Studies on the role of Parainfluenzavirus Type 3 and Adenovirus in Respiratory Disease of Sheep.

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CONTENTS

Page

DECLARATION

The work reported in this thesis was part of a larger project concerned with investigations of respiratory diseases of sheep, and consequently many of the experimental findings were obtained in collaboration with my colleagues at the Moredun Institute. Nevertheless, most of the work presented in this thesis was carried out by myself and where conjoint experiments were necessary, a full role was played in the design of the experiments and in the interpretation of the results.

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SUMMARY

Investigations were undertaken to isolate viruses from sheep, and to assess their role in respiratory diseases by means of epidemiological observations and experimental studies of their pathogenesis.

Viruses were isolated from 0·7 per cent. of samples taken at necropsies and from 16 per cent. of sheep in 3 of 4 flocks currently experiencing outbreaks of respiratory disease, but not from 7 flocks with few or no signs of clinical illness.

An adenovirus (strain 7769) was isolated from rectal swabs, but not nasal swabs, from 3 of 15 lambs during an outbreak of pneumonia in a group of 4 to 10-week-old lambs. This adenovirus differed antigenically from other ovine, bovine and human adenoviruses, and the species of erythrocytes that were agglutinated by strain 7769 differed from those agglutinated by porcine, canine, equine and murine adenoviruses. It was concluded that strain 7769 was a previously unreported type of adenovirus and was designated ovine adenovirus type 4 (OA4).

Although antibodies to the adenovirus group specific antigen were detected in only 6 per cent. of 661 sheep sera, neutralizing antibodies to 4 serotypes of ovine adenovirus were more common. Neutralizing antibodies to ovine adenovirus types 1 - 4 were more prevalent in animals over 12 months of age.

Following exposure of specific pathogen-free (SPF)

lambs to an aerosol of OA4 virus, replication of this virus occurred in the respiratory and alimentary tracts and liver, and neutralizing antibodies could be detected in the serum and nasal secretions as early as 8 days after inoculation. Infection was associated with a mild clinical illness, detectable by auscultation only, and accompanied by lesions in the lungs and liver. The lesions found in the lungs of infected lambs were pulmonary oedema and peribronchiolar accumulations of mononuclear cells and in the livers were focal necrosis, lymphangitis and occlusive cholangitis.

The clinical disease and pneumonic lesions observed in SPF lambs infected with both ovine adenovirus type 4 and Pasteurella haemolytica were no more severe than those in lambs infected with P.haemolytica alone.

Enzootic pneumonia was induced consistently in SPF lambs inoculated with parainfluenza virus type 3 (PI3) followed by P.haemolytica 4 or 7 days later. Seventy-eight per cent. of lambs developed severe respiratory disease by this method, 54 per cent. died and 95 per cent. had macroscopic lung lesions. The illness and lesions were more marked in lambs inoculated with both PI3 virus and P.haemolytica than in lambs inoculated with either agent alone and were associated with rapid multiplication of P.haemolytica within the lung.

REVIEW OF LITERATURE

Pneumonia is an important disease which has been recognised as a problem in sheep for many years. It has an almost worldwide distribution and is regarded as a major cause of economic loss to the sheep industry. The range of microorganisms associated with respiratory disease in man and the domestic animals is broad, and numerous unsuccessful attempts to reproduce the clinical condition experimentally with individual organisms have led to the view that this disease has a complex aetiology.

As a preliminary to this work it was thought that it would be useful to have some measure of the importance of pneumonia to the national flock. Unfortunately, this is an area where accurate data are lacking but some indication may be gained by assessing two parameters, viz. the number of deaths attributable to pneumonia and the number of lungs containing pneumonic lesions at slaughter.

Several surveys have been carried out on the causes of death in sheep and, from these, pneumonia appears to be a recurring problem which has fluctuated little over the years. For example, Rowlands (1955) ascribed death in 19 of 374 (5 per cent) adult sheep to "septic pneumonia" and, in a more recent report (Hughes, 1964), death in 10 per cent. of 715 sheep of all ages from 14 farms was attributed to pneumonia. A more extensive survey, conducted over a 2 year period by Veterinary Investigation Centres in England and Wales, showed that 8 per cent. of 9,960 deaths were due

to respiratory disease (Hughes, 1964). The interpretation of these findings is limited by the failure of the surveys to take into account those animals which recover. Presumably, pneumonic lesions in recovered animals would be obvious at slaughter and, therefore, a more balanced view of ovine pneumonia would be obtained at the abattoir thereby offsetting the deficiencies of the above surveys. However, accurate records of pneumonic lesions are not kept at abattoirs. The only figures available are those for the number of lungs which are condemned, and data obtained from 3 Scottish abattoirs show that, during a 12 month period, 0·7 per cent. of 524,838 lungs were seized (Table 1). This figure grossly underestimates the true situation as, in many cases, individual lobes containing pneumonic lesions may be resected without condemnation of the whole lung. It has been estimated that the percentage of sheep lungs with pneumonic lesions varies between 5 and 80 on any one day and is, on average, about 20.

Whilst the above figures give some indication of the of the more obvious manifestations of respiratory disease in sheep, they do not take into account the effects of clinical and subclinical respiratory disease on the food conversion ratio and growth rate of the affected animals. For these reasons, it is extremely difficult to estimate the prevalence of respiratory disease in sheep and make some appraisal of its economic importance.

There is, at present, a general trend towards more

TABLE 1

NUMBER OF LUNGS CONDEMNED FOR PNEUMONIA AT THREE SCOTTISH ABATTOIRS, DURING 1973.

Abattoir	Number of sheep slaughtered	Number of lungs condemned	Per cent. condemned
Dundee	31,738	263	0·8
Edinburgh	186,287	763	0·4
Glasgow	306,813	1,244	0·4
Total	524,838	2,270	0·4

The data in this table were kindly supplied by Messrs. Dunn, McCance and Norval.

intensive methods of animal husbandry and, consequently, a greater potential for transmission of disease. Such problems have already been encountered in the pig and poultry industries where, despite controlled environment houses and a high standard of husbandry, the potential for explosive outbreaks of disease remains. In the British Isles, the situation is best exemplified by the recent epidemics of Newcastle disease in poultry. There are now indications that traditional methods of sheep husbandry are being supplemented, and sometimes replaced, by intensive methods including the housing of sheep. One report indicated that no fewer than 76,957 breeding sheep and 23,673 fattening lambs were housed, mainly in adapted buildings, on 356 farms in the British Isles (Farm Buildings Centre, 1967). Clearly, if this trend continues it would be reasonable to expect a rise in the number of sheep affected with respiratory disease and, in these circumstances, adequate control measures would be required.

The aetiology of respiratory disease is complex and attempts to identify the important agents based on pathological descriptions of the lesions, have contributed little towards its understanding, because the lung can respond only in a limited number of ways and several agents can produce similar lesions (Stevenson, 1968). Consequently, the solution to the problem appears to lie in investigations of the involvement of the microorganisms which are associated with respiratory diseases of sheep. Much of the published work to date has centred on the role

of bacteria, particularly Pasteurella haemolytica, which can be isolated frequently at necropsy from the lungs of sheep which have died from respiratory disease. However, it should be emphasized that inoculation of Pasteurella haemolytica into clinically healthy sheep generally fails to reproduce the clinical illness and these inconclusive results have initiated a search for other agents or factors, such as chlamydiae, mycoplasmas and viruses.

Although the first virus to be isolated from the respiratory tract of sheep, affected with acute respiratory disease, was parainfluenza virus type 3 in 1966, the aetiological significance of this finding is not yet clear and our knowledge of other viral infections of the ovine respiratory tract is meagre. In contrast to this, the catalogue of viruses isolated from the respiratory tracts of other domestic animals closely resembles that described for humans, and there is no reason why this should not be the case for sheep. From the available information, and by extrapolation from other species, it is possible to compile a list of those viruses which may be present in the respiratory tract of sheep, and which may play a role in the aetiology of ovine respiratory disease (Table 2).

For these reasons, it was considered important in the present investigation to review adequately the literature relating to the presence of viruses in sheep and, where possible, examine their aetiological role in respiratory infections.

TABLE 2

VIRUSES ASSOCIATED WITH RESPIRATORY DISEASES OF SHEEP.

Virus Group	Type	Date of isolation	Author	Serological evidence
Orthomyxovirus	influenza type A	1962	Romvary *et al*	-
Paramyxovirus	parainfluenza 1	-		±
	parainfluenza 2	-		±
	parainfluenza 3	1966	Hore	+
Pneumovirus	respiratory syncytial virus	-		+
Picornavirus	enterovirus	1969	McFerran *et al*	-
	rhinovirus	-		-
	calicivirus	-		-
Reovirus	type 1	-		+
	type 2	-		+
	type 3	1969	McFerran *et al*	+
Lentivirus	maedivirus	1964	Sigurdardottir & Thormar	+
Coronavirus		-		-
Adenovirus	ovine type 1 type 2 type 3	1969	McFerran *et al*	+
	unknown	1970	Snowdon	
Herpesvirus	jaagsiekte	1969	Mackay	+
	others	-		-

ORTHOMYXOVIRUS

There are only two reports in the literature concerning the isolation of influenza virus from sheep. Romvary, Takatsy, Barb and Farkas (1962) and Barb, Farkas, Romvary and Takatsy (1962) reported the isolation of influenza A2 from a ewe with respiratory disease, and her mature foetus, by inoculating lung suspensions into the amniotic cavity or yolk sac of 9 to 11-day-old chick embryos. The isolate appeared to be related antigenically to influenza A2/Singapore 1/57, but the authors failed to indicate whether the virus was re-isolated from the original material, and did not exclude the possibility of laboratory contamination. The same authors also reported clinical respiratory illness, together with a serological response and pneumonic lesions, in lambs inoculated intratracheally with human (PR8 and Sing.1/57) and porcine (Shope) strains of influenza A virus. Virus was re-isolated from each lung, but no reference was made to the volume and infectivity of the inoculum, the amount of virus in the lungs, or to the absence of lesions in the lungs of any control lambs.

McQueen and Davenport (1963) detected an antibody response in sheep inoculated intratracheally with high concentrations of either influenza A/PR8 or influenza A/Bor (Hungarian isolate from sheep), but failed to produce clinical respiratory illness. No virus was isolated from nasal swabs taken 48 and 72 hours post-inoculation, or suspensions of lung and tracheal mucosa from 2 lambs killed

72 hours post-inoculation. Schmidt, Maassab and Davenport (1974) have shown by *in vitro* experiments that compared to homologous cell-virus interactions, heterologous interactions required 10 times more influenza virus to establish infections of cell cultures and lower yields of virus were obtained. If such a mechanism is operative *in vivo*, then infections of sheep with influenza viruses may not occur or only very rarely.

PARAMYXOVIRUSES

Parainfluenza virus type 3 (PI3).

The first evidence that PI3 virus was associated with naturally occurring respiratory disease in sheep was provided by Hore (1966) who isolated the virus from nasal swabs obtained from a group of lambs showing signs of respiratory illness. One of these isolates, designated the G2 strain, was shown by haemagglutination-inhibition (HI) and neutralization tests to be related to, but distinct from, the T1 strain of bovine PI3 virus (Hore, 1966, 1968). Subsequent to this report, PI3 virus has been isolated from the respiratory tract of sheep in other countries (Ditchfield, 1966; Erhan and Martin, 1969; St.George, 1969a; Giauffret and Russo, 1972).

Parainfluenza virus type 3 infections of sheep have a worldwide distribution and workers in many countries have reported the presence of HI and neutralizing antibodies in the sera of healthy sheep and those affected with respiratory disease (Table 3). Although the majority of these surveys have not been comprehensive, the available evidence suggests that antibody to PI3 virus is prevalent within the indigenous sheep population. For example, in Britain, Hore (1969) examined 500 sera from 35 farms and found not only that 53 per cent.of sheep possessed HI antibody with a geometric mean titre of 81, but also that animals in 28 of the 35 flocks, which he examined, possessed antibody to the virus. Similar results have been reported

TABLE 3

WORLD DISTRIBUTION OF SERUM ANTIBODY TO PI3 IN SHEEP

Country	Serum Antibodies		Per cent. positive	Author
	Haemagglutination Inhibiting	Neutralizing		
Australia		13/23*	57	St.George & French, 1966.
		630/816	77	St.George, 1971.
Central Africa	23/29		79	Provost et al, 1967.
Egypt	10/64	9/64	16	Singh & Ata, 1967.
France	24/30		80	Faye et al, 1967.
Great Britain	265/500		53	Hore, 1968.
South Africa	34/35		97	Erasmus et al, 1967.
Syria	59/90		66	Lange, 1967.
Turkey	269/333		80 81	Erhan & Martin, 1969.
U.S.A.	97/121	80/121	80	Fischman, 1967.

* no. positive / no. tested.

from Australia, where St.George (1971) observed that 77 per cent. of 816 sera contained neutralizing antibody with titres of $\geqslant 5$, and 87 per cent of flocks possessed antibody. In Turkey also, Erhan and Martin (1969) found that no fewer than 80 per cent. of 333 sera contained HI antibody.

Hore (1968, 1969) also observed that there was little variation between age groups as regards the percentage of animals with serum antibody to PI3 virus, although there was a decrease in the geometric mean HI antibody titre in sheep 2 years of age or older. He regarded these observations as evidence for recent infections in young sheep and that older sheep are seldom exposed to reinfections with PI3 virus. Conversely, in Australia, although most sheep became infected during their first year of life, the percentage of sheep with neutralizing antibodies in their sera increased with age, indicating that infection with PI3 virus can occur in sheep up to 42 months old (St.George, 1971).

Parainfluenza virus type 3 is a well recognised respiratory pathogen in humans, which is capable of producing a spectrum of disease ranging from inapparent infection to serious illness of the lower respiratory tract (Chanock, Parrott, Johnson, Kapikian and Bell, 1963). There is evidence that PI3 virus is associated with a similar spectrum of naturally occurring disease in sheep. Although St.George (1971) considered that many PI3 virus infections in sheep were of a subclinical nature, he arrived at this

conclusion indirectly, by noting that antibody to the virus was present in 80 per cent. of sheep despite the fact that the majority had not shown signs of clinical respiratory disease. More direct evidence was reported by Fischman (1967) who detected a rise in serum antibody titres to PI3 virus in a flock of sheep when there was no exacerbation of an existing mild respiratory disease. More recently, St.George and Liefman (1972) obtained similar results in a flock of lambs in Australia. The fact that PI3 virus may also be associated with a mild form of respiratory disease was reported by Hore (1966) who isolated the virus from the anterior nares of lambs affected with mild nasal and ocular discharges. In addition to the mild and inapparent types of infections, PI3 virus has been associated with more severe forms of respiratory disease. The virus has been isolated from the pneumonic lungs of individual animals (Hore, 1968; Erhan and Martin, 1969) and from the respiratory tract of lambs during outbreaks of respiratory disease involving such clinical signs as pyrexia, dullness, tachypnoea, dyspnoea and death (Ditchfield, 1966; Hore, Stevenson, Gilmour, Vantsis and Thompson, 1968; St.George, 1969a, 1972; Giauffret and Russo, 1972).

The clinical response of lambs following the experimental inoculation of the G2 strain of ovine PI3 has been variable. Simultaneous intratracheal and intranasal inoculation of colostrum-deprived and colostrum-fed lambs within 48 hours of birth with $10^{5.7}$ $TCID_{50}$ of the G2 strain

resulted in a mild respiratory infection with a slight mucoid nasal discharge and a transient pyrexia. By the fifth or sixth day post-inoculation, the lambs exhibited a slight degree of respiratory distress and occasional coughing (Hore and Stevenson, 1967, 1969; Stevenson, 1968). The response of weaned lambs to combined intratracheal and intranasal inoculation with either a large dose ($10^{6.7}TCID_{50}$) or small dose ($10^{5.0}TCID_{50}$) of PI3 virus also was generally mild, and was characterized by a slight mucoid nasal discharge with an excessive cellular content (Hore, 1968). Inoculation of specific pathogen-free lambs with PI3 virus by the intranasal route or by aerosol did not induce clinical illness (Smith, 1975).

At necropsy, dull red areas of consolidation were present in the lungs of neonatal lambs inoculated with PI3 virus, and the lesions were most extensive in lambs killed between the sixth and eighth days post-inoculation (Hore and Stevenson, 1969; Stevenson, 1968). The essential histological features of these lesions consisted of hyperplasia of the bronchiolar epithelium, infiltration of inter-alveolar septa by mononuclear cells and cellular exudate in the bronchiolar lumen. The presence of acidophilic intracytoplasmic inclusions in the bronchiolar epithelium was considered to be pathognomonic of the disease but this was a transient feature. The severity of the lesions progressed from the third day post-innoculation and reached a maximum between the sixth and eigth days, when alveolar epithelialization, foci of alveolar necrosis and neutrophil

infiltration were also present (Hore and Stevenson, 1969). From the ninth day, the lesions began to resolve (Stevenson, 1968), as judged by the reappearance of a recognisable alveolar structure, numerous mitotic figures in the bronchiolar epithelium and the absence of cytoplasmic inclusions. Nevertheless, there were residual lesions of mild interstitial pneumonia and focal alveolar epithelialisation in most lungs up to 28 days post-inoculation.

The effect of experimental infection with PI3 virus in conjunction with Pasteurella haemolytica biotype A and their involvement in enzootic pneumonia of sheep, has been examined by Biberstein, Shreeve, Angus and Thompson (1971). Although the number of lambs involved was small, the inoculation of PI3 virus three days before the inoculation of P. haemolytica appeared to cause a more serious type of illness together with more severe lesions than were obtained by inoculation of Pasteurella alone. Despite these findings, the authors did not consider that they had obtained convincing evidence of synergism or involvement of PI3 virus in the aetiology of enzootic pneumonia.

Several authors have investigated the infection of sheep and cattle with the heterologous PI3 virus. Woods, Sibinovic and Marquis (1965), Faye and Charton (1967), St.George (1969a) and Stevenson and Hore (1970) successfully infected lambs with strains of bovine PI3 virus and re-isolated the virus. However, only Faye and Charton (1967) and Stevenson and Hore (1970) succeeded in inducing clinical illness and pneumonic lesions. It has also proved possible

to infect calves with the G2 strain of ovine PI3 virus and produce typical histological lesions in the lungs (Stevenson and Hore, 1970).

Parainfluenza virus types 1 (PI1) and 2 (PI2).

In addition to PI3 virus, other parainfluenza viruses may be involved in respiratory disease of sheep. Both PI1 virus and PI2 virus have been associated with croup in young children (Chanock, Parrott, Johnson, Kapikian and Bell, 1963; Chanock and Parrott, 1965a) while respiratory illness and infection can be produced experimentally in adult volunteers (Reichelderfer, Chanock, Craighead, Huebner, Turner, James and Ward, 1958; Taylor-Robinson and Bynoe, 1963). Sendai virus, a strain of PII, can exist as a latent infection in mice and may occasionally be activated to produce pneumonia (Chu, Lian and Wen, 1957). Also, Sendai virus has been shown to produce pneumonia in pigs (Sasahara, 1955), although recent evidence suggests that this virus is not present in pigs in Britain (Harkness, Chapman and Darbyshire, 1971).

Infections of cattle with parainfluenza virus type 2 have not been considered to any extent but may be quite common as, in Germany, HI antibodies have been detected in 71 per cent. of 1557 bovine sera (Woernle, Brunner and Kussmaul, 1973). In sheep, Fischman (1965) failed to detect HI antibody to PI1 virus or PI2 virus in 5 and 9 sera respectively, whereas Cuadrado (1965) detected HI antibody to PI1 virus in 31 per cent. of 100 ovine sera, and to PI2 virus in 95 per cent. Cuadrado (1965) also found a

correlation between HI, neutralizing, complement-fixing and precipitating antibodies to mumps virus in the same sheep sera. Much of the literature relating to parainfluenza virus infections of sheep and cattle is of questionable value in the light of the heterotypic serological responses of humans to infections with paramyxoviruses (Chanock, Wong, Heuber and Bell, 1960; Parrott, Vargosko, Kim, Bell and Chanock, 1962).

PNEUMOVIRUS.

Respiratory Syncytial Virus.

Respiratory syncytial (RS) virus was first isolated from a chimpanzee with upper respiratory tract illness (Morris, Blount and Savage, 1956) and subsequently shown to produce inapparent infections in several species of laboratory animals (Coates and Chanock, 1962). Respiratory syncytial virus is now known to be the most important cause of severe viral respiratory illness in young children, particularly infants less than 6 months old in whom it has been associated with approximately 50 per cent. of bronchiolitis cases and up to 40 per cent. of pneumonic cases (Chanock, Roizman and Meyers, 1957; Chanock, Kim, Vargosko, Deleva, Johnson, Cumming and Parrott, 1961; Chanock and Parrott, 1965b).

Infection of cattle with RS virus was suggested first by the detection of an inhibitory substance, most probably immunoglobulin, in bovine sera (Taylor-Robinson and Dogget,

1963; Dogget, Taylor-Robinson and Gallop, 1968). The association of RS virus with respiratory disease in cattle has been confirmed in several countries by the isolation from the upper respiratory tract of cattle with respiratory disease of cytopathic agents closely related antigenically to human RS virus (Paccaud and Jacquier, 1970; Inaba, Tanaka, Omori and Matumoto, 1970; Wellemans, Leunen and Luchsinger, 1970; Jacobs and Edington, 1971).

During the course of this thesis, evidence became available which suggested that RS virus could infect sheep. Berthiaume, Joncas, Boulay and Pavilanis (1973) detected complement-fixing antibody in sera from 25 of 31 (81 per cent.) sheep in Canada, and Smith, Frey and Dierks (1975) claimed that neutralizing antibodies were widespread in sera of sheep in the U.S.A., although mean antibody titres were 2 to 4-fold lower than in cattle.

PICORNAVIRUSES.

Picornaviridae is the family name which includes 3 genera, Enterovirus, Rhinovirus and possibly Calicivirus. Several members from each of these genera have been shown to produce or be associated with respiratory disease in a number of species of animals.

Enteroviruses, such as Coxsackieviruses and echoviruses, have been associated with mild upper and severe lower respiratory tract illnesses in humans (Dalldorf and Melnick, 1965; Melnick, 1965). In cattle, Thomas (1973) isolated

an enterovirus from a pharyngeal swab from a calf during an outbreak of respiratory disease, and Spradbrow and Cole (1971) showed that a picornavirus isolated from bovine faeces could produce pneumonia in calves when inoculated intratracheally in conjunction with E.coli.

Rhinoviruses are associated with the common cold syndrome in humans (Andrewes and Tyrrell, 1965). Two serotypes of rhinovirus have been isolated from the respiratory tract of horses affected with rhinitis and pharyngitis (Ditchfield and Macpherson, 1965) and also two antigenic types, other than foot-and-mouth disease virus, from the bovine respiratory tract (Bögel and Böhm, 1962; Reed, Tyrrell, Betts and Watt, 1971; Ide and Darbyshire, 1972b). The bovine rhinoviruses have been associated with both spontaneous and experimentally-induced mild respiratory illness (Rosenquist, 1971; Betts, Edington, Jennings and Reed, 1971; Ide and Darbyshire, 1972a).

Calciviruses can be frequently isolated from cats with upper, and occasionally lower, respiratory tract diseases (Crandell and York, 1966; Gillespie and Scott, 1973). Experimental infections in both conventional and specific pathogen-free kittens have produced variable clinical responses. The typical disease is a mild upper respiratory tract illness, although some strains also have produced an interstitial pneumonia (Povey and Hale, 1974).

In 1969, McFerran, Nelson, McCracken and Ross reported the isolation from sheep faeces of cytopathic agents, which morphologically resembled picornoviruses, and a selected

strain was subsequently classified as an enterovirus on the basis of its morphological, physical and biological properties.

In India also, cytopathic agents were isolated from 6 of 32 ovine rectal swabs (Rao, Saraswat and Pathak, 1972). The CPE appeared after 3-4 days incubation and was characterized by syncytial formation, cytoplasmic vacuolation and other degenerative changes, but these findings are not consistent with the authors' claim that the agent is an enterovirus. In the absence of any further information, their claim must remain unsubstantiated.

REOVIRUSES.

The relationship of reoviruses to respiratory disease is uncertain as they appear to be ubiquitous in nature and are most often recovered from faeces. However, reoviruses have been isolated from the upper respiratory tract of spontaneously and experimentally infected humans (Rosen, Hovis, Mastrota, Bell and Huebner, 1960a, b; Jackson, Muldoon, Johnson and Dowling, 1963), cattle (Rosen and Abinanti, 1960; Kurogi, Inaba, Takahashi, Sato, Goto, Omori and Matumoto, 1974) and dogs (Lou and Wenner, 1963; Thompson, Wright and Cornwell, 1970). In addition, reoviruses have been associated with the presence of interstitial pneumonitis in dogs (Lou and Wenner, 1963), mice (Stanley, Leak, Walters and Joske, 1964), and a child (Joske, Keall, Leak, Stanley and Walters, 1964). Inhibitors of

haemagglutination by reovirus types 1,2 and 3 were first recognised in sheep sera by Stanley, Leak, Grieve and Perret (1964). The inhibitor of reovirus type 3 haemagglutinin was present in 54 of 56 sera (96 per cent.) and those of reovirus types 1 and 2 in approximately 15 per cent. In a large proportion of the sera that were examined these inhibitors were present at titres of 80 or greater. Ten sera were examined also for virus neutralizing antibody, which was detected and found to correlate quite closely with the inhibitor of haemagglutination. For these reasons, and also because inhibition of haemagglutination by one serotype did not result in inhibition of another serotype, these authors considered that the inhibitors were specific antibodies.

Similar findings have been reported in Scotland by Pringle and Cartwright (1969), who observed that the addition of pooled sheep sera to cell culture media not only depressed yields of reovirus type 3 but also delayed the cytopathic effects of the virus in cell culture. Fractionation of the pooled sera by DEAE-cellulose or Sephadex G200 showed that the inhibitory activity occurred mainly in the immunoglobulin M region while the neutralizing activity was largely confined to those fractions which contained most of the immunoglobulin G. The authors concluded that their results indicated extensive exposure of sheep to reovirus infection.

These observations have been supported by the isolation of reovirus type 3 from the faeces of sheep in Northern

Ireland (McFerran, Nelson and Clarke, 1973), reovirus type 1 from lambs in Hungary (Belak and Palfi, 1974) and reovirus type 2 from lambs in Scotland (Snodgrass, Burrells and Wells, 1976). Forty-six per cent. of 155 sera from adult sheep, slaughtered at abattoirs throughout Northern Ireland contained inhibitors of haemagglutination by reovirus type 3, but none to reovirus types 1 or 2 (McFerran, Nelson and Clarke, 1973). There was a good correlation between the haemagglutination-inhibition and neutralizing activities in 30 of these sera and the authors considered that they were detecting specific antibody. These observations suggest not only that infections by reovirus type 3 are prevalent but also that most are asymptomatic, which is borne out by experimental studies of its pathogenicity. Combined intranasal and oral inoculation of six-month-old lambs with reovirus type 3 failed to produce clinical disease, although the virus appeared to replicate in the respiratory and alimentary tracts, and stimulated a serological response (McFerran, Baskerville and Nelson, 1974). The presence of non-specific neutralizing substances in the sera of some inoculated lambs did not influence the course of the infection nor the serologic response.

These observations are in contrast to those made with reovirus type 1, which was isolated from nasal and rectal swabs from lambs with nasal discharge, conjunctivitis and diarrhoea (Belak and Palfi, 1974a). In this report, intranasal or intratracheal inoculation of 2 to 4-week-old

lambs with $10^{3.0}$ $TCID_{50}$ of the Hungarian isolate of reovirus type 1 resulted in lambs developing disease similar to that encountered in the spontaneous outbreak. The virus was isolated from nasal and rectal swabs between days 4 and 9 post-inoculation, and specific neutralizing antibodies were detected 3 weeks after infection (Belak and Palfi, 1974a).

CORONAVIRUSES.

Coronaviruses have been shown to be the cause of a variety of diseases, but in humans and chickens, particularly, they are associated with infections of the respiratory tract.

In man, coronaviruses have been associated with acute upper respiratory tract illness in adults (Tyrrell and Bynoe, 1965; McIntosh, Kapikian, Turner, Hartley, Parrott and Chanock, 1970) and their ability to produce a common cold-like syndrome in adult volunteers also has been demonstrated (Bradburne, Bynoe and Tyrrell, 1967). The situation in children is not so clear, as coronavirus infections are commonly detected in young people admitted to hospital with lower respiratory tract disease, but no more so than in equivalent controls. Nevertheless, coronavirus strains have been recovered from the lungs of 2 infants during the acute stage of pneumonia (McIntosh, Chao, Krause, Wasil, Mocega and Mufson, 1974).

Coronavirus infection of chickens can be very serious and birds less than 4 weeks old may suffer from severe

bronchitis, with a mortality rate approaching 90 per cent. (Hungerford, 1969). It appears that more severe illness develops when the coronavirus acts in combination with other agents, notably M.gallisepticum and certain serotypes of E.coli (Jordan, 1972). There is as yet, however, no information regarding infection of sheep with coronaviruses.

ADENOVIRUSES.

Adenoviruses have been associated with infections and diseases of the respiratory tract in several species, including humans (Ginsberg and Dingle, 1965; Brandt, Kim, Vargosko, Jeffries, Arrobio, Rindge, Parrott and Chanock, 1969), cattle (Darbyshire and Pereira, 1964; Phillip and Darbyshire, 1972), horses (Johnston and Hutchins, 1967; McChesney, England, Adcock, Stackhouse and Chow, 1970) and dogs (Thompson, Wright and Cornwell, 1975).

All serotypes of adenovirus from various mammalian species have been shown to possess a common group-specific antigen by either complement-fixation or gel-diffusion tests (Andrewes and Pereira, 1972). This property was first employed as a means of detecting antibody to adenovirus in the sera of several mammalian species by Darbyshire and Pereira (1964). They used a gel-diffusion technique and detected precipitating antibody to the group specific antigen in one of 103 ovine sera. The technique has been adopted by other workers. For example, in Iran, Afshar (1969) detected antibody in 46 of 326 (14 per cent.) ovine

sera, while in Macedonia, Paschaleri-Papadopoulou (1968) found 32 of 102 (31 per cent.) ovine sera contained antibody. The high percentage of sheep with antibody in these countries contrasts markedly with that found in Britain and Eire, where both Darbyshire and Pereira (1964) and Timoney (1971) recorded that less than one per cent. of sheep possessed antibodies to adenovirus. The reasons for this discrepancy are not immediately apparent, but it should be emphasized that Darbyshire and Pereira (1964) alone stated that a "positive" reaction was confirmed by the demonstration of a line of identity with a known positive reaction in the gel-diffusion test. All of these authors gave few details of the source or age of the sheep from which sera were taken. This omission may be important in the light of observations by McFerran, Clarke, Knox and Connor (1972), who found that adenoviruses could be isolated frequently on particular farms.

Following the demonstration of antibody in sheep sera, McFerran, Nelson, McCracken and Ross (1960) reported the isolation of 8 strains of adenovirus from the faeces of sheep in Northern Ireland. They later were able to assign 7 of these isolates to 3 antigenic serotypes, but the 8th strain produced little infectious virus and was not typed (McFerran, Nelson and Knox, 1971).

Since starting work for this thesis two further serotypes of adenovirus have been isolated from sheep. One serotype, antigenically similar to bovine adenovirus type 2, was isolated in Hungary from nasal swabs from lambs affected

with respiratory illness and enteritis (Belak and Palfi, 1974b). Intratracheal inoculation of lambs with this virus resulted in replication of the virus in the respiratory and alimentary tracts, accompanied by clinical respiratory disease and enteritis similar to that observed in the natural outbreak. The other adenovirus was isolated from the faeces of healthy lambs in Turkey (Bauer, Müller and Gürtürk, 1975).

Isolations of cytopathic agents resembling adenoviruses have also been made from sheep in Australia but details are not available of the antigenic and physicochemical characteristics of these isolates (Snowdon, 1970, 1971). Recently, Kretzschmar (1973) reported the isolation of an adenovirus from the brain of a lamb with lymphocytic meningoencephalitis, but was unable to exclude the possibility that the virus was a contaminant in his cell cultures.

HERPESVIRUSES.

Herpesviruses have long been associated with naturally occuring and experimentally produced respiratory disease in a number of domestic species, notably equid herpesvirus 1 in equine rhinopneumonitis, bovid herpesvirus 1 in infectious bovine rhinotracheitis (IBR) and feline herpesvirus 1 in feline rhinotracheitis (Burrows, 1968; Phillip and Darbyshire, 1972; Crandell, 1973).

Several herpesviruses that are unrelated antigenically

to IBR virus have been isolated from the respiratory tract of cattle (Bartha, Juhasz, Lieberman, Hantschel and Schulze, 1967; Mohanty, Hammond and Lillie, 1971; Smith, Cutlip, Ritchie and Young, 1972). The DN-599 and FTC-2 strains, which are antigenically related (Potgieter and Mare, 1974), were able to produce mild respiratory disease following experimental inoculation of calves. Recently, IBR virus and another unrelated herpesvirus have been associated with respiratory disease and enteritis in goats, a species closely related to sheep (Mohanty, Lillie, Corselius and Beck, 1972; Saito, Gribble, Berrios, Knight and McKercher, 1974). The herpesvirus that was associated with enteritis failed to produce disease following inoculation of two 1 to 2-week-old lambs, and was reisolated on one occasion only.

Apart from the herpesvirus associated with jaagsiekte, our knowledge of herpesvirus infections of sheep is confined to the findings of Howe, Woods and Marquis (1966) and Parks and England (1974). These authors failed to detect neutralizing antibodies in the sera of 2 and 29 Rocky Mountain bighorn sheep (Ovis canadensis) respectively.

PROGRESSIVE PNEUMONIAS.

The foregoing viruses have been associated with acute and mild forms of respiratory disease from which the affected animal usually recovers. At present, there exist in sheep two forms of chronic progressive pneumonia, sheep pulmonary

adenomatosis (SPA) or jaagsiekte and maedi, which almost invariably culminate in the death of the animal. Maedi is caused by an RNA virus, which is closely related to the RNA tumour viruses but, as it does not occur in this country, it will not be considered further.

Although SPA was not studied during the course of this thesis, a herpesvirus and an RNA tumour virus have been recovered from affected lungs and it would be valuable to review these 2 agents.

Sheep pulmonary adenomatosis is a contagious neoplasm of unknown aetiology (Wandera, 1971). A herpesvirus has been isolated from such tumours in Britain (Mackay, 1969 a and b), Africa (Malmquist, Krauss, Moulton and Wandera, 1972; de Villiers, Els and Verwoerd, 1975) and Jugoslavia (Cvjetanovic, Forsek, Nevjestic and Rukavina, 1972), but attempts to produce adenomata with this virus, by experimental inoculation of sheep, have met with mixed success. Cvjetanovic et al (1972) claimed that adenomatous lesions were present in the lungs of lambs inoculated by the intra-trachial route, 10-22 months previously, with herpesvirus grown in cell cultures, whereas Mackay and Nisbet (1972), de Villiers et al (1975) and Martin, Robinson and Angus (1976) did not find such lesions in the lungs of experimentally inoculated lambs. The results of Cvjetanovic et al (1972) must be viewed with caution as they conflict with those of all the other reports, in addition to which, these workers also claimed to have passaged the virus in guinea-pigs, with the production of typical epithelial proliferations.

Recently, evidence has been presented that C-type particles of an RNA tumour virus, with a buoyant density of 1·15-1·20g/ml, containing 60-70S RNA and an RNA-dependent DNA polymerase (reverse transcriptase), are present in adenomatous tissue, but not lungs from unaffected sheep (Perk, Michalides, Spiegelman and Schlom, 1974). The presence of a similar agent in the adenomatous tissue of affected sheep in Scotland also has been reported (Martin, Scott, Sharp, Angus and Norval, 1976).

The role of the C-type particles in the aetiology of SPA is not clear, but it is possible that they may act together with the herpesvirus. Evidence supporting this view was reported by Martin et al (1976) who found that adenomatous foci were commoner and more extensive in sheep which were inoculated with both agents, than in sheep inoculated with either agent alone.

ABBREVIATIONS

BA - Bovine adenovirus eg. BA2, bovine adenovirus type 2.

CK - Calf kidney.

CPE - Cytopathic effect.

FLK - Foetal lamb kidney.

HA2 - Human adenovirus type 2.

H & E - Haematoxylin and eosin.

HI - Haemagglutination-inhibition.

OA - Ovine adenovirus eg. OA1, ovine adenovirus type 1.

PBS - Phosphate-buffered saline.

pi - Post-inoculation.

PI3 virus Parainfluenza virus type 3.

RS virus- Respiratory syncytial virus.

SPF - Specific pathogen-free.

STh - Sheep thyroid.

TC - Tissue culture.

$TCID_{50}$ - Tissue culture infective dose $_{50}$.

GENERAL MATERIALS AND METHODS.

Cell Culture Procedures.

Cultures of sheep thyroid (STh) cells were prepared by the method of Plowright and Ferris (1961) and calf kidney (CK) and foetal lamb kidney (FLK) as described by Paul (1965).

The growth medium for all cells was Hanks' saline supplemented with 0·5 per cent. lactalbumin hydrolysate, 0·2 per cent. glucose, 0·1 per cent. yeast extract and 10 per cent. heat-inactivated calf serum. For maintenance purposes, the same medium, supplemented with 2 per cent. heat-inactivated horse serum instead of calf serum was used in the experiments described in Chapters 1, 2 and 3, whereas Eagle's medium with one per cent. foetal bovine serum was used in Chapter 4.

Cytological Examinations.

Cell cultures, grown on coverslips, were inoculated with specimens for virus isolation, or stock viruses. At intervals after inoculation, coverslips were removed and stained with haematoxylin and eosin (Hore, 1968) or acridine-orange (Anderson, Armstrong and Niven, 1959) and examined for the presence of inclusions.

Virological Techniques.

a) Isolation Procedures: Nasal and rectal swabs from sheep were placed in transport medium and kept on ice whilst

in transit to the laboratory. Faeces were suspended in Hanks' saline at 10 per cent. (w/v) and either homogenised in an MSE blender or shaken with sterile glass beads. Tissues taken at necropsy were chopped finely and a 10 per cent. (w/v) suspension was prepared in Hanks' saline. The faeces and tissue suspensions were clarified by centrifugation at 2000g for 30 min. at 4°C and the supernates were used to inoculate cell cultures which had been washed 3 times with phosphate-buffered saline (PBS) before inoculation. Swabs and suspensions that were not inoculated immediately were stored at -70°C. All specimens in Chapters 1, 2 and 3 were examined for cytopathic agents by inoculating 0·2 ml into each of 3 or 4 STh tube cultures, which were incubated at 37°C and examined regularly. In Chapter 4, and occasionally in Chapter 1, specimens were inoculated into tube cultures of FLK cells.

b) Infectivity Titrations: Primary monolayers of STh were used for all infectivity titrations involving adenovirus. PI3 virus titrations were performed in secondary FLK cultures. Serial 10-fold dilutions of virus in PBS were inoculated in 0·2 ml amounts into each of 4 tubes per dilution. Virus was allowed to absorb for 60 min. at 37°C before the addition of one ml of maintenance medium. Following incubation of the cultures at 37°C in the stationary position, end points were determined for adenovirus on the eighth day after inoculation by the presence of the typical cytopathic effect (CPE), and for PI3 virus on the fifth day after inoculation by the haemadsorption

technique of Vogel and Shelokov (1957). Infectivity titres were calculated as a 50 per cent. end point according to the method of Kärber (1931). The above methods were used to determine the infectivity of all stocks of virus, swabs and suspensions.

c) Strains of Virus: The viruses used in this thesis were as follows:-

(i) ovine adenovirus types 1, 2 and 3 (OA1-3), which were kindly supplied by Dr. J.B. McFerran, Stormont, N. Ireland.

(ii) ovine adenovirus type 4 (OA4), strain 7769, which was isolated during this work,

(iii) bovine adenovirus type 2 (BA2), from Dr. J.I.H. Phillip, Central Veterinary Laboratory, Weybridge.

(iv) human adenovirus type 2 (HA2), which was in general use at the Moredun Institute as an antigen in the gel diffusion test,

(v) parainfluenza virus type 3 (PI3), strain G2 (Hore, 1968).

Ovine adenovirus types 1-3 and PI3 virus were propogated in FLK cells, BA2 and HA2 viruses in CK cells, and OA4 virus in STh cells.

There is general agreement that only 2-6 per cent. of adenovirus virions are released spontaneously from cultured cells following replication (Ginsberg, 1958). For this reason working stocks of adenovirus were obtained from infected cells by alternately freezing and thawing the cultures 6 times. Cellular debris was removed by centrifugation at 1000g for 30 min. at $4^{o}C$. Antigens for use in gel diffusion tests were prepared in the same manner

except that infected cells were suspended in a small volume of medium before freezing and thawing. This procedure was calculated to give a 50-100 fold concentration of antigen.

d) Preparation of Antisera: Antiserum to OA1-4 viruses was prepared in rabbits. By means of centrifugation, debris was removed from an extract of infected cells, which had been prepared by 6 cycles of freezing and thawing, and the supernatant fluid removed and stored at -70°C. Two rabbits each were inoculated intravenously, once per week for 6 weeks, with one ml of this fluid. Two weeks after the last inoculation, the rabbits were exsanguinated.

Antisera to all the other viruses were donated as follows. Those to BA1-3 viruses were given by Dr. Phillip, Weybridge, and to BA4-8 viruses from Dr. A. Bartha, Budapest, Hungary. The antisera to the human adenoviruses were provided by Dr. M.S. Pereira, Virus Reference Laboratory, Central Public Health Laboratory, London.

Bacteriological Techniques.

a) Isolation and Identification: Specimens were inoculated into nutrient broth No.2* and on to 7 per cent. sheep blood agar, then incubated overnight at 37°C. The following day, bacteria were identified on the basis of their colony morphology and other characteristics on solid medium. If few or no colonies were visible on the blood agar, the broth culture was sub-cultured on to blood agar, which was

* Oxoid.

incubated overnight at 37°C. Colonies, which were suspected of being P.haemolytica, were examined further by selecting several colonies and growing them in a broth culture. The strains of P.haemolytica were typed serologically by means of the indirect haemagglutination test (Thompson, 1973).

b) Cultivation of P.haemolytica: Strains of P.haemolytica biotype A, serotype 1 were grown overnight in nutrient broth No.2. The organisms were concentrated 10-fold by centrifuging the cultures at 12,000g for 30 min. at 10°C and resuspending the resultant pellet in PBS.

c) Titration of P.haemolytica: The numbers of viable bacteria in inocula, and homogenates prepared from tissues taken at necropsies, were estimated by the technique of Miles and Misra (1938).

Mycoplasmological Procedures.

The isolation and typing of mycoplasmas were kindly undertaken by Dr. A. Foggie and Mr. G.E. Jones. Mycoplasmas were isolated by inoculation of samples into broth or on to solid media, and typed by the growth-inhibition or metabolic-inhibition tests (Jones, Foggie, Mould and Livitt, 1976).

Serological Tests.

Four types of serological test were used in this work. Antibodies to the adenovirus group specific antigen were detected by means of a gel diffusion test, and those to the

various serotypes of adenovirus by neutralization tests. The haemagglutination-inhibition (HI) test was used to estimate the amount of antibodies to PI3 virus and the complement-fixation test for antibodies to RS virus. In all tests, except gel-diffusion and complement-fixation, sera were inactivated at 56°C for 30 min. Nasal secretions, which were examined for the presence of neutralizing antibodies, were not heat-inactivated.

a) Gel-diffusion Test: This test was similar to that used by Darbyshire and Pereira (1964) except that one per cent. agar in PBS plus 0·08 per cent. sodium azide was used. An extract from CK cells infected with HA2 virus was employed as antigen to detect antibodies in sera from sheep. The test was read at 24 hours and 72 hours and "doubtful" reactors (Darbyshire and Pereira, 1964) were considered negative. A positive result was verified by the demonstration of a line of identity with a positive control serum.

b) Complement-fixation Test: This was based on the method described by Bradstreet and Taylor (1962), but was adapted to microtitre plates. All antigens and sera were diluted in veronal buffer with 0·08 per cent. (w/v) sodium azide. Haemolytic serum and complement were purchased from Burroughs Wellcome & Co. The RS virus antigen was prepared from a strain of human RS virus that had been passaged twice in CK cells and once in Hep-II cells. The supernatant culture medium was centrifuged at 3000g for 30 min. at 4°C to remove cellular debris, dispensed in small

aliquots and stored at -70°C. All sera were treated to remove anti-complementary factors by incubating 0·4 ml of serum with 0·1 ml of undiluted complement for 30 min. at 37°C. The serum was then diluted 1/8 and heated at 60°C for 30 min. to inactivate excess complement. The test was then carried out as described by Bradstreet and Taylor (1962), except that 4·5 units of complement were used, and the reagents were allowed to react overnight at 4°C, to allow fixation of complement.

c) Haemagglutination-Inhibition Test: The HI activity of sera was estimated by the method of Smith, Wells, Burrells and Dawson (1976). Four haemagglutinating units of viral antigen were incubated with dilutions of the serum that was being tested, and the antibody titre of the serum was expressed as the reciprocal of the highest dilution of serum to completely inhibit haemagglutination.

d) Neutralization Tests: (i) constant serum-varying virus. One ml aliquots of a 10-fold dilution series of virus were prepared and an equal volume of a 1/10 dilution of serum was added to each. After incubation at room temperature for one hour, 0·2 ml of the mixture was inoculated on to each of 4 cell cultures in tubes and allowed to absorb for one hour at 37°C. Thereafter, one ml of maintenance medium was added and the cultures incubated for 7 days before they were examined for the presence of adenovirus CPE.

(ii) constant virus-varying serum. This test was performed using either tube cultures or in microtitre plates. For tests in microplates, 0·025 ml

volumes of a 2-fold dilution series of the serum that was being examined were prepared in duplicate in microplates and an equal volume of virus containing approximately 100 $TCID_{50}$ was added. After incubation at room temperature for one hour, 0·1 ml of a suspension of FLK cells at a concentration of $1{\cdot}4 \times 10^5$ per ml was added to each well. The plates were incubated at 37°C for 7 days, at which time the end points were determined by observation of adenovirus CPE, and calculated by the method of Kärber (1931).

The test, performed on tube cultures, was similar in that one ml aliquots of the dilution series of serum were mixed with an equal volume of virus containing approximately 100 $TCID_{50}$/0·1 ml. After incubation for one hour at room temperature, 0·2 ml of the mixture was inoculated on to each of 4 tube cultures and allowed to absorb for one hour at 37°C. Thereafter, one ml of maintenance medium was added and the cultures incubated at 37°C for 7 days before the test was read.

Electronmicroscopy.

a) Negative Contrast Staining: Cultures showing widespread CPE were selected, the supernatant fluid discarded and the monolayer washed 2 or 3 times with PBS. The cells were scraped off the glass and resuspended in a few drops of distilled water before disrupting the cells further by placing them in an ultrasonic bath for 10 secs. The preparations were applied to carbon-coated grids, stained with one per cent. potassium phosphotungstic acid (pH 6·0)

and examined in a Siemens Elmiskop I microscope.

b) Examination of Thin Sections: Infected cells, from cultures showing widespread CPE, were scraped into the supernatant fluid and formed into a pellet by centrifugation. The pellet of cells was fixed with one per cent. glutaraldehyde followed by one per cent. osmium tetroxide in phosphate buffers and embedded in Araldite. Sections were stained with a saturated aqueous solution of uranyl acetate followed by lead citrate and examined as above.

Histological and Ultrastructural Procedures.

Tissues for histological examination were post-fixed in mercuric chloride formaldehyde, dehydrated in alcohols and then processed in a series of mixtures of alcohol and toluene before being embedded in paraffin wax. Sections, which were cut at 6μ, were stained routinely with haematoxylin and eosin (H & E), but also by the phloxine tartrazine and Feulgen methods to demonstrate inclusions, Stein's procedure for bile (Culling, 1963) and with Martius scarlet blue for fibrin (Drury and Wallington, 1967).

Glutaraldehyde-fixed tissues were washed and transferred to one per cent. osmium tetroxide for 2 hours before being dehydrated in a series of alcohols of ascending strength. The tissues were then immersed in epoxy propane prior to embedding in Araldite, and stained with uranyl acetate and lead citrate.

CHAPTER 1

ISOLATION OF CYTOPATHIC VIRUSES ASSOCIATED WITH OVINE RESPIRATORY DISEASE

It was apparent from the review of the literature not only that sheep may be infected with a great variety of viruses but that few of these viruses have been isolated. At the outset of this thesis, only PI3 virus, 3 serotypes of adenovirus and reovirus type 3 had been recovered from the respiratory or alimentary tract of sheep. Investigations were undertaken, therefore, which were designed to isolate viruses from sheep and to evaluate their role in ovine respiratory disease.

It is sometimes claimed that outbreaks of respiratory disease in sheep can be related to some "stressful" factor, and one example, which is frequently quoted, is that of inclement weather. Smith, Hugh-Jones and Jackson (1972) suggested that an important meteorological factor might be the rapid decline in ambient temperature. They found that the development of clinical signs in 27 outbreaks of respiratory disease in kittens was closely associated with 2 such periods of rapid temperature decline. The interval between these periods was approximately equal to the incubation period of the virus infection. L.P. Smith (personal communication) has also found that the same holds true for outbreaks of respiratory disease in cattle. During the present investigations, several outbreaks of

disease occurred and the opportunity was taken to examine the hypothesis of Smith et al (1972) in relation to these outbreaks.

MATERIALS AND METHODS

The techniques used to isolate viruses, bacteria and mycoplasmas, and to detect antibodies to viruses have been described previously (General Materials and Methods). Specimens for virus isolation generally were inoculated on to primary STh monolayers, although in some cases primary lamb testis and secondary FLK cells were used. Each specimen was subjected to 3 serial transfers in cell cultures and each passage was made by scraping cells into the medium, which was inoculated on to fresh monolayers.

Attempts to isolate cytopathic agents were made using material drawn from two sources:-

a) Necropsy specimens, which were mainly lungs, other respiratory tissues and faeces from sheep with pneumonia. These were supplied by the Scottish Veterinary Investigation Service.

b) Outbreaks of acute respiratory disease, which were referred to the Moredun Institute by the Veterinary Investigation Service and Mr. A. Whitelaw, Hill Farming Research Organisation. Each outbreak was investigated with the minimum of delay after it was referred to the Moredun Institute. A detailed history was obtained from the farmer or shepherd and nasal swabs were taken for examination for

viruses, bacteria and mycoplasmas, and rectal swabs for viruses. Swabs for virological and mycoplasmological examination were broken off into transport medium and placed on ice during transport to the laboratory; swabs for bacteriological examination were immersed in peptone water. On arrival at the laboratory, usually within 3 hours, the samples were inoculated into appropriate media as described in General Materials and Methods. A blood sample for serological tests also was obtained from the sheep that were swabbed.

Meteorological Data.

Daily maximum and minimum ambient temperatures for the periods under study were made available through the courtesy and cooperation of Messrs Armour and Dunsyre of the Meteorological Office, Climatological Service (Scotland), Edinburgh. These recordings were made 8 miles from flock B, and one mile from flock H.

Cold stress days were identified, as defined by Mr. L.P. Smith (personal communication), as days when the minimum temperature was $0^{o}C$ or below, and the fall in temperature between the day maximum and the night minimum (diurnal range) was 1·5 times the mean monthly diurnal range.

RESULTS

a) Samples obtained at necropsy.

During the 18 months between October, 1971 and July, 1973, 423 specimens from ovine necropsies were examined by serial passage in cell cultures. The various specimens are enumerated in Table 1.1, and cytopathic agents were isolated on 3 occasions, which are described below.

Carcases of all sheep that died on a farm practicing intensive husbandry were examined routinely. Post-mortem examinations of one of these, a 5-week-old lamb, revealed extensive pneumonic lesions from which a cytopathic agent (strain GD1) was recovered during the first passage in secondary FLK monolayers. The first indication of a cytopathic effect was the appearance, after 3-4 days' incubation, in the supernatant fluids of inoculated monolayers, of numerous, small, round, refractile cells. These increased in number without any apparent disruption of the confluence of the monolayer. When these cultures were tested with an 0·5 per cent suspension of guinea pig erythrocytes, haemadsorption occurred.

Examination of coverslips of secondary FLK cells infected with strain GD1 revealed numerous syncytia, incorporating up to 15 nuclei and containing eosinophilic intracytoplasmic inclusions. Cells infected with strain GD1 were disrupted in a few drops of distilled water and stained with phosphotungstic acid (pH 7·0) for electron-microscopic examination. A few pleomorphic virions, many

TABLE 1.1

ISOLATION OF CYTOPATHIC AGENTS FROM NECROPSY SPECIMENS

Specimens investigated		Cytopathic agents isolated	
Source	Number examined	Isolates	Isolation rate (%)
lung	177	1 x PI3 virus (strain GD1)	0·6
faeces	110	1 x adenovirus (strain 1248)	0·9
rectal swab	21	-	-
tonsil	26	-	-
nasal swab	12	1 x PI3 virus (strain CH2)	8·3
pulmonary lymph node	10	-	-
tracheal mucosa	6	-	-
miscellaneous	61	-	-
TOTAL	423	3	0·7

of them disrupted, were seen, but more common were strands of helical ribonucleoprotein, approximately 18nm diameter, similar to those described for human and other ovine strains of PI3 virus (Waterson, Jensen, Tyrrell and Horne, 1961; Hore, 1968). The agent was confirmed as PI3 virus by inhibition of haemadsorption with a 1 in 20 dilution of a specific antiserum to the G2 strain of ovine PI3 virus.

The circumstances surrounding the isolation of PI3 virus (strain CH2) from a nasal swab are described in section b in relation to an acute outbreak of respiratory disease (Flock B).

Another cytopathic agent (strain 1248) was isolated from the faeces of a lamb during the first passage in primary STh monolayers. The cytopathic effect (CPE) cytology and morphology of this agent resembled that of a member of the adenovirus group. The lamb came from the same farm from which another adenovirus (ovine adenovirus type 4, strain 7769) had been isolated previously (Section b, Flock D; Chapter 2). The antigenic similarity of these 2 strains was established when the same neutralizing titre of a hyperimmune serum to strain 7769 was obtained using both homologous virus and strain 1248.

b) Outbreaks of respiratory disease.

Investigations into outbreaks of respiratory disease in flocks of sheep were made on 11 farms, and these are summarized, chronologically, in Table 1.2. At the time when each farm was visited and the sheep examined, clinical

TABLE 1.2

INVESTIGATION INTO OUTBREAKS OF OVINE RESPIRATORY DISEASE.

Date of first visit	Location of flock	Signs of respiratory disease	Age/ breed	Number swabbed	Result of virus isolation
15.2.72	A Roxburgh-shire	none	11m. H/B*	12	negative
25.2.72	B W.Lothian	deaths coughing tachypnoea nasal discharge	4-5 yrs. BF**	see text	1 isolate of PI3 virus
28.2.72	C Roxburgh-shire	coughing	9-10m. BF	30	negative
20.3.72	D Midlothian	deaths coughing tachypnoea	8-10w. Olden-berg X	see text	3 isolates of adenovirus
1.11.72	E Midlothian	none	various	see text	7 isolates of PI3 virus
25.1.73	F Midlothian	coughing	1-4 yrs. BF	12	negative
22.4.73	G Dumfries-shire	deaths pneumonic-lesions	4 yrs.	28	negative
17.5.73	H Midlothian	coughing nasal & conjunctival-discharge tachypnoea	4-6w. BF	see text	3 isolates of PI3 virus
21.5.73	J Argyll-shire	none	2-6w. BF	48	negative
1.6.73	K Midlothian	none	12w. 1-4 yrs. BF	6	negative
28.6.73	L Perthshire	none	3-4m. BF	10	negative
6.7.73	M E.Lothian	none	2-5 yrs. BF	19	negative

* H/B Half Breed
** BF Blackface.

signs of respiratory disease were not evident on 5 farms, and coughing was the sole sign on 2 further farms. Acute or subacute outbreaks of respiratory disease occurred on the remaining 4 farms, and a virus was isolated from affected sheep on 3 of these farms (B,D, and H).

On one farm (E), various breeds of sheep, which were subjected to regimens of varying ambient temperature, were sampled on a regular basis.

Flock B.

History: During the autumn of 1971, a group of 158, 5-year-old cast ewes was purchased from a single hill farm, and 3 tup lambs from 2 other sources. The ewes were run at grass as a single flock, together with ewes bought from the same farm the previous year. At the time of tupping, some of the ewes, particularly those purchased in 1971, and the new tups were noticed to be coughing. Coughing continued despite treatment with procaine penicillin* during January. Following anthelmintic treatment (tetramisole and oxyclozanide+), one ewe died and there was an exacerbation of the respiratory problem in the flock. Tissues from the respiratory tract of the dead ewe were submitted for microbiological examination.

Clinical Observations: Although the ewes appeared to be in good bodily condition, they were coughing frequently and occasionally paroxysmally, even when at rest. Some appeared dull, and most had a copious nasal discharge that varied from serous to mucopurulent, with encrustation around the nares. None of the ewes examined was febrile. Nasal

* Propen, Glaxo.
+ Nilzan, I.C.I.

and rectal swabs, and a blood sample were taken from 13 ewes.

At a further visit, 5 weeks later, all the ewes appeared well, and occasional coughing was heard only when the ewes were disturbed. There had been 4 further deaths, from which no material was obtained. Nasal and rectal swabs, and a blood sample were taken from 15 animals, although ewes previously sampled could not be identified.

Microbiological Examinations: Examination of respiratory tissues taken from the dead ewe resulted in the isolation from a nasal swab of a cytopathic agent (strain CH2). This was identified as PI3 virus by tests previously described (see Section a)). Further cytopathic agents were not isolated from swabs taken during the 2 visits to the flock. Pasteurella haemolytica was recovered from 8 of 13 nasal swabs, on the first occasion, and Haemophilus influenzae from 3 nasal swabs. A species of mycoplasma was isolated from nasal swabs from 6 animals.

Serum samples taken during the first visit showed that none of the 13 sheep had HI antibody to PI3 virus at a titre greater than 10. However, 15 of 16 sera, taken during the second visit, had HI titres between 16 and 32.

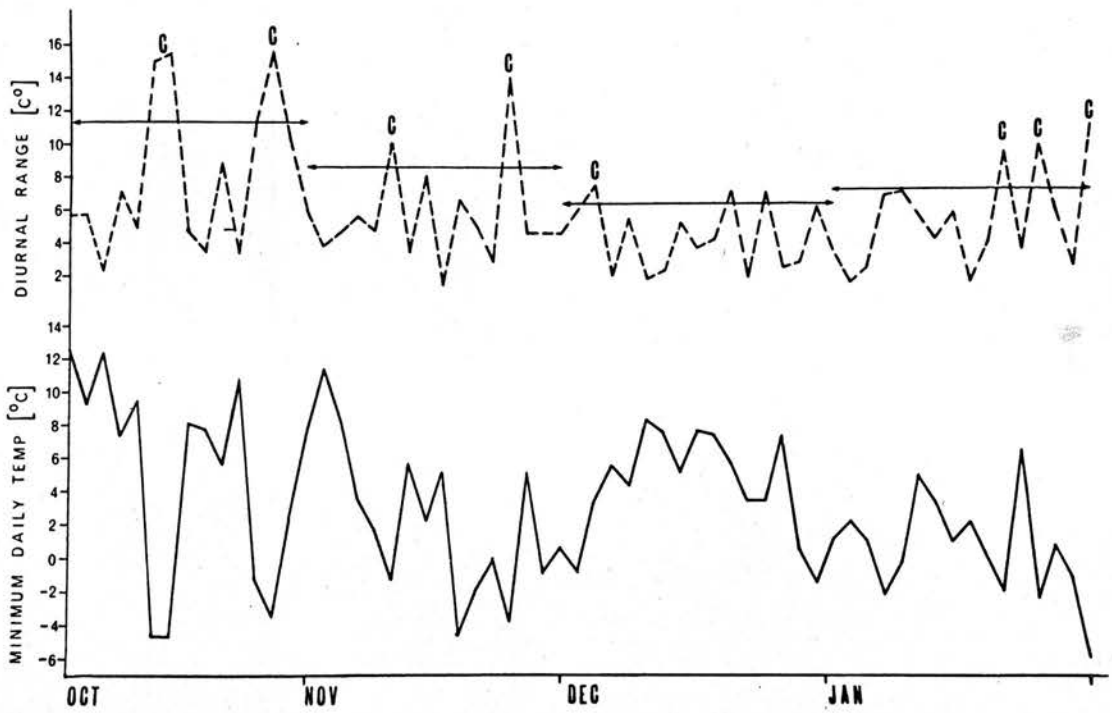
Meteorological data: The minimum daily temperature and diurnal range during the 4 months preceding the outbreak are presented in Fig. 1.1. There were several periods of cold stress in October and November and the interval between these stresses varied between 8 and 15 days. There were

Figure 1.1

Meteorological data for flock B showing minimum daily temperature and diurnal range during the 4 month period (October - January) preceding an outbreak of respiratory disease in February.

C = cold stress day

⟷ = mean monthly diurnal range x 1.5


DIURNAL RANGE [C°]
MINIMUM DAILY TEMP [°C]
C
OCT
NOV
DEC
JAN

no cold stress days between early December and mid January, but in the 3 weeks immediately before the outbreak there were 3. The last cold stress day occurred about 9 days before the start of the outbreak, and involved a minimum temperature of $-10^{o}C$.

Flock H.

History: A small number of ewes, forming part of a flock of Blackface sheep, were moved temporarily to another farm during the summer. Here they were in contact with other sheep. In November, after they had returned to the main flock, coughing was observed in this group and progressed gradually through the remainder of the flock. The coughing continued throughout the winter, particularly in the sheep on 2 hirsels, and some ewes required treatment with antibiotics. Several weeks before lambing, the ewes were moved to low ground and, approximately 4 weeks after the first lambs were born, a few lambs were noticed to be listless and coughing.

Clinical Observations: The flock was examined about one week after the first lambs became ill. Clinical signs of acute respiratory disease were confined to the lambs, most of which appeared to be affected. The salient features were copious nasal and conjunctival discharges, tachypnoea and coughing, particularly after exercise. It was apparent from the history and clinical examination that the discharges started as serous and progressed to mucopurulent with encrustation around the nares and on the cheeks.

Nasal, conjunctival and rectal swabs, but not blood,

were taken from 2 ewes and 7 lambs, 6 of which had been observed to be ill for less than 2 days.

A further lamb (HM8), which had not appeared to be ill the previous day, was removed from the flock and observed in isolation. The clinical condition of this lamb deteriorated during the next 24 hours until, immediately preceding necropsy, it was dull and had a marked respiratory "lift". Post-mortem examination of the lamb revealed extensive consolidation of the apical and cardiac lobes of both lungs, with no evidence of pleural effusion or adhesions. A second visit to the flock 2 months later, showed that many lambs were still coughing and had failed to thrive. Two of these lambs were killed and the appearance of the lung lesions at necropsy appeared similar to those of lamb HM8 which had been killed earlier.

Microbiological Examination: Cytopathic agents were isolated from nasal swabs, but not rectal or conjunctival swabs, from 3 of 7 lambs. The first indications of CPE were seen 3 days after inoculation of the samples on to primary STh monolayers, areas of which assumed a "ground glass" and slightly refractile appearance. At this stage, haemadsorption with guinea pig erythrocytes could be demonstrated throughout the monolayer. The agents were identified as PI3 virus by the methods described previously. Pasteurella haemolytica was present in 6 of 9 nasal swabs, and Mycoplasma ovipneumoniae in all 9 nasal swabs.

No cytopathic agents were recovered from the lungs of 3 lambs which were killed, but Pasteurella haemolytica and

Mycoplasma ovipneumoniae were isolated on each occasion.

Histology: A prominent feature of the consolidated portions of lung obtained from lamb HM8 was an extensive cellular exudate, comprised of polymorphonuclear cells, in the lumena of bronchioles. These cells were supplemented in affected alveoli by mononuclear cells, which occasionally appeared to form syncytia. Partial alveolar epithelialization was fairly extensive. There was frequent infiltration by polymorphs of the bronchiolar epithelium, which was hyperplastic in some areas. Peribronchiolar accumulations of lymphoid cells were minimal and no intracytoplasmic inclusions were seen when sections were stained with H & E, or with Pollack's trichrome.

Meteorological Data: Data for the 4 months preceding the outbreaks are presented in Fig. 1.2. There were many periods of cold stress in the months leading up to the outbreak, and the intervals between these varied from 6 to 12 days. About 8 - 10 days before the start of the outbreak, there were 2 cold stress days, separated by an interval of 3 days.

Flock E.

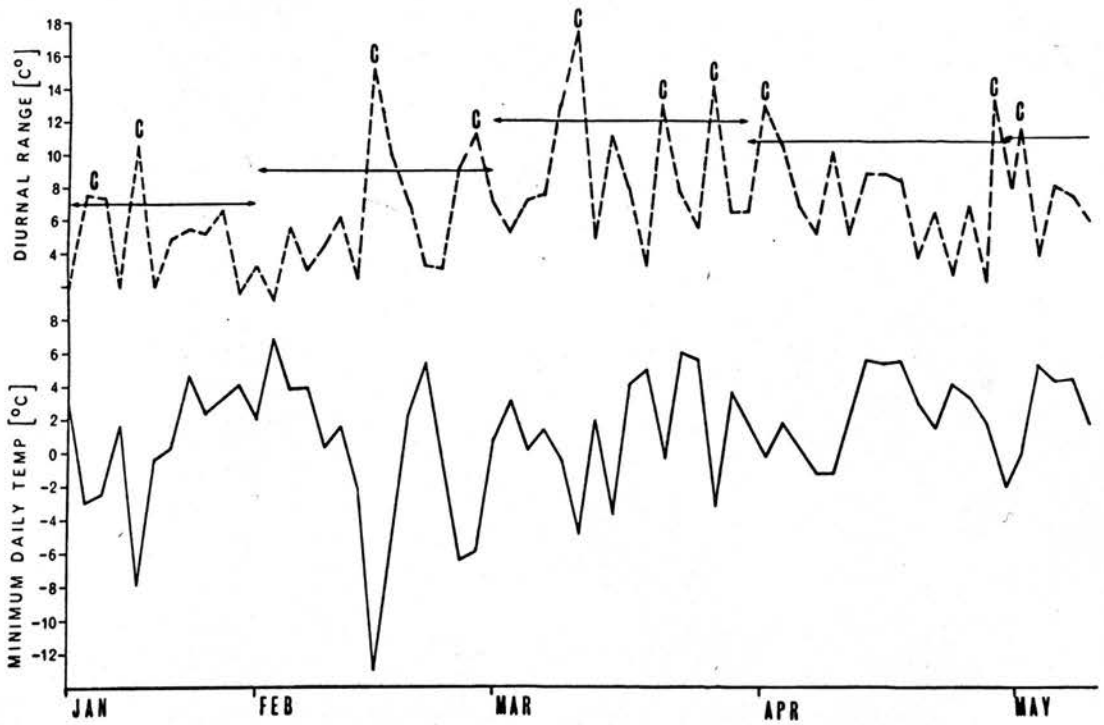
History: Sheep were drawn from several flocks owned by a research organization and used in experiments centred on the animals physiological response to variations in ambient temperature. Before and after experimental procedures, all the sheep were kept in individual pens in a large holding room. During the experiments, up to 8 sheep were kept in individual pens in a climate chamber (Slee, personal communication).

Figure 1.2

Meteorological data during the 4 months (January - May) preceding an outbreak of respiratory disease in flock H in early May.

C = cold stress day

⟷ = mean monthly diurnal range x 1.5


DIURNAL RANGE [C°]
MINIMUM DAILY TEMP [°C]
C
JAN
FEB
MAR
APR
MAY

Nasal and rectal swabs, and a blood sample were collected routinely from each group of sheep, before, during, and after the experimental procedures.

Microbiological Examinations: Parainfluenza virus type 3 was isolated on primary STh monolayers from nasal swabs from 7 of 12 sheep in groups 1C and 1F (Table 1.3).

None of the sheep from which this virus was isolated showed a 4-fold or greater rise in serum HI antibody to PI3 virus (Table 1.3) although such a rise was detected in one other sheep (2s3). Pasteurella haemolytica and Mycoplasma ovipneumoniae were isolated also from several sheep. The virus, Pasteurella and Mycoplasma were isolated 5 days before the sheep were subjected to regimes outlined in Table 1.4 but the sheep did not exhibit any clinical signs at this time, nor during the subsequent experiment.

No cytopathic agents were recovered from nasal or rectal swabs taken from 13 sheep in groups 1W and 2F. However, sera from 6 of these sheep, taken whilst they were in the climate chamber, showed a 4-fold increase in HI antibody titre to PI3 virus. In group 1W, 5 of 8 sheep showed an increase, and the geometric mean titre for the group increased from 3·6 before they went into the chamber (27.11.72) to 18·6 whilst they were in the chamber (28.12.72). In group 2F, only 1 sheep showed an increase, and the geometric mean titre for the group increased from 9·1 to 18·2 (Table 1.5). Pasteurella haemolytica was not recovered from any of the sheep at the begining of the experiment but was present in 4 of 13 nasal swabs after 8 weeks (Table 1·5).

TABLE 1.3

MICROBIOLOGICAL EXAMINATIONS OF SAMPLES

TAKEN FROM 2 GROUPS OF SHEEP IN FLOCK E

Group	Sheep	First Visit (27.10.72)				Second Visit (27.11.72)				Third Visit (20.12.72)			
		Bacterium	Mycoplasma	Virus	Serology	Bacterium	Mycoplasma	Virus	Serology	Bacterium	Mycoplasma	Virus	Serology
1C	1624	A7+* Haem.	-	-	8⊕	-	-	-	-	-	+	-	8
	1655	A7+ Haem.	+	+	-	-	-	-	8	-	+	-	8
	1656	NR	-	-	8	-	-	-	8	A2	+	-	8
	2S3	Staph.	+	-	-	Haem.	-	-	16	T4	+	-	16
	2S5	-	-	+	-	-	-	-	8	-	+	-	8
	2S6	-	+	+	8 (8·0)	-	-	-	8 (9·2)	-	+	-	16 (10·0)
1F	55	A2	+	+	8	-	-	-	-	-	+	-	-
	60	A7	-	+	8	-	-	-	8	A7	+	-	8
	65	A7	-	+	-	-	-	-	8	A7	+	-	-
	2B18	A2	-	+	-	Haem.	-	-	-	-	+	-	-
	2B34	A7+ Haem.	-	-	32	-	-	-	-	Died: urinary calculi			
	2B45	A7+ Haem.	-	-	32 (16·0)	Staph.	-	-	- (8·0)	A7+ T3	+	-	- (8·0)

* = P.haemolytica serotype eg. A7, T3, NR (non reactive).

Haem. = Haemophilus

Staph. = Staphylococcus spp

⊕ = titre of serum HI antibody to PI3 virus

( ) = geometric mean titre of serum HI antibody.

\+ = isolation of PI3 virus or M.ovipneumoniae.

\- = No isolations of bacteria, mycoplasmas or cytopathic agents

TABLE 1.4

EXPERIMENTAL REGIMES FOR SHEEP IN FLOCK E

Group No.	Sheep No.	Breed	4 Weeks Chamber Treatment	Date of Commencement in climate chamber (Day 0)	Date of Acute Cold Exposure and Exit from Chamber (Day 28)
1C	1624) 1655) 1656) 2S3) 2S5) 2S6)	Finn South-down	Cold +10°C	1/11/72	29/11/72
1F	55) 60) 65) 2B18) 2B34) 2B45)	Suffolk Black-face	Fluctuating +28°C to +2°C	3/11/72	1/12/72
1W	2S 8) 2S12) 2S15) 2S91) 2S94) 1675) 1676) 1687)	South-down Soay Finn	Warm + 28°C	30/11/72	28/12/72
2F	2S95) 2S98) 2S101) 2RO23) 2R185)	Soay Welsh	Fluctuating +28°C to 2°C	2/12/72	30/12/72

N.B. Food and water (both recorded) is offered ad lib. for 4 weeks prior to entry to the climatic chamber and throughout the period in chamber.

TABLE 1.5

MICROBIOLOGICAL EXAMINATIONS OF SAMPLES TAKEN FROM 2 GROUPS OF SHEEP IN FLOCK E

Group	Sheep	First Visit (27.11.72)				Second Visit (26.12.72)				Third Visit (10.1.73)			
		Bacterium	Mycoplasma	Virus	Serology	Bacterium	Mycoplasma	Virus	Serology	Bacterium	Mycoplasma	Virus	Serology
1W	258	-	+	-	-⊕	-	-	-	32	-	-	-	16
	2S12	Staph.	+	-	8⊕	-	+	-	16	T10*	+	-	32
	2S15	-	+	-	-	-	+	-	16	A2	+	-	32
	2S91	-	+	-	8	Haem.	+	-	8	Staph.	+	-	16
	2S94	-	-	-	8	-	+	-	16	-	+	-	-
	1675	-	+	-	8	-	+	-	32	A7	+	-	16
	1676	-	-	-	8	Haem.	+	-	32	-	+	-	16
	1687	-	-	-	-	Haem.	+	-	16	-	+	-	16
					(8·0)				(18·6)				(19·5)
2F	2S95	Staph.	+	-	8	-	+	-	16	A2	+	-	16
	2S98	-	-	-	8	-	-	-	32	-	+	-	64
	2S101	-	+	-	8	-	-	-	16	-	+	-	16
	2R028	-	-	-	16	T2	+	-	16	-	+	-	16
	2R185	Staph.	-	-	8	Staph.	+	-	16	Staph.	+	-	16
					(9·1)				(18·2)				(20·9)

* = P.haemolytica serotype eg. T2, A2.
Haem. = Haemophilus
Staph. = Staphylococcus spp
⊕ = titre of serum H1 antibody to PI3 virus.
( ) = geometric mean titre of serum H1 antibody.

\+ = isolation of M.ovipneumoniae
\- = no isolations of bacteria, mycoplasmas or cytopathic agents.

Mycoplasma ovipneumoniae was present in the nasal swabs of all sheep by the eighth week.

Flock D.

History: During the late winter of 1971/2 an outbreak of respiratory disease occurred in one shed on a large commercial breeding establishment, which housed its sheep for varying periods. This particular outbreak occurred during one lambing spell when the shed contained in-lamb ewes, ewes with lambs at foot, and freshly weaned lambs. Approximately 50 lambs had died during the 6 weeks preceding the first visit. Lambs between the ages of 4 and 10 weeks, particularly Oldenberg-cross lambs, were the most severely affected.

Clinical Observations: The precise nature of the clinical manifestations was difficult to define as they were superimposed on a background of insidious respiratory disease. This background respiratory disease was characterized by various degrees of coughing, nasal discharge and tachypnoea, The outbreak became apparent by an increase in the number of lambs that were coughing and tachypnoeic, together with an increased mortality-rate in the 4 - 10 week age group. Nasal and rectal swabs, and a blood sample were taken from lambs in several pens, including the one most recently affected (pen 74). The lambs in pen 74 were weaned the day before the first visit to the farm, and 4 days after the most recent deaths in the pen attributable to acute pneumonia. Subsequent to this visit, one lamb (8366) died, but no tissues were received for examination. At a second visit, 14 days later, repeat samples were taken from the

lambs previously examined.

Microbiological and Serological Results: Cytopathic agents were not detected in nasal or rectal swabs taken at the first visit nor at a second visit 14 days later (Table 1.6). On the first occasion, 6 different serotypes of Pasteurella haemolytica were recovered from nasal swabs from 10 lambs, and Mycoplasma arginini from nasal swabs from 13 lambs. A glucose fermenting organism, probably M.ovipneumoniae, was isolated from nasal swabs from the 2 remaining lambs. At the second visit, 4 different serotypes of Pasteurella haemolytica were isolated from 6 lambs, and Pseudomonas aeruginosa from another 2.

Evidence of a current adenovirus infection in the group was indicated by an increasing number of sera giving positive results in gel diffusion tests between the first and second visits (Table 1.6). At the first visit, 3 sera were positive, and at the second visit, 6 sera were positive. Two of the sera, which gave positive results only at the second visit, were from lambs in pen 74 and, at this time, sera from 4 of the 5 lambs in this pen were positive (Tables 1.6 and 1.7).

A third series of samples was taken from all the lambs in this pen, 20 days after the first investigations. Cytopathic agents were recovered from rectal swabs from 3 of 15 lambs, but not from nasal swabs (Table 1.7). The initial isolations were made on primary STh monolayers rolled at 34°C. The earliest changes were seen 5 days after inoculation of the monolayers and consisted of

TABLE 1.6

MICROBIOLOGICAL EXAMINATIONS OF SAMPLES

TAKEN DURING THE FIRST 2 VISITS TO FLOCK D

Pen	Sheep	First visit (20.3.72)				Second visit (6.4.73)		
		Bacterium	Mycoplasma	Virus	Serology	Bacterium	Virus	Serology
45	8366	A5*	Ma	-	-	DIED. no samples received		
63	5677	A2	Ma	-	-	A2	-	+
	7258	A2	Ma	-	-	-	-	-
73	7860	A2 + T3	Ma	-	+	A2 + A6	-	+
	9625	A2 + T10	Ma	-	-	T10	-	-
	9630	A1	Ma	-	-	T3	-	-
74	7703	T3	Ma	-	+	-	-	+
	7751	-	Ma	-	-	A2 + A6	-	+
	7798	-	Ma	-	-	-	-	-
	7839	A7	Ma	-	+	A2 + A6	-	+
	7841	-	G	-	-	-	-	+
77	7984	A1	G	-	-	-	-	-
	9652	-	Ma	-	-	-	-	NS
	9658	T10	Ma	-	-	Pseud.	-	-
	9663	-	Ma	-	-	Pseud.	-	-

* = Pasteurella haemolytica serotype ie, A1, A2, A7, T3, T10.

Pseud. = Pseudomonas aeruginosa

Ma. = Mycoplasma arginini

G = glucose fermenting organisms.

NS = not sampled.

\+ = precipitation line formed in gel diffusion test against HA2 antigen.

\- = no cytopathic agents or bacteria isolated.

TABLE 1.7

PRECIPITATING AND NEUTRALIZING ANTIBODIES IN THE SERA OF LAMBS IN PEN 74 OF FLOCK D

Sheep	Day 1		Day 16		Day 22		Day 83	
	GD*	Neut+	GD	Neut	GD	Neut	GD	Neut
7703	+	64⊕	+	22	ND		ND	
7751	-	6	+	16	ND		ND	
7798	-	6	-	6	+	256	-	32
7839	+	11	+	16	+	16	-	22
7841	-	16	+	64	+	64	-	32
7678					-	22	-	64
7739					ND		-	64
7741					-	256	-	32
7746					-	64	-	256
7753					-	64	-	64
7767					-	ND	+	ND
7768	ND		ND		-	64	-	32
7769**					+	32	+	32
7815**					+	64	-	32
7816					-	6	+	178
7824					+	89	-	22
7837**					+	64	+	45

* = Gel diffusion test.

\+ = Microneutralization test.

⊕ = Antibody titre expressed as a reciprocal of the serum dilution.

ND = Not done.

** = Lambs from which a cytopathic agent was isolated.

increased refractility and rounding of cells in one or two small foci. The CPE progressed slowly from the original foci of affected cells both by local extension and also the development of a few new foci, but rarely involved more than 50 per cent. of the monolayer. Further passages of the cytopathic agents were made by scraping cells into the supernatant medium and inoculating both cells and medium on to fresh STh monolayers.

Precipitating antibodies to adenovirus were detected in 7 of the 14 sera obtained from lambs at a third visit, 22 days after the first visit (Table 1.7). Serum samples from only 2 of these 7 lambs, obtained at a fourth visit 2 months later, contained precipitating antibodies, but those from 2 further lambs were positive. Neutralizing antibodies to one of the cytopathic agents (strain 7769) were detected in the sera of lambs in pen 74. A 4-fold rise in titre was demonstrated in 4 lambs (Table 1.7) and, in 3 of these lambs, the increased titre in the convalescent serum was associated with the appearance of antibodies detectable by the gel diffusion test.

DISCUSSION.

The present work has emphasized the advantages of acute outbreaks and fresh samples as a source of material for investigating the aetiology of respiratory disease and also has indicated a role for viruses in this type of outbreak. For example, viruses were isolated from 16 per cent.

of sheep in 3 of 4 flocks currently experiencing outbreaks of respiratory disease, whereas a virus was isolated from less than one per cent. of tissues taken at post mortem examinations. The discrepancy between these 2 approaches probably arises from differences in the freshness of the material examined and the time of sampling during the course of the virus infection. Also, the isolation of viruses from 3 of 4 flocks with current respiratory disease, but not 7 other flocks with few or no signs of respiratory illness, has contributed further to the view that viruses, particularly PI3 virus, are involved in acute outbreaks of respiratory disease.

During this study, only PI3 virus and adenovirus were isolated, but other viruses discussed in the introduction were not detected. It was hoped that some of these viruses, particularly respiratory syncytial (RS) virus, might have been isolated during outbreaks of respiratory disease and, therefore, precautions were taken to preserve the infectivity of any viruses that might have been present in the swabs. For example, all specimens were kept cool, transported to the laboratory as soon as possible and inoculated into cell cultures before storage at $-70^{o}C$. It is unlikely that the CPE's produced by enteroviruses or reoviruses would have been overlooked, had they been present, but this is not so with RS virus. Consequently, all sera were screened for complement-fixing antibodies to RS virus, but with negative results.

In addition to these general points, more specific

observations have been made with regard to certain aspects of the epidemiology of PI3 virus.

It has been assumed generally that infection of an animal with a virus results in a detectable rise in serum antibody titre and, in most tests, only increases of 4-fold or greater are regarded to be outwith the errors of the test. However, the observations on flock E clearly show that PI3 virus infections of sheep can occur in the absence of such a rise. This observation agrees with those of Gates, Cesario, Ebert, Kriel, Wulff, Poland, Gutekunst and Chin (1970) and Thomas (1973). These authors have suggested that, in calves, the failure to detect a rise in serum antibody stems from pre-existing antibody levels of 20 or greater, which may interfere with or suppress the serological response. It is unlikely that such events occurred in Flock E because Hore (1968) found that HI antibody titres of 8 or less did not interfere with the serological response of sheep to PI3 virus infection and similar titres were present in the sera of sheep in Flock E. It is possible that local antibody only was elicited but again, this is regarded as unlikely because both Hore (1968) and Smith (1975) have shown that multiplication of PI3 virus in the respiratory tract stimulates the production of serum antibody.

Thomas (1973) has drawn attention to the limitations of paired sera as a means of diagnosing viral infections and has suggested that the timing of the samples may be crucial. This view was supported by later work in which

it was found that the rate of decline in serum antibody in cattle to certain respiratory viruses could be as rapid as the increase (Thomas and Collins, 1974). In flock E, there was a lapse of one month between the first and second samples, and the observations in cattle could be applicable to this situation. The results obtained by Smith (1975) favour this hypothesis. In both specific pathogen-free (SPF) and conventionally-reared lambs, with no detectable serum antibody to PI3 virus at the time of inoculation, the HI antibody response to PI3 virus infection reached peak titres of 20 and 40 between 6-18 days post-inoculation (pi) and declined rapidly to titres around 10 by 4 weeks pi. Smith regarded this to reflect a primary infection with PI3 virus, and the same may have occurred in flock E. However, Thomas and Collins (1974) observed many such minor fluctuations in serum antibody titres in cattle and suggested that, in the field situation, it is more likely that the timing of the samples influences the detection of a serological response.

It is often suggested that pathogens operate in conjunction with each other, and with environmental conditions, to produce overt disease. In flock E, 5 days before the sheep in groups 1C and 1F went into the climate chamber, replicate nasal swabs contained Pasteurella haemolytica, Mycoplasma ovipneumoniae and PI3 virus. All these organisms have been demonstrated to be pathogenic for experimentally infected sheep (Smith, 1964; Gilmour, Thompson, Smith and Angus, 1975; Foggie, Jones and Buxton, 1976;

Hore, 1968) and one might have expected the combination of these organisms, and the fluctuating temperature in one group, to have resulted in overt respiratory disease. Respiratory illness did not develop but the absence of any disease in these sheep does not refute this theory, since a local immunity may have developed. Specific local antibody has been demonstrated in the lower respiratory tract of mice as soon as 3 days pi (Blandford and Heath, 1972) and in nasal secretions from SPF lambs infected with PI3 virus or adenovirus at 8 days pi (Smith, 1975; chapter 3). Thus, the sheep in flock E could well have been immune before they were subjected to fluctuating ambient temperatures and cold stress.

The observations on flocks B and H provide further evidence of the association of PI3 virus with ovine respiratory disease. Most reported isolates of this virus have been obtained from sheep less than 12 months old, so the outbreak in flock B was unusual in that 5-year-old cast ewes were involved. The susceptability of these sheep to PI3 virus can probably be attributed to their origin from a hill farm in Ayrshire and the comparative isolation which this imposed on the flock.

PI3 virus was intimately associated with the onset of upper respiratory disease in flock H, as virus was isolated from nasal swabs from 3 of 6 lambs, which were considered by the shepherd to have been showing signs of respiratory illness for less than 2 days. The involvement of PI3 virus in the disease of the lower respiratory tract is not

so clear, because virus was not recovered from the pneumonic lesions of a lamb which had apparently developed severe respiratory disease over a period of 48 hours. However, the histology of the pneumonic lesions revealed several features in common with those described by Stevenson (1968) suggestive of PI3 virus infection and corresponded to those observed in lambs 7-8 days after inoculation. No intracytoplasmic inclusions were seen, but these generally were not detected by Stevenson in lambs killed at this stage. Further, the presence of inclusions was closely associated with recovery of virus from the lung.

Although the histological findings provide equivocal evidence that PI3 virus was involved in the pneumonia, the results are consistent with the hypothesis that infection with PI3 virus predisposes to a severe pneumonia caused by secondary bacteria. Similar observations have been reported by Hore (1968), who found that, in sheep naturally infected with PI3 virus, clinical disease was not always detected at the time of virus isolation and illness could occur several days later. Also, in lambs infected experimentally, clinical signs were first seen when the titre of virus in nasal swabs was declining 6-7 days following inoculation. Hore considered the more severe clinical signs to be due to secondary bacterial complications.

The temporal relationship of cold stress days to the outbreaks in flocks B and H appears to agree with the hypothesis of Smith, Hugh-Jones and Jackson (1972). However, in both flocks, cold stress days, separated by an

appropriate interval were also present in the preceding months, yet no outbreaks occurred (Figs. 1.1 and 1.2). It can be contested that in flock H a susceptible population was not present until the lambs were born, but this does not appear to apply to flock B. In this flock, a group of 5-year-old cast ewes were purchased in the autumn and run as a single flock with the resident ewes until the outbreak occurred the following February. During this period no new animals were introduced and, presumably, no new organisms either. It follows from this that the organisms responsible for the outbreak were present concurrently with appropriate cold stress periods during the months preceding the outbreak. Consequently, if the hypothesis is correct and "suitable weather" can influence the occurrence of outbreaks of disease, then cold stress by itself is apparently insufficient and other factors need to be examined.

Alternatively, one could conjecture that the cast ewes were immune to infection with the offending organisms in the autumn when the sheep were introduced to the flock. This immunity may have waned during the winter months, when the organisms were perhaps circulating slowly within the flock, or even establishing persistent or latent infections in a few animals. The first appropriate cold stress period after the flock became susceptible to infection occurred in February and the outbreak then developed.

Thus, the difficulties associated with this type of <u>ad hoc</u> investigation are clearly emphasized by the results

of this one outbreak. It appears, therefore, that the validity of the hypothesis proposed by Smith, Hugh-Jones and Jackson (1972) can only be tested by comprehensive longitudinal studies in selected flocks.

Cytopathic agents resembling adenoviruses were isolated from rectal swabs from 3 lambs during the outbreak of respiratory disease in flock D. That these agents originated from this flock of sheep was confirmed by the detection of rising antibody titres to one of the agents (strain 7769) in sera from 4 lambs.

The sera of the 3 lambs from which the agents were isolated contained moderately high titres of neutralizing antibody to strain 7769. Presumably, these titres represented residual colostral-derived antibody, and any local antibody had waned to such an extent that it did not interfere with the recovery of virus.

It is difficult to assess the relationship of these agents to the clinical disease because adenoviruses have been recovered from faeces of healthy, as well as diseased sheep (McFerran, Nelson and Knox, 1971). Furthermore, the agents in flock D were isolated from rectal but not nasal swabs; but this point was examined and is recorded in Chapter 3.

CHAPTER 2

CHARACTERIZATION OF AN ADENOVIRUS ISOLATED FROM SHEEP.

On the basis of the CPE produced in primary STh cultures, the cytopathic agents isolated from lambs in flock D (Chapter 1, section b) were tentatively identified as adenoviruses. One isolate, designated strain 7769, was selected for further study and its cultural, physical and biological characteristics were examined in greater detail. Although the presence of a group specific antigen has been clearly established in all mammalian adenoviruses, other features of the antigenic relationship of ovine adenoviruses to other mammalian serotypes have not been examined and, for this reason, an attempt was made to investigate some aspects of this with strain 7769.

MATERIALS AND METHODS.

Most of the methods used in the work described in this chapter have been detailed in General Materials and Methods, but the procedures used to characterize strain 7769 are as follows.

Comparison of Virus Growth at 34°C and 37°C in both Stationary and Rolled Cultures.

Aliquots (0·1 ml) of strain 7769 at its eighth passage level in STh cells, containing approximately $10^{3\cdot0}$ $TCID_{50}$, were inoculated on to several STh cultures in tubes. The cultures were incubated both in the stationary position and

by rolling (8 revs/hr) at both 34°C and 37°C. The virus was harvested after 3 and 7 days incubation by freezing and thawing the cultures 6 times, and titrating the fluids on STh cells.

Electronmicroscopy.

Material from the second and ninth passages of the original isolate in primary STh monolayers was examined by the procedure given in General Methods.

Haemagglutination.

The ability of adenovirus strain 7769 to agglutinate erythrocytes was examined using goose, chicken, guinea pig, rat, mouse, rhesus monkey, horse, cattle, sheep and human 'O' cells.

Rat, mouse, rhesus monkey and human 'O' erythrocytes were used as a one per cent. suspension in 0·85 per cent. saline, and all other cells as a 0·5 per cent. suspension in PBS. Each species of erythrocyte was incubated at 4°, 24° and 37°C with two wells per dilution of the 2-fold dilution series of virus, until the control cells had settled. The end point was taken as the highest dilution of virus to completely agglutinate the erythrocytes.

Gel-Diffusion Studies.

Strain 7769 was compared with HA2 virus by means of the gel-diffusion test, using convalescent sera from specific pathogen-free lambs infected with strain 7769 (see Chapter 3) and hyperimmune sera prepared in rabbits, against strain 7769 and HA2 virus.

In the tests used to examine the physicochemical characteristics, strain 7769 was examined simultaneously with

the G2 strain of ovine PI3 virus whose characteristics were known.

Sensitivity to Chloroform.

The method used was that described by Feldman and Wang (1961). Equal volumes of virus and chloroform were shaken together for 5 mins. at room temperature. The supernatant fluid was then removed and titrated on STh cells.

Stability at Low pH.

An 0·1M citrate buffer was prepared at pH 2·7 and 6·1 according to the method of Gomori (1955). An equal volume of virus and citrate buffer were mixed and maintained at 37°C for one hour before being titrated and inoculated on to STh monolayers.

Stability at 56°C.

Aliquots (0·5 ml) of a virus pool were placed in thin-walled ampoules and placed in an ice bath. The ampoules were transferred simultaneously to a water bath at 56°C. Three ampoules were removed at intervals of 0·5, 5, 10, 20, 30 and 60 minutes and replaced in the ice bath. Thereafter, the virus fluids from each triad of ampoules were pooled and titrated on STh cultures.

Inhibition of Growth by 5-Iodo-2'-deoxyuridine (IUDR).

Monolayers of STh cells were washed 3 times with PBS and inoculated with 0·1 ml amounts of a dilution of virus containing approximately $10^3 TCID_{50}$. After one hour of incubation at 37°C, the fluid was removed and replaced with either a) Eagle's medium, b) medium plus 10^{-4}M IUDR or

c) medium plus 10^{-4}M IUDR plus 50μg/ml of thymidine. When virus controls, which did not contain IUDR, showed advanced CPE, all cultures were harvested, frozen and thawed 6 times and titrated.

Pathogenicity for Laboratory Animals.

The pathogenicity of strain 7769 for small laboratory rodents was investigated using mostly hamsters, but also rats, mice and guinea pigs (Table 2.1). In each experiment the animals were examined for evidence of clinical illness and, at the end of the experiment, a necropsy was performed to detect any macroscopic abnormalities.

a) hamsters; following inoculation, these were examined over a period of 9 months, after which they were killed and necropsies performed.

b) rats; 10 rats were killed at each of 3 and 5 days after inoculation, and the lungs removed for virus isolation.

c) mice; these were killed 13 days after inoculation and at necropsy examined for macroscopic lesions.

d) guinea pigs; these were examined for signs of illness for 10 days after inoculation, at which time they were killed and a necropsy carried out.

RESULTS.

Cultural Characteristics.

The nature of the CPE produced by strain 7769 was typical of an adenovirus and, as a general observation, the appearance and distribution of the cellular changes were largely influenced by the amount of virus in the inoculum.

TABLE 2.1

LACK OF PATHOGENICITY OF STRAIN 7769 FOR HAMSTERS, RATS, MICE AND GUINEA PIGS

Species	Type	Number in Group	Route	Inoculum ($log_{10}TCID_{50}$)	Result (Death/ Illness)
Hamster (*Mesocricetus auratus* W.)	newly weaned	15	0·1 ml/cheek pouch	4·7*	-
"	"	15	0·2 ml/sub-cutaneously	5·0*	-
"	"	10	0·1 ml/ cheek pouch	-	-
"	"	10	0·2 ml/sub-cutaneously	-	-
Hamster	newborn (less than 24 hrs)	56	0·1 ml/sub-cutaneously	4·7⊕	-
"	"	14	"	- ∓	-
Porton mouse	adult	10	0·2 ml/intraperitoneally	6·0⊕	-
" "	newly weaned	10	"	6·0⊕	-
" "	newborn	14	0·1 ml/intraperitoneally	5·7⊕	-
Wistar rat	newly weaned	20	0·2 ml/intrathoracically	5·0*	-
Guinea pig	newly weaned	4	1·0 ml/intraperitoneally	6·0⊕	-

* = virus used at the 6th pass.

⊕ = virus used at the 10th pass.

∓ = uninfected tissue culture fluid.

\- = no deaths nor clinical illness observed and no lesions observed at necropsy.

For example, dilutions of virus near the end point produced one or 2 foci of CPE whereas undiluted virus produced CPE, which involved most of the monolayer, within 24 hours of inoculation (figs. 2.2 and 2.3).

Infected coverslip cultures of STh were removed at intervals after inoculation with strain 7769, stained with H & E and examined. The CPE in the stained monolayers was similar to that observed in unstained cultures, except that intranuclear inclusion bodies could be seen at higher magnifications. The first changes were seen in cultures that contained only a few foci of CPE. In the nucleus of a few cells was a central, granular, basophilic mass surrounded by a clear area (Fig.2.4a). As the CPE became more extensive, intranuclear inclusions were present in an increasing number of cells and many nuclei became so distorted and pyknotic, that the details of any internal structures were obscured (Fig.2.5). However, it was possible in a few instances to discern a central, very densely basophilic mass with strands radiating from it to the nuclear membrane (Fig.2.4b).

Acridine-orange staining of virus infected cells produced an intense intranuclear greenish yellow fluorescence (Fig.2.6a), indicating an accumulation of DNA material. A few nuclei contained inclusions that were similar in appearance to the earliest changes seen in cultures stained by H & E (Fig.2.6b).

The data in Table 2.2 indicate that strain 7769 could replicate equally well at 34^{o}C and 37^{o}C, and that the yield

Figure 2.1

Uninfected monolayer of STh cell.

Magnification X 20.

Figure 2.2

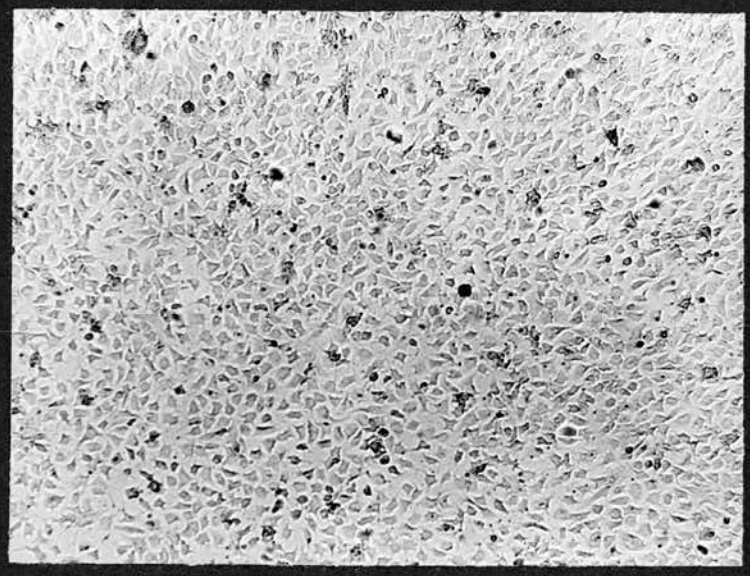
Cytopathic effect of strain 7769 in STh cells showing a focus of round, refractile cells, 4 - 5 days after inoculation.

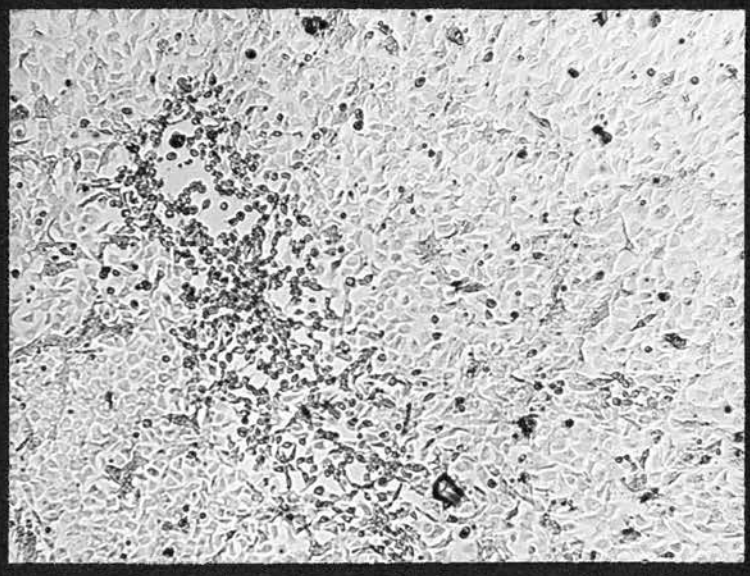
Magnification X 20.

Figure 2.3

Cytopathic effect of strain 7769 in STh cells. Advanced CPE involving most cells. Many infected cells have become detached from the glass, leaving "islands" of highly refractile cells, 6 days after inoculation.

Magnification X 20.





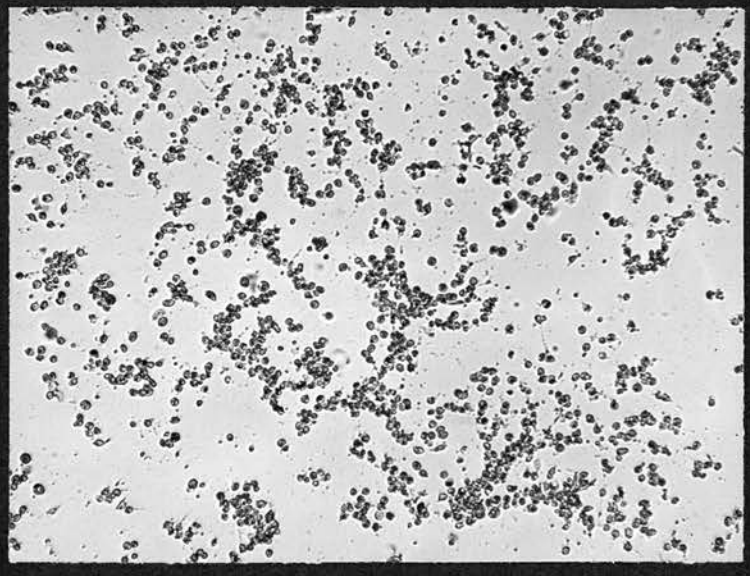


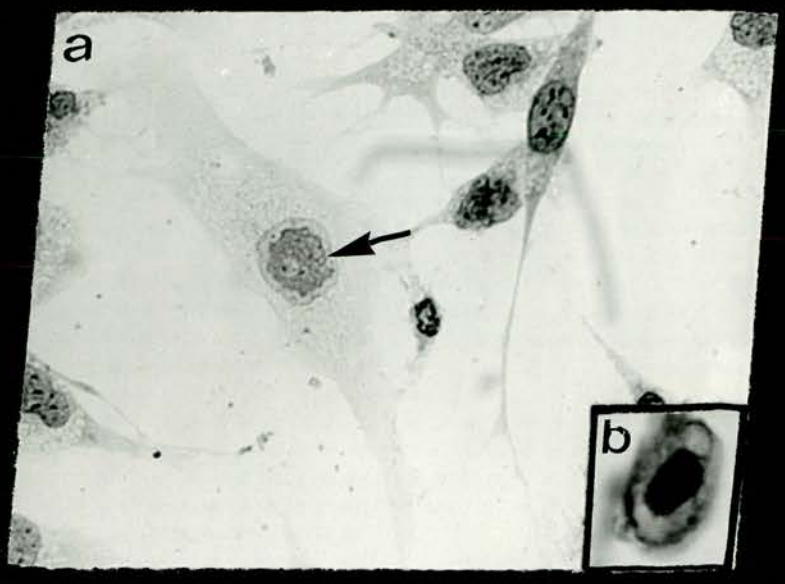
Figure 2.4

a) Early intranuclear inclusion (arrow) produced by strain 7769 in STh cells stained with H & E.
Magnification X 480.

b) Mature intranuclear inclusion showing strands radiating from central mass to nuclear membrane. Stained with H & E.
Magnification X 750.

Figure 2.5

A group of infected cells showing morphological changes at various stages of infection. Early inclusions (solid arrows) are present in a few cells, whose morphology is similar to that of uninfected cells. Several other cells, at a later stage of infection, have shrunken, pyknotic nuclei (open arrow) and little cytoplasm is evident. Stained with H & E.
Magnification X 330.


a
b

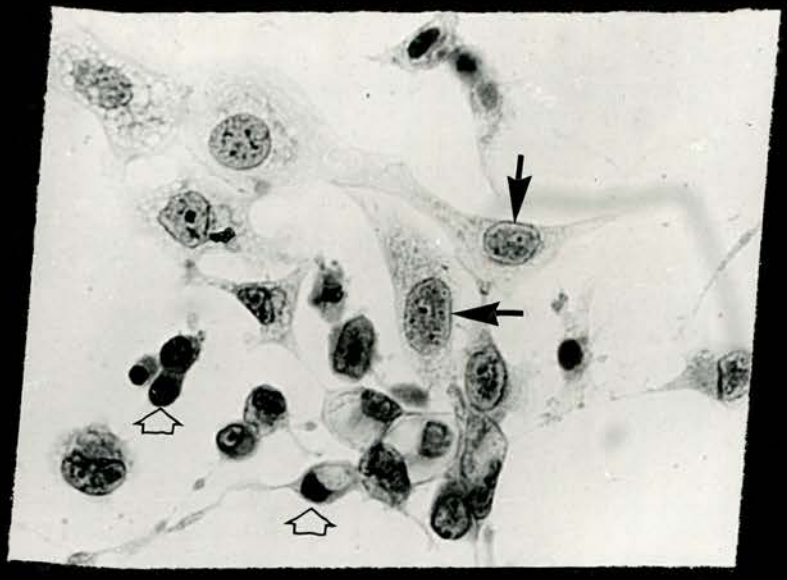
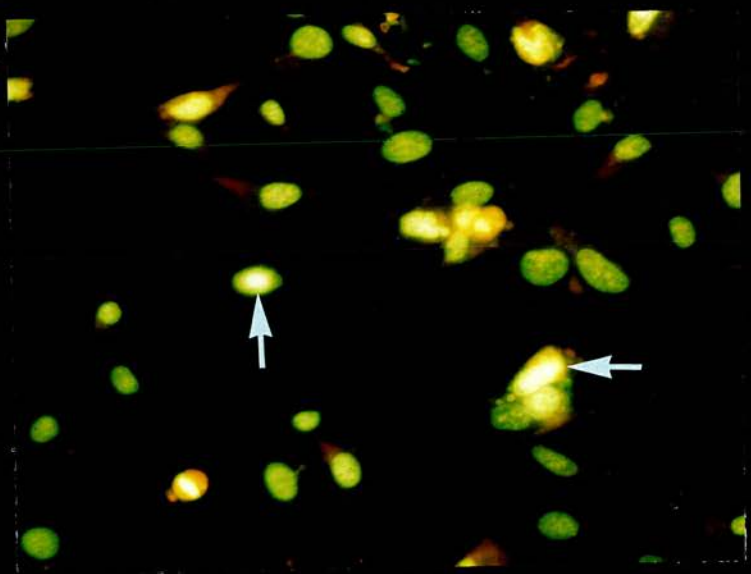


Figure 2.6

a) FLK cell cultures infected with strain 7769. The nuclei of infected cells (arrows) show intense greenish yellow fluorescence when viewed by ultra-violet light. Stained with acridine-orange.
Magnification X 330.

Figure 2.6

b) FLK cell cultures infected with strain 7769. One nucleus contains a large inclusion body (arrow), corresponding to that shown in Fig. 2.4a, with margination of chromatin. Stained with acridine-orange.
Magnification X 330.



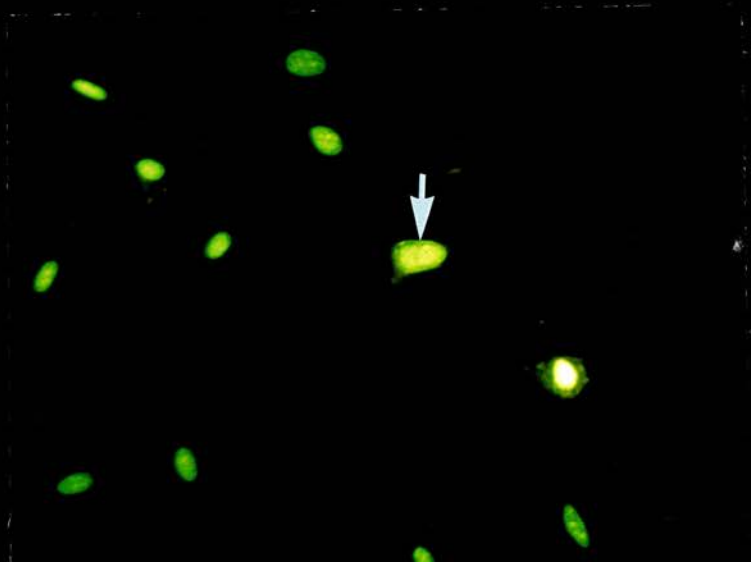


TABLE 2.2

PROPAGATION OF STRAIN 7769 IN PRIMARY SHEEP THYROID MONOLAYERS

Incubation Time (days)	Conditions of Incubation			
	34°C		37°C	
	Rolled	Stationary	Rolled	Stationary
3	5·5 *	5·3	5·5	5·3
7	5·5	6·0	5·8	5·3

* virus titre expressed as log $_{10}TCID_{50}$/0·2 ml.

of virus was not increased by either rolling the cultures or incubating beyond the third day after inoculation.

Physicochemical Characteristics.

Electronmicroscopy by negative contrast staining revealed strain 7769 to have non-enveloped virions showing icosahedral symmetry with the size ranging from 71 - 75 nm (Fig.2.7). Along the edge of each triangular facet were 6 hollow, roughly spherical capsomeres, and hexons and pentons were easily distinguishable. Thin sections of infected cells showed groups of spherical virus particles in the nuclei. Most of the virions had an electron-dense core and the size ranged from 66 - 71 nm diameter (Figs. 2.8, 2.9, 2.10).

The data in Table 2.3 show that the infectivity of strain 7769 was not affected by treatment with chloroform or when held at pH 2·7 for 60 mins, but was completely lost after 20 mins. at 56°C. Presumptive evidence of the nucleic acid type of strain 7769 was indicated by inhibition of replication by IUDR, which could be reversed by the addition of thymidine (Table 2.3).

The same tests performed simultaneously with the G2 strain of ovine PI3 virus showed this virus to be inactivated by chloroform, low pH and after 5 mins. at 56°C. Replication of PI3 virus was not inhibited by IUDR (Table 2.3).

Biological Characteristics.

Strain 7769 agglutinated erythrocytes from rats and mice at 4°, 22° and 37°C, and from cattle at 4° and 22°C; the highest titres being obtained at 4°C. Haemagglutination

Figure 2.7

Electron micrograph of strain 7769, stained with phosphotungstic acid. The virions are icosahedral with a size of 71 - 75 nm. Magnification X 300,000.

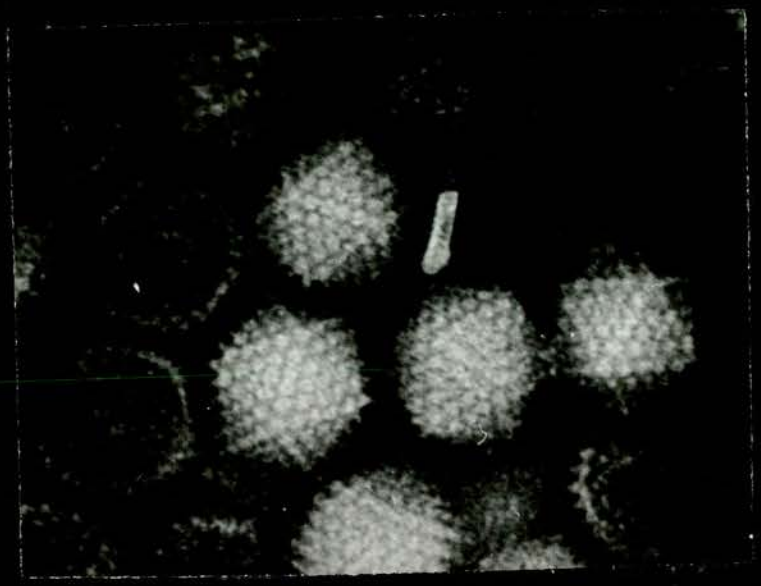


Figure 2.8

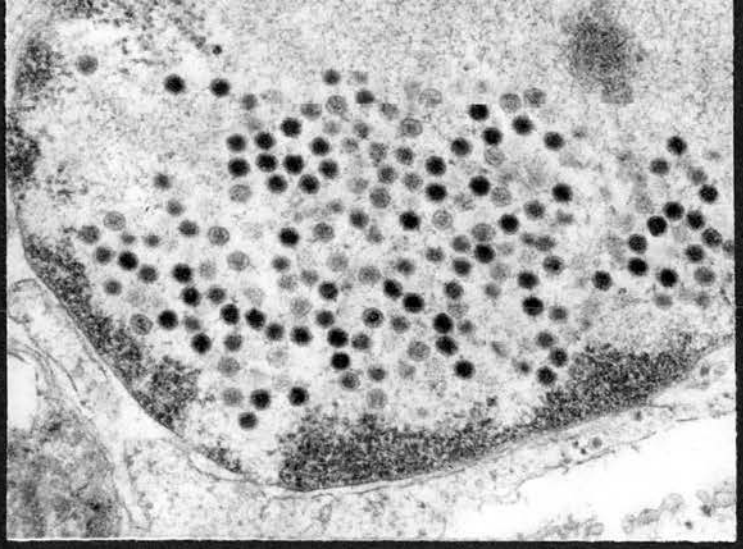
Nucleus of a STh cell, infected with strain 7769, showing margination of chromatin and a group of virions within the nucleus. All of the virions have an electron-dense core of varying intensity. Magnification X 45,000.

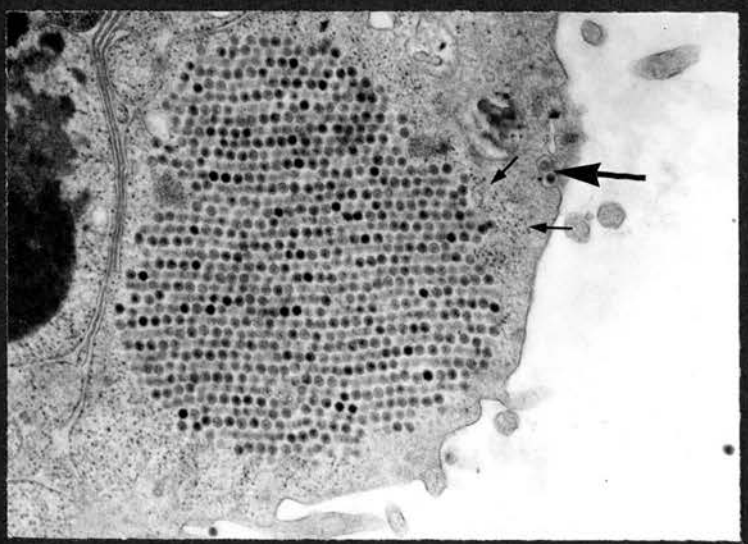
Figure 2.9

Nucleus of a STh cell, infected with strain 7769, showing virions arranged in a crystalline array. The nuclear membrane is indicated by small arrows. Two particles (large arrow) can be seen contained within an invagination of the cytoplasmic membrane, and may represent virus release from the cell.
Magnification X 22,500.

Figure 2.10

Higher magnification of the "crystal" shown in Fig. 2.9, showing particles of 66 - 71 nm diameter, with electron-dense cores.
Magnification X 90,000.





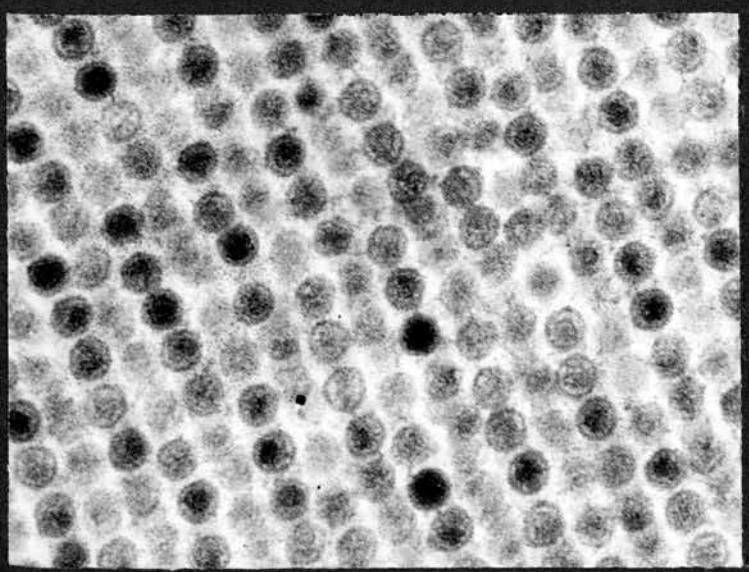


TABLE 2.3

EFFECT OF CHLOROFORM, pH, HEAT AND IUDR ON THE INFECTIVITY OF STRAIN 7769 AND PARAINFLUENZA TYPE 3 (PI3) VIRUSES

Treatment	Virus	
	Strain 7769	PI3 virus (strain G2)
Chloroform		
PBS (control)	4·8*	7·0
chloroform	4·5	2·3
pH		
6·1	4·3	5·5
2·7	4·0	$\leq$ 0·5
Heat (56°C)		
0·5 mins.	4·5	4·0
5 mins.	2·8	-
10 mins.	0·8	-
20 mins.	-	-
IUDR		
medium alone	5·0	7·5
medium + IUDR	-	7·5
medium + IUDR + thymidine.	5·5	7·5

* = Virus titre expressed as $\log_{10}TCID_{50}/0{\cdot}1$ ml.

\- = No virus detectable.

titres of 128, 64 and 16 respectively were obtained when using rat, mouse and cattle erythrocytes.

In gel-diffusion tests, convalescent serum from a specific pathogen-free (SPF) lamb infected with strain 7769 produced a single line of precipitation with extracts prepared from cells infected with homologous virus. This line was confluent with that formed between the convalescent serum and extracts prepared from cells infected with HA2 virus (Fig.2.11). Confluence was also shown with the lines of precipitation formed between HA2 virus and strain 7769 and their homologous antisera (Fig.2.12). Sera taken at the same time, from control lambs inoculated with supernatant fluids from uninfected cultures failed to produce precipitation lines with HA2 virus or strain 7769.

Relationship of Strain 7769 with other Mammalian Adenoviruses.

The antigenic similarity between strain 7769 and strain 7837, which also was isolated from a lamb in flock D during the outbreak of disease (Chapter 1, section b), was established when the same neutralizing titre of a hyper-immune serum to strain 7769 was obtained with either homologous virus or strain 7837. Strain 7815 could not be transferred beyond the second passage level and, therefore, its relationship to strain 7769 could not be ascertained. Cross-neutralization tests showed that strain 7769 differs from the 3 serotypes of adenovirus isolated from sheep in Northern Ireland. Specific antisera to OA1-3 viruses failed to neutralize 100 $TCID_{50}$ of strain 7769 and vice versa,

Figure 2.11

Demonstration of Adenovirus Group Specific Antigen in Strain 7769 by the Gel Diffusion Test.

A and C 90 day convalescent sera from specific pathogen-free lambs inoculated with uninfected tissue culture fluid.

B 90 day convalescent serum from specific pathogen-free lamb infected with strain 7769.

D extract of cells infected with human adenovirus type 2.

E extract of cells infected with strain 7769.

Figure 2.12

Demonstration of Adenovirus Group Specific Antigen in Strain 7769 by the Gel Diffusion Test.

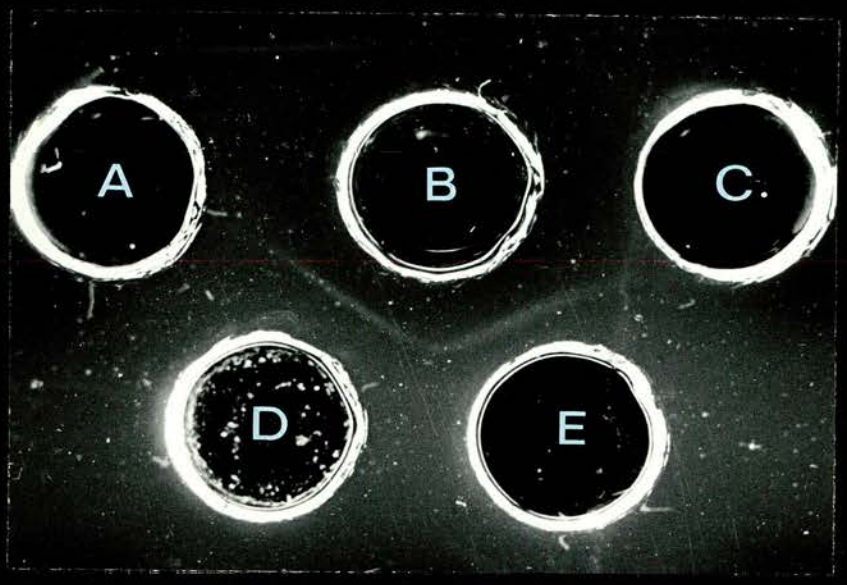
A hyperimmune serum to human adenovirus type 2, prepared in rabbits.

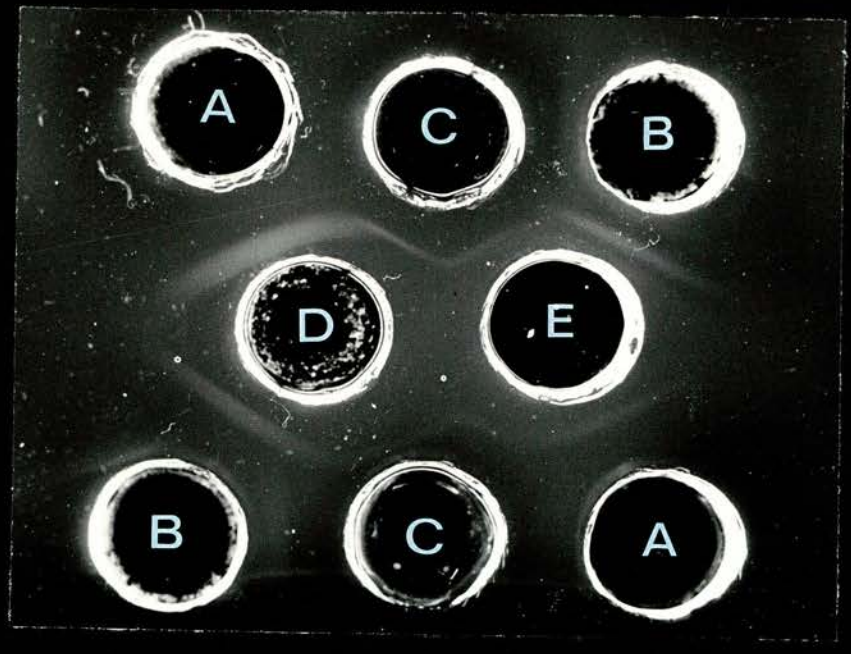
B hyperimmune serum to strain 7769, prepared in rabbits.

C 90 day convalescent sera from 2 specific pathogen-free lambs infected with strain 7769.

D extract of cells infected with human adenovirus type 2.

E extract of cells infected with strain 7769.


A
B
C
D
E


A
C
B
D
E
B
C
A

(Table 2.4).

Strain 7769 also differs antigenically from the bovine adenoviruses. Using the constant serum-varying virus method, 1 in 10 dilutions of antisera to BA1 virus and BA3-8 viruses failed to reduce the titre of strain 7769, whereas antiserum to BA2 virus reduced the titre by 1·5 $\log_{10}$ (Tables 2.5 & 2.6). In a cross-neutralization test, a slight one-way cross-reaction was observed between BA2 virus and strain 7769 (Table 2.7).

Strain 7769 also differs from those human adenoviruses which share with it the ability to agglutinate rat erythrocytes. Undiluted antisera to human adenovirus serotypes 1,2,4,5,6,8,9,10,13,15,17,19,22,23,24,26,27,29 and 30 were unable to neutralize 10 $TCID_{50}$ of strain 7769 in a neutralization test in microplates.

Pathogenicity for Laboratory Animals.

None of the hamsters inoculated with strain 7769 or uninfected tissue culture fluid appeared ill or died during the 9 months observation period (Table 2.1). Also, no palpable tumours developed near any of the inoculation sites, nor were observed during post-mortem examination of each hamster.

Neither clinical disease nor macroscopic lesions developed in mice, rats and guinea pigs inoculated with strain 7769, and the virus was not reisolated from the lungs of the rats (Table 2.1).

TABLE 2.4

CROSS-NEUTRALIZATION TESTS BETWEEN 3 OVINE ADENOVIRUS SEROTYPES AND STRAIN 7769.

Virus	Antiserum			
	7769	OA1	OA2	OA3
7769	1496	<10	<10	<10
OA1	<10	10240	<10	<10
OA2	<20	<10	2138	<10
OA3	<10	<10	<10	1496

Titres expressed as the reciprocal of the serum dilution.

TABLE 2.5

NEUTRALIZATION OF STRAIN 7769 BY ANTISERA TO BOVINE ADENOVIRUS TYPES 1,2,3 AND 5

	Antiserum					
	BA1	BA2	BA3	BA5	7769	CONTROL
titre of strain 7769 ($log_{10}TCID_{50}$)	4·5	3·3	5·0	4·8	$\leq$0·5	4·8

TABLE 2.6

NEUTRALIZATION OF STRAIN 7769 BY ANTISERA TO BOVINE ADENOVIRUS TYPES 4 - 8

	Antiserum						
	BA4	BA5	BA6	BA7	BA8	7769	CONTROL
titre of strain 7769 ($log_{10}TCID_{50}$)	4·0	4·5	4·3	4·0	3·8	$\leq$0·5	4·3

TABLE 2.7

CROSS-NEUTRALIZATION TEST BETWEEN STRAIN 7769 VIRUS AND BOVINE ADENOVIRUS TYPE 2 (BA2)

Virus	Antiserum	
	7769	BA2
7769	8192	< 4
BA2	128	708

Antibody titres expressed as a reciprocal of the serum dilution.

Survey of sheep sera for antibodies to strain 7769 and ovine adenovirus types 1 - 3.

Precipitating antibody to the adenovirus group specific antigen was detected in only 38 of 661 sheep sera but neutralizing antibodies to each serotype of ovine adenovirus were detected in a much higher proportion (Table 2.8). Antibodies to strain 7769 and OA3 virus were more prevalent than those to OA2 virus and OA1 virus.

Both precipitating and neutralizing antibodies were present in sheep sera obtained from a high proportion of farms (Table 2.8).

The sera were divided into 2 groups on the basis of the age of the donor sheep; one group contained sera from sheep older than 12 months and the other, sera from animals younger than 12 months of age. There was no difference between the proportion of sheep in each group with precipitating antibody, but neutralizing antibodies to each serotype were significantly more common in older animals (Table 2.9).

DISCUSSION.

The physicochemical and biological characteristics of strain 7769 are similar to those described for viruses of the adenovirus group (Pereira, Heubner, Ginsberg and van der Veen, 1963; Wildy, 1971).

The cytopathic effect produced by strain 7769 closely resembled that described originally for adenoviruses by

TABLE 2.8

ANTIBODIES TO OVINE ADENOVIRUSES IN SERA FROM SHEEP IN SCOTLAND

Test	Number of Sera Tested	Number of Sera Positive	Percentage of Sera Positive	Number of farms positive / Number of farms tested.
Gel Diffusion				
HA2	661	38	5·8	12/25
Neutralization				
OA1	621	129	20·8	22/25
OA2	589	238	40·4	23/25
OA3	633	429	67·8	21/24
Strain 7769	627	446	71·1	20/25

TABLE 2.9

A COMPARISON OF ANTIBODIES TO OVINE ADENOVIRUSES IN SERA FROM ADULT SHEEP AND LAMBS

Test	Adults			Lambs			Value obtained in Chi squared Test
	Number of Sera Tested	Number of Sera Positive	Percentage of Sera Positive	Number of Sera Tested	Number of Sera Positive	Percentage of Sera Positive	
Gel Diffusion							
HA2	397	16	4·0	226	17	7·5	2·8
Neutralization							
OA1	371	96	25·9	216	24	11·1	18·4
OA2	344	168	48·8	214	66	30·8	17·5
OA3	381	307	80·6	218	94	43·1	87·9
Strain 7769	376	295	78·5	214	125	58·4	25·0

Rowe, Huebner, Gilmore, Parrott and Ward (1953). Further, the formation of intranuclear inclusion bodies and other nuclear changes, seen in cultures stained with H & E, were similar to those described for human adenovirus types 3 and 4 (Boyer, Leuchtenberger and Ginsberg, 1957) and ovine adenovirus types 1 - 3 (McFerran, Nelson and Knox, 1971). Basophilic intranuclear inclusions corresponding to the early and middle stages noted by Boyer et al (1957) were commonly seen in cultures infected with strain 7769, but the late type of inclusion, characterized by strands radiating to the nuclear membrane, was produced less frequently. The eosinophilic structures observed in the nuclei of cells infected with human adenovirus type 3 were not seen in cells infected with strain 7769. The absence of these crystals can probably be attributed to the use of Bouin's fluid as fixative, which was considered by Boyer et al (1957) to reduce the number of crystals and distort them, making identification difficult.

The morphology of the virions of strain 7769 as seen by negative contrast staining and in thin sections of infected cells, was similar to that described for adenoviruses and the size of the virus particles was within the range reported for this genus (Melnick, 1971; Wildy, 1971).

Strain 7769 possessed several other features in common with adenoviruses. For example, infectivity was not affected by treatment with chloroform or by low pH, but was rapidly reduced by heating at 56^{o}C. Evidence that the nucleic acid was DNA was provided by inhibition of replication

by IUDR, and the presence of greenish-yellow fluorescence in the nuclei of infected cells after staining with acridine orange. In common with many other adenoviruses, strain 7769 was not pathogenic for laboratory animals.

Greater difficulty was encountered in demonstrating the adenovirus group-specific antigen by gel diffusion in strain 7769 than in HA2 virus. This observation confirmed the previous experience of McFerran, Nelson and Knox (1971) working with OA1, OA2 and HA2 viruses. The main reason for this difficulty may be due to a relatively inefficient production of virus antigens, because the infectivity obtained with strain 7769 did not exceed $10^{5 \cdot 5}TCID_{50}/0 \cdot 1$ ml, whereas with human adenoviruses the infectivity that can be expected is about $10^{8 \cdot 0}TCID_{50}/0 \cdot 1$ ml (Pereira and Valentine, 1958).

As a result of these findings it is concluded that strain 7769 is a member of the adenovirus group. Moreover, the results of neutralization tests with specific antisera indicate that strain 7769 differs antigenically from the ovine and bovine adenoviruses and those human adenoviruses which share with it the ability to agglutinate rat erythrocytes, although there is a small amount of cross-reaction with BA2 virus.

Further, the ability of strain 7769 to agglutinate erythrocytes from rats, mice and cattle is of interest on two accounts. Firstly, human adenoviruses have been divided into 3 groups by means of their ability to agglutinate various erythrocytes and it has been suggested

that haemagglutination could form the means of rapid, tentative identification of wild strains (Hierholzer, 1973). On the basis of published results, the species of erythrocytes agglutinated by strain 7769 differ from those agglutinated by porcine, canine, equine and murine adenoviruses (Hartley and Rowe, 1960; Espmark and Salenstedt, 1961; Kasza, 1966; Clarke, Sharp and Derbyshire, 1967; England, McChesney and Chow, 1973) and also OA1 virus (Knox, 1974). Thus strain 7769 appears to be a previously unreported type of adenovirus and will be referred to in the remainder of this work as ovine adenovirus type 4 (OA4).

This conclusion is supported by the findings of Adair and McFerran (1976) who examined the antigenic relationships, by cross-neutralization tests, of several bovine, porcine, ovine and human adenoviruses. These authors reported that OA4 virus (strain 7769) differed from the other adenoviruses which they were examining. However, unlike the present work, they did not report any cross-reaction between OA4 and BA2 viruses. It may be that such relationships are revealed only when antisera with high neutralizing titres are used.

A number of adenoviruses are capable of inducing tumours in hamsters, and several of these viruses originate from domestic animals e.g. CELO virus (Sarma, Heubner and Lane, 1965), infectious canine hepatitis virus (Sarma, Vass, Heubner, Igel, Lane and Turner, 1967) and bovine adenovirus types 3 and 8 (Darbyshire, 1966; Rondhuis, 1973). The present work has shown that OA4 virus does not appear

to be oncogenic in hamsters. The virus titre used was lower than that employed in many studies of oncogenic adenoviruses, but was comparable with those reported for human adenovirus types 12 and 18 (Girardi, Hilleman and Zwickey, 1964), siman virus 38 (Hull, Johnson, Culbertson, Reimer and Wright, 1965) and bovine adenovirus types 3 and 8.

The survey of sheep sera for antibodies to adenovirus has shown that precipitating antibodies to the group specific antigen are not very prevalent, which might be interpreted as indicating that infections by adenovirus are not common. However, because neutralizing antibodies to each serotype of ovine adenovirus are widespread, this rather suggests that infections by adenoviruses, in fact, are common. The different results obtained by means of these 2 tests probably arises from the insensitivity of serological procedures used for the detection of antibodies to the group specific antigen, as has been commented on by other workers. For example, Ludwig and Liebermann (1970) reported that only 18 of 617 cattle sera contained complement-fixing antibody to human adenovirus type 5, but 161 sera contained neutralizing antibody to bovine adenovirus type 3. Similarly, in sheep, Knox (1974) found that neutralizing antibody to OA1 virus was much more common than either complement-fixing or precipitating antibody and, in horses, Harden, Pascoe, Spradbrow and Johnston (1974) and Studdert, Wilks and Coggins (1974) considered that gel-diffusion or complement-fixation tests were less sensitive than neutralization or haemagglutination-inhibition tests. Thus, although serological tests for the group specific antigen

are useful for screening sera rapidly, they present an erroneous picture of the prevalence of infections by adenoviruses.

The proportion of sheep with precipitating antibodies to adenovirus was higher in the present work than that previously reported for sheep in the British Isles (Darbyshire and Pereira, 1964; Timoney, 1971; Knox, 1974). There is no adequate explanation for this difference, other than that different populations of sheep were sampled. However, this may be an important feature because McFerran, Clarke, Knox and Connor (1972) found that adenoviruses could be isolated frequently on particular farms.

There are no other surveys of neutralizing antibodies to ovine adenoviruses except that of Knox (1974), who found antibody to OA1 virus in 50·3 per cent. of 400 sheep sera. This is higher than the figure of 20·8 per cent. for OA1 virus in the present suevey, but compares favourably with the prevalence of antibodies to the other serotypes.

The results in Table 2.9 show that neutralizing antibodies are commoner in older animals, which is similar to the findings of Harden et al (1974) and Studdert et al (1974) for equine adenovirus in horses, and of St.George (1971) for PI3 virus in sheep. Such evidence has been taken to indicate that animals continue to be infected as adults, although it also might suggest that latent or persistent infections may be established.

CHAPTER 3

INVESTIGATION OF THE PATHOGENICITY OF OVINE ADENOVIRUS TYPE 4 IN SPECIFIC PATHOGEN-FREE LAMBS.

Although infection of sheep by adenoviruses has been recorded for many years (Darbyshire and Pereira, 1964), there is little information regarding their pathogenicity in this species (see Introduction). Therefore, following the isolation and identification of a new serotype of ovine adenovirus, it was considered important to examine its pathogenicity for sheep. As this work was part of a larger project on ovine respiratory disease, and OA4 virus was isolated during an outbreak of pneumonia in young lambs, it was decided to investigate its pathogenicity for lambs, following inoculation into the respiratory tract.

MATERIALS AND METHODS.

The preparation of cell cultures, stocks of viruses and the serological tests employed have been described in General Materials and Methods. Three experiments were performed:-

EXPERIMENT 1.

DESIGN OF EXPERIMENT.

The first experiment was designed to ascertain whether OA4 virus could replicate in sheep, and the type of clinical disease and lesions which would be produced. Nine specific

pathogen-free (SPF) lambs were obtained by the method of Hart, Mackay, McVittie and Mellor (1971) and maintained in strict isolation (Brotherston, 1968). At 6 days old, 7 lambs were exposed for 10 minutes to an aerosol of OA4 virus, at the third passage level in STh cultures and the next day received a further 10 minutes exposure. Lambs included as controls were exposed for 10 minutes to an aerosol of uninfected tissue culture (TC) fluid. The apparatus used to generate the aerosol (Fig. 3.1) has been described by Smith (1975). Briefly, air was passed at 10 pounds per square inch through a Wright's nebuliser containing the inoculum and the aerosol collected in a 23 litre reservoir maintained at atmospheric pressure. The lambs inhaled from this reservoir by means of a face mask attached to a non-return valve. Using the formula described by Smith (1975) it was estimated that each lamb could have inhaled $10^{3 \cdot 3}TCID_{50}$ of OA4 virus. Clinical observations, rectal temperature and nasal, rectal and conjunctival swabs were taken daily for 14 days after inoculation (pi). The lambs were killed serially (Table 3.1) by means of intravenous pentobarbitone. Samples for microbiological examination were taken from liver, kidney, spleen and several sites in the respiratory and alimentary tracts, using a different set of sterile instruments for each tissue. Portions of these tissues were immersed in formol-saline for histological examination, and glutaraldehyde for electron microscopy.

Figure 3.1

Diagram of apparatus used to generate aerosols of virus in Experiment 1.

F_1), F_2)	filters
FM	flow meter
C	control valve
IS	all-glass impinger
R	reservoir
N	nebuliser
MN	manometer
T	tap
V	three-way non-return valve
M	face mask
AVP	adjustable vacuum pump

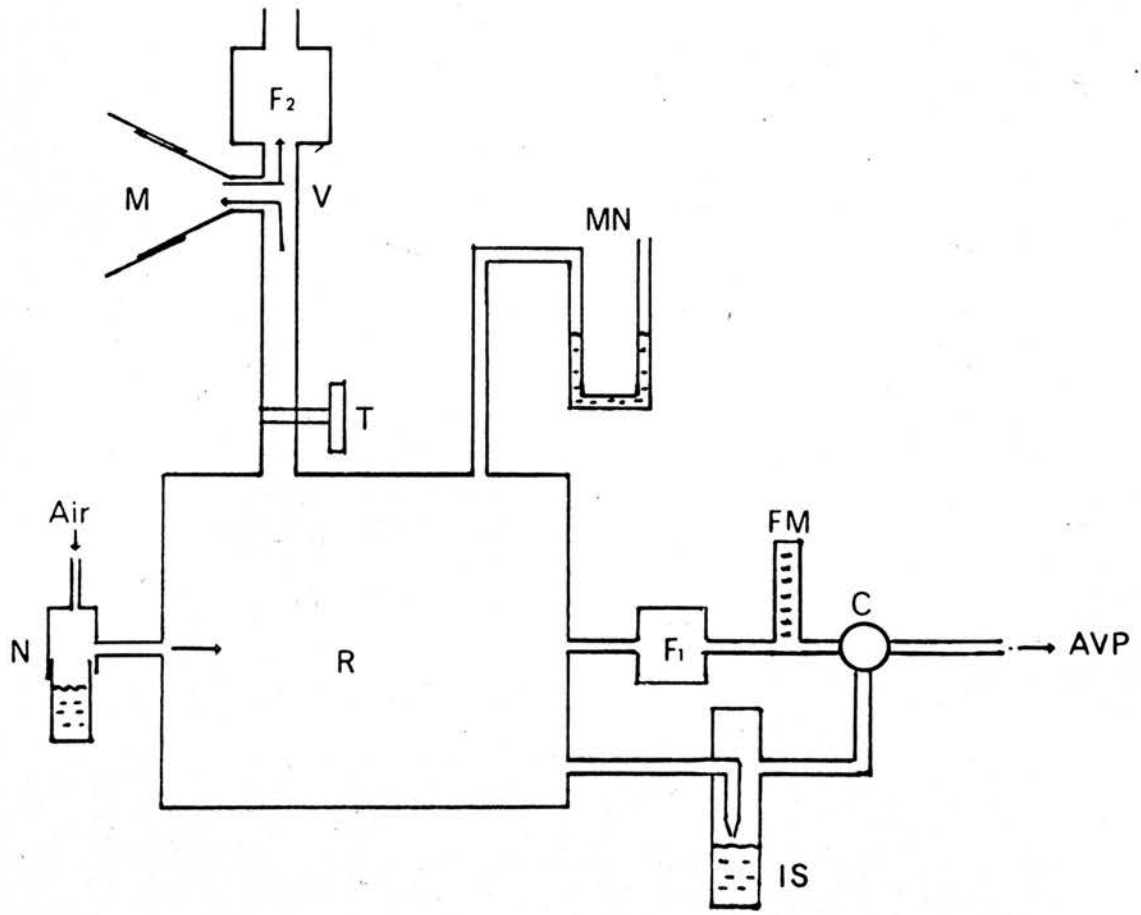

F2
M
V
MN
T
Air
FM
C
N
R
F1
AVP
IS

TABLE 3.1

PATHOGENICITY OF OA4 VIRUS IN SPF LAMBS

DESIGN OF EXPERIMENT 1

Days post-inoculation	Number of lambs killed	
	Infected group	Control group
0		
4	1	
6		1
7	2	
9	2	
11	1	
14	1	1

RESULTS

Clinical Observations.

Following exposure to the aerosol of OA4 virus or uninfected TC fluid, none of the lambs inhibited overt signs of respiratory or enteric disease. There was no evidence of nasal or conjunctival discharges, alteration in rate or character of the respirations, diarrhoea or pyrexia (rectal temperature $\geqslant 105^{o}F$). However, auscultation revealed adventitious sounds in the lower anterior regions of the thorax of 4 of 7 infected lambs. The sounds consisted of occasional high pitched squeaks which were first detected on day 3 pi in 3 lambs and on day 4 pi in one further lamb. Thereafter, the sounds could be detected intermittently in each of these lambs until they were killed.

Virus Recovery.

The isolation of OA4 virus from nasal and rectal swabs taken from lambs is presented in Table 3.2. Isolation of the virus was generally made during the first passage in primary STh monolayers, as judged by the appearance of a typical CPE but swabs taken after day 8 pi required prolonged culture, or a second passage, before CPE became apparent. Virus was recovered daily between days 1 - 7 pi from nasal swabs from each infected lamb that remained alive and from 2 lambs up to day 9. Virus could not be recovered from rectal swabs taken on the first 2 days pi, and from only one of 7 lambs on day 3 pi. Between days 5 and 7 pi, it

TABLE 3.2

RECOVERY OF OA4 VIRUS FROM SWABS

Lamb	Days post-inoculation													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
21	N	N	N											
10	N	N	N	N	NR	NR								
18	N	N	N	NR	NR	NR								
13	N	N	N	N	NR	NR	NR	-						
19	N	N	NR	NR	NR	NR	NR	-						
12	N	N	N	NR	NR	NR	NR	NR	NR	-				
20	N	N	N	NR	NR	NR	NR	NR	N	-	nt	R	-	-

Cytopathic agents were not recovered from conjunctival swabs nor from swabs from control lambs.

N = virus isolated from nasal swab

R = virus isolated from rectal swab

- = virus not isolated from nasal or rectal swab

nt = not tested

was possible to isolate virus daily from rectal swabs from each lamb that was not killed. OA4 virus was not isolated at any time from conjunctival swabs. Recoveries of OA4 virus from tissues taken at necropsies are presented in Table 3.3 and showed that the distribution of virus was limited to the respiratory and alimentary tracts. OA4 virus was isolated from the respiratory tissues of only 2 lambs, and from the colon of 4 of 5 infected lambs killed up to 9 days pi. In contrast to this, virus was recovered from the tonsils of all but one infected lamb, including the lamb killed on day 14 pi.

Cytopathic agents were not recovered from swabs or tissues taken from control lambs inoculated with uninfected tissue culture fluid.

Neither mycoplasma nor bacteria were isolated from the selected tissues of any lamb, except one. Secondary broth cultures from the diaphragmatic lobes of this lamb yeilded a coagulase positive strain of Staphylococcus aureus.

Serology.

Precipitating antibodies to the group specific antigen could not be demonstrated in sera from any of the lambs. Neutralizing antibodies were detected at low titre (1/4) in the serum of only one infected lamb killed 7 days pi.

Post-mortem observations.

No significant macroscopic abnormalities were noted in the organs of the 2 control lambs. In the infected group of lambs, lesions were confined to the lungs and liver.

TABLE 3.3

ISOLATION OF OA4 VIRUS FROM TISSUES TAKEN AT NECROPSY FROM EXPERIMENTALLY INFECTED LAMBS

Tissue examined	Virus Isolations from Tissues of 7 Lambs killed after (days)						
	4	7	7	9	9	11	14
Nasal mucosa	+	-	-	-	-	-	-
Tonsil	+	+	+	+	+	-	+
Retropharyngeal lymph node	-	-	-	-	-	-	-
Tracheal mucosa	+	+	-	-	-	-	-
Bronchial lymph node	-	-	-	-	-	-	-
Mediastinal lymph node	-	-	-	-	-	-	-
Lung	+	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-
Jejunum	+	-	-	-	-	-	-
Colon	+	+	+	+	-	-	-

Cytopathic agents were not recovered from tissues taken from control lambs.

+ = virus isolated

- = no virus isolated.

In the lungs of 4 lambs there was a variable degree of purple discolouration and oedema, as judged by the moist appearance of the lungs and expression of fluid from the lung parenchyma.

The diaphragmatic lobes of the lamb killed 4 days pi were slightly swollen and showed a diffuse mauve discolouration of the diaphragmatic lobes. Of the 2 lambs killed 7 days pi, only one presented macroscopic changes in the lungs. The posterior portions of the diaphragmatic lobes were a much darker pink colour than the rest of the lung, and were separated by a sharp line of demarcation (Figs. 3.2 & 3.3). A frothy exudate was present in the trachea, bronchi and larger bronchioles and could be expressed from the cut surface of the lung. Two lambs were killed 9 days pi. The lungs of one appeared normal, but those of the other were slightly discoloured and contained small areas of consolidation in the right apical lobe. The lungs of the lamb killed 11 days pi, appeared normal, but those of the lamb killed on day 14 pi contained well-defined areas of discolouration in the apical and diaphragmatic lobes which appeared moist on their cut surfaces.

In the liver of one lamb killed 9 days pi small grey spots were seen in one area of the diaphragmatic curvature.

Histology.

Uppper Respiratory Tract.

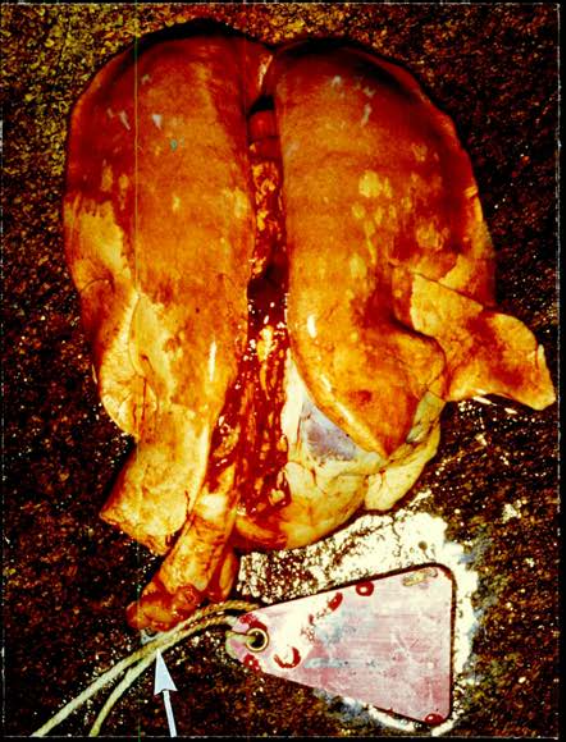
Microscopic abnormalities were not detected in the

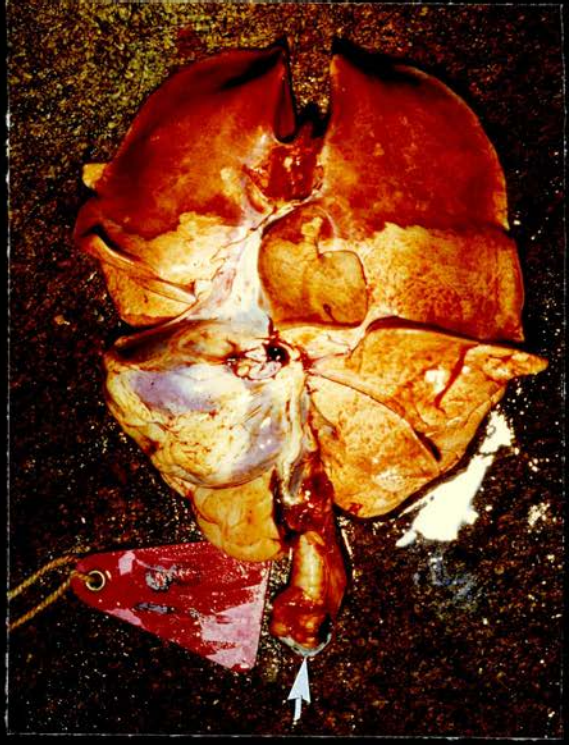
Figure 3.2

Dorsal view of the lungs from a lamb infected with OA4 virus and killed 7 days pi. The posterior portions of the diaphragmatic lobes are discoloured and sharply demarcated from the remainder of the lungs. Froth can be seen exuding from the trachea (arrows).

Figure 3.3

Ventral view of the lungs displayed in Fig. 3.2.





nasal cavities and tracheas of either the control lambs or the 2 infected lambs killed 7 days pi, and one lamb killed 9 days pi. In the other lambs, one side only of the nasal septum contained foci of sloughing epithelial cells together with focal infiltration of the epithelium and submucosa by polymorphonuclear leukocytes. In the lambs killed 11 and 14 days pi, a few eosinophils and plasma cells were seen in the submucosa.

No inclusion bodies were observed in any of the fields examined.

Lung.

No lesions were observed in the lungs of one control lamb, but those of the other lamb contained foci in which the alveolar walls were congested.

The lungs of all infected lambs contained lesions of varying severity.

Microscopic evidence of oedema was seen in all 4 lungs which had appeared discoloured at necropsy. In the lambs killed on days 4, 7 and 14 pi, there were large perivascular and peribronchiolar spaces which contained an amorphous eosinophilic substance. Alveoli and interstitial tissues were distended, and a cryostat section from the posterior diaphragmatic lesion of the lungs showed the alveoli to be filled with a faintly eosinophilic fluid. The lungs of 2 other lambs, killed 7 and 11 days pi, and which did not show discolouration at necropsy, contained foci of alveoli and interstitial tissues that were slightly distended with fluid.

Infiltration of interalveolar septa by mononuclear cells, consisting mainly of lymphocytes, was present in 2 lambs killed 9 days pi and one lamb killed on each of 7 and 11 days pi. The cellular infiltration was slight in 2 lambs but in the other lambs (killed 9 and 11 days pi) interalveolar septa were thickened markedly (Fig. 3.4). In the lamb killed on day 9 pi, the infiltration was confined to the right apical lobe in which depressed areas of consolidation had been noted at necropsy.

There was no involvement of the bronchiolar epithelium or peribronchiolar accumulations of lymphoid tissue in any of the lambs. Rigorous examination of all fields failed to detect the presence of inclusion bodies.

Liver.

The small foci observed at necropsy in the liver of one lamb killed on day 9 pi were identified as areas of necrosis. Most foci consisted of degenerating hepatocytes mingled with mononuclear cells, but some were composed of ghost outlines of hepatocytes and occasional masses of erythrocytes surrounded by a zone of mononuclear cells (Fig. 3.5). In sections stained with H & E, basophilic inclusion bodies (IB's) were seen in the nuclei of many degenerating hepatocytes (Figs. 3.6 & 3.7a) whilst in sections stained by Feulgen's method or with phloxine tartrazine the IB's were, respectively, bright red or bronze-coloured (Figs. 3.7 c & b). These IB's varied in size, shape and tinctorial qualities. Most were small, centrally placed and deeply stained, whereas others were

Figure 3.4

Lung from lamb infected with OA4 virus and killed 11 days pi showing thickened inter-alveolar septae due to infiltration by mono-nuclear cells. Stained with H & E. Magnification X 25.

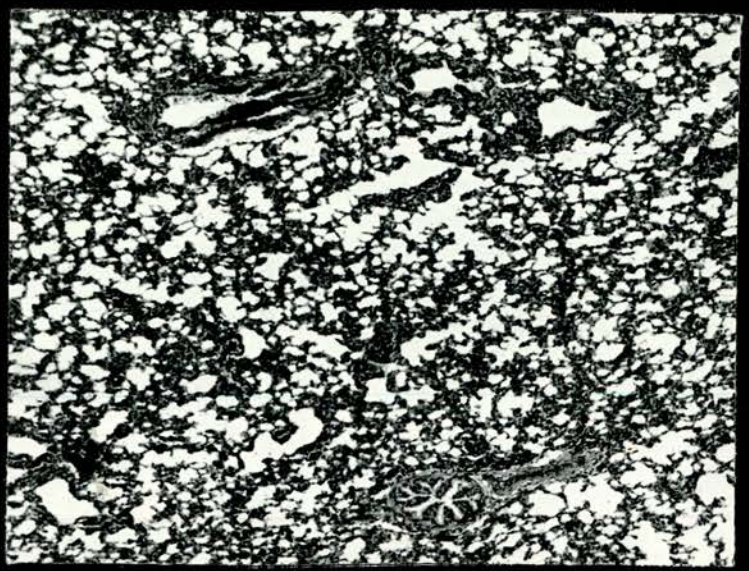
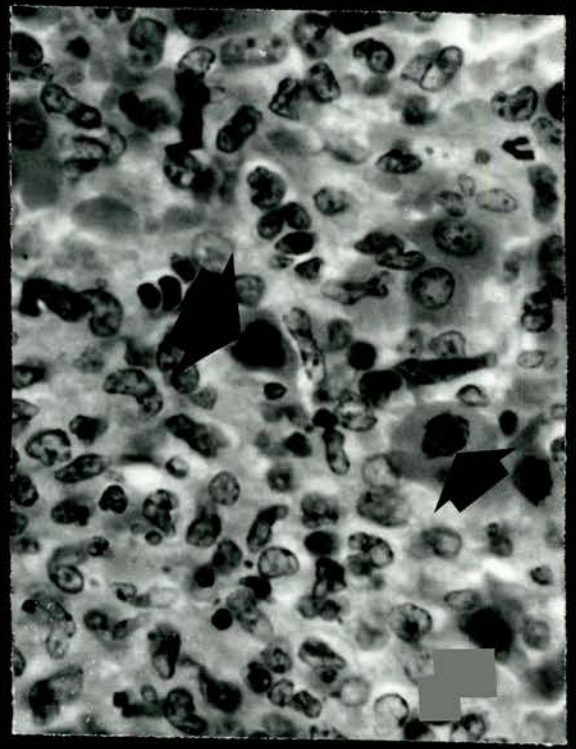


Figure 3.5

Hepatic foci consisting in A of dead and dying hepatocytes mingled with mononuclear cells, and in B of ghost outlines of cells with an outer zone of mononuclear cells. Stained with H & E. Magnification X 55.

Figure 3.6

Area of hepatic necrosis showing hepatocytes containing basophilic intranuclear inclusion bodies. In one nucleus (large arrow) there is margination of chromatin and a deeply staining inclusion body, and in another nucleus (small arrow) an indistinct early-type of inclusion. Stained with H & E.
Magnification X 350.



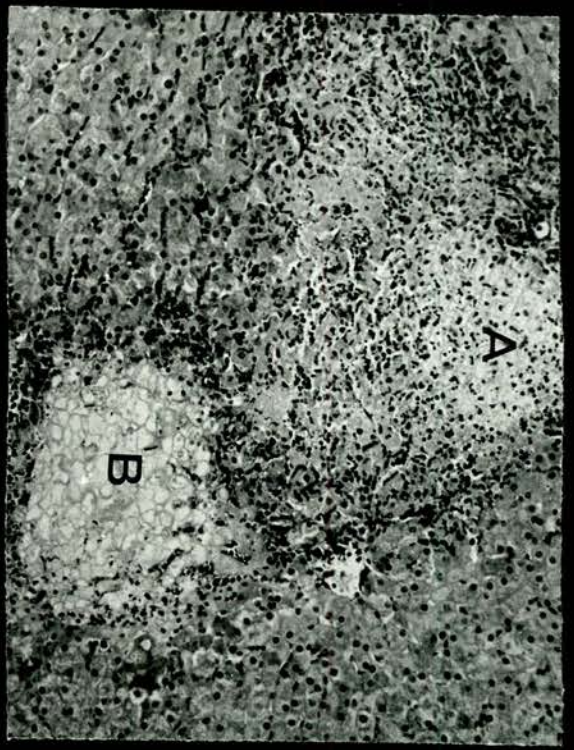

A
B

Figure 3.7

Areas of hepatic necrosis showing the appearance of the intranuclear inclusion bodies (arrows) with different stains.

a) Stained with H & E.

Magnification X 380.

Figure 3.7

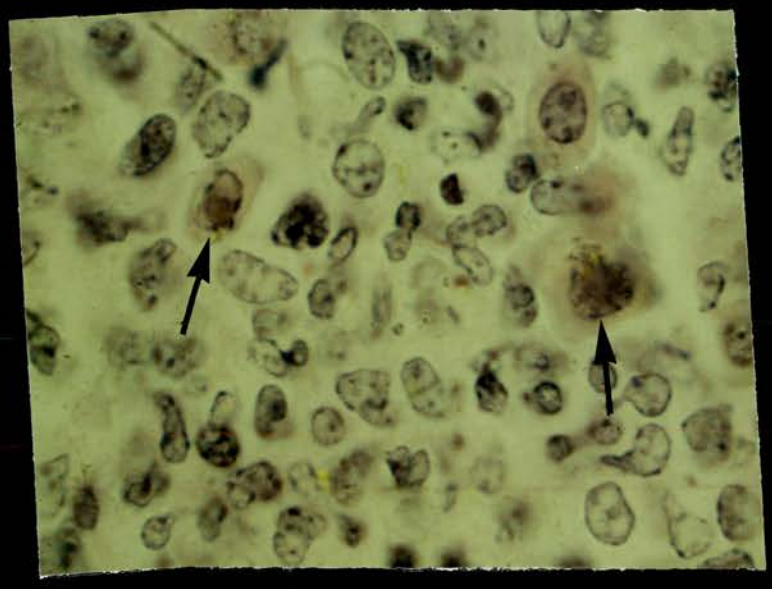
b) Stained with phloxine-tartrazine.

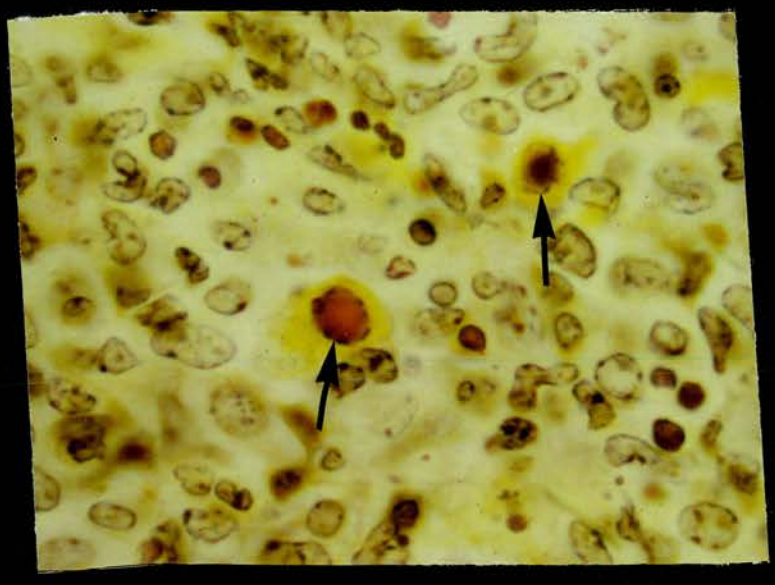
Magnification X 380.

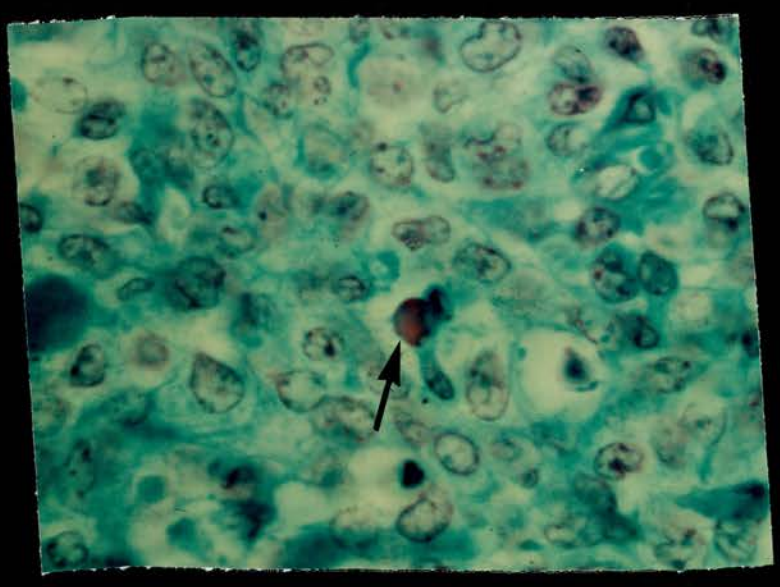
Figure 3.7

c) Stained with Feulgen.

Magnification X 380.







large and occupied most of the nucleus.

The portal tracts of this lamb showed a number of changes of the same type. In most tracts, secondary bile ducts had been replaced by circumscribed masses of mononuclear cells and a lesser number of cells with pale kidney-shaped nuclei (Figs. 3.8 & 3.9). At the centre of this cellular reaction was a substance which appeared dark brown when stained by Stein's procedure, and was considered to be bile. There was atrophy of peripheral hepatocytes and bile ductule formation. In a few tracts, the epithelium of secondary bile ducts was intact but dystrophic. There was slight infiltration of surrounding tissues by mononuclear cells and the peribiliary arterial plexus was congested (Fig. 3.10). In other tracts, there was more extensive infiltration by mononuclear cells and the dystrophic epithelium had been shed, allowing an influx of mononuclear cells. An inclusion body was identified in the nucleus of one dystrophic epithelial cell.

Lymphatic endothelial cells in the portal tracts of this lamb also were enlarged and protruded into the duct lumen. In a few places, the endothelium was disrupted by mononuclear cells and small thrombi, which occluded the duct. Inclusion bodies were seen not only in the nuclei of the endothelial cells but also in endothelial-like cells lying within the thrombi (Fig. 3.11).

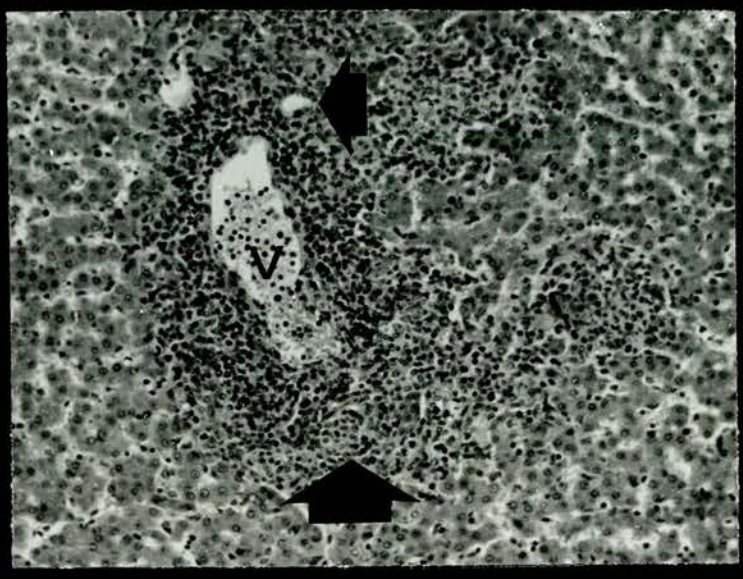
A few foci of necrotic hepatocytes containing intranuclear IB's and mild portal tract infiltration were observed in both lambs killed on day 9 pi.

Figure 3.8

Mononuclear cells infiltrating a portal canal and the surrounding parenchyma. Within the canal are a portal vein (V), a mass of cells replacing a bile duct (large arrow) and a bile duct with a dystrophic epithelium (small arrow). Stained with H & E.
Magnification X 55.

Figure 3.9

Mononuclear cells and cells with kidney-shaped nuclei replacing a secondary bile duct. The central material (arrow) is Stein's positive. Stained by Stein's method.
Magnification X 220.


V

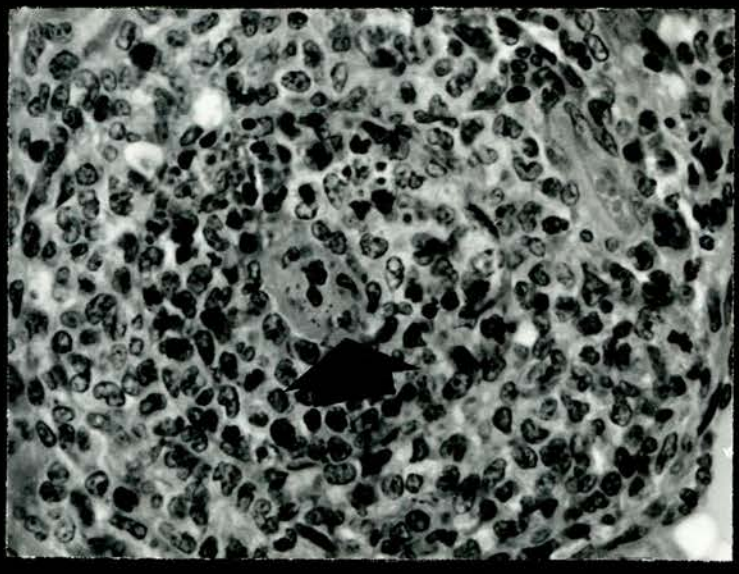
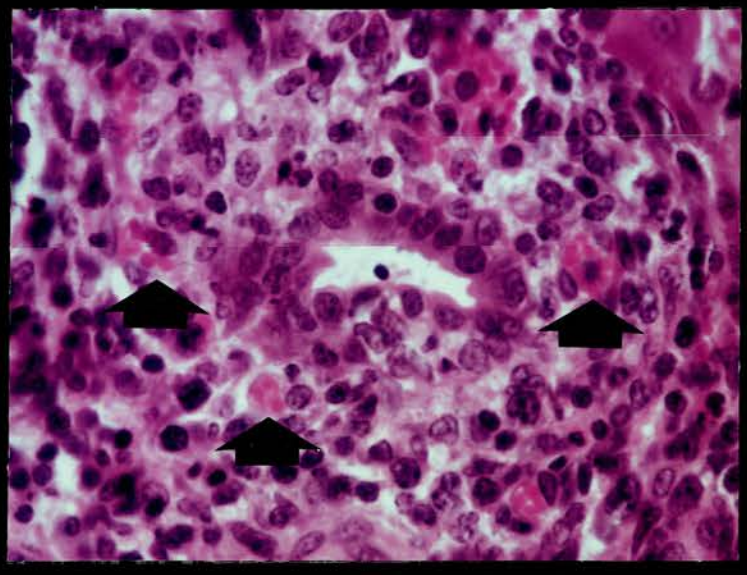


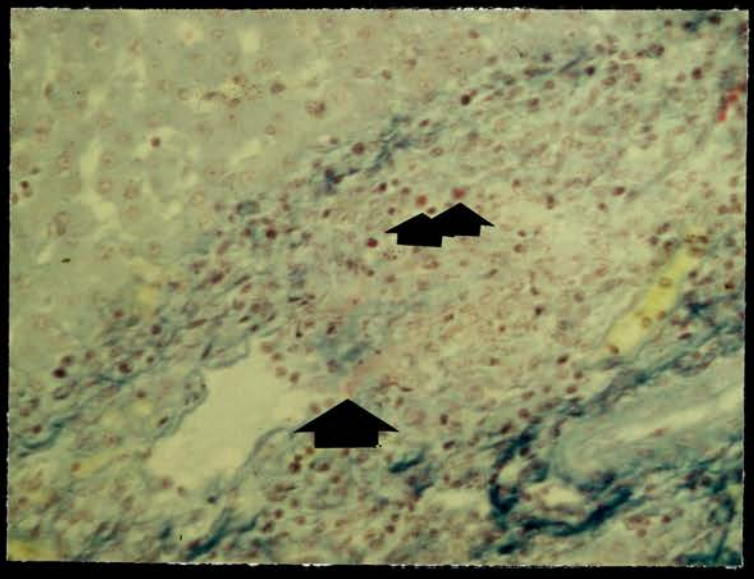
Figure 3.10

A secondary bile duct with a dystrophic epithelial lining that is being shed in one place. Surrounding the duct are mononuclear cells and the peribiliary arterial plexus (arrows), which is distended with red blood cells.
Stained with H & E.
Magnification X 220.

Figure 3.11

A portal tract lymphatic duct occluded by mononuclear cells and strands of fibrin (large arrow). Two cells containing intranuclear inclusion bodies (small arrows) can be seen within the thrombus. Stained with Martius-Scarlet-Blue.
Magnification X 95.





Degenerate hepatocytes from the necrotic areas were examined by electronmicroscopy. In the nuclei of these cells were numerous virions, identified as adenovirus by their hexagonal shape and diameter of 70 - 80 nm (Fig. 3.12).

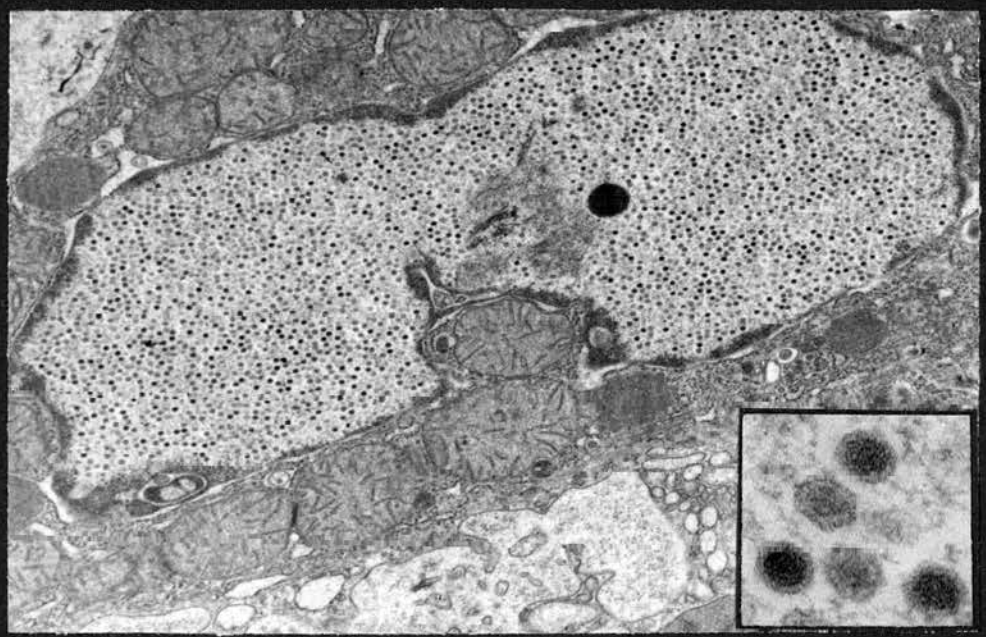
COMMENT

In view of the prolonged excretion of virus in nasal and rectal swabs, and the isolation of virus from tonsils up to 14 days pi, it seems that OA4 virus has replicated in the SPF lambs. The infection was associated with a mild clinical disease, detectable by ausculation only, accompanied by lesions in the lung and liver. The focal necrotic hepatitis and lymphangitis seen in the livers of infected lambs were almost certainly caused by OA4 virus as intranuclear inclusion bodies and adenovirus particles were seen in cells in these lesions. The role of the virus in the development of cholangitis and pulmonary oedema is less certain because OA4 virus was not isolated from these tissues nor were inclusion bodies detected.

Clearly, further work was necessary to determine more fully the pattern of virus replication and dissemination following inoculation of lambs with OA4 virus and to ascertain the involvement of OA4 virus in the development of the lung and liver lesions.

Figure 3.12

Electron micrograph of a section of an hepatocyte (magnification X 15,000). The nucleus contains many hexagonal, electron-dense particles measuring 70 - 80 nm in diameter (insert, magnification X 120,000).



EXPERIMENT 2.

Experiment 1 has shown that infection of SPF lambs with OA4 virus resulted in focal necrotic hepatitis, lymphatic thrombosis and possibly, occlusive cholangitis.

Although the main theme of this thesis was concerned with the role of viruses in the aetiology of respiratory disease, the presence of lesions in the livers of lambs inoculated with an aerosol of AO4 virus was an interesting feature that merited further investigation. An experiment therefore was designed to confirm whether OA4 virus was involved in the development of these lesions, particularly cholangitis, and to examine viral replication and dissemination following intravenous inoculation.

DESIGN OF EXPERIMENT.

At 10 weeks of age, 2 SPF lambs were inoculated intravenously with 1·5 ml of OA4 virus (titre = $10^{4\cdot2}TCID_{50}$/ml) at the 7th passage level in sheep cells (STh6 FLKI). Nasal and rectal swabs were taken at daily intervals during the first 9 days pi, and blood samples were collected in heparin on each of the first 3 days pi. Liver biopsies were obtained on days 7 and 14 pi by the method described by Dick (1944, 1952). On day 21 pi, both lambs were killed by intravenous inoculation of pentobarbitone and portions of liver taken for virological and histopathological examinations.

RESULTS.

Clinical Observations.

Following inoculation with OA4 virus, neither lamb developed clinical disease.

Virus Isolation.

OA4 virus was not detected in nasal or rectal swabs, nor in the heparinised blood samples. Virus was not isolated from the liver biopsy material nor from samples of liver collected at necropsy.

Serology.

When an extract of cells infected with OA4 virus was used as antigen in the gel diffusion test, precipitating antibodies were first detected, in the serum of one lamb on day 17 pi. When an extract of cells infected with HA2 virus was used as antigen, precipitating antibodies were not detected in this lamb until day 21 pi, and even then the line of precipitation was faint. However, precipitating antibodies were not detected in the second lamb.

Pathology.

a) biopsy material:- no macroscopic abnormalities were seen in the liver biopsy samples taken from each lamb at 7 and 14 days pi.

b) necropsy samples:- in one lamb, a small area (5 cm in diameter) on the diaphragmatic curvature of the liver contained small pale-grey foci. Microscopically, these were seen to consist of necrotic hepatocytes, similar to

those described in Experiment 1. There was no evidence of the cholangitis or lymphatic thrombosis described in Experiment 1. No microscopic or macroscopic abnormalities, other than the biopsy scars, were seen in the liver of the other lamb. Inclusion bodies were not detected in any of the biopsy or necropsy samples.

COMMENT.

The results of Experiment 1 indicated that the most likely manner by which OA4 virus reached the liver was a viraemia, which was simulated in Experiment 2 by intravenous inoculation of virus. Following this route of challenge, OA4 virus was not isolated from nasal or rectal swabs, indicating that virus had not spread from the blood to the mucosal surfaces. However, replication of virus was confirmed because precipitating antibodies were detected in sera taken at necropsy and lesions were present in the liver of one lamb. These lesions were areas of necrotic hepatitis and their presence supports the view that the hepatitis was caused by OA4 virus.

The failure to detect IB's or lesions in the biopsy material can be attributed to the fact that the small area of affected liver was not included in the biopsy samples.

EXPERIMENT 3

The results of Experiment 1 showed that OA4 virus was present in the respiratory and alimentary tracts of experimentally infected lambs up to 12 days pi. Although the virus appeared to produce pulmonary oedema in 4 of the 7 infected lambs it was isolated from the lungs of only one of the lambs, and then at low titre.

The present experiment was designed to obtain further information about viral replication and dissemination and, if possible, to confirm that the development of pulmonary oedema was associated with the infecting agent. The persistence of OA4 virus in infected lambs was examined also.

DESIGN OF EXPERIMENT.

When they were 14 days old, 6 SPF lambs were exposed to an aerosol of uninfected TC fluid and 10 lambs to an aerosol of OA4 virus at its 6th passage level in STh cells. The apparatus used to generate the aerosol was modified so that 4 lambs could be exposed simultaneously and the relative humidity of the aerosol could be controlled (Figs. 3.13 a & b). Eight litres/min. of aerosol were mixed with 50 litres/min. of moist air in order to maintain relative humidity above 90 per cent. to preserve viral infectivity (Miller and Artenstein, 1967). The aerosol was passed into a 300 litre exposure box with four 16 cm diameter holes in its sides. Each lamb's head was passed

Figure 3.13 a & b.

Diagram and photograph of the apparatus used to generate aerosols in Experiment 3.

P pump

B air-bleed

FM flow meter

WR water reservoir

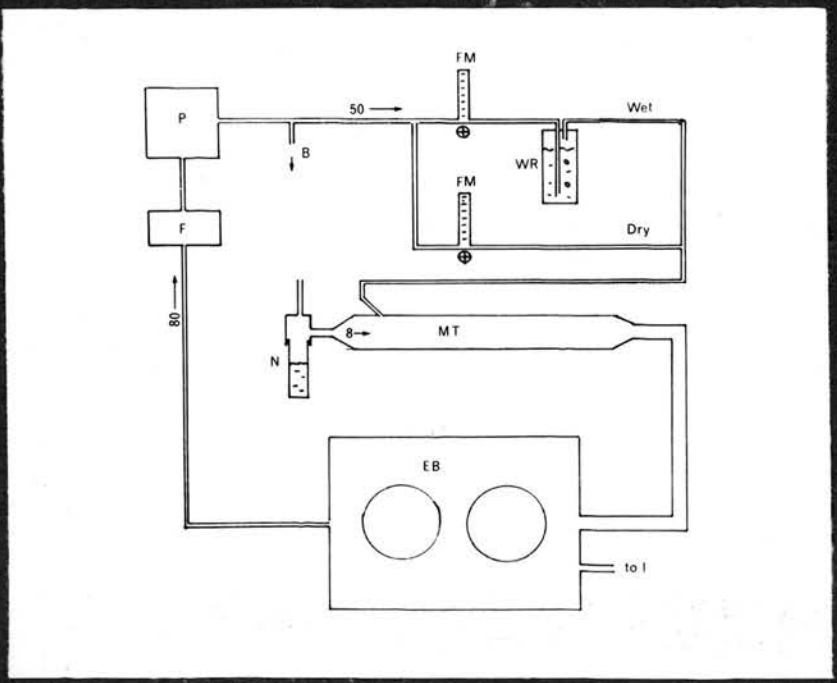
N nebuliser

MT mixing tube

EB exposure box

F filter.

Numbers indicate the flow rate of gases in litres/min.


FM
P
50
Wet
B
WR
FM
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Dry
80
8
MT
N
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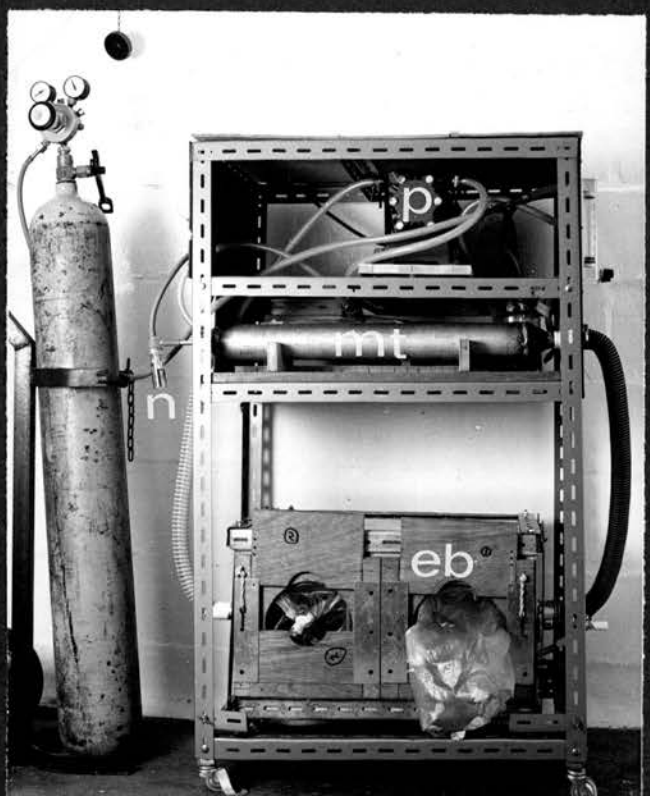
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through a hole, and the aperture sealed by taping a polythene sleeve round the lambs neck. Aerosol was extracted from the box at 80 litres/min. to reduce further any leakage. The lambs were exposed for 30 minutes and it was estimated that each could have inhaled $10^{4 \cdot 6} TCID_{50}$ of OA4 virus.

The experiment was divided into 2 parts (Table 3.4). In the first, following infection by aerosol, clinical observations, rectal temperature, and nasal and rectal swabs were taken daily for 11 days pi. Also, 7 infected and 4 control lambs were killed serially and the tissues removed and examined as described previously.

In the second part of the experiment, blood and nasal secretions were obtained from the remaining 3 infected and 2 control lambs on days 3,5,8,12,17 and 25 pi, and blood only on days 20 and 31 pi (Table 3.6). These lambs were then maintained in isolation until 80 days pi when they were treated with corticosteroid to stimulate recrudescence of viral excretion. Each lamb was inoculated intravenously for 7 consecutive days with one mg dexamethasone* and swabs were collected as before.

Lambs were killed by intravenous pentobarbitone and exsanguinated by severing the axillary vessels. The lungs of those lambs killed on days 1 - 3 pi and following dexamethasone treatment, were allowed to deflate before removal from the thorax. Tissues for microbiological examination were taken using separate sterile instruments and those required for histopathological examination were

* Dexadreson, Intervet.

TABLE 3.4

DESIGN OF EXPERIMENT 3

Treatment	Day post-inoculation	Number of lambs killed	
		Infected group	Control group
Aerosol of OA4 virus or uninfected TC fluid	0		
	1	1	1
	2	1	
	3	1	1
	7	2	1
	11	2	1
Start treatment with dexamethasone	80		
	81	1*	
	90	2	2

* Died

fixed in formol-saline. The procedure differed slightly with lambs killed on days 7 and 11 pi. The tracheas of these lambs were clamped and the inflated lungs removed from the thorax within 20 mins. of death. After the left primary bronchus had been clamped, tissues from the left lobes were taken for microbiological examination and representative portions fixed in formol-saline. The right lung was fixed in the inflated state by intra-bronchial perfusion with 3 per cent. glutaraldehyde (pH 7·5) before portions were taken for histopathological examination.

RESULTS.

Clinical Observations.

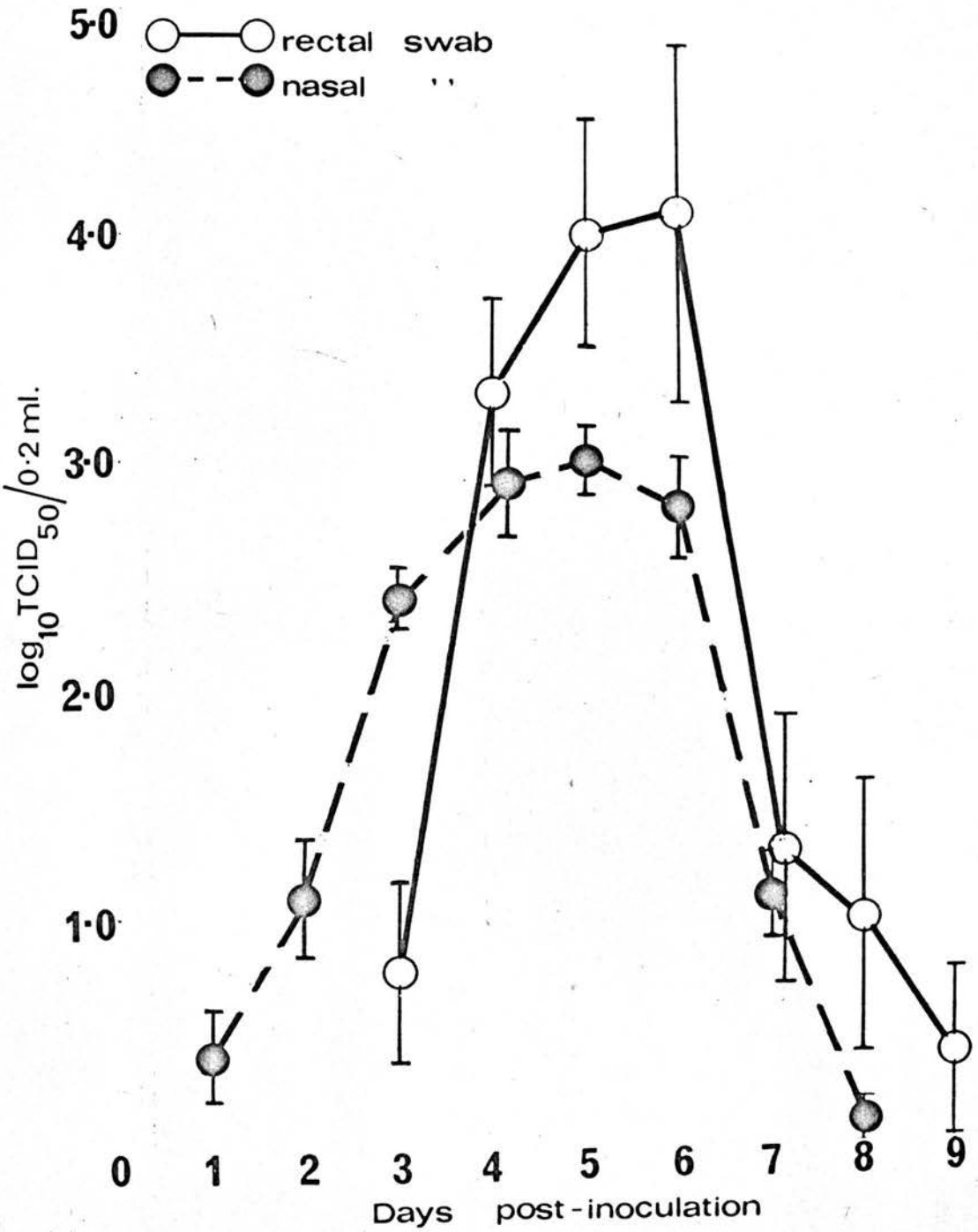
Clinical disease was not observed in infected or control lambs, with one exception. One lamb, infected 80 days previously, appeared slightly dull and had a temperature of 105·6^{o}F one day after the start of dexamethasone treatment and was found dead the following morning.

Virus Isolation.

Isolations of OA4 virus from nasal and rectal swabs are presented in Fig. 3.14. As in Experiment 1, most of the isolates produced a CPE during the first passage in primary STh monolayers. Virus was obtained from nasal swabs from each lamb between days 1 - 8 pi and the viral titres increased daily from day one pi to days 4 - 6 pi and then declined rapidly. OA4 virus was recovered from rectal

Figure 3.14

Isolation of OA4 virus from nasal and rectal swabs. Mean virus titre ($\pm$ SE).


5·0
4·0
3·0
2·0
1·0
rectal swab
nasal ''
log10TCID50/0·2 ml.
0 1 2 3 4 5 6 7 8 9
Days post-inoculation

swabs from each lamb between days 3 and 9 pi, with maximum titres on days 5 and 6 pi. Virus was not isolated from peripheral blood leucocyte preparations during the first 5 days pi.

Isolations of OA4 virus from tissues taken at necropsy are presented in Table 3.5. Virus was isolated from the upper and lower respiratory tract of all infected lambs killed up to 11 days pi, but was found in the alimentary tract in only 3 lambs, which were killed 7 and 11 days pi.

It was also recovered from the tonsils of 5 of the 7 lambs killed up to 11 days pi and the mesenteric lymph nodes of both lambs killed on day 7 pi. OA4 virus was isolated on one further occasion, namely, from a rectal swab from one infected lamb, 80 days after inoculation, and immediately before injection with dexamethasone.

Following the administration of dexamethasone, OA4 virus was not recovered from nasal and rectal swabs, or tissues taken at necropsy from lambs which had been infected more than 80 days previously and had excreted virus at that time.

Cytopathic agents were not isolated from nasal and rectal swabs, or tissues from control lambs. Mycoplasmas were not cultured from any of the tissues, but Escherichia coli was grown from several tissues of the lamb which died.

Immunology.

Three infected and 2 control lambs were examined for antibody to OA4 virus. Antibodies were not detected in undiluted sera or nasal secretions from control lambs but,

TABLE 3.5

RECOVERY OF OA4 VIRUS FROM TISSUES TAKEN AT NECROPSY FROM EXPERIMENTALLY INFECTED LAMBS

Tissue examined	Virus Recovery ($log_{10}TCID_{50}/g$) from tissues of 7 lambs killed after (days)						
	1	2	3	7	7	11	11
Nasal mucosa	-	+	-	+	+	+	-
Oropharynx	-	+	-	NS	NS	NS	NS
Tonsil	-	+	-	+	+	+	+
Retropharyngeal lymph node	-	-	-	NS	NS	NS	NS
Tracheal mucosa	3·6	+	+	NS	NS	NS	NS
Left apical lung	3·2	+	2·3	-	-	+	+
Left cardiac lung	4·6	1·8	+	-	+	+	-
Left diaphragmatic lung	-	+	2·5	-	+	-	-
Pulmonary lymph node	-	-	-	-	-	-	-
Jejunum	-	-	-	+	-	+	-
Colon	-	-	-	+	4·2	+	-
Mesenteric lymph node	-	-	-	+	+	-	-
Spleen	-	-	-	NS	NS	NS	NS

\+ = OA4 virus recovered in low concentration after prolonged incubation.

\- = OA4 virus not recovered.

NS = Not sampled.

at 8 days pi, neutralizing antibody was evident in both serum and nasal secretions from each infected lamb. Mean serum antibody titres increased from 10 to 708 by 90 days pi and mean nasal antibody titres rose from 14 to 20 at 12 days pi then fell to 14 at 25 days pi (Table 3.6). Serum precipitating antibodies were not detected at 20 days pi but were present at 31 days pi when OA4 antigen was used. At 90 days pi, precipitating antibody was detected using both OA4 antigen and HA2 antigen.

Post-mortem observations.

Macroscopic abnormalities were confined to the lungs of those lambs killed on days 3 - 11 pi and were essentially the same as those described in Experiment 1. The changes consisted of a variable degree of purple discolouration and oedema, as judged by the moist appearance of the lungs and expression of fluid from the cut surfaces of the lung parenchyma. The lung of the lamb killed 3 days pi also had areas of consolidation in the right apical and cardiac lobes and a discrete firm lesion (3 cm in diameter) which appeared mottled grey on its cut surface and was slightly raised above the dorsum of the right diaphragmatic lobe. The lungs of 3 of the 4 lambs killed on days 7 and 11 pi appeared oedematous and discoloured. In 2 lambs the subpleural lymphatics were dilated and copious frothy exudate was present in the trachea and smaller airways. At the posterior pole of each diaphragmatic lobe of one of these lambs was an extensive area of tissue which was markedly congested and sharply demarcated from the other lung tissue.

TABLE 3.6

NEUTRALIZING ANTIBODY TITRES TO OA4 VIRUS IN NASAL SECRETIONS AND SERA OF INFECTED LAMBS

	Lamb	Days post-inoculation									
		Pre-inoculation	3	5	8	12	17	20	25	31	90
Nasal Antibody	23	-	-	-	22	22	ND	ND	ND	ND	ND
	24	-	-	-	11	11	16	ND	32	ND	ND
	32	-	-	-	11	32	8	ND	6	ND	ND
Geometric Mean Titre		-	-	-	14	20	11	ND	14	ND	ND
Serum Antibody	23	-	-	-	6	89	45	128	128	256	708
	24	-	-	-	11	64	45	64	128	256	708
	32	-	-	-	16	89	128	128	64	128	ND
Geometric Mean Titre		-	-	-	10	80	64	102	102	203	708

Titres expressed as the reciprocal of the serum dilution.

\- = no antibodies detected.

ND = not done.

No macroscopic changes were observed in the organs of control lambs, of infected lambs killed on days 1 and 2 pi, or of infected lambs treated with dexamethasone, with the exception of the lamb which died. The pleural cavity and pericardial sac of this lamb contained an excessive amount of straw-coloured fluid, tinged with blood. The lungs were of a dark purple colour and were congested.

Histopathology.

Upper Respiratory Tract.

There were no microscopic abnormalities in the nasal turbinates or trachea of control or infected lambs and inclusion bodies could not be detected.

Lung.

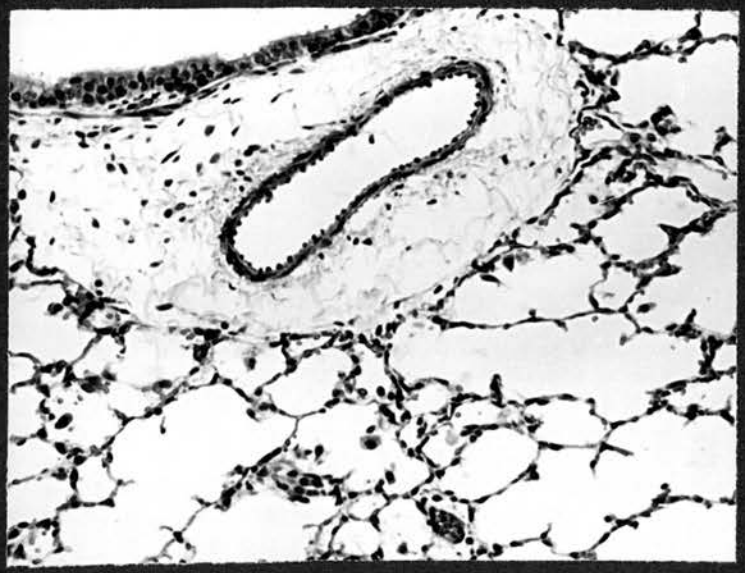
Microscopic lesions were seen only in the lungs from the 4 infected lambs which showed visible evidence of oedema. The most prominent lesion was a perivascular accumulation of fluid, which stained faintly eosinophilic (Figs. 3.15 & 3.16) and a similar change was present around the bronchioles of one lamb killed 7 days pi. In 2 lambs killed 7 and 11 days pi, there was intra-alveolar and intra-bronchiolar fluid and a few free mononuclear cells (Fig. 3.17 & 3.18). These changes were present in both perfused and non-perfused lobes, but perivascular oedema was more obvious in the former. In 4 lambs, there were small accumulations of mononuclear cells around bronchioles and occasionally small blood vessels. In the lamb killed 3 days pi the areas of consolidation were composed of alveoli filled with exudate containing mononuclear and polymorphonuclear cells (Fig. 3.19).

Figure 3.15

Area from glutaraldehyde-perfused lung of an infected lamb, killed 7 days pi, showing perivascular oedema and a few mononuclear cells within alveoli. Stained with H & E. Magnification X 95.

Figure 3.16

Area from formalin-fixed, unperfused lung from the same lamb as in Fig. 3.15, showing extensive perivascular oedema. Stained with H & E. Magnification X 95.



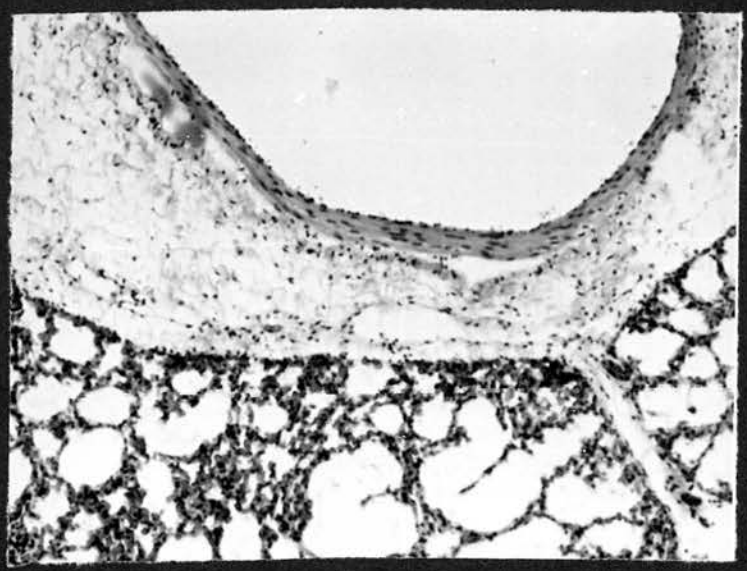
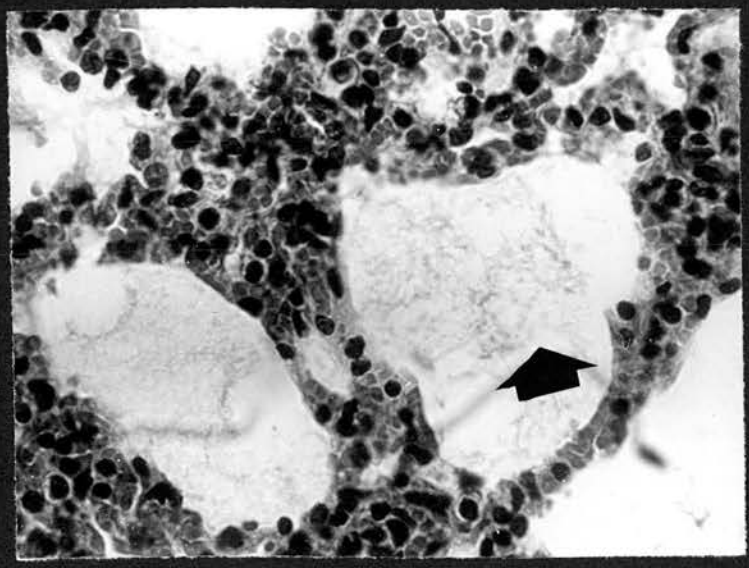


Figure 3.17

Alveoli in the unperfused lung of an infected lamb killed 7 days pi. The alveolar walls appear thickened due to infiltration by mononuclear cells and to the lungs collapsing after removal from the thorax. Within the alveoli is a faintly eosinophilic staining oedema fluid (arrow). Stained with H & E. Magnification X 250.

Figure 3.18

Bronchiole in the unperfused lung of an infected lamb killed 11 days pi. Oedema fluid (arrow) appears to be occluding the lumen, but there is no bronchiolitis. Stained with H & E. Magnification X 65.



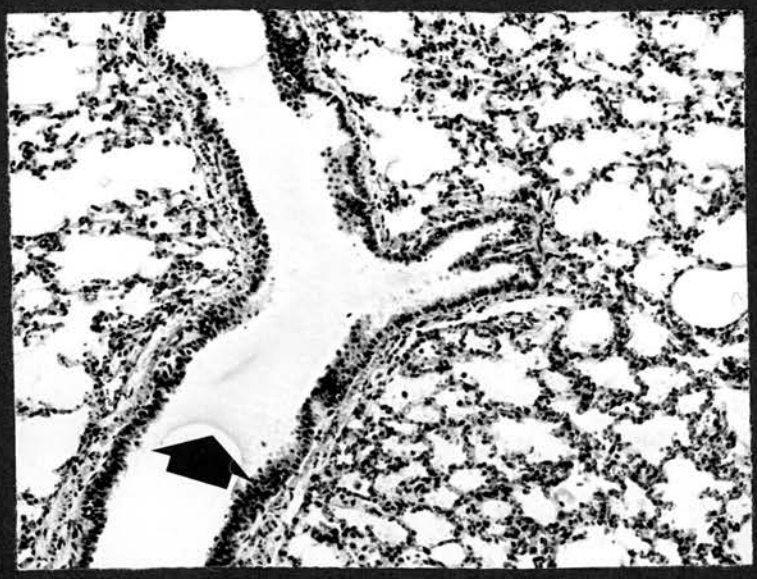
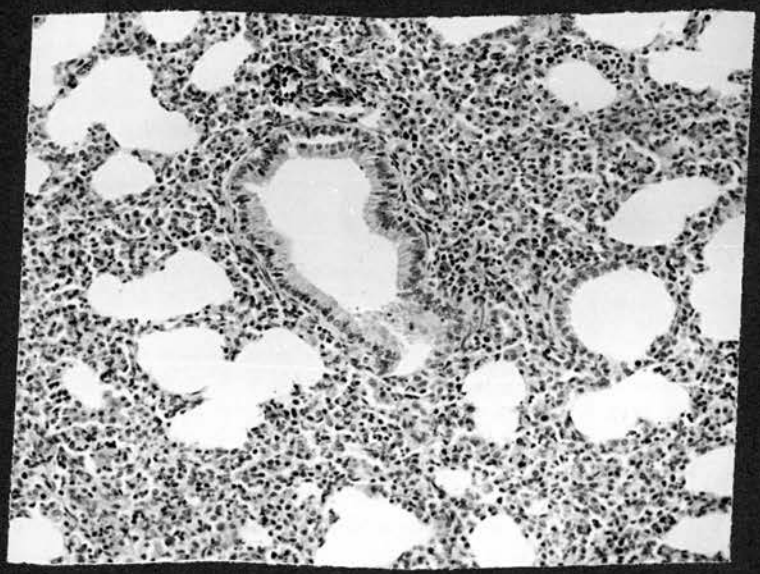


Figure 3.19

Area of consolidation in lungs of a lamb killed 3 days pi. The alveoli contain mononuclear and polymorphonuclear cells, but there is no bronchiolitis. Some interalveolar septae are thickened by an infiltration of mononuclear cells. Stained with H & E. Magnification X 65.



There was no evidence, in any lamb, of an alteration in the integrity of the bronchiolar epithelium, nor were intranuclear inclusion bodies, which are generally present in adenovirus infections, detected in sections stained by H & E or Pollack's trichrome.

No lesions were detected in the lungs of control lambs or those lambs treated with dexamethasone. The lungs from the lamb which died showed severe autolytic changes and were very congested. Many blood vessels contained small numbers of polymorphonuclear leukocytes, but there were no bacterial emboli or foci of necrosis.

COMMENT.

As in Experiment 1, there was prolonged excretion of OA4 virus in nasal and rectal swabs, as well as in tissues taken at necropsy. The titres of virus in swabs confirmed that replication had occurred and peak titres were attained just before nasal and serum antibodies were detected. Infected lambs showed no overt clinical disease, but pulmonary oedema was present in about half of them. Recovery of OA4 virus from one lamb, 80 days pi, indicated that a persistent infection could occur, but recrudescence of virus excretion was not stimulated by administration of corticosteroid to infected lambs.

DISCUSSION.

The sequence of virus recovery from nasal and rectal swabs in Experiments 1 and 3, and necropsy tissues in Experiment 3 indicated that OA4 virus replicated first in the respiratory tract. Thereafter the virus, which is stable at pH 2·7 (Chapter 2), was probably swallowed and infected the alimentary tract. Other forms of virus dissemination may take place in infected lambs but these do not seem to be involved in the spread of OA4 virus from respiratory tract to the alimentary tract because intravenous inoculation of virus did not result in virus excretion in nasal or rectal swabs.

The isolation of OA4 virus from the tonsils and mesenteric lymph nodes of infected lambs, and the presence of adenovirus particles and an associated hepatitis in the liver of a lamb infected by aerosol suggest that the virus might be disseminated via the cardiovascular or lymphatic system. This possibility is supported by the finding of hepatitis in a lamb, which was inoculated intravenously with OA4 virus, although virus was not isolated from heparinised blood samples. Furthermore, the systemic spread of adenoviruses after experimental inoculation of the respiratory tract has also been reported in dogs, cattle, pigs and mice. For example, following inoculation of dogs with canine adenovirus type 1, virus was phagocytosed in the pharynx by macrophages and carried in the macrophages through the tonsils to the blood by which it was transported

to the liver (Wright, 1973). Adenovirus also has been isolated from the livers of calves which had been inoculated intratracheally with a strain of bovine adenovirus (Cole, 1971) and from the kidneys and spleens of mice inoculated intranasally with a mouse adenovirus (Van der Veen and Mes, 1973). Other workers have described intranuclear inclusions in the kidneys and thyroids of germ-free or SPF piglets inoculated intranasally with porcine adenovirus type 4 (Shadduck, Koestner and Kasza, 1967). The dissemination of OA4 virus could be more precisely determined in further experiments by means of an immunofluorescent test, designed to detect viral antigens in the tissues of infected lambs.

Although OA4 virus replicated well in the respiratory tract, it was isolated originally from rectal swabs (Chapter 1) and two features of the experimental infection suggest that it is primarily an enteric virus. It replicated more readily and attained higher titres in the alimentary tract, and also appeared to persist in this site, because it was isolated from a rectal swab from one lamb 80 days pi.

Persistence of infection is a feature of adenoviruses in many species, but the reported duration and sites of infection vary with each host. The isolations of bovine adenovirus type 3 from the jejunum of a calf after 3 months (Darbyshire, Jennings, Dawson, Lamont and Omar, 1966) and porcine adenovirus type 2 from the contents of the colon of pigs after 50 days (Sharpe and Jesset, 1967) are similar to the results obtained with OA4 virus. On the other

hand, murine adenovirus can be isolated from the urine and kidneys of mice up to 2 years after inoculation (Ginder, 1964; van der Veen and Mes, 1973). The means by which adenoviruses can persist in animals has been discussed by Pereira (1972), who concluded that there were most likely low levels of infection which were asynchronous, and that the spread of virus was inhibited by antibody or other factors.

In both Experiments 1 and 3, the interval between inoculation of the tissue suspensions and the appearance of CPE in cultures was prolonged for a variable time with suspensions prepared from the tissues of lambs killed on or after day 7 pi. It is known that both serum and local antibody can interfere with the recovery of virus from infected hosts (Pereira, 1972; van der Veen and Mes, 1973; Zalan, Pukitis, Rhodes and Labzoffsky, 1973). In Experiment 3, both nasal secretion and serum antibody were detectable by day 8 pi when viral titres in swabs were decreasing, and it seems probable that they combined to influence adversely the recovery of OA4 virus from tissues taken at necropsy.

The failure to detect antibody in the sera of infected lambs in Experiment 1 cannot be explained adequately. The results of Experiment 3 suggest that there must have been an immunological response to OA4 virus. There may have been antibody production at the mucosal surface only, without any serological response, but the presence of virus particles in the liver of infected lambs tends to go against this hypothesis.

In Experiment 3, neutralizing antibodies to OA4 virus were first detected in the serum on day 8 pi and the geometric mean titre rose to 203 by day 31 pi. The appearance of precipitating antibodies was much later and their detection was influenced by the choice of antigen in the test. Homologous OA4 antigen detected precipitating antibodies first on day 31 pi, whereas antibodies could not be detected at this time to the group specific antigen present in HA2 virus. A similar pattern developed in Experiment 2. Precipitating antibody was detected at 17 days pi to homologous OA4 antigen, but not until day 31 pi, and then only faintly, to HA2 antigen. These results agree with those of Darbyshire et al (1966) who found that precipitating antibodies to the group specific antigen did not appear until the fourth week after inoculation of calves with bovine adenovirus type 3. From these findings, it can be seen that there is a more rapid response to the type specific antigens than to the group specific antigens, and the results reinforce the view expressed in Chapter 2 that the neutralization test is a more sensitive procedure, both for screening sera and as a diagnostic test.

Adenoviruses have been shown experimentally to be pathogenic in most domestic species. They produce hepatitis in dogs, pigs, cattle, horses and chickens (Shadduck et al, 1967; Phillip and Darbyshire, 1972; Aghakhan, 1974; McChesney, England, Whiteman, Adcock, Rick and Chow, 1974; Thompson, Wright and Cornwell, 1975), and a haemorrhagic

disease in calves known as 'weak calf syndrome' (Cutlip and McClurkin, 1975). The reported involvement of adenoviruses in any enteric disease must await confirmation in view of the recent work with rotaviruses.

Lambs infected with OA4 virus did not develop overt clinical disease, but lesions were present in their lungs and livers. In contrast, lambs inoculated intratracheally with either bovine adenovirus type 2 or a Hungarian strain of ovine adenovirus type 1 developed respiratory and enteric disease (Belak and Palfi, 1974b; Belak, personal communication).

The lesions found in the lungs of infected lambs were pulmonary oedema and peribronchiolar accumulation of mononuclear cells. There was no evidence of necrosis of bronchiolar epithelium, which is a feature of adenovirus infections of the respiratory tract in pigs, dogs, cattle and Arabian foals (Betts, Jennings, Lamond and Page, 1962; Darbyshire et al, 1966; McChesney et al, 1974; Thompson et al, 1975). Pulmonary oedema was observed both macroscopically and microscopically, in 8 of 14 infected lambs killed up to 11 days pi in Experiments 1 and 3, but not in 6 control lambs killed at the same time ($p = 0{\cdot}024$). It appears, therefore, that there is a statistically significant relationship between the presence of pulmonary oedema and infection with OA4 virus. The true significance of these findings must be assessed cautiously as it has been shown that fluid can accumulate in the lung after death (Staub, 1974), but there are several other points to support

this relationship. All lambs were exsanguinated and the lungs removed from the thorax shortly after death, procedures which reduced the post-mortem development of pulmonary oedema. In Experiment 1, four lambs were assessed by auscultation as having pulmonary lesions and these same four lambs were found at necropsy to have pulmonary oedema. Lastly, although inclusion bodies were not detected in the lungs in either experiment, virus was isolated from the lungs of infected lambs in Experiment 3. For these reasons, it would appear that the pulmonary oedema was associated with infection with OA4 virus.

The way in which the oedema was initiated remains unknown, but the mechanisms seem to be transient because oedema was not observed in lambs killed 90 days pi. However, the presence of oedema may be associated with circumstances operating at this time, such as the stage of the infection or the emergence of the immune response and its interaction with the virus.

Although interstitial pneumonia has been described in other species infected with adenoviruses, this lesion was seen in only 4 of 16 infected lambs killed up to 11 days pi in Experiments 1 and 3, which is not significantly different from the control group.

There were 3 types of lesion in the livers of lambs infected with OA4 virus. Three lambs infected by aerosol, and one by intravenous inoculation, had hepatitis which consisted of small foci of necrosis. One of the 3 lambs also had thrombotic lymphangitis and occlusive cholangitis.

The necrosis and lymphangitis appear to have been caused by OA4 virus as many necrotic hepatocytes and lymphatic endothelial cells contained intranuclear inclusion bodies, which were shown, in hepatocytes, to contain adenovirus particles. The involvement of OA4 virus in the development of the cholangitis is less clear. Occlusive cholangitis was seen in one lamb and slight mononuclear cell infiltration of portal tracts in 2 of 7 lambs, in Experiment 1, infected with an aerosol of OA4 virus. Only one inclusion body was identified in an epithelial cell in a bile duct and cholangitis did not develop after intravenous inoculation of lambs with OA4 virus. However, examination of more than 120 SPF and 30 conventional lambs reared at the Moredun Institute, infected with a variety of ovine respiratory pathogens, did not reveal any abnormalities in their bile ducts (Rushton, personal communication). Therefore, the possibility remains that OA4 virus may have induced the cholangitis.

Hepatitis is a well recognised sequel to infection of dogs with canine adenovirus type 1 (Wright, 1973) and of chickens with inclusion body hepatitis virus (Fadly and Winterfield, 1975). The focal hepatic necrosis induced in lambs by OA4 virus resembles that produced in these 2 species, but infective virus could not be isolated. The failure to recover virus from the lesions could be due to the presence of specific antibodies. However, antibody was not detected in the sera of lambs in this experiment, but, for reasons previously discussed, this possibility

cannot be dismissed. Anternatively, an abortive infection with the formation of inclusion bodies and the assembly of incomplete virus particles may have occurred. There are precedents for abortive infections by adenoviruses, both *in vitro* and *in vivo*. Carmichael (1965) described the production of viral antigens and inclusions, but not infective virus, in canine cell cultures as well as in the lymph nodes of dogs inoculated with human adenovirus type 4. Postlethwaite (1973) reported a fatal hepatitis in mice inoculated intravenously with high infectivities of human adenovirus type 5, and it was the opinion of Mautner and Willcox (1974) that incomplete virus replication occurred. Both these reports describe adenoviruses in heterologous culture systems, but there are no reports of abortive infections in homologous systems.

There does not seem to be any report of an occlusive cholangitis developing during adenovirus infections of other species. However, a small amount of evidence indicates that adenoviruses may replicate in the biliary tract and induce lesions. Kawamura and Horuichi (1964) isolated virus from the bile and saw intranuclear inclusions in the epithelial cells of the intralobular bile tubule of a chicken infected with the Ote strain of CELO virus. Other workers have reported bile duct hyperplasia (Cook, 1974) and cellular infiltration of the portal areas of chickens infected with adenovirus (Gallina, Winterfield and Fadly, 1973), while Tury, Belak and Palfi (1975) described proliferation of the bile duct epithelium in lambs infected

with bovine adenovirus type 2. The occlusive cholangitis associated with OA4 virus can be compared with 2 others, one in primary biliary cirrhosis in man (Rubin, Schnaffner and Popper, 1965) and the other in fascioliasis in sheep (Rushton, 1974). In each condition, intermediate-sized ducts undergo a process which starts as a pericholangitis and proceeds to complete destruction of the duct and its replacement by mononuclear cells.

The presence of neutralizing antibodies to OA4 virus in a high proportion of sheep indicates that many sheep have been infected with the virus (Chapter 2). The experiments in this chapter have cast some light upon the pathogenesis of OA4 virus, but the significance of the lesions is, as yet, not known. However, it is possible that these lesions may predispose the sheep to other more serious infections, which may have occurred when OA4 virus was isolated originally from sheep dying from enzootic pneumonia (Chapter 1).

CHAPTER 4

THE PATHOGENICITY FOR SPECIFIC PATHOGEN-FREE LAMBS, OF OVINE ADENOVIRUS TYPE 4 OR PARAINFLUENZA VIRUS TYPE 3 IN COMBINATION WITH PASTEURELLA HAEMOLYTICA.

The aetiology of respiratory disease is complex, and disease is generally considered to be the result of the interactions among organisms, host and environment. This hypothesis has arisen partly as a result of the inconsistent results obtained following inoculation of animals with single types of organism, and the repeated failure to induce, by experimental inoculation, respiratory disease as severe as that observed in the field. For example, Pasteurella haemolytica is commonly associated with respiratory disease in sheep, but attempts to produce illness with this organism have met with variable success (Smith, 1964; Gilmour, Thompson, Smith and Angus, 1975). Similarly, PI3 and OA4 viruses have been associated with ovine respiratory disease. Experimental infections with PI3 virus produce a transient mild disease (see Review of Literature) whereas OA4 virus produces subclinical pneumonia and hepatitis (Chapter 3). It should be recalled, however, that OA4 virus was isolated originally from lambs during an outbreak of enzootic pneumonia, and that both PI3 virus and P. haemolytica can be isolated from the same sheep with respiratory disease. It was decided, therefore, to ascertain whether OA4 virus or PI3 virus could influence the response of SPF lambs to a superimposed infection with P. haemolytica.

MATERIALS AND METHODS

The preparation of cell cultures, and serological tests for the detection of antibodies have been described previously (General Materials and Methods).

Experiments.

This chapter comprises the results of four experiments. The first experiment was designed to examine the effect of OA4 virus on a superimposed infection of P.haemolytica and the remaining three experiments were to investigate the same effect using PI3 virus.

Bacterial Cultures.

Recently isolated strains of P.haemolytica biotype A, serotype 1 (subsequently referred to as type A1) were grown overnight in nutrient broth No.2. The organisms were concentrated 10-fold by centrifuging the cultures at 12,000g for 30 min at 10°C and resuspending the resultant pellet in PBS.

Necropsy Procedure.

In each experiment, lambs that did not die, nor were sufficiently ill to be killed, were allowed to survive until 7 - 10 days after exposure to the aerosol of P.haemolytica. At this time, they were anaesthetized with intravenous pentobarbitone and exsanguinated by severing the axillary vessels. The lungs were removed aseptically and portions of any lesions were immersed in formol-saline for histological examination. The remaining lung tissue was excised from the trachea and homogenised with 300 ml. of

peptone water in an MSE Atomix blender for 2 minutes. The number of viable P.haemolytica organisms in this homogenate was estimated by the technique of Miles and Misra (1936), and representative colonies were typed serologically (Shreeve, Biberstein and Thompson, 1972).

EXPERIMENT 1

DESIGN OF EXPERIMENT

Twenty-four SPF lambs were divided into 3 groups of 8, which were treated as shown in Table 4.1. At 2 weeks of age, each lamb in group A was exposed for 30 mins. to an aerosol of noninfective tissue culture fluid and those in groups B and C to an aerosol of OA4 virus. It was estimated that each lamb in groups B and C could have inhaled $10^{3 \cdot 4}$ $TCID_{50}$ of virus. Four days later, each lamb in group A, then in group B was exposed for 15 mins. to an aerosol of P.haemolytica and it was estimated that each lamb could have inhaled $10^{6 \cdot 0}$ viable organisms. Lambs in group C were exposed to an aerosol of P.haemolytica 8 days after exposure to the aerosol of OA4 virus. The aerosol of P.haemolytica was produced from a different broth culture to that used at 4 days, and it was found that each lamb could have inhaled only $10^{3 \cdot 3}$ viable organisms. All lambs were examined clinically during the period between receiving the aerosol of OA4 virus and necropsy, and nasal swabs for the isolation of virus were taken daily for the first 6 days after exposure to the aerosol of virus.

TABLE 4.1

PATHOGENICITY OF OA4 VIRUS IN COMBINATION WITH *P.HAEMOLYTICA*

DESIGN OF EXPERIMENT 1

Group		Number of lambs	Age of lambs at time of OA4 aerosol (weeks)	Interval between OA4 aerosol and *P.haemolytica* aerosol (days)	Estimated number of viable *P.haemolytica* inhaled by each lamb (log_{10})
A	TC fluid + *P.haemolytica*	8	4*	4	6·0**
B	OA4 virus + *P.haemolytica*	8	4	4	6·0**
C	OA4 virus + *P.haemolytica*	8	4	8	3·3

* uninfected TC fluid.

** lambs in these groups received *P.haemolytica* aerosol on the same day.

RESULTS

Clinical Observations.

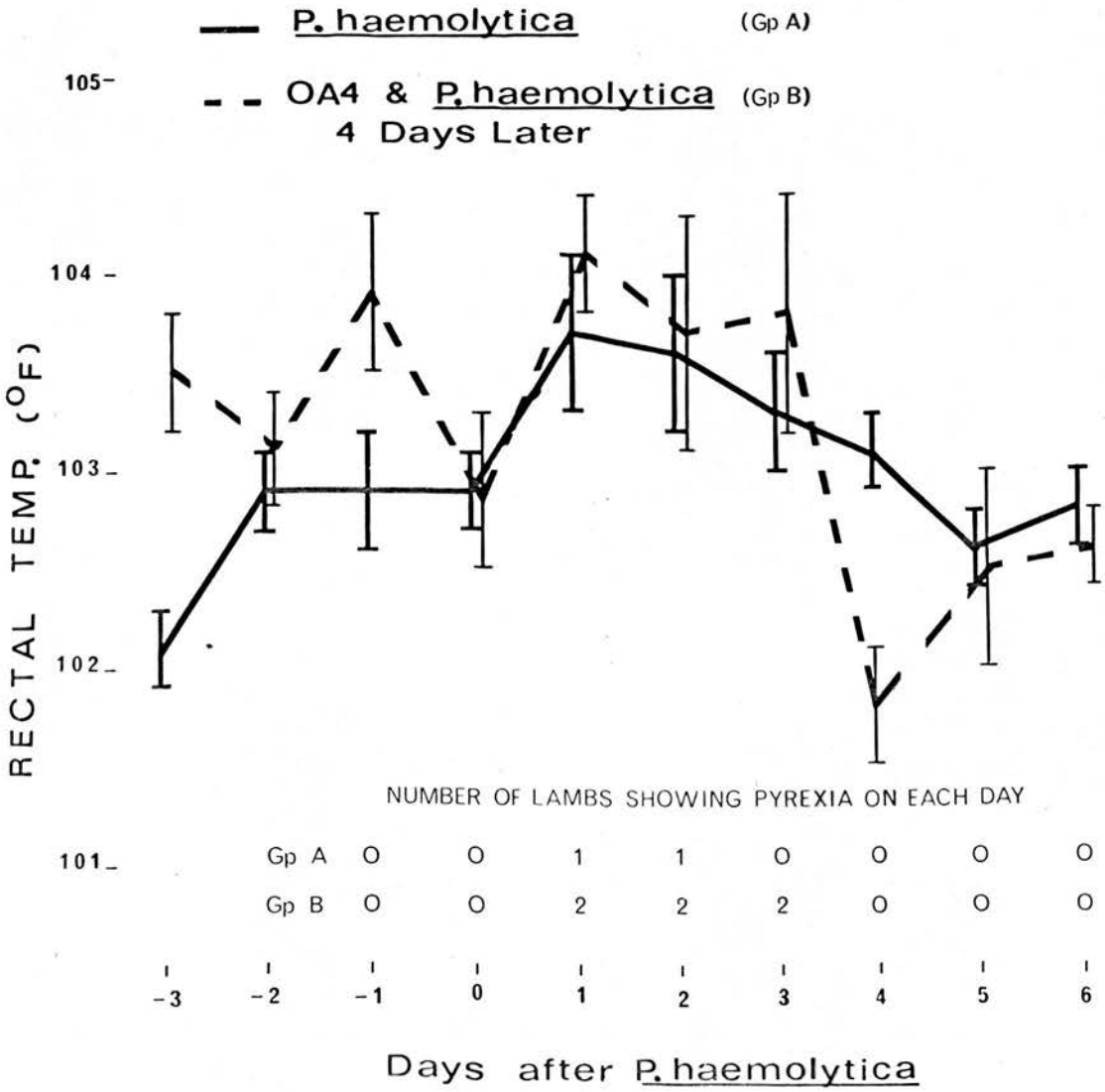
After exposure to an aerosol of OA4 virus only one lamb, in group C, showed overt clinical disease. Five days after exposure to OA4 virus, this lamb was listless and anorexic, with obvious abdominal discomfort. It was found dead the following morning.

Following infection with aerosols of *P.haemolytica*, clinical signs in all 3 groups of lambs were confined largely to those which subsequently died or were killed *in extremis*. In group A, on the day after exposure to the aerosol of *P.haemolytica* one lamb appeared dull but without obvious respiratory signs or pyrexia. The next day it was moribund and died on the way to the post-mortem room. Another lamb had pyrexia (temperature 105°F) for 2 days following the aerosol although the mean rectal temperature for the group did not rise above 103·7 $\pm$ 0·4°F (Fig. 4.1). The remaining lambs in group A did not show any signs of illness. In group B, 2 lambs were dull on the day after infection with *P.haemolytica*. By day 2, these lambs were dyspnoeic and recumbent, and they were killed *in extremis*. Three other lambs had pyrexia of up to 2 days duration in the first 3 days after infection with *P.haemolytica*, but the mean rectal temperature for the group did not exceed 104·1 $\pm$ 0·3°F (Fig. 4.1).

In group C, which received a different aerosol of *P.haemolytica* to groups A and B, one lamb developed pneumonia.

Figure 4.1

Mean rectal temperature ($\pm$ SE) of lambs in groups A and B following exposure to an aerosol of P.haemolytica.


P. haemolytica (Gp A)
OA4 & P. haemolytica (Gp B)
4 Days Later
RECTAL TEMP. (°F)
105
104
103
102
101
NUMBER OF LAMBS SHOWING PYREXIA ON EACH DAY
Gp A 0 0 1 1 0 0 0 0
Gp B 0 0 2 2 2 0 0 0
-3 -2 -1 0 1 2 3 4 5 6
Days after P. haemolytica

On day one after exposure to the aerosol, this lamb had pyrexia. By day 2 it showed pyrexia, depression, tachypnoea (100 respirations/min.), an "abdominal lift" and coughed occasionally. It was killed at this time. Four other lambs developed pyrexia of 1 - 2 days duration in the first 5 days after exposure to P.haemolytica, but the maximum mean rectal temperature for the group was 104·7 $\pm$ 0·3°F on the second day.

Microbiology.

OA4 virus was isolated from nasal swabs from lambs in groups B and C between days one and 6 pi. It was recovered from 6 of 16 lambs on day one pi, 10 on day 2, 11 on day 3 and from all lambs thereafter up to day 6. Virus was recovered at necropsy from the tissues only of those lambs which had died within 6 days of the virus aerosol. It was isolated from the lungs of the 2 lambs in group B which died, and also from the lungs and gut contents of the lamb in group C which died before exposure to the aerosol of P.haemolytica. Virus was not isolated from the lamb in group C which was killed 10 days after the virus aerosol, nor from swabs or tissues from lambs in group A.

The isolation of P.haemolytica from samples taken at necropsy are presented in Table 4.2. P.haemolytica type A1, were cultured from the lungs of 2 lambs in group A, 3 in group B and 4 in group C, and also from the pleural fluid of the 4 lambs which were killed in extremis. They were isolated from nasal swabs from most lambs in each group

TABLE 4.2

ISOLATION OF P.HAEMOLYTICA FROM VARIOUS TISSUES OBTAINED AT NECROPSY FROM SPF LAMBS INFECTED BY AEROSOL

Group	Number of lambs	Lung	Pleural Fluid	Tonsil	Bronchial Lymph Node	Mediastinal Lymph Node	Nasal Swabs
A	8	2	1	7*	1	0	6
B	8	3	2	6*	1	0	6
C	7	4	1	7	2	2	4

* tonsils from only 7 lambs in group A, and 6 in group B were examined.

and invariably from the tonsils. Large numbers of organisms were cultured from the lungs (mean, $10^{10.9} \pm 10^{0.7}$ organisms/lung) and pleural fluid (mean, $10^{9.6} \pm 10^{0.7}$ organisms/ml) of the 4 lambs which were killed in extremis, but they were isolated from the lungs (mean, $10^{7.3} \pm 10^{1.9}$ organisms/lung) of only 5 of 19 surviving lambs.

Pathology.

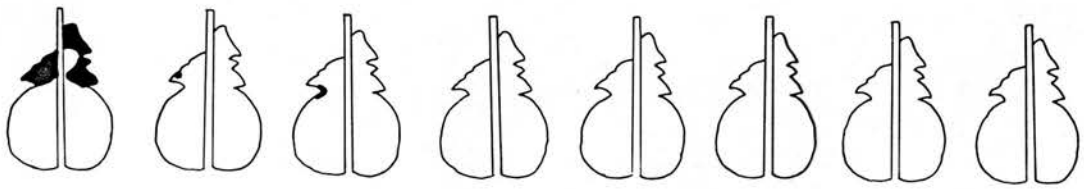
The extent of gross lung lesions observed at necropsy is depicted in Fig. 4.2.

In group A, lung lesions were seen in 3 lambs. In the lamb which was killed in extremis there was a large volume of serous fluid in the pleural cavity and many fibrinous adhesions between lobes, and between the visceral and parietal pleurae. There was total consolidation of the apical lobes. In one of the other 2 lambs the lesions consisted of a small area of adhesions between the left cardiac and diaphragmatic lobes and in the other of a small abscess in the left cardiac lobe. In group B, lesions were observed only in the lungs of the 2 lambs which were killed in extremis. There was a copious pleural effusion, extensive pleurisy and patchy consolidation of the apical and cardiac lobes in both lambs.

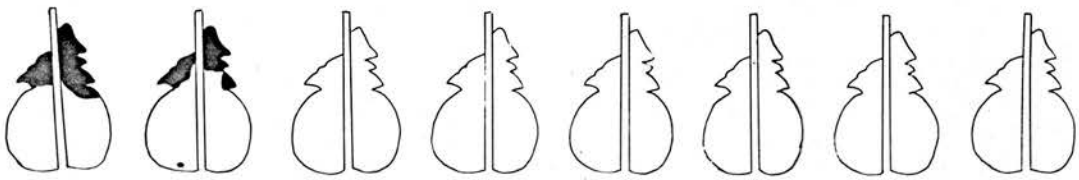
Lesions were seen in the lungs of 5 lambs in group C. Extensive consolidation of the apical and cardiac lobes was present in the lungs of the lamb which was killed in extremis. The pleural cavity contained a large volume of fluid and overlying much of the visceral and parietal pleurae was a thick gelatinous exudate (Fig. 4.3). Large

Figure 4.2

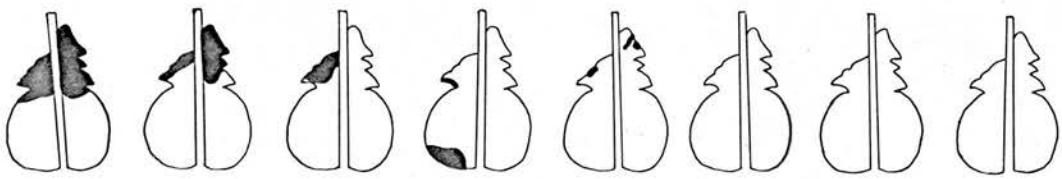
Macroscopic lesions in the lungs of lambs inoculated with OA4 virus and *P.haemolytica*, and *P.haemolytica* alone.



P. HAEMOLYTICA ONLY



OA4 & P. HAEMOLYTICA 4 DAYS LATER



OA4 & P. HAEMOLYTICA 8 DAYS LATER

areas of consolidation with overlying pleurisy and adhesions were present in the lungs of 3 other lambs (Fig. 4.4) and in one of these, there were small abscesses (Fig. 4.5). In one further lamb there were small areas of collapse in the apical lobes.

Histopathology.

In group A, microscopic lesions were seen in the lungs of 4 lambs. In the lamb that died there was an acute necrotic pneumonia consisting of a purulent pleurisy overlying areas of consolidation, with necrosis of the bronchial mucosa. In 2 other lambs that had macroscopic lesions, one showed pleural adhesions without involvement of the lung parenchyma, and the other had a single abscess in the left cardiac lobe. One further lamb had small foci of consolidation in the left apical lobe.

In group B, microscopic lesions were seen in the lungs of 3 lambs. In the 2 lambs which were killed *in extremis*, the predominant lesion was a purulent pleurisy which extended into the underlying lung parenchyma resulting in foci of exudative pneumonia. In the third lamb there were a few scattered foci of consolidation and necrosis.

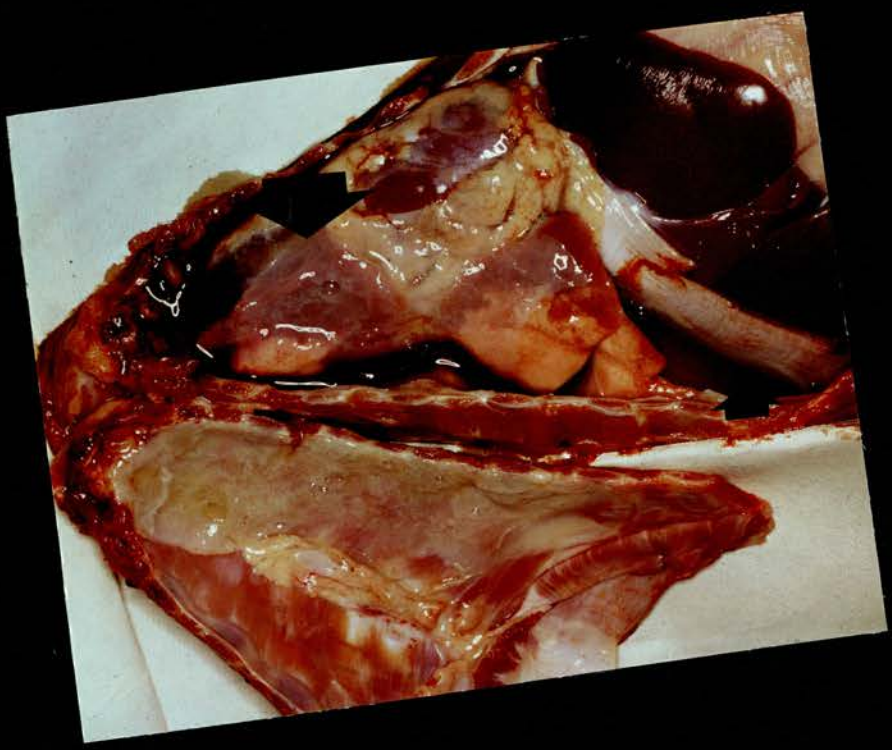
In group C, microscopic lesions were seen in the lungs of 6 of the 7 lambs. Purulent pleurisy was present in 3 lambs and, in 2 of the 3, there was underlying consolidation and necrosis. Small areas of consolidation were seen in 3 further lambs and a necrotic abscess was identified in one of these. The apical lobes of the remaining lamb contained areas in which desquamated epithelial cells

Figure 4.3

Thoracic cavity and lungs of lamb in group C, killed 2 days after exposure to P.haemolytica aerosol, showing extensive consolidation (large arrow) of apical and cardiac lobes, with overlying pleurisy. The pleural cavity contains an excess amount of fluid (small arrow) and on the parietal pleura is a thick gelatinous exudate.

Figure 4.4

Thoracic cavity and lungs of a lamb in group C, killed 14 days after exposure to an aerosol of P.haemolytica showing fibrinous adhesions between the visceral and parietal pleurae.



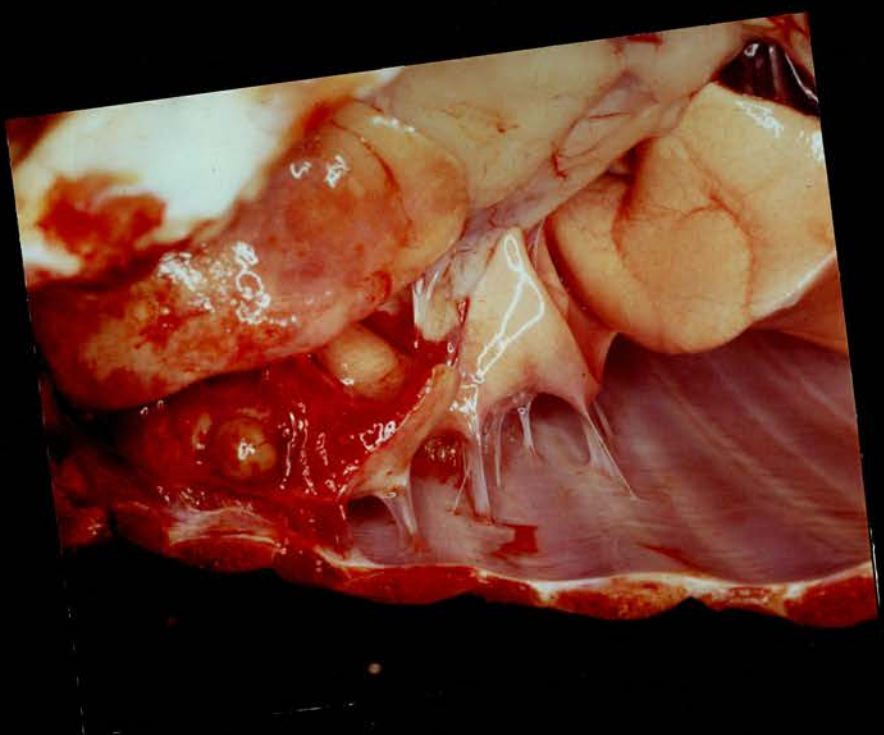
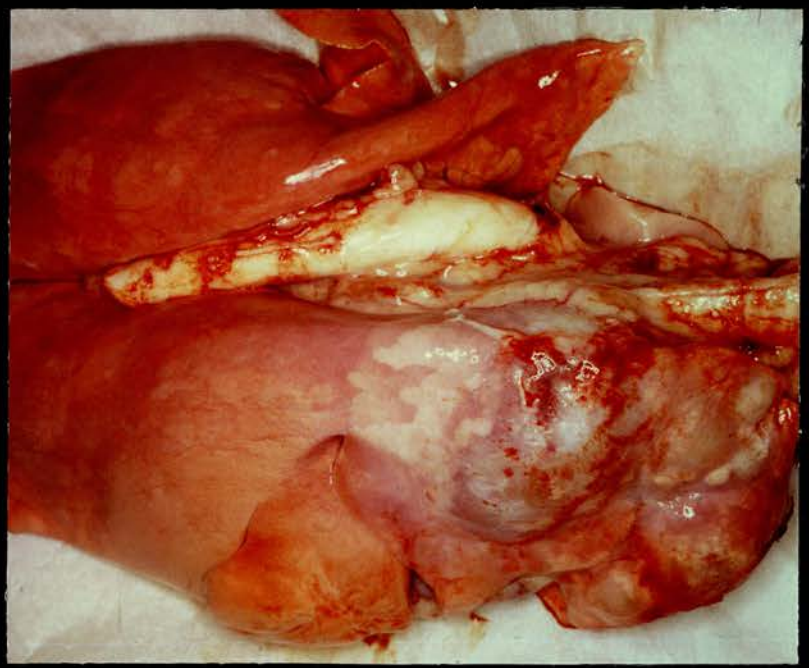


Figure 4.5

Lungs from lamb in group C, showing small abscesses in the right apical lobe.



were suspended in mucus within the bronchioles.

COMMENT

The macroscopic and microscopic lung lesions in most of the lambs in each group resembled the lesions induced experimentally in sheep by P.haemolytica (Smith, 1964; Gilmour, Thompson, Smith and Angus, 1975) except that pleurisy was more prominent in the present work. Ten of 23 lambs in the 3 groups had macroscopic lesions and, in 7 pleurisy and adhesions were evident. Thirteen lambs had microscopic lesions in their lungs and in 10 there were areas of pleurisy with underlying focal necrosis and consolidation; P.haemolytica was recovered from 8 of these. Thus it seems that the lung lesions can be attributed to P.haemolytica.

Whilst interpreting other results of this experiment, several points must be remembered. The lambs in groups A and B were exposed to the same aerosol of P.haemolytica, therefore the results from these 2 groups can be compared directly. However, because lambs in group C were exposed to a different aerosol of P.haemolytica which contained 500 times fewer organisms than that received by lambs in groups A and B, it is difficult to make direct comparisons between group C and the other two groups.

The number of lambs in group A, infected only with P.haemolytica, that developed pyrexia as well as macroscopic and microscopic lung lesions is similar to that in

another report of SPF lambs exposed to an aerosol of P.haemolytica (Gilmour et al, 1975). The number affected, however, does not differ greatly from the number in group B, in which lambs were infected with virus and P.haemolytica (Table 4.3). Therefore, infection of lambs with OA4 virus 4 days before exposure to an aerosol of P.haemolytica was not shown to enhance the clinical disease and lung lesions induced by P.haemolytica.

In contrast, 5 of 7 lambs in group C had pyrexia, 5 had macroscopic lung lesions and 6 had microscopic lesions (Table 4.3). Although not exposed to the same aerosol of pasteurella as lambs in groups A and B, the response to P.haemolytica in group C was more severe than that obtained with greater numbers of P.haemolytica in groups A and B. Thus it seems that infection with OA4 virus 8 days before an aerosol of P.haemolytica may have increased the number of lambs with pyrexia and lung lesions. Further support for this hypothesis is gained from previous experience at the Moredun Institute with aerosols of P.haemolytica. Gilmour et al (1975) reported macroscopic and microscopic pneumonia in only 4 of 9 SPF lambs exposed to an aerosol containing 32 times as many organisms as in the present work. In another experiment, Gilmour, Thompson and Angus (personal communication) exposed 7 SPF lambs to 3 aerosols of P.haemolytica at intervals over a period of 6 days; it was estimated that the lambs inhaled $10^{7\cdot7}$, $10^{6\cdot7}$ and $10^{6\cdot7}$ viable organisms at each exposure respectively. No clinical illness developed, other than a transient pyrexia in 2 lambs

TABLE 4.3

SUMMARY OF CLINICAL OBSERVATIONS AND PATHOLOGICAL FINDINGS IN LAMBS FOLLOWING EXPOSURE TO AEROSOLS OF P.HAEMOLYTICA

Group	Number in Group	Number with Pyrexia	Number Died or Killed	Number with Lung Lesions	
				Macroscopic	Microscopic
A	8	1	1	3	4
B	8	4	2	2	3
C	7	5	1	5	6

after the first aerosol, and pneumonic lesions were present in only 4 of the 7 lambs.

The ability of adenovirus infections to potentiate the pathogenicity of other organisms has not been investigated extensively. Ginder (1964) has shown, in mice, that acute or chronic adenovirus infection of the kidney predisposes this organ to acute pyelonephritis after challenge with E.coli. In gnotobiotic piglets, it has been reported that porcine adenovirus type 4 can produce a more severe pneumonia in combination with Mycoplasma hyopneumoniae than either agent alone (Kasza, Hodges, Betts and Trexler, 1969) but that it cannot enhance the response to P.septica (Smith, Betts, Watt and Hayward, 1973). The latter observation agrees with the conclusion, from groups A and B in the present work, that OA4 virus does not predispose the lung to acute pasteurella pneumonia. On the other hand, if the conclusion derived from the results in group C is correct, then the interval between infection with OA4 virus and infection with P.haemolytica may be critical, and should be examined in further experiments.

One lamb died 6 days after inoculation with OA4 virus, but before exposure to the aerosol of P.haemolytica. At necropsy, this lamb was found to have an intussusception of the small intestine. The possibility that OA4 virus induced this lesion must be borne in mind because adenoviruses have been attributed with causing intussusception in children (Potter, 1964; Younis, Atchison, Michaels and De Cicco, 1975) and monkeys (Lange, Apodaca and Kohler, 1969).

However, the role of OA4 virus in the development of this lesion is not clear because, although virus was recovered from the gut contents of the affected lamb, it was also isolated from rectal swabs of infected lambs between days 3 and 9 pi (Chapter 3) and intussusception did not occur in another 31 infected lambs.

EXPERIMENTS 2 - 4

Each of these 3 experiments was designed to investigate the influence of an existing infection with PI3 virus on a subsequent infection with P.haemolytica. In each experiment, SPF lambs were inoculated intratracheally with 8 ml. and intranasally with 2 ml. of ovine PI3 virus (G2 strain), followed at intervals by exposure for 15 min. to an aerosol of P.haemolytica type A1. The virus was given by intratracheal and intranasal inoculation in preference to aerosol because Hore and Stevenson (1969) had shown that this method of inoculation of young lambs with PI3 virus produced a mild respiratory disease whereas Smith (1975) failed to induce illness in SPF lambs with aerosols of the same strain of virus.

The details of the design of each experiment are presented in Table 4.4.

EXPERIMENT 2

This experiment was designed to determine, first whether PI3 virus predisposed SPF lambs to a subsequent

TABLE 4.4

DESIGN OF EXPERIMENTS 2 - 4, IN WHICH SPF LAMBS WERE INOCULATED WITH PI3 VIRUS AND *P.HAEMOLYTICA*

Experiment	Inocula	Group	Number of lambs	Age of lambs at time of PI3 virus inoculation (weeks)	Infectivity of PI3 inoculum (log_{10}/10 ml)	Interval between inoculation of PI3 virus and bacterium (days)	Estimated number of viable *P.haemolytica* inhaled by each lamb (log_{10})
2	PI3 virus + *P.haemolytica*	A	8	4	8·2	4	7·7
		B	8	4	8·2	7	7·7
3	PI3 virus + *P.haemolytica*		14	12	8·2	7	6·0
4	PI3 virus + *P.haemolytica*	A	11	12	7·5	7	6·1
	P.haemolytica	B	4	12	-*	7	6·1
	PI3 virus	C	3	12	7·5	7	-**

* sterile tissue culture fluid

** aerosol of sterile broth.

infection with P.haemolytica and, second, whether the interval between infection with the 2 agents affected the response of the lambs to P.haemolytica.

Sixteen 4-week-old SPF lambs were divided into 2 groups of 8 (Table 4.4). Group B was inoculated with PI3 virus (at its 5th passage level in FLK cell cultures) 3 days before the inoculation of group A with aliquots from the same pool of virus, which was stored at -70°C. Four days later, both groups were exposed to an aerosol of P.haemolytica type A1, that was generated from a single broth culture. All lambs were examined for clinical illness during the period between the inoculation of PI3 virus and necropsy. Nasal swabs for the isolation of virus were taken daily for the first 8 days after the inoculation of PI3 virus.

RESULTS

Clinical Observations.

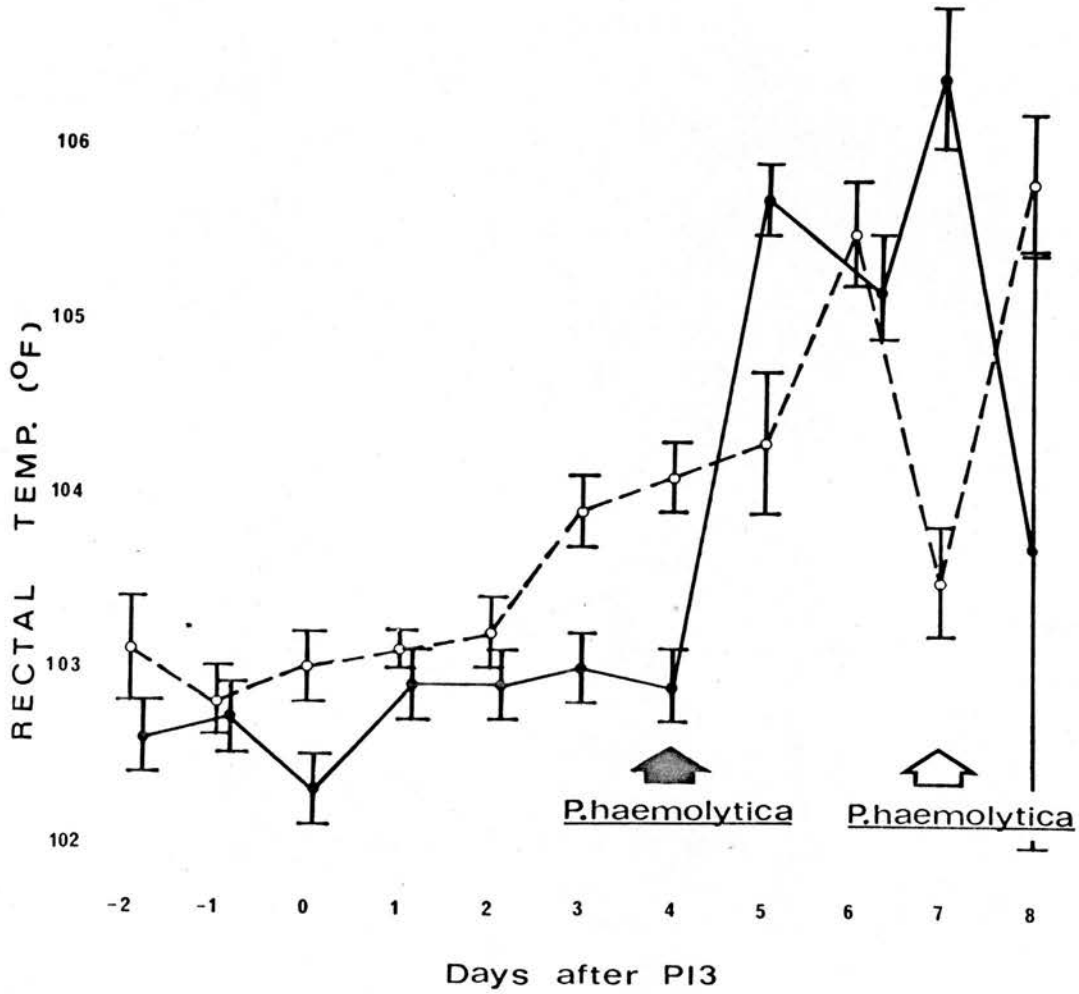
Following inoculation with PI3 virus, all 8 lambs in group B developed respiratory disease. On the fourth day after inoculation of virus a few lambs showed slight depression, hyporexia, and an "abdominal lift". By day 5 - 6 pi, most lambs were pyrexic (Fig. 4.6), the respiratory signs were more marked and were present in all lambs. Seven days after inoculation of PI3 virus, when they were exposed to P.haemolytica, the lambs appeared to be recovering as they were no longer dull or pyrexic, and their appetite had returned. Two lambs in group A were slightly depressed and had an "abdominal lift" on day 4 pi, when

Figure 4.6

Mean rectal temperature (± SE) of lambs in Experiment 2, inoculated with PI3 virus followed by exposure to an aerosol of P.haemolytica 4 or 7 days later.

●——● group A, PI3 virus + P.haemolytica 4 days later.

○– – –○ group B, PI3 virus + P.haemolytica 7 days later.


106
105
104
103
102
RECTAL TEMP. (°F)
-2
-1
0
1
2
3
4
5
6
7
8
Days after PI3
P.haemolytica
P.haemolytica

lambs in this group were exposed to P.haemolytica.

The day after the lambs were exposed to the aerosol of P.haemolytica, there was no difference between the severity of respiratory disease in each group. All but 3 of the 16 lambs in groups A and B were very depressed with anorexia, an "abdominal lift" and coughing. Thirteen lambs were pyrexic (mean 105·7 $\pm$ 0·2°F in group A; 105·8 $\pm$ 0·4°F in group B) 2 had subnormal temperatures and 4 lambs were killed in extremis. Next morning, 2 lambs were dead, 4 were killed in extremis and 4 of the 6 survivors continued to show severe respiratory disease. On the third day after they were exposed to the aerosol of P.haemolytica 2 further lambs were killed, but the 4 survivors (2 in each group) looked bright with only minor respiratory signs and were killed at the end of the experiment, 4 days later.

Microbiology.

PI3 virus was recovered from nasal swabs from lambs in group A between days one and 7 pi and from lambs in group B between days one and 8 pi (Fig. 4.7). The mean titres of virus in the nasal swabs from the lambs in both groups were not significantly different except on days 5 and 6 pi when the titres in the lambs infected 7 days before the aerosol of P.haemolytica were significantly higher than those in the lambs infected 4 days before the aerosol (day 5, $p < 0{\cdot}05$; day 6, $p < 0{\cdot}05$).

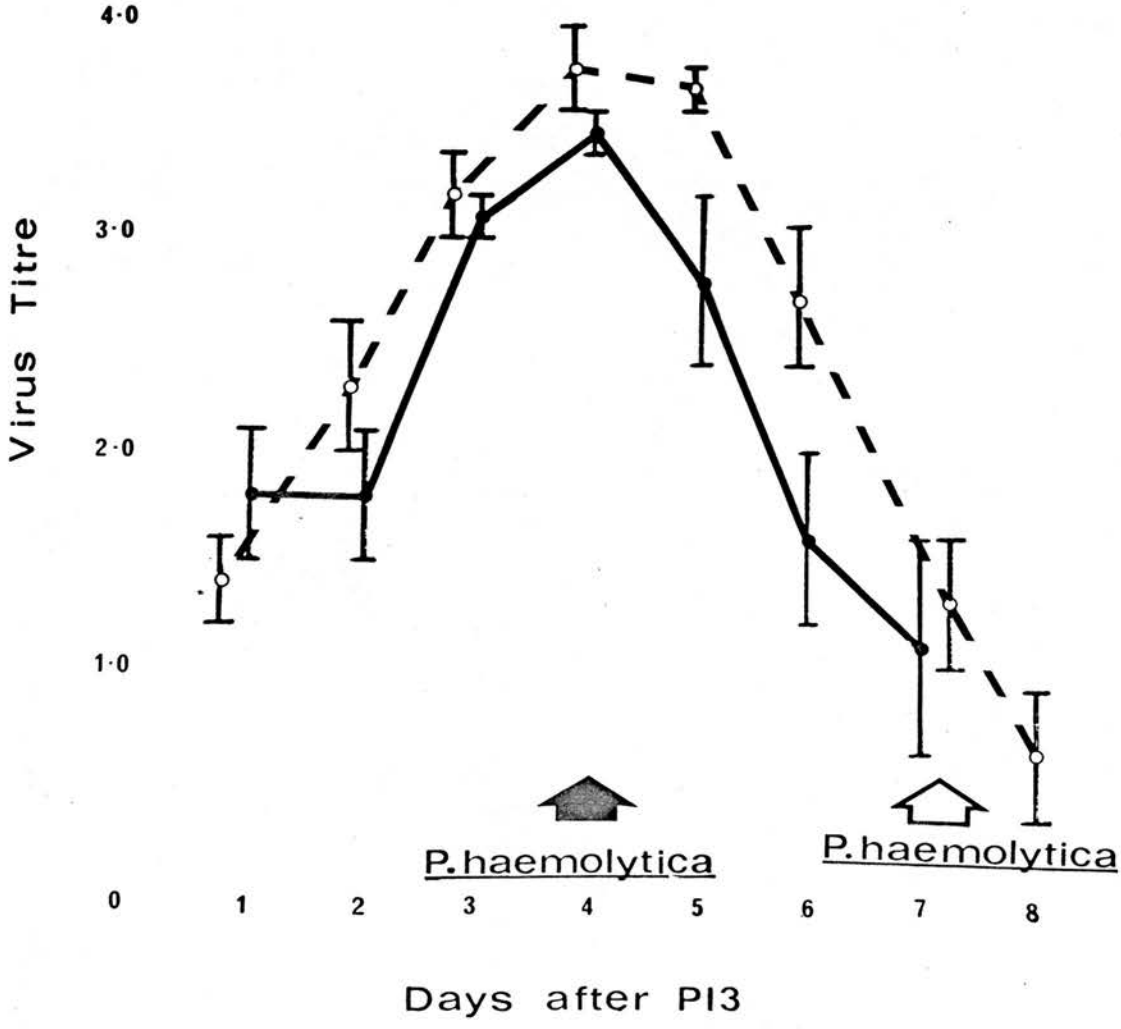
High titres ($\geqslant 10^{6{\cdot}0}$ $TCID_{50}/g.$) of PI3 virus were recovered from the lungs of all 6 lambs that were killed in

Figure 4.7

Titres of virus (± SE) isolated from nasal swabs from lambs inoculated with PI3 virus, followed by exposure to an aerosol of P.haemolytica 4 or 7 days later.

●——● group A, PI3 virus + P.haemolytica 4 days later.

○- - -○ group B, PI3 virus + P.haemolytica 7 days later.


4·0
3·0
2·0
1·0
0
Virus Titre
1
2
3
4
5
6
7
8
P.haemolytica
P.haemolytica
Days after PI3

group A, and at lower titres ($10^{2\cdot0}$ to $10^{3\cdot7}$ $TCID_{50}/g$) from the lungs of the 6 lambs which were killed in group B. Virus was not recovered from the lungs of the 4 surviving lambs. Haemagglutination-inhibiting antibody to PI3 virus was detected only in the post-inoculation sera from each lamb in group B (geometric mean titre, 29).

P.haemolytica type A1, was recovered from the lungs of all lambs. In group A, the mean number of organisms recovered from whole lung homogenates from lambs which died or were killed in extremis was $10^{10\cdot1} \pm 10^{0\cdot5}$ and from 2 lambs which survived was $10^{9\cdot5}$. In group B, the numbers of organisms were $10^{11\cdot1} \pm 10^{0\cdot4}$ and $10^{7\cdot8}$ respectively.

Pathology.

At necropsy, the lungs of all 16 lambs contained large areas of deep-red to purple consolidation. The lesions were as extensive in the lambs which survived as they were in those which died or were killed.

COMMENT

Although no lambs were exposed to P.haemolytica only, previous experience with aerosols of this bacterium (Experiment 1) indicated that PI3 virus enhanced both the clinical disease and pneumonic lesions produced by P.haemolytica. The illness and death following the bacterial aerosol seemed to be associated with rapid multiplication of P.haemolytica because all lambs developed severe

disease within 24 hours of the aerosol, and the number of organisms in the lungs of lambs which died were much higher than the numbers they could have inhaled.

The duration of PI3 virus excretion in the present experiment is similar to that reported by other workers (Hore and Stevenson, 1969; Smith, 1975) and the growth curve approximates that were reported by Smith (1975). The amount of virus in nasal swabs from lambs in group A was lower than that in swabs from lambs in group B on days 5 and 6 pi (Fig. 4.7). Lambs in group A were exposed to P.haemolytica at the peak of virus replication and the rapid multiplication of the bacterium, with ensuing damage to cells and the resulting pyrexia, may have reduced the ability and/or number of cells to support virus replication.

EXPERIMENT 3

Experiment 2 has shown that clinical respiratory disease and pneumonic lesions can be produced in a high proportion of 4-week-old SPF lambs infected with PI3 virus and P.haemolytica. In terms of further investigation of the pathogenesis of the disease and, more particularly prophylaxis, it would be more advantageous to use older animals. Experiment 3 investigated whether clinical respiratory disease and pneumonic lesions could be induced in 12-week-old lambs. Fourteen lambs were inoculated with PI3 virus, at the 5th passage level in FLK cell cultures, 7 days before they were infected with an aerosol of P.haemolytica, type A1 (Table 4.4). The interval of 7 days between inoculation

with the virus and that with P.haemolytica was chosen because the disease caused by PI3 virus would be abating at this time and any subsequent clinical signs could be attributed clearly to P.haemolytica.

RESULTS

After inoculation with PI3 virus, 13 of the 14 lambs developed the same type of respiratory disease as described in Experiment 2. The disease was most severe on day 6 pi when most of the lambs showed pyrexia (Fig. 4.8), depression, anorexia and an "abdominal lift". When they were exposed to P.haemolytica, 7 days after inoculation with the virus, the lambs were no longer depressed nor pyrexic, and their appetite was restored.

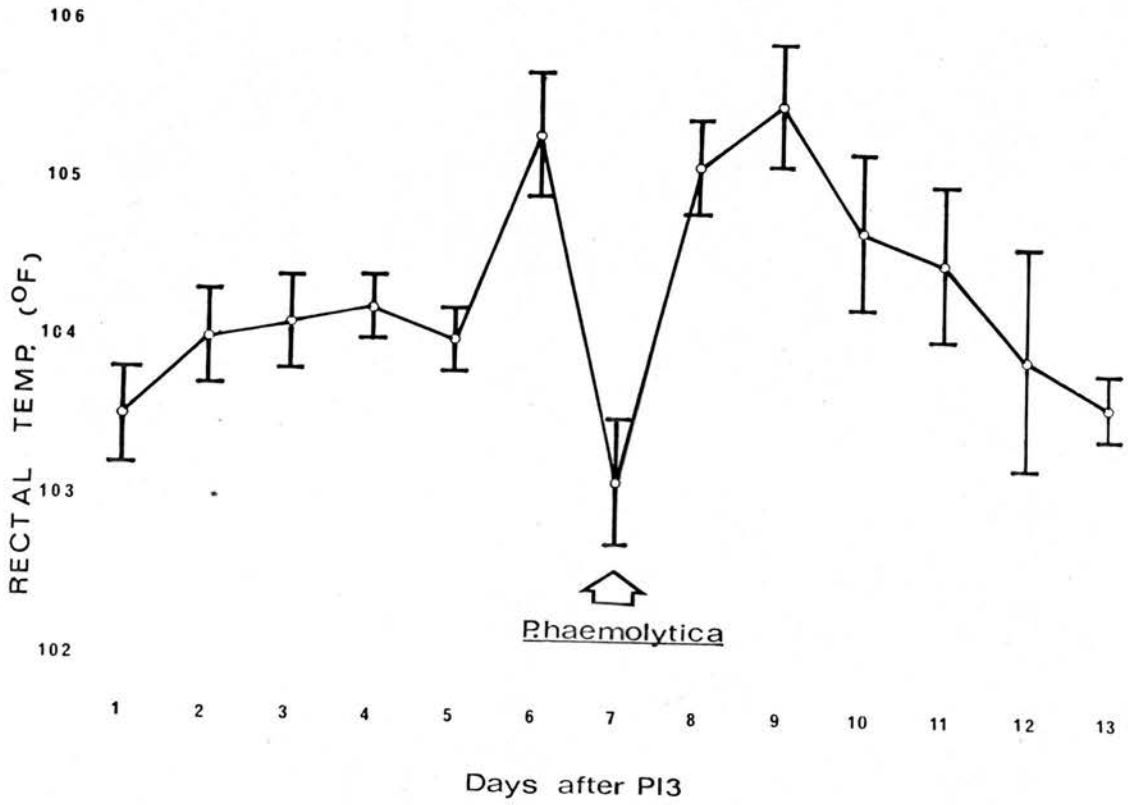
Following exposure to the aerosol of P.haemolytica, 11 of the 14 lambs showed severe respiratory disease as described in Experiment 2 and 7 lambs died between one and 5 days after the aerosol.

PI3 virus was isolated from nasal swabs from every lamb between days 1 - 6 pi, but swabs were not taken later than this. P.haemolytica was recovered from the lungs of 13 lambs. The mean number of bacteria in whole lung homogenates from lambs that died was $10^{8 \cdot 6} \pm 10^{0 \cdot 5}$ and from lambs that survived was $10^{5 \cdot 9} \pm 10^{1 \cdot 0}$.

At necropsy, the lungs of 11 lambs contained large areas of consolidation, with abscesses and pleurisy.

Figure 4.8

Mean rectal remperature ($\pm$ SE) of 12-week-old lambs, in Experiment 3, inoculated with PI3 virus followed by exposure to an aerosol of P.haemolytica 7 days later.


106
105
104
103
102
RECTAL TEMP. (°F)
P.haemolytica
1
2
3
4
5
6
7
8
9
10
11
12
13
Days after PI3

COMMENT

This experiment has confirmed that inoculation of SPF lambs with both PI3 virus and P.haemolytica produces severe clinical disease and lung lesions. It has further demonstrated that this disease can be induced in 12-week-old SPF lambs.

EXPERIMENT 4

In Experiments 2 and 3, it was concluded that the high proportion of disease induced by P.haemolytica was due to the influence of PI3 virus. Experiment 4 was designed to confirm this assumption by means of appropriate control groups.

Eighteen, 12-week-old SPF lambs were divided into 3 groups (Table 4.4). One group of 11 lambs (group A) and another of 3 lambs (group C), were inoculated with PI3 virus, at its 6th passage level in FLK cell cultures. Seven days later, group A and another group of 4 lambs (group B) were exposed to an aerosol of P.haemolytica, type A1.

RESULTS

Clinical Observations.

(a) Following inoculation with PI3 virus: Nine of 11 lambs in group A and all 3 lambs in group C developed the same type of respiratory disease as lambs in Experiments 2 and 3. Again, the disease was most severe on day 6 pi, and on day 7

lambs were alert and the respiratory signs seemed to be abating. However, although the rise and fall in mean rectal temperature followed the same pattern in both groups, the maximum mean temperature occurred on day 4 pi, and only in group C did it exceed 105°F (Fig. 4.9). The lambs in group B, which were inoculated with tissue culture fluid, did not show any clinical signs.

(b) Following exposure to the aerosol of P.haemolytica: Nine lambs in group A, which were inoculated with both PI3 virus and P.haemolytica, showed pyrexia and the mean rectal temperature for the group remained above 105°F for the first 4 days after exposure to the aerosol of P.haemolytica (Fig. 4.9). Seven lambs showed severe respiratory disease and a further 2 lambs were depressed but without obvious respiratory signs. Three lambs died, one of which had no signs of respiratory disease other than a frequent cough. None of the lambs in group B, which were inoculated with P.haemolytica only, developed respiratory disease, but 2 lambs were slightly depressed and hyporexic for 1 - 3 days. One lamb had pyrexia, which was reflected in a mean rectal temperature for the group of 105 $\pm$ 0·5°F on the second day after the aerosol (Fig. 4.9).

The respiratory signs due to PI3 virus in the 3 lambs in group C, which had been inoculated with PI3 virus but exposed only to an aerosol of sterile broth, continued to abate and the lambs were clinically normal by day 9 after inoculation with virus.

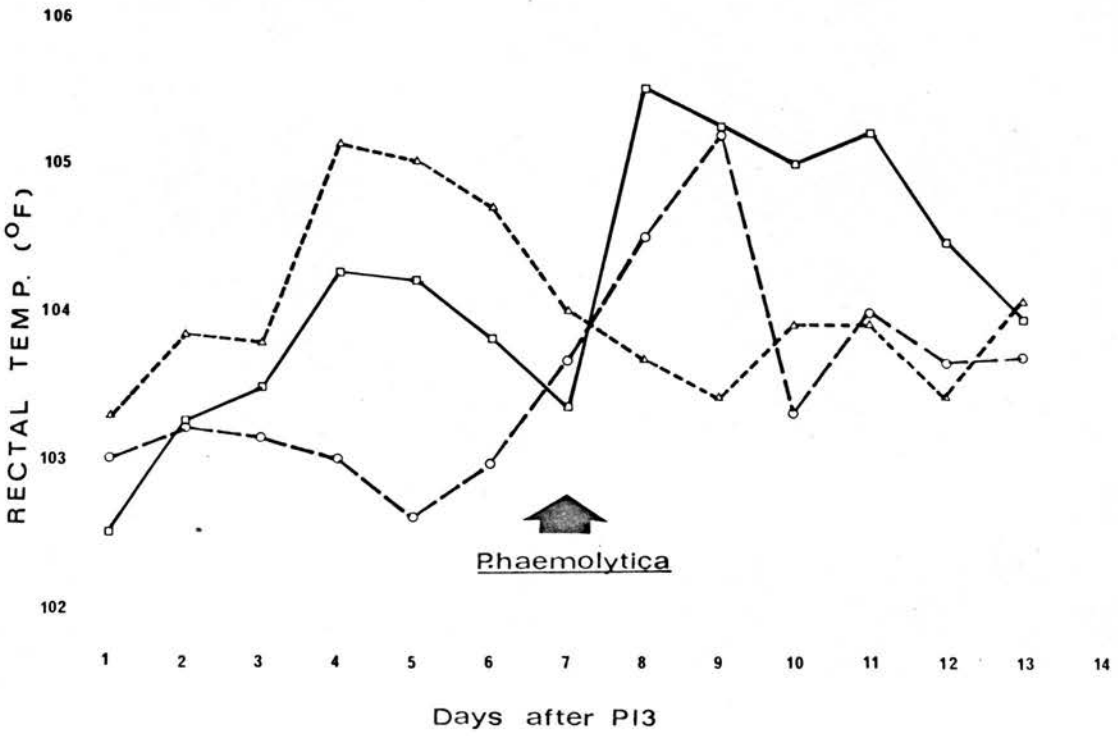
Figure 4.9

Mean rectal temperatures of lambs in Experiment 4, inoculated with PI3 virus or *P.haemolytica* or PI3 virus and *P.haemolytica*. Standard errors have been omitted for clarity.

□——□ group A, PI3 virus + *P.haemolytica*.

○— —○ group B, *P.haemolytica* only.

△- - -△ group C, PI3 virus only.


106
105
104
103
102
RECTAL TEMP. (°F)
P.haemolytica
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days after PI3

Microbiology.

PI3 virus was isolated from nasal swabs from lambs in groups A and C between days one and 7 pi.

P.haemolytica was cultured from the lungs of 10 lambs in group A. The mean number of organisms in the lungs of the 3 lambs which died was $10^{12.2} \pm 10^{0.3}$, and in the 8 survivors $10^{7.8} \pm 10^{1.1}$. The bacterium was recovered from the lungs of 3 of the 4 lambs in group B, and the mean number of organisms/lung was $10^{6.9} \pm 10^{2.3}$. P.haemolytica was not isolated from the lungs of any lamb in group C, which were inoculated only with virus.

Pathology.

Ten of the 11 lambs in group A had lesions in their lungs (Fig. 4.10). There was extensive consolidation with necrosis and pleurisy in 6 lambs (Fig. 4.11) and lesser consolidation or a single focus of necrosis in 4 further lambs (Fig. 4.12). Each of the lambs in group B had a single area of necrosis in one lobe (Fig. 4.13), but one lamb in group C had a few small linear depressions in an apical lobe.

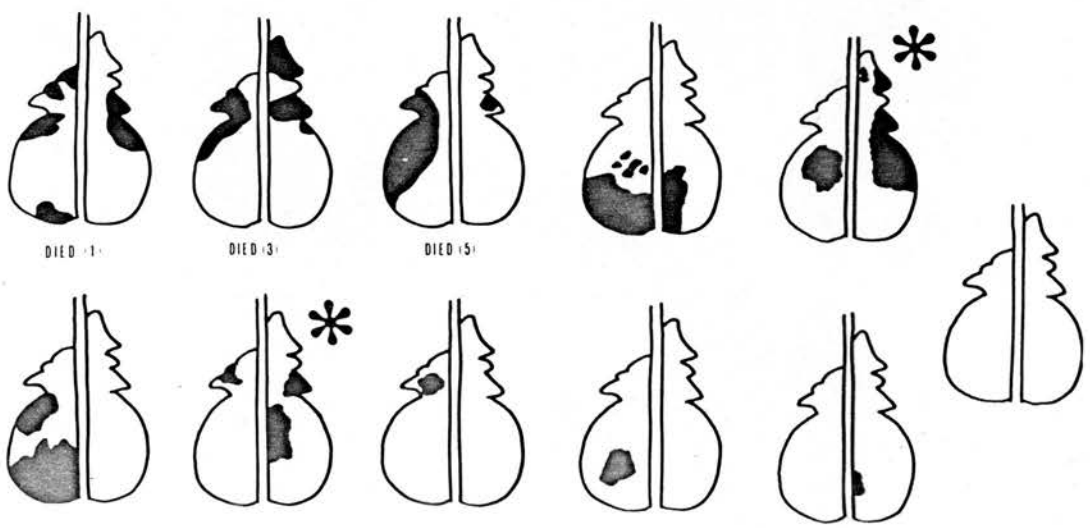
COMMENT

The results of this experiment have confirmed those obtained in Experiments 2 and 3. The disease and lesions in lambs inoculated with both PI3 virus and P.haemolytica were much more severe than in lambs inoculated with either agent alone, thus proving that PI3 virus can predispose the

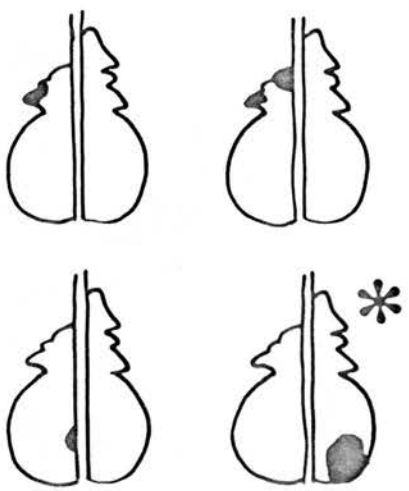
Figure 4.10

Macroscopic lesions in the lungs of lambs inoculated with PI3 virus and P.haemolytica or P.haemolytica alone. Lesions were not seen in the lungs of lambs inoculated with PI3 virus alone.

* details of these lungs are shown in Figs. 4.11, 4.12 and 4.13.



PI3 VIRUS & P. Haemolytica



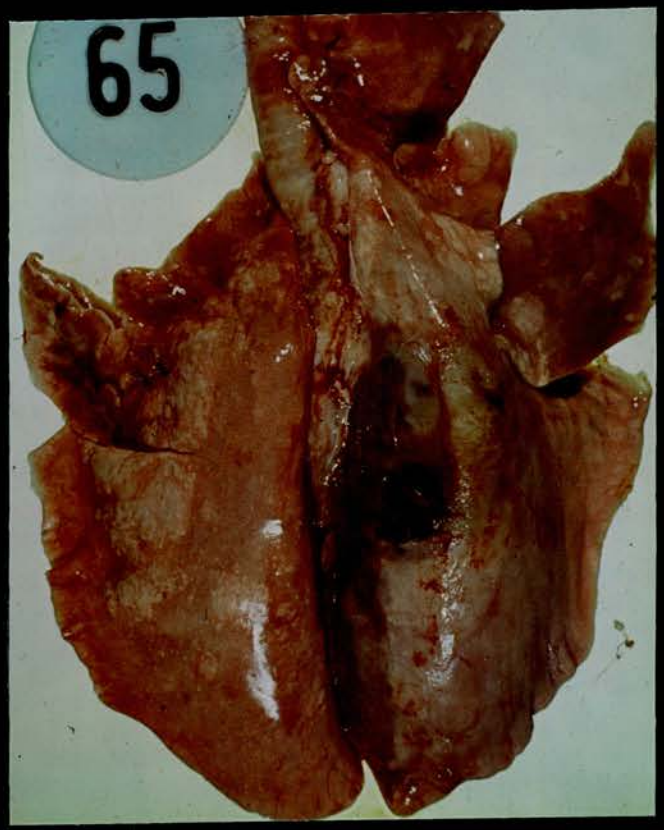
P. Haemolytica ALONE

Figure 4.11

Dorsal view of the lungs from a lamb inoculated with PI3 virus and P.haemolytica, type A1, and killed 7 days after the aerosol of Pasteurella. Extensive consolidation is evident in both diaphragmatic and the right cardiac lobes, and the centres of these lesions appear necrotic (arrows).

Figure 4.12

Dorsal view of the lungs from a lamb inoculated with PI3 virus and P.haemolytica, type A1, and killed 7 days after the aerosol of Pasteurella. The area of consolidation in the diaphragmatic lobe has been cut to show the haemorrhagic and necrotic centre of this lesion. There is no necrosis within the consolidation in the right cardiac lobe.


65

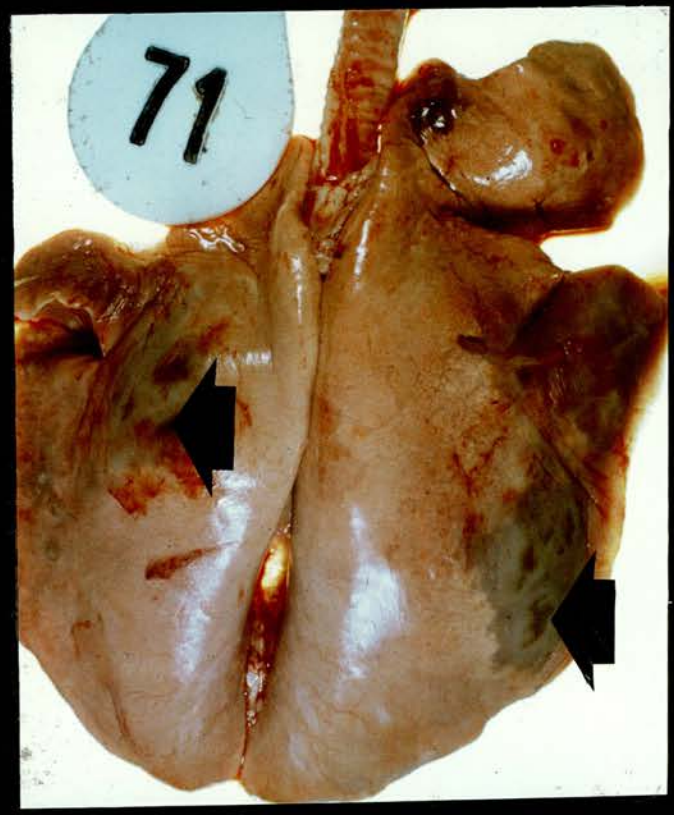
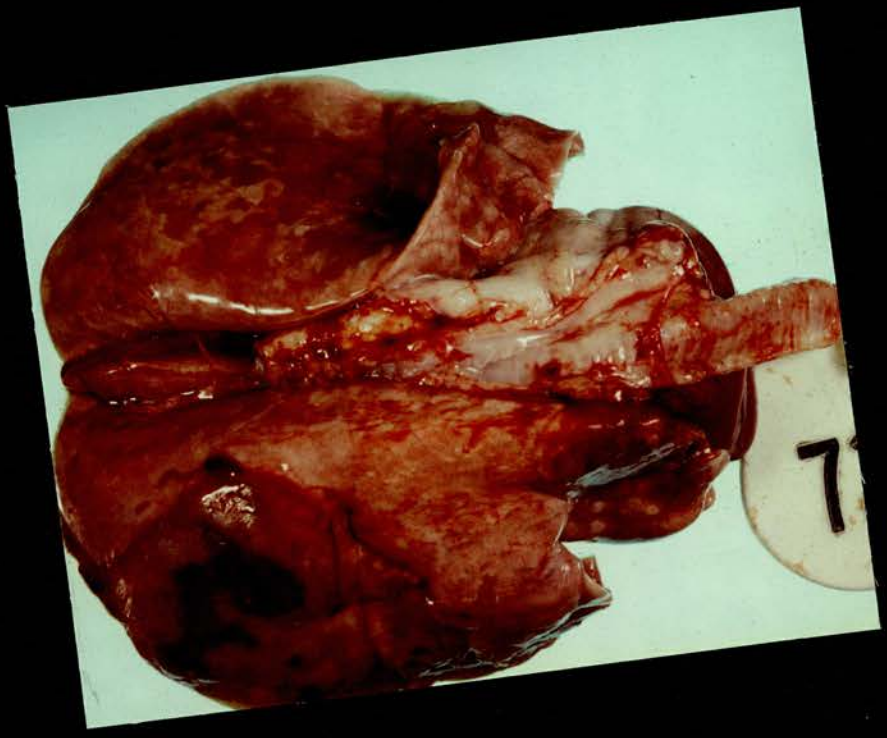

71

Figure 4.13

Dorsal view of the lungs from a lamb exposed to an aerosol of P.haemolytica only. There is a single area of consolidation, with central necrosis, in the right diaphragmatic lobe.



respiratory tract of sheep to disease induced by P.haemolytica.

DISCUSSION

The outstanding feature of the experiments in this chapter has been the production of severe respiratory disease and lung lesions in a high proportion of SPF lambs by a combination of PI3 virus and P.haemolytica. The response of lambs receiving both agents in each of Experiments 2 - 4 was similar and in total, 32 of 41 (78 per cent.) lambs developed severe respiratory illness, 22 (54 per cent.) died and 39 (95 per cent.) had macroscopic lung lesions (Table 4.5). Previous experiments, in which calves and sheep have been inoculated with combinations of PI3 virus and P.haemolytica and/or P.multocida, have not produced such severe disease. The results have often been inconsistent, with no clear differences between animals inoculated with the combination of PI3 virus and Pasteurella and those inoculated with either agent alone (Heddleston, Reisinger and Watko, 1962; Hamdy, Trapp, Gale and King, 1963; Hetrick, Chang, Byrne and Hansen, 1963; Baldwin, Marshall and Wessman, 1967; Biberstein, Shreeve, Angus and Thompson, 1971). The success of the method, reported in the present work, that consistently induces severe pneumonia, can probably be attributed to the use of SPF lambs in all experiments, whereas previous workers have inoculated calves and sheep, which were not reared in isolation and which may have encountered pasteurellae

TABLE 4.5

SUMMARY OF THE CLINICAL SIGNS, LESIONS AND NUMBER OF ORGANISMS RECOVERED AT AUTOPSY FROM LAMBS AFTER EXPOSURE TO THE AEROSOLS OF PASTEURELLA HAEMOLYTICA

Experiment	Group	Number of lambs	Number with respiratory disease	Number dead and killed in extremis	Number with lung lesions	P.haemolytica in whole lung homogenates from lambs which	
						died/killed in extremis	survived
2	A	8	7 (88)	6 (75)	8 (100)	10·1 ± 0·5*	9·5
	B	8	7 (88)	6 (75)	8 (100)	11·1 ± 0·4	7·8
3		14	11 (79)	7 (50)	13 (93)	8·6 ± 0·5	5·9 ± 1·0
4	A	11	7 (64)	3 (27)	10 (91)	12·2 ± 0·3	7·8 ± 1·1
	B	4	0	0	4 (100)	-	6·9 ± 2·3
	C	3	0	0	0	-	0

( ) = per cent. * = $\log_{10}$ mean number of organisms (± SE)

before inoculation. For similar reasons, although it was clearly apparent from the results of Experiment 4 that a pre-existing infection by PI3 virus enhanced the pathogenicity of P.haemolytica, such a relationship was not obvious from previous reports.

The experiments described in this chapter have provided no indication of which factors enable PI3 virus to influence the pathogenicity of P.haemolytica.

Work with laboratory animals has indicated that the interval between virus infection and inoculation of the bacterium may influence the severity of the respiratory disease and that the optimum interval is about 7 days. Reports which suggest that a similar relationship may exist in calves are of limited value, because small numbers of calves were used and only one report investigated the effect of infecting calves with pasteurellae more than 48 hours after inoculation with PI3 virus (Heddleston et al, 1962; Hetrick et al, 1964; Baldwin et al, 1967). In the present work, the response of lambs in Experiment 2 exposed to P.haemolytica 4 days after PI3 virus inoculation was as severe in lambs exposed 8 days pi, which indicated that the interval may not be critical. On the other hand, the results of Experiment 1 have shown that infection with OA4 virus 4 days before P.haemolytica does not enhance the pathogenicity of this bacterium, whereas an interval of 8 days may do so. At the moment, the potentiating influence of OA4 virus is speculative and, therefore, no conclusions can be made with respect to the

relative effects of OA4 and PI3 viruses.

Lambs, infected with both PI3 virus and P.haemolytica, developed severe respiratory disease and depression within 24 hours of exposure to the aerosol of bacteria. This response seemed to be associated with rapid multiplication of P.haemolytica, as the numbers of bacteria isolated from the lungs of lambs which died, particularly in the first 2 days after exposure to the aerosol, greatly exceeded the numbers which they could have inhaled (Tables 4.4 and 4.5). Further, as P.haemolytica is a Gram-negative organism which contains large quantities of endotoxin (Keiss, Will and Collier, 1964) it seems likely that some of the clinical signs can be attributed to the effects of this substance.

The number of bacteria in the lungs of lambs which died after inoculation with PI3 virus and P.haemolytica was usually 2 - 3 $\log_{10}$ higher than in those lambs which survived (Table 4.5). The difference may reflect an inherent ability of lambs that survive to restrict bacterial multiplication to sub-lethal levels. A more likely possibility, in view of the uniform clinical response on the day after exposure to the aerosol of P.haemolytica, is that rapid bacterial multiplication occurred in each lamb and, that during the next 2 - 3 days, equilibrium was achieved between bacterial multiplication and inactivation by the lung defence mechanisms. Those lambs which could tolerate the large numbers of bacteria during this period did not die and, subsequently, developed an immunity,

which reduced the numbers of bacteria recovered at necropsy.

The macroscopic and microscopic (Rushton, personal communication) lung lesions in lambs infected with PI3 virus and P.haemolytica were similar to those described in naturally-occurring cases of enzootic pneumonia (Dungal, 1931; Stamp and Nisbet, 1963).

Thus, these experiments have shown that on clinical, microbiological and pathological grounds, enzootic pneumonia can be produced consistently in SPF lambs by a combination of PI3 virus and P.haemolytica. This method therefore may be used in future experiments to investigate the pathogenesis and immunity of this disease.

Another notable feature in Experiments 2 - 4 was the marked, but transient, respiratory illness produced in SPF lambs by the G2 strain of ovine PI3 virus. This result contrasts with previous experiments in which sheep, inoculated intranasally or by aerosol with the G2 strain of PI3 virus, did not develop respiratory illness (Gilmour, Drysdale, Stevenson, Hore and Brotherston, 1968; Smith, 1975). Hore and Stevenson (1969) inoculated lambs by combined intranasal and intratracheal routes, as in Experiments 2 - 4, and only produced a mild respiratory illness. Similarly, the response of calves to experimental inoculation of bovine PI3 virus has been, generally, mild (Phillip and Darbyshire, 1972) although some reports describe illness (St.George, 1969b; Frank and Marshall, 1971). The report of Frank and Marshall (1971) is of interest because they describe a reproducible illness in calves

exposed to the SF-4 strain of bovine PI3 virus, a procedure that previously had produced only mild illness (Reisinger, Heddleston and Manthei, 1959). The more severe clinical response obtained during the present work can probably be attributed to the greater infectivity of the inoculum and the larger volume of inoculum injected into the trachea.

In each of Experiments 2 - 4, the sequence in which clinical signs appeared, progressed and then disappeared was predictable. No clinical signs were apparent during the first 2 days after inoculation of virus, but on days 3 - 4 pi a few lambs appeared slightly ill. During days 4 - 5 pi, the clinical signs became obvious in an increasing number of lambs, until on day 6 pi over 90 per cent. of the lambs appeared very ill. They were depressed, anorexic, pyrexic and had marked "abdominal lifts". Nevertheless, within 24 hours every lamb had made a dramatic recovery; they were no longer dull, anorexic or pyrexic, although the "abdominal lift" remained and did not disappear until about day 9 pi. By comparing these results with those of other workers it is possible to speculate further on the pathogenesis of PI3 virus.

The clinical illness in Experiments 2 - 4 can be divided into 2 types; those which arose from damage to the respiratory tract (abdominal lift) and those which reflected systemic involvement (depression, pyrexic, anorexia). The development of the "abdominal lift" can be explained in terms of damage to the lower respiratory tract (Stevenson, 1968; Hore and Stevenson, 1969). On the other hand, certain

aspects of these experiments suggest that the systemic illness was closely associated with the virus itself. For 5 days following inoculation with PI3 virus, the appearance and progression of both types of clinical signs paralleled the increasing virus titres in nasal swabs. After this time, the relationship was not so precise but a general trend could be detected. As the virus titres decreased, the signs of systemic illness disappeared abruptly, whereas the "abdominal lift" abated over a longer period. Thus there was a close relationship between the systemic signs, and the amount of virus recovered from nasal swabs. Furthermore, the improvement in the systemic illness, associated with decreasing virus titres, occurred between days 6 - 7 pi, the time when nasal and serum antibodies to the virus would be first detectable (Smith, 1975; Chapter 3). St.George (1969b) reported similar observations in calves inoculated intratracheally with high infectivities of PI3 virus. The fall in pyrexia and clinical improvement occurred at the same time as neutralizing antibody first appeared. Signs of pneumonia, however, did not disappear at this time and were attributed to the presence of lung lesions. The coincidence of these observations point to the systemic illness originating not from tissue damage in the respiratory tract with release of inflammatory substances, nor to the actions of secondary bacteria, but to some other effect of the virus.

GENERAL DISCUSSION

It was stated in the introduction to this work that, although few viruses had been recovered from sheep before this study began, there was no reason to doubt that further types of viruses could be isolated. This premise has been borne out by the isolation of OA4 virus from lambs during the present study, and other reports have described the isolation of further serotypes of adenovirus (Bauer, Müller and Gürtürk, 1975; Belak and Palfi, 1974b; Davies, 1975), reovirus types 1 and 2 (Belak and Palfi, 1974a; Snodgrass, Burrells and Wells, 1976), enterovirus (Snodgrass and Sharp, in preparation) and rotavirus (Snodgrass, Smith, Gray and Herring, 1976).

To date, there have been few reports concerning the epidemiology in sheep of these viruses and, consequently, their involvement in respiratory diseases remains unclear. Most isolates have been recovered from faeces of healthy lambs, and such infections appear to be common. For example, in Britain, antibodies to OA1-4 viruses, reovirus type 3 and rotavirus are widespread (Chapter 2; Knox, 1974; McFerran et al, 1973; Snodgrass, personal communication) and adenoviruses can be isolated frequently from faeces of healthy sheep on certain farms (McFerran, Clarke, Knox and Connor, 1972; Sharp and Snodgrass, unpublished observations). Thus, the isolation from sheep of any of these viruses, particularly adenoviruses, during an outbreak of disease should not be unexpected and their involvement in such

outbreaks must be viewed cautiously until detailed epidemiological evidence is available. Nevertheless, it seems that in Hungary, when lambs from various sources are gathered together and housed intensively, some strains of adenovirus and reovirus can be pathogenic. In these circumstances, reovirus type 1, OA1 and BA2 viruses have been isolated from nasal and rectal swabs of lambs with respiratory and enteric disease (Belak and Palfi, 1974a & b; Belak, Palfi and Palya, 1976) and BA2 virus was shown, by means of a vaccination trial, to be involved in the naturally occurring disease (Belak et al, 1976). The contrasting experiences of the epidemiology of these viral infections in Britain and Hungary may arise from the different systems of flock management.

Although field observations have failed, as yet, to demonstrate an association between most of these viruses and naturally occurring diseases, experimental inoculation of lambs has provided some evidence as to their potential pathogenicity. McFerran et al (1974) reported that combined intranasal and oral inoculation of lambs with reovirus type 3 did not produce clinical disease, whereas Belak and Palfi (1974a) found that colostrum-deprived lambs inoculated intranasally and intratracheally with a Hungarian strain of reovirus type 1 developed respiratory and enteric disease similar to that observed in the original outbreak. Similarly, in the present work, SPF lambs exposed to an aerosol of OA4 virus did not become ill (Chapter 3) but colostrum-deprived lambs inoculated intranasally and intratracheally

with Hungarian strains of OA1 and BA2 viruses developed disease (Belak and Palfi, 1974b; Belak, personal communication). Clearly, the route of inoculation may be important in whether lambs develop disease because both reovirus and adenovirus produced illness in the experiments reported by Belak and Palfi, but not in the present work nor that of McFerran et al (1974). Further support for this view is that PI3 virus has been found to produce inapparent infections following intranasal or aerosol inoculation (Smith, 1975), but a marked clinical pneumonia develops after intratracheal inoculation (Chapter 4). However, the different results obtained by British and Hungarian workers may be due, in part, to the use of different virus serotypes or strains, because combined intranasal, intratracheal and intravenous inoculation of SPF lambs with the N. Ireland strain of OA1 and OA2 viruses failed to induce clinical illness (Sharp, Rushton and Scott, unpublished results).

In Chapter 1, the isolation of viruses from 3 of 4 flocks currently experiencing acute outbreaks of respiratory disease, but not from 7 other flocks with few or no signs of respiratory disease, has contributed further to the view that viruses are involved in acute outbreaks of respiratory disease. The viruses involved were PI3 virus and OA4 virus yet, following experimental inoculation of susceptible sheep, neither of these viruses by themselves has produced a disease resembling the naturally occurring disease. Consequently, the view has emerged that in out-

breaks of respiratory disease, viruses act in conjunction with other organisms and, in the case of sheep,the most important bacterium is P.haemolytica.

In Chapter 4, such an hypothesis was examined experimentally using OA4 and PI3 viruses. The ability of OA4 virus to produce disease with P.haemolytica was not confirmed in the present experiments and so bears out the field observations. On the other hand, PI3 virus greatly enhanced the disease produced by P.haemolytica and both clinical signs and lung lesions were similar to naturally occurring enzootic pneumonia. Thus, for the first time, a method of repeatedly recreating enzootic pneumonia in the laboratory has been achieved and may prove useful in future investigations of the pathogenesis and immunity of this disease.

Virus infections have been shown to predispose the respiratory tract to bacterial infections in several species. In laboratory rodents, mainly mice, the mortality rate due to a variety of bacteria is greater when animals are infected previously with influenza virus or parainfluenza virus type 1 (Francis and Vicente de Torregrosa, 1945; Harford, Leidler and Hara, 1949; Gerone, Ward and Chappell, 1957; Jansen, Chappell and Gerone, 1963; Degre and Glasgow, 1968; Jakab and Dick, 1973). In domestic animals, viruses have been shown to enhance the pathogenicity of bacteria in pigs and cattle. For example, Shope (1931) demonstrated that inoculation of pigs with a bacteria-free filtrate of pneumonic lesions and Haemophilus influenzae induced

swine influenza whereas, independently, the 2 agents produced little illness. In cattle there is no clear evidence that mixed infections by PI3 virus and pasteurellae induce more severe disease than either agent by itself. However, Jericho, Magwood and Stockdale (1976) reported that IBR virus enhanced the illness induced in calves by Pasteurella and Spradbrow and Cole (1971) reported that calves inoculated intratracheally with a bovine picornavirus and E.coli developed more severe illness than calves inoculated with either agent alone. The experiments in Chapter 4 have shown that such a relationship obtains in sheep, at least with respect to PI3 virus and P.haemolytica.

The mechanisms by which viruses influence the susceptibility of the respiratory tract to bacteria have been investigated most extensively in laboratory rodents. Bacteria that are inhaled into the normal respiratory tract are rapidly inactivated and removed due to the phagocytic and bactericidal actions of alveolar macrophages and, to a lesser extent, by the mechanical clearance and bactericidal action of the mucociliary apparatus (Rylander, 1973). These defence systems, particularly the alveolar defences, may be impaired by a variety of agents such as noxious gases, inert particles and microorganisms, including viruses, so that bacteria may multiply and possibly cause disease (Kass, Green and Goldstein, 1966; Rylander, 1973). It seems likely that these observations in rodents may apply also to calves. Lillie and Thompson (1972) have

shown, in a small number of animals, that the pattern of clearance of P.haemolytica and S.aureus from the lungs of calves was similar to that of mice. Further, Gilka, Thomson and Savan (1974) have reported that pulmonary clearance of P.haemolytica by calves was reduced by pulmonary oedema induced by histamine, endotoxin or croton oil, and also by treatment with hydrocortisone, although it was not affected by infection with PI3 virus. However, the same group of workers have since reported a series of experiments in which infection of calves with PI3 virus reduced pulmonary clearance of P.haemolytica (Lopez, Thomson and Savan, 1976).

It would be interesting to investigate whether the same type of defence systems operated in the respiratory tract of sheep and the extent to which these defences were influenced by agents such as PI3 virus. For example, in the experiments described in Chapter 4, at the time when the lambs were exposed to aerosols of P.haemolytica, the respiratory tract, and in particular the mucociliary apparatus, would have been damaged due to the effects of PI3 virus (Hore and Stevenson, 1969). The bactericidal activity of the alveolar macrophages also may have been impaired, as has been described above in rodents and calves, which may have allowed P.haemolytica to multiply rapidly. However, owing to the high cost of SPF lambs, this apsect was not investigated and the answers to these points must await the development of techniques which allow alveolar macrophages to be obtained from SPF lambs at intervals over

a number of days in order to assess *in vitro*, the ability of these cells to phagocytose and inactivate *P.haemolytica*.

At the moment, there are insufficient data to indicate whether PI3 virus and *P.haemolytica* together are responsible for some outbreaks of respiratory disease in sheep. Also, it must be recalled that other infectious and non-infectious agents have been implicated as factors which precipitate outbreaks of disease. For example, Hamdy and Pounden (1959) have shown, experimentally, that mycoplasmas produce more severe disease when inoculated together with *P.multocida* than either agent alone. Thus, those organisms and non-infectious factors that are important in the aetiology of ovine respiratory diseases can be identified only by extensive epidemiological surveys.

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A New Adenovirus from Sheep

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SUMMARY. An adenovirus has been isolated from lambs in Scotland. It differs antigenically from the 3 known serotypes, and should be classified as a fourth serotype of adenovirus from sheep.

IN 1969, MCFERRAN *et al.* reported the ioslation from sheep faeces of 8 adenoviruses, which were subsequently classified into 3 antigenic types (McFerran *et al.* 1971). Adenoviruses of unknown type have been isolated from sheep in Australia (Snowdon, 1970, 1971). This communication reports the isolation of an adenovirus from sheep in Scotland and its relationship to those types isolated in Northern Ireland.

MATERIALS AND METHODS

Sheep thyroid (STh) monolayer cultures were prepared as described by Plowright & Ferris (1961), except that the medium was Hanks' saline supplemented with 0·5% lactalbumin hydrolysate, 0·2% glucose and 0·1% yeast extract. Ten % calf serum was included for growth and 2% horse serum for maintenance purposes. Monolayers were washed 3 times with phosphate-buffered saline before inoculation.

Gel diffusion tests were performed as described by Darbyshire & Pereira (1964) using human adenovirus type 2 as antigen.

The method of Feldman & Wang (1961) was used to examine the sensitivity of virus to chloroform.

Sera, to be used in neutralization tests, were heated at 56°C for 30 min and 2-fold dilution series were prepared. Equal volumes of the serum dilution and a dilution of virus containing 100 $TCID_{50}$ were mixed and incubated at 37°C for 1 h before inoculation on to monolayers.

RESULTS

An outbreak of respiratory disease occurred in a housed group of 4–10-week-old lambs, some of which had died a few days previous to the first visit. Cytopathic agents were not detected in nasal or rectal swabs taken at the first visit, nor at a second visit 14 days later. Evidence of a current adenovirus infection in the group was indicated by an increasing number of sera giving positive gel diffusion test results between the first and second visit. A third series of samples was taken 20 days after the first investigation and cytopathic agents were recovered from rectal swabs from 3 of 15 lambs.

The initial isolations were made on primary STh monolayers rolled at 34°C. The earliest changes were seen 5 days after inoculation of the monolayers and consisted of increased refractility and rounding of cells in one or two small foci. The effect progressed slowly from the original foci by local extension and the development of a few new foci. Further passages of the cytopathic agents were made by scraping cells into the medium and inoculating this on to fresh STh monolayers.

The agents were identified by the nature of the cytopathic effect, the presence of basophilic intranuclear inclusions and the morphology of the virion (Fig. 1). Later, the adenovirus group specific antigen was demonstrated in all isolates by gel diffusion. One virus, designated 7769, was shown to be insensitive to treatment with chloroform. Acridine-orange staining of virus infected cells produced an intense intranuclear greenish yellow fluorescence indicative of DNA.

Tests showed that virus 7769 was not neutralized by antisera to bovine adenoviruses 1, 3 and 5, and that it differed from the 3 serotypes of adenovirus previously isolated from sheep in Northern Ireland (Table I).

DISCUSSION

The reported findings strongly suggest that 7769 is an adenovirus, which is antigenically distinct from

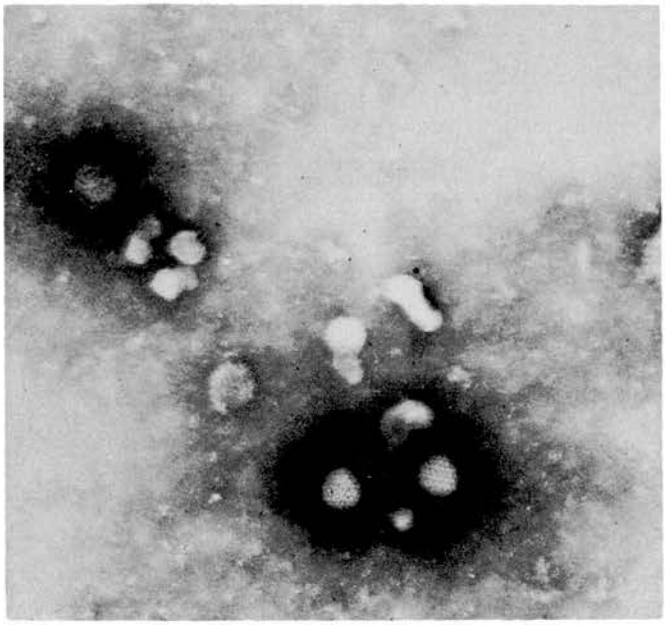

FIG. 1. Particles of adenovirus strain 7769 are shown. The particles ranged from 71 to 75 nm in diameter. Stain used, 4% sodium phosphotungstate, pH 6·5.

TABLE I

CROSS NEUTRALIZATION TESTS BETWEEN 3 OVINE ADENOVIRUS SEROTYPES AND STRAIN 7769

	Antiserum			
Virus	7769	OA1	OA2	OA3
7769	1/1500*	< 1/12	1/24	< 1/12
OA1	< 1/12	1/3000	< 1/12	< 1/12
OA2	1/24	< 1/12	1/1500	< 1/12
OA3	< 1/12	< 1/12	< 1/12	1/768

* Initial serum dilution which protected 2 out of 4 cultures. Final readings taken 7 days after inoculation.

the serotypes isolated in Northern Ireland (McFerran *et al.*, 1971).

The isolation of the virus 20 days after the first investigation can probably be attributed to the virus slowly circulating within the group of lambs. At the first and second visits, only 5 lambs were swabbed, but on the third occasion, all 15 lambs in the pen were sampled.

It is difficult to assess the relationship of this virus to the clinical disease at the time of its recovery as adenoviruses can be recovered from the faeces of healthy, as well as diseased sheep (McFerran *et al.*, 1971). Following inoculation in pathogen-free lambs, adenovirus strain 7769 has been shown to replicate and stimulate an immunological response without the development of clinical signs (Sharp & Rimer, in preparation). It may be that 7769 will only produce disease when in conjunction with other agents or factors. The epidemiology of infection and further pathogenicity of this virus are being examined.

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EXPERIMENTAL INFECTION OF SPECIFIC PATHOGEN-FREE LAMBS WITH OVINE ADENOVIRUS TYPE 4

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INTRODUCTION

Infection of sheep by adenoviruses has been recorded (Darbyshire and Pereira, 1964) and one bovine and five ovine serotypes have been isolated from sheep (McFerran, Nelson and Knox, 1971; Sharp, McFerran and Rae, 1974; Belak and Palfi, 1974; Bauer, Müller and Gürtürk, 1975). The ovine serotypes have been isolated only from faeces and are of uncertain pathogenicity. In contrast, the bovine serotype was isolated from nasal and rectal swabs from diseased sheep and, on experimental inoculation, produced respiratory and enteric disease in lambs (Belak and Palfi, 1974).

A preliminary experiment had indicated that ovine adenovirus type 4 (OA4) could replicate in sheep and produce a mild pulmonary oedema (Sharp, unpublished observations). The present communication describes infection of specific pathogen-free lambs with this virus.

MATERIALS AND METHODS

Cell cultures. Cultures of sheep thyroid (STh) cells were prepared by the method of Plowright and Ferris (1961) and calf kidney (CK) and foetal lamb kidney (FLK) as described by Paul (1965).

The medium for all cells was Hank's saline supplemented with 0·5 per cent. lactalbumin hydrolysate, 0·2 per cent. glucose and 0·1 per cent. yeast extract. Ten per cent. heat-inactivated calf serum was included for growth and 2 per cent. heat-inactivated horse serum for maintenance purposes. All cultures were washed 3 times with phosphate-buffered saline (PBS) before inoculation.

Virus. Ovine adenovirus type 4, strain 7769, was used at the sixth passage in STh monolayers. When the cytopathic effect (CPE) was maximal, generally 5 days after inoculation, virus was harvested by scraping the cells into the medium which contained 2 per cent. sheep serum. The cells were pelleted by centrifuging at 180 **g** at 4 °C. for 20 min. and the supernate was removed except for 2 ml. in which the cells were resuspended. Virus was liberated from the cells by 6 cycles of freezing and thawing, and the cellular debris deposited by centrifuging at 10 000 **g** for 30 min. The supernate, containing the virus, was made up to 15 ml. with the original supernate and stored at −70 °C. This preparation served as the infecting inoculum, and a control inoculum was derived in the same way from uninoculated STh cultures. Neither mycoplasma nor bacteria were detected in either inoculum.

Virus isolation. Nasal and rectal swabs from experimental lambs were placed in transport medium. Tissues taken at necropsy were chopped finely and a 10 per cent. (w/v) suspension was prepared in Hank's saline. Suspensions were clarified by

centrifuging at 2000 **g** at 4 °C. for 30 min. and the supernate was used to inoculate cell cultures. All swabs and suspensions that were not inoculated immediately were stored at −70 °C. Virus was also sought in leucocytes, a rich preparation of which was obtained by a method similar to that of Naylor and Little (1975). All specimens were examined for cytopathic agents by inoculating 0·2 ml. into each of 3 or 4 STh tube cultures, which were examined regularly for the next 28 days. One further passage was made if no CPE was seen.

Immunological tests. Sera, to be used in neutralization tests in microplates, were heated at 56 °C. for 30 min., whereas nasal secretions were not heat-inactivated.

For the micro-neutralization test, 0·025 ml. volumes of a 2-fold dilution series of each serum were prepared in microplates and an equal volume of virus containing approximately 100 $TCID_{50}$ was added. After incubation at room temperature for 1 h., 0·1 ml. of a suspension of FLK cells at a concentration of $1{\cdot}4 \times 10^5$ per ml. was added to each well. The plates were incubated at 37 °C. for 7 days, at which time the end-points were determined by observation of adenovirus CPE.

The gel diffusion test used was based on that described by Darbyshire and Pereira (1964). The size and pattern of the wells was as descibed by these authors but 1 per cent. agar in PBS plus 0·08 per cent. sodium azide was used. An extract from CK cells infected with human adenovirus type 2 (HA2) was used as antigen to detect antibody in sera from sheep. The test was read at 24 and 72 h. and "doubtful" reactors (Darbyshire and Pereira, 1964) were considered negative. A positive result was verified by the demonstration of a line of identity with a positive control serum.

Experimental design. Sixteen specific pathogen-free lambs were obtained by the method of Hart, Mackay, McVittie and Mellor (1971) and maintained in strict isolation (Brotherston, 1968). At 14 days of age, 10 lambs were exposed for 30 min. to an aerosol of OA4, and 6 lambs to an aerosol of non-infective tissue culture fluid. The apparatus used to generate the aerosols and the techniques of estimating the amount of virus inhaled by each lamb were those employed by Smith (1975). Relative humidity in the apparatus was maintained above 90 per cent. to preserve viral infectivity (Miller and Artenstein, 1967). It was estimated by the formula described by Smith (1975) that each lamb could have inhaled $10^{4{\cdot}6}$ $TCID_{50}$ of OA4.

The experiment was divided into 2 parts (Table 1). In the first part, clinical observations, rectal temperature, and nasal and rectal swabs were taken daily for 11 days after inoculation (pi). Also, 7 infected and 4 control lambs were killed serially and tissues taken. Blood for serology was collected by jugular venopuncture before inoculation and immediately prior to euthanasia.

In the second part, in addition to blood, nasal secretions were obtained from the remaining 3 infected and 2 control lambs on days 1, 3, 5, 8, 12, 17 and 25 pi. These lambs were then maintained in isolation until 80 days pi when they were treated with corticosteroid to stimulate recrudescence of viral excretion. Each lamb was inoculated intravenously for 7 consecutive days with 1 mg. dexamethasone* and swabs were collected as before.

Necropsy procedure. Lambs were killed by intravenous pentobarbitone and exsanguinated by severing the axillary vessels. In lambs killed on days 1 to 3 pi and following dexamethasone treatment, the lungs were allowed to deflate before removal from the thorax. Tissues for microbiological examination were taken with separate sterile instruments and tissues for histopathology were fixed in formol-saline. In lambs killed on days 7 and 11 pi, the trachea was clamped and the inflated lungs were removed from the thorax within 20 min. of death. After the left primary bronchus had been clamped, tissues from the left lobes were taken for microbiological examination and representative portions fixed in formol-saline. The right lung was fixed in the inflated state by intrabronchial perfusion with 3 per cent. glutaraldehyde (pH 7·5) before portions were taken for histopathology.

* Dexadreson, Intervet.

TABLE 1

EXPERIMENTAL DESIGN

Treatment	*Day after inoculation*	*No. of lambs killed*	
		Infected	*Control*
Aerosol of OA4 or TC fluid	0		
	1	1	1
	2	1	
	3	1	1
	7	2	1
	11	2	1
Start treatment with dexamethasone	80		
	81	1*	
	90	2	2

* Died.

RESULTS

Clinical Observations

Clinical disease was not observed in infected or control lambs, with one exception. One lamb, infected 80 days previously, appeared slightly dull and had a temperature of 40·9 °C. a day after the start of dexamethasone treatment, and was found dead the following morning.

Virus Recovery

The recovery of OA4 from nasal and rectal swabs is presented in Fig. 1 and most isolates produced a CPE during the first passage in primary STh monolayers. Virus was isolated from nasal swabs from each lamb between days 1 to 8 pi and the viral titre increased daily from day 1 pi to days 4 to 6 pi and then declined rapidly. OA4 was recovered from rectal swabs from each lamb between days 3 and 9 pi, with maximum titres on days 5 and 6 pi. Virus was not isolated from peripheral blood leucocyte preparations during the first 5 days pi.

Recovery of OA4 from tissues taken at necropsy is presented in Table 2. Virus was isolated from the upper and lower respiratory tract of all infected lambs killed up to 11 days pi, but only from 3 of the 4 lambs killed on days 7 or 11 pi was it recovered from the alimentary tract. OA4 was isolated on one further occasion, 80 days after inoculation, from a rectal swab from one infected lamb.

Following treatment with dexamethasone, OA4 was not recovered from nasal and rectal swabs, or from tissues taken at necropsy from lambs which had been infected more than 80 days previously and had excreted virus at that time.

Cytopathic agents were not isolated from nasal and rectal swabs, or tissues from control lambs. Mycoplasma were not cultured from any of the tissues, but *Escherichia coli* was grown from several tissues from the lamb that died.

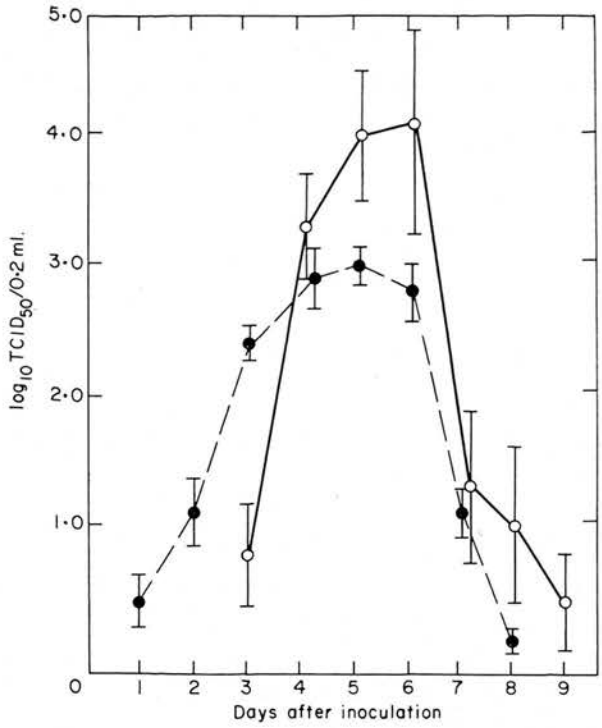

Fig. 1. Recovery of OA4 from nasal and rectal swabs. Mean virus titre (± standard error). (○——○) Rectal; (●---●) nasal.

TABLE 2

RECOVERY OF OA4 FROM TISSUES TAKEN POST-MORTEM FROM EXPERIMENTALLY INFECTED LAMBS

Tissue examined	*Virus recovery (log_{10} $TCID_{50}$/g) from tissues of 7 lambs killed after (days)* 1	2	3	7	7	11	11
Nasal mucosa	—	+	—	+	+	+	—
Oropharynx	—	+	—	NS	NS	NS	NS
Tonsil	—	+	—	+	+	+	+
Retropharyngeal lymph node	—	—	—	NS	NS	NS	NS
Tracheal mucosa	3·6	+	+	NS	NS	NS	NS
L. apical lung	3·2	+	2·3	—	—	+	+
L. cardiac lung	4·6	1·8	+	—	+	+	—
L. diaphragmatic lung	—	+	2·5	—	+	—	—
Pulmonary lymph node	—	—	—	—	—	—	—
Jejunum	—	—	—	+	—	+	—
Colon	—	—	—	+	4·2	+	—
Mesenteric lymph node	—	—	—	+	+	—	—
Spleen	—	—	—	NS	NS	NS	NS

\+ OA4 recovered in low concentration after prolonged incubation.
— OA4 not recovered.
NS Not sampled.

Immunology

Three infected and 2 control lambs were examined for antibody to OA4. Antibodies were not detected in undiluted sera or nasal secretions from control lambs but, at 8 days pi, neutralizing antibody was evident in both serum and nasal secretions from each infected lamb. Mean serum antibody titres increased from 10 to 708 by 90 days pi and mean nasal antibody titre rose from 14 to 20 at the 12th day pi then fell to 14 at the 25th day pi. Serum precipitating antibody was not detected at the 20th day pi but was present 31 days pi when OA4 was used as antigen. At the 90th day pi, precipitating antibody was detected with both OA4 and HA2 antigens.

Post-mortem Observations

Macroscopic abnormalities were confined to the lungs of those infected lambs killed on days 3 to 11 pi. Changes consisted of a variable degree of purple discolouration and oedema, as judged by the moist appearance of the lungs and expression of fluid from the cut surface of the lung parenchyma. The lung of the lamb killed 3 days pi also had areas of consolidation in the right apical and cardiac lobes and a discrete firm lesion (3 cm. diameter) which appeared mottled grey on its cut surface and was slightly raised above the dorsum of the right diaphragmatic lobe. The lungs of 3 of the 4 lambs killed on days 7 and 11 pi appeared oedematous and discoloured. In 2 lambs the subpleural lymphatics were dilated and a copious frothy exudate was present in the trachea and smaller airways. In one of these lambs, there was an extensive, sharply demarcated, dark red congested area at the posterior pole of each diaphragmatic lobe.

No macroscopic changes were observed in the organs of control lambs, infected lambs killed on days 1 and 2 pi, or infected lambs treated with dexamethasone.

Histopathology

Microscopic lesions were not seen in organs from infected lambs other than those lungs which macroscopically showed oedema. The essential lesion was a perivascular accumulation of fluid, which was faintly eosinophilic. A similar change was present around the bronchioles of one lamb and, in 2 lambs, there was intra-alveolar fluid and a few free mononuclear cells. These changes were present in both perfused and non-perfused lobes, but perivascular oedema was more obvious in perfused lobes. In 4 lambs there were small accumulations of mononuclear cells around bronchioles and occasionally small blood vessels. In the lamb killed 3 days pi the areas of consolidation were composed of alveoli filled with exudate containing mononuclear and polymorphonuclear cells. There was no evidence, in any lamb, of an alteration to the integrity of the bronchiolar epithelium nor were intranuclear inclusion bodies, which might be expected in an adenovirus infection, detected in sections stained by HE.

No lesions were detected in the lungs of control lambs.

DISCUSSION

The pattern of virus recovery from nasal and rectal swabs and post-mortem tissues indicates that OA4 replicated first in the respiratory tract. Thereafter the virus, which is stable at pH 2·7 (Sharp, unpublished observations), was probably swallowed to infect the alimentary tract. However, OA4 was isolated originally from rectal swabs (Sharp *et al.*, 1974) and two features of the experimental infection suggest that it is primarily an enteric virus. It replicated more rapidly and attained higher titres in the alimentary tract and may also have had a tendency to persist in this site, as it was isolated from a rectal swab from one lamb 80 days pi. Persistence of virus is a feature of adenovirus infection in many species and the isolation of OA4 80 days after the initial inoculation is similar to the results obtained with bovine adenovirus type 3 in calves and porcine adenovirus type 2 in pigs (Darbyshire, Jennings, Dawson, Lamont and Omar, 1966; Sharpe and Jesset, 1967).

The interval between the inoculation of tissue homogenates into tissue culture and the appearance of a CPE was prolonged for variable periods in respect to homogenates prepared from lambs killed on days 7 and 11 pi. It is known that both serum and local antibody can interfere with the recovery of virus from infected hosts (Pereira, 1972; Van der Veen and Mes, 1973; Zalan, Pukitis, Rhodes and Labzoffsky, 1973). In the present experiment, both nasal secretion and serum antibody were detectable by day 8 pi when viral titres in swabs were decreasing, and it seems probable that they combined to influence adversely the recovery of OA4 from tissues of dead animals.

The lesions found in the lungs of infected lambs were pulmonary oedema and peribronchiolar accumulations of mononuclear cells. There was no evidence of the necrosis of bronchiolar epithelium that is a feature of adenovirus infections of the respiratory tracts of pigs, dogs and cattle (Betts, Jennings, Lamont and Page, 1962; Wright, Thompson and Cornwell, 1970; Darbyshire *et al.*, 1966). Pulmonary oedema was seen, macroscopically and microscopically, in 4 of 7 infected lambs killed during the first 11 days pi, but not in 4 control, lambs killed at the same time. The significance of these findings must be assessed cautiously as it has been shown that fluid can accumulate in the lung after death and that water-miscible fluids perfused via the trachea can enter the pulmonary interstitial compartment (Staub, 1974). In the present experiment, perivascular oedema was seen in both perfused and unperfused lobes, but was more obvious and extensive in perfused lobes. These results indicate that the oedema was not produced by the perfusion technique although intratracheal perfusion of glutaraldehyde can cause this fluid to enter the interstitial compartment. Further, the lambs were exsanguinated and the lungs removed from the thorax shortly after death. Therefore, it appears that the pulmonary oedema was associated with infection with OA4.

The way in which the oedema was initiated remains obscure, but the mechanism seems to be transient as oedema was not observed in lambs killed 90 days pi. However, the presence of oedema in 3 of 4 lambs killed 7 to 11 days pi suggests that the development of oedema may be associated with circum-

stances at this time, such as the stage of the infection or the emergence of the immune response and its interaction with the virus.

SUMMARY

Specific pathogen-free lambs were exposed to an aerosol of ovine adenovirus type 4 (OA4). The virus replicated both in the respiratory and the alimentary tracts, and was recovered from one lamb 80 days after inoculation. Although infection with OA4 did not produce clinical disease, it appeared to be associated with the development of pulmonary oedema in approximately half of the infected lambs. The oedema was mainly perivascular, but in severe cases was present in alveoli and bronchioles. Small accumulations of mononuclear cells were present around bronchioles and small blood vessels, but inclusion bodies were not detected.

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