was shaken, intermittently, until the compounds were dissolved after which 8 ml of 1N NaOH and a further 12 ml of distilled water were added. The stain solution was thoroughly mixed and filtered before use.

b) Uranyl acetate stain

Supplied as a saturated solution in absolute methanol and filtered before use.

3. Staining procedure of thin sections for electron microscopy

A drop of saturated solution of uranyl acetate, in methanol, was placed in a paraffin wax covered petri dish and the grids (plus thin section) were inverted (thin section down) onto the stain and left for 5 minutes. After staining, the grids were washed with absolute methanol, dried on blotting paper and finally washed with 0.01N NaOH. The grids were then stained for 5 minutes with lead citrate then washed in 0.1N NaOH, rinsed in sterile distilled water and finally dried on blotting paper.

4. Cytospin preparation of cell smears

Cytospin cell smears were prepared in a Shandon Elliot haematocrit centrifuge (Fig. A.II. 1). Cell suspensions were diluted to a concentration of approximately $5 \times 10^5$ cells/ml and 0.1 ml of the cell suspension was pipetted into the specially designed bucket which was previously clamped to a clean slide (Fig. A.II. 2) inside the haematocrit centrifuge and subsequently centrifuged (200 rpm, 5 mins). After this time the slide was removed and the cell deposit on the slide was allowed to air dry before staining of the cells with
Wright's or Giemsa stain (Fig. A.II. 3).

5. **Ammonium chloride/tris (ACT) solution** (Symons and Binns, 1975)
   
   Nine volumes of 0.83% (w/v) NH₄Cl plus 1 volume of tris buffer (20.594 g/1000 ml of distilled water, adjust to pH 7.6 with NHCl). Adjust the pH of the ACT solution to 7.2 with NHCl.

6. **Preparation of lymphocytes using ACT** (Symons and Binns, 1975)
   
   To 1 volume of heparinised venous blood was added 5 volumes of ACT solution, thoroughly mixed together and placed at 37°C for 3 minutes. After the incubation period, 6 volumes of phosphate buffered saline were added and then centrifuged (300 g, 5 mins, 4°C). The cell pellet was resuspended in 5 ml of LYH and layered onto a ficoll/hypaque gradient (1.0774 g/cm³ at 25°C), centrifuged (200 g, 20 mins, 4°C) and the cell band (predominantly lymphocytes) at the interface of LYH and gradients was removed.
APPENDIX II

Figures
Figure Aii. 1  Shandon Elliott cytospin
Figure AII. 2  Centrifuge buckets for Shandon Elliott cytospin
Figure AII. 3 Cell deposit (stained with giemsa) after centrifugation in cytospin centrifuge

The migration test on circulating bovine leucocytes and its possible application in the diagnosis of Johne's disease.

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