STUDIES OF THE IMMUNOLOGY AND EPIDEMIOLOGY OF ORF

by

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"Infectious disease is one of the few genuine adventures left in the world. The dragons are all dead, and the lance grows rusty in the chimney corner.... About the only genuine sporting proposition that remains unimpaired by the relentless domestication of the once free-living human species is the war against these ferocious little fellow creatures which lurk in the dark corners and stalk us in the bodies of rats, mice, and all kinds of domestic animals; which fly and crawl with the insects, and waylay us in our food and drink and even in our love."

Zinsser (1935) Rats, Lice and History.

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List of Abbreviations

APC	=	Antigen presenting cell
BSA	=	Bovine serum albumin
BPS	=	Bovine Papular Stomatitis
°C	=	Degrees Centigrade
CFA	=	Complete Freunds adjuvant
CFT	=	Complement Fixation Test
cm	=	centimetre/s
CMI	=	Cell Mediated Immunity
CPE	=	Cytopathic Effect
CTL	=	Cytotoxic T Lymphocyte
DH	=	Delayed hypersensitivity
DNA	=	Deoxyribonucleic acid
DNCB	=	Dinitrochlorobenzene
DNFB	=	Dinitrofluorobenzene
EDTA	=	Ethylenediaminetetracetate
ELISA	=	Enzyme Linked Immunosorbent Assay
°F	=	Degrees Farenheit
$F'ab_2$	=	Antigen binding portion of the immunoglobulin molecule
FBS	=	Foetal bovine serum
FDNB	=	Fluorodinitrobenzene
FITC	=	Fluorescene isothiocyanate
fl	=	fentolitre/s
FLM	=	Foetal lamb muscle
FMD	=	Foot and Mouth Disease
g	=	gram/s
xg	=	relative centrifugal force
G+C	=	Guanine + Cytosine

GIT	=	Guanidinium isothiocyanate
HFBS	=	Hank's Balanced Salts Solution + 1% FBS
hr	=	hour/s
IgA	=	Immunoglobulin A
IgG	=	Immunoglobulin G
IgM	=	Immunoglobulin M
IU	=	International Units
kb	=	1000 base pairs
kD	=	kiloDalton/s
kV	=	kiloVolt/s
2ME	=	2-mercaptoethanol
mm	=	millimetre/s
MHC	=	Major Histocompatibility Complex
MNV	=	Milker's Nodule Virus
µ	=	micron/s
µg	=	microgram/s
µl	=	microlitre/s
ND	=	not done
nm	=	nanometre/s
NP40	=	Nonidet P 40
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate Buffered Saline
PFU	=	Plaque Forming Units
PVC	=	Polyvinylchloride
RIA	=	Radioimmunoassay
RNA	=	Ribonucleic acid
SDS	=	Sodium dodecyl sulphate
SPF	=	Specific Pathogen Free

SSC	=	Standard Saline Citrate
ST	=	Sheep Thyroid
$TCID_{50}$	=	50% Tissue Culture Infectivity Dose
UV	=	Ultraviolet

Declaration

Except where specifically defined in the text,the work reported in this thesis was carried out entirely by myself.

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ABSTRACT OF THESIS (Regulation 7.9 ~~6.9~~)

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Title of Thesis Studies of the Immunology and Epidemiology of Orf

The responses of seven sheep to orf virus infection were examined by cannulating the efferent supramammary lymphatic before infecting the drainage area of the node. All animals developed lesions typical of orf virus infection, and responded after an initial lag period with an increase in total cell output paralleled by an increased proportion of lymphoblast cells. The majority of these lymphoblasts contained immunoglobulin, with a predominance of the IgG class. When efferent lymph cells from four of the sheep were cultured in vitro they were found to produce measurable amounts of virus specific antibody. When assessed in two of the sheep, the proportion of cells which stained with a T-cell-specific monoclonal antibody was found to decrease as the response developed. In contrast, analysis of afferent lymph (derived from efferent prefemoral lymphatics cannulated eight weeks after removal of the node) in four sheep revealed no marked cellular changes following experimental infection.

The serological responses of naturally and experimentally infected lambs to orf virus infection was analysed using an enzyme-linked immunosorbent assay (ELISA) together with the Western Blotting technique. The combination of these methods permitted a quantitative and qualitative assessment of humoral responses, which revealed considerable variation between animals. However, all post-exposure sera reacted with a polypeptide which appears to be a component of the surface tubules which are characteristic of the virus. Despite high titres in some animals, there was no apparent association between humoral responses and recovery or protection.

The heterogeneity of the orf virus population within the UK was examined using the restriction enzyme EcoRI by digesting viral DNA prepared from scab material derived from various outbreaks, and separating the fragments by electrophoresis in polyacrylamide gels. This technique revealed obvious genomic differences among the isolates which were examined, extending even to those derived from animals undergoing synchronous infections on the same premises.

By subjecting orf scab material to various contrived environmental conditions both indoors and outdoors, it was shown that exposure to rainfall can abrogate infectivity of the virus, which questions the significance of virus persisting on pasture as a source of infection.

Two rams which were shown to suffer from chronic orf infection suggest that the virus is capable of surviving inter-epizootic periods within the animal population. These animals had high serum antibody titres, but exhibited reduced delayed hypersensitivity responses to intradermal challenge with orf virus when compared with control animals.

It was concluded that humoral responses play little or no part in recovery and protection from of virus infection, and that immunity is manifested by an accelerated reaction which is the result of a delayed hypersensitivity response.

CHAPTER ONE

REVIEW OF THE LITERATURE

1) HISTORICAL BACKGROUND

The introduction of the term "orf" into scientific literature can be attributed to Walley (1890), who described it as a colloquial term for a contagious dermatitis which occurred in some Lothian and Border counties of Scotland. The condition was seen most commonly in sheep grazing old pastures, and was predominantly a disease of the feet and legs, spreading frequently to the skin of the face.

Descriptions of similar syndromes had been published earlier. Two years previously, Walley (1888) wrote of malignant aphtha, a skin condition which had been prevalent that year among the sheep populations of England and Scotland, and which was characterised by the development of pustular lesions on the udders of newly lambed ewes, and on the lips and nostrils of suckling lambs. He considered the term malignant aphtha inappropriate and proposed pustular fever or contagious ecthyma as alternatives. Over a half century before that, a disease known as black-muzzle was described by Youatt (1837) as being "a pimpled or scabbed eruption about the nose of the sheep sometimes extending up to the eyes and ears"

McFadyean (1901) described both malignant aphtha and orf, classifying the former as a disease affecting the nose or lips of lambs less than a few weeks old and the latter as one affecting the legs and face of lambs up to one year old. His concern was their significance in the differential diagnosis of foot-and-mouth disease. Berry (1901), preferring the term contagious pustular dermatitis, described the forms of orf seen in England, Scotland and Wales. He noted some synonyms for the disease, such as crusta labialis, carbuncle of the coronary band, "hair and hoof disease" and "mouth and foot disease".

Although the early investigators of the disease were aware of its infectious nature, it would appear (Hoare, 1913) that they believed the bacterium Fusiformis necrophorus to be the agent responsible. It was not until 1921 in France, that Marcel Aynaud, referring to the disease as contagious pustular stomatitis, concluded that a filterable virus was responsible (Aynaud, 1921). In a subsequent, more comprehensive study, he demonstrated that the virus could pass the Berkefeld V and Chamberland L1 and L1 bis candles, and noted its marked resistance to dessication (Aynaud,1923). He described the progression of experimental lesions through macule, papule, vesicle and pustule stages to thick crusts, which contained abundant quantities of virus, and noted that lesions were histologically similar to those of vaccinia. Indeed he remarked that "one could not fail to be struck by number of similarities between the two viruses". Glover (1928) examined the disease as it occurred in Great Britain for any resemblance to that described by Aynaud, and repeated much of the latter's work, obtaining similar results.

Reports of a similar disease occurring in other parts of the world had been appearing in the literature about this time. Zeller (1920) had described a variola-like condition of goats in south west Africa. Blanc, Melanidi and Caminopetros (1922) investigated a viral skin disease of goats in Greece which they considered analagous to that of Zeller and Aynaud. Moussu (1923) wrote of the disease in France, preferring the term contagious ecthyma to Aynaud's contagious pustular stomatitis, since stomatitis was not a constant feature. Lanfranchi (1925) reported the occurrance of the same disease in Italy, and Jacotot (1926), describing a "contagious ecthyma of the lips" of goats in Annam (Vietnam), considered it to

be identical to the disease of sheep described by Aynaud. Theiler (1928) reported the disease in South Africa, referring to it as ecthyma contagiosum , and mentioned that it had been known there for some time under the name "vuilbek". In 1929, Howarth documented its existence in California (Howarth, 1929), and in the same year it was reported as being prevalent in western Texas (Schmidt and Tunnicliff 1929) and Australia (Seddon and Belschner, 1929). By this time the disease had been well defined, and it has since been recognised throughout the world wherever sheep and goats are raised.

2) NOMENCLATURE

Despite various propositions by the early workers, no single term predominates as a name for the disease, which is not surprising since over 83 synonyms have been used (Nurnberg, 1942). The condition is today known in Great Britain as orf or contagious pustular dermatitis (Watt, 1982), in the United States of America (USA) as soremouth or contagious ecthyma, (Jensen & Swift, 1982), and in Australia and New Zealand it is commonly referred to as scabby mouth (Seddon and Belschner, 1929; Robinson, 1983).

The origins of the term orf are not clear, and although it is defined in many modern English dictionaries and dictionaries of old English as being derived from an Anglo-Saxon word for cattle, it is difficult to relate this to the disease in sheep. Jamieson's Scottish Dictionary (1880), however defines the word as a Lothian term for "a puny creature, one who has a contemptible appearance", and supplies "warf" as the Lanarkshire equivalent. Wright (1903) quotes this definition as one alternative, but further suggests that orf is a derivation of an old Yorkshire term "hurf", meaning

scurf. Websters Third New International Dictionary (1961) favours the latter option, stating that the word "hurf" is probably of Scandinavian origin, akin to the Old Norse word "hrufa" meaning a scab, or crust on a wound. The supplement to the Oxford English Dictionary (1982) describes orf as a virus disease of sheep and goats, and suggests that the word is a variation of the dialect term "hurf" which is probably from the Old Norse "hrufa" meaning a crust, scab or boil. Since the word orf was originally described as being colloquial in the Border areas of Scotland (Walley 1890), it is reasonable to assume that it is indeed derived from the old Yorkshire term.

3) THE DISEASE

3.1) General

Orf is a contagious disease of the skin and mucous membranes of sheep and goats, and is characterised by eruptive lesions which occur most commonly around the lips and nostrils (Aynaud, 1923) of lambs and kids in their first year of life. In uncomplicated cases, lesions form dry crusts which drop off in about 4 weeks leaving no scar (see Robinson and Balassu, 1981), but lesions may become extensive, involving large areas of the lips and muzzle (Belschner, 1950). The buccal cavity may become invaded, with involvement of the gums, tongue, palate, dental pad and cheek (Aynaud, 1923; Moussu, 1923; Howarth, 1929; Glover, 1930; Belschner, 1950; Greig, 1956b; Jensen and Swift, 1982), where rasberry-like proliferative lesions may be found (Moussu, 1923; Glover, 1928; Belschner, 1950; Greig, 1956b). Spread may occur to the skin and mucous membranes of other parts of the body, such as the thigh, axilla, coronet (Glover, 1928) and genitalia (Aynaud 1923; Greig, 1956b; Jensen and Swift 1982), and these lesions often

take the form of verrucose masses which are easily abraded and prone to haemorrhage (Glover, 1930; Watt, 1982). Nursing ewes can derive udder lesions from infected lambs (Aynaud, 1923; Moussu, 1923; Theiler, 1928; Howarth, 1929; Glover, 1930; Boughton and Hardy, 1934; Greig, 1956a) and these can give rise to severe mastitis causing them to wean and abandon young lambs (Howarth, 1929; Watt, 1971; Jensen and Swift, 1982).

Within a given outbreak, morbidity tends to be high, approaching 100 per cent (Aynaud 1923; Jensen and Swift, 1982), but mortalities rarely exceed 5 per cent (Greig, 1956b),and are usually due to secondary bacterial infections. Aynaud (1923) noted that suppurative bronchopneumonia was a common cause of death in lambs with extensive buccal lesions, and Watt (1982) states that Fusiformis necrophorus is a common invader of lesions in the buccal cavity and may spread to the viscera.

Outbreaks can result in serious economic wastage, the main source of which is loss of condition (Hart, Hayston and Keast, 1949) which may extend over a period of five to six weeks (Glover, 1928), at a critical period in the animal's life (Boughton and Hardy, 1934). Although losses to the individual farmer may not be great, the cost of the disease to the sheep industry as a whole is high (Jensen and Swift, 1982).

The disease has a further significance in that zoonotic infections can occur in man (Beck and Taylor, 1974; Johannessen, Krogh, Solberg, Dalen, van Wijngaarden and Johansen, 1975), and has caused concern in the meat industry in New Zealand (Robinson, 1982, 1983), and resulted in the refusal by Australian shearers to handle affected flocks (Carne, Wickham, Whitten and Lockley, 1946).

Lesions in man are usually benign (Robinson, 1983) but can give rise to complications such as lymphadenitis, lymphangitis, erythema multiforme (Johannessen and others,1975) and even ocular lesions resulting in permanent blindness (Beck and Taylor, 1974).

3.2) Lesions

In the most frequently seen benign form of the disease, lesions start as discrete papular swellings which progress through a vesicular phase to form pustules within a few days. These rupture giving rise to ulcers which form a thick crust, and this is shed within three to four weeks, leaving no scar (Aynaud, 1923; Glover, 1928; Howarth, 1929; Boughton and Hardy, 1934; Greig, 1956b; Buxton and Fraser, 1977; Jensen and Swift 1982). Premature removal of scabs reveals a raw bleeding hypertrophied dermis with raised papillae, (Moussu, 1923) sometimes covered with a purulent exudate (Aynaud, 1923). More severe manifestations of the disease are characterised by multiple coalesced lesions (Moussu, 1923,) and the development of papilloma-like outgrowths which may persist for several weeks (Glover, 1928; Howarth, 1929; Greig, 1956b; Buxton and Fraser, 1977). During the course of natural infection, the vesiculo-pustular nature of the lesions is not always obvious due to the constant trauma to which they may be subjected (Aynaud, 1923) or to the presence of secondary microorganisms (Glover, 1930). The developmental sequence of lesions is therefore best studied in susceptible animals which have been experimentally inoculated with the disease (Theiler, 1928; Glover, 1930; Boughton and Hardy, 1934).

The first attempt at a histological examination of an orf lesion was made by Walley (1890), who could discern little more than

enlargement of the dermal papillae due to "a small celled infiltration and distension of the blood vessels". He remarked that each papilla was covered by an irregular crust which he considered to be composed of degenerated epithelium and extravasated blood. Since that time, the microscopic appearance of lesions has been well documented by several workers. Aynaud (1923) considered its main characteristics to be a "ballonisante" degeneration of the cells of the Malpighian layer (stratum germinativum) of the epidermis, followed by infiltration with leucocytes, and cellular infiltration of the dermal papillae. Glover (1928) in an account of the histological features of lesions experimentally induced in sheep, described three stages. The first, or papulovesicle phase was dominated by proliferation of the cells of the rete Malpighii (stratum germinativum), the most superficial layers of which were seen to undergo degeneration, becoming swollen and rounded. Cellular infiltration of the dermis with polymorphonuclear cells was also evident. Small vesicles, represented by collections of polymorphonuclear leucocytes could be seen directly below the stratum lucidum. The second, or vesiculopustular stage saw a continuation of the proliferation and degeneration of this epidermal layer, which resulted in the development of an irregular network resembling the "ballonisante" stage seen in vaccinia lesions. The vesicles, by now considerably larger, became further infiltrated with leucocytes and formed pustules. Continued cellular infiltration of the dermis was also seen. The third, or scab stage was characterised by an enlargement of the pustule, and its rupture through the stratum lucidum, giving rise to a a crust composed of fibrin and cellular debris.

Wheeler and Cawley (1956) in a more comprehensive histological study of experimental lesions reported no changes until the 4th day after inoculation. They described ballooning degeneration in the upper layers of the stratum spinosum, which after a week had adopted a basket weave appearance. This reticular degeneration was seen to give rise to tiny vesicles which by the eleventh day had developed into multilocular pustules that comprised the surface of the lesion. Marked pseudoepitheliomatous hyperplasia was a feature of the underlying epidermis, and the dermis became densely infiltrated with round cells, reticulo-endothelial cells and fibroblasts. By the 17th day the pustule had disintegrated, and the lesion was covered by a parakeratotic and hyperkeratotic crust. This subsequently became thicker, and beneath it a finger like papillomatous appearance was produced by downward growth of the epidermal pegs and upward growth of the dermal papillae. The lesion involuted gradually between the 22nd and 40th day after inoculation.

Abdussalam (1957a) described similar features, but considered the degenerative changes in the epidermis to be reticular rather than "ballonisante" or ballooning. He also recorded the presence of cytoplasmic inclusions 4-8μ in diameter which were not seen by previous workers. Nisbet (1954) also favoured the term reticular degeneration, and recorded involvement of the hair follicles.

Kluge, Cheville and Peery (1972) in a more recent study, detected thickening of the epidermis at 31 hours after experimental inoculation, and markedly ballooned cells were seen between the 55th and 72nd hour. They also reported the presence of eosinophilic inclusions in degenerating cells, and concluded that

they were composed of viral DNA and viral protein, together with cellular debris. Using an immunofluorescent technique, they detected viral antigen as early as 31 hours after inoculation. Ultrastructurally, the early stages of infection were characterised by increased numbers of polyribosomes, and greatest quantities of virions were observed intissues sampled between the 72nd and 143rd hour after infection. Infected cells were severely depleted of organelles and contained virions in various forms which were interpreted as different stages of maturity.

Pospischil and Bachmann (1980) also described ultrastructural changes in experimental orf infection and made similar observations, but in addition recorded filamentous nuclear changes, attributing them to by-products of virus replication.

3.3) Epidemiology

Aynaud (1923) observed that the virus was highly resistant and present in considerable quantities in scab material shed from infected animals, and saw this as being responsible for the recurrence of infections on some holdings. This has given rise to the traditional concept that virus which persists in the environment is the source of new infections (Glover, 1928; Theiler, 1928; Boughton and Hardy, 1934; Belschner, 1950; Buxton and Fraser, 1977; Blood, Henderson and Radostits, 1979; Morin and Baas, 1981; Jensen and Swift, 1982; Watt, 1982). Gaining entrance through small wounds or epidermal abrasions, (Theiler, 1928; Boughton and Hardy, 1934; Jubb and Kennedy, 1970; Buxton and Fraser, 1977; Jensen and Swift; 1982; Watt, 1982), the virus replicates in susceptible animals, from which spread may occur by direct or indirect contact (Blood, Henderson and Radostits, 1979; Morin and

Baas, 1981). Scabs are eventually shed from infected animals, and contribute to the environmental pool of virus (see Robinson and Balassu, 1981).

Results of several workers have however been at variance with this conventional view. Using electron microscopy and complement fixation and immunoprecipitation tests, Romero-Mercado, McPherson, Laing, Lawson and Scott (1973) found that the viral content of scab material decreased with the age of the lesion, and were unable to detect virus in scabs taken within a few days of healing. Boughton and Hardy (1934) questioned the absolute requirement for epidermal damage for viral entry, having observed disease after application of inoculum to unbroken skin, and indeed Walley (1890) produced the first experimental transmission of the disease by application of material from a lesion to unbroken interdigital skin.

3.4) Host Range

Although most commonly seen in the domestic sheep and goat, natural orf infection has been reported in Rocky Mountain bighorn sheep (Ovis canadensis) (Connell, 1954; Samuel, Chalmers, Stelfox, Lowen, and Thomsen, 1975; Lance, Adrian and Widhalm, 1981), Rocky Mountain goats (Oreamnos americanus) (Samuel and others, 1975; Herbert, Samuel and Smith, 1977), musk-oxen (Ovibos moschatus) (Kummeneje and Krogsrud 1978; Dieterich, Spencer, Burger, Gallina and Vanderschalie, 1981; Zarnke, Dieterich, Neiland and Ranglack, 1983), Dall Sheep (Ovis dalli) (Dieterich and others, 1981; Smith, Heinier and Foreyt, 1982; Zarnke and others, 1983), chamois (Rupicapra rupicapra) (Grausgruber, 1964), tahr (Hemitragus emlaicus) (Kater and Hansen, 1962), reindeer (Rangifer tarandus) (Kummenege and Krogsrud, 1979), steenbock (Raphicerus campestris)

(Robertson, 1976) and Japanese serow (Capricornis Crispus) (Okada, Okada, Numakunai and Ohshima, 1984). An apparent outbreak in a pack of foxhounds has also been described, and attributed to the feeding of entire infected sheep carcases (Wilkinson and Prydie, 1970).

Infection in man has been well documented (Hatziolos, 1930; Newsom and Cross, 1934a; MacDonald and Bell 1961; Nagington and Whittle, 1961; Beck and Taylor, 1974; Johannessen and others, 1975) and is associated with direct or indirect contact with sheep and their products. Transmission from person to person is not known to occur (Nagington and Whittle, 1961).

Attempts by many workers to establish experimental orf infection in laboratory animals have given conflicting results. Much early attention was focused on the susceptibility of the rabbit, an animal widely used in the study of poxviruses (Abdussalam, 1957b). Blanc and others (1922), Aynaud (1923), Jacotot (1926), Howarth (1929), Glover (1930) and Boughton and Hardy (1934) all failed to produce lesions on the inoculated skin of rabbits, although Lanfranchi (1925) claimed to have infected the lips of young rabbits, and Selbie (1944) described multiple passages of the virus in this species. Bennett, Horgan and Haseeb (1944) compared rabbit lesions of sheep-pox, goat-pox and orf, and histological features of orf in sheep, rabbits and man were described by Wheeler and Cawley (1956). A report by Abdussalam (1957b) described 22 passages of the disease in rabbits, and claims that material from three of these passages produced typical lesions in lambs. This author found that the breed of rabbit did not affect susceptibility, but a higher dose of virus was required for

successful transmission than is the case with sheep. He was sceptical of Selbie's claim (Selbie, 1944) of experimental transmission to rabbits and attributed it to the dermatophyte Trychophyton gypseum, which he isolated from material supplied by Selbie, and with which he produced a disease in rabbits indistinguishable from that described in the latter's report.

Of other laboratory animals, guinea pig (Aynaud, 1923; Lanfranchi, 1925; Jacotot, 1926; Howarth, 1929; Boughton and Hardy, 1934; Selbie, 1944; Abdussalam, 1957b; Trueblood and Chow, 1963), rat (Aynaud, 1923; Jacotot, 1926; Selbie, 1944) and mouse (Aynaud, 1923; Selbie, 1944, Greig, 1956a; Abdussalam, 1957b; Trueblood and Chow 1963) have consistently proved to be refractory, and Trueblood and Chow(1963) failed to infect hamsters.

Several other species have been shown to be resistant to experimental infection with orf virus. Aynaud (1923) and Jacotot (1926) attempted transmission to the horse, chicken and pigeon without success, and efforts to establish infection in the frog (Aynaud,1923) and hedgehog (Abdussalam,1957b) met with similar failure.

The results of attempts at experimental transmission in cattle have proved to be rather equivocal. Orf does not occur in cattle that are run with sheep (see Robinson and Balassu, 1981), and several workers have failed to transmit the disease to this species (Glover ,1930; Howarth, 1929; Boughton and Hardy, 1934). However, Aynaud (1923), Lanfranchi (1925), Jacotot (1926), Bennet and others (1944) all report experimental lesions in cattle.

More recently, Zarnke and others (1983), succeeded in infecting a moose calf (Alces alces), and Lance, Hibler and DeMartini (1983) transmitted the disease to mule deer (Odocoileus hemionus), white tailed deer (Odocoileus virginianus), pronghorn (Antilocapra americana), and wapiti (Cervuselaphus nelsoni).

4) IMMUNITY

4.1) Active Immunity

4.1.1) Natural

The early investigators of orf were satisfied that recovery from the disease was accompanied by an immunity to reinfection, although reports on the duration of this immunity vary. Altara (1925) considered that it might disappear after five to eight months. Jacotot (1926) found that immunity following natural infection was still absolute after two and a half years, although experimentally infected animals were susceptable after one year, and Glover (1928) concluded that experimental disease in lambs conferred a high degree of immunity which lasted at least eight months. Seddon and Belschner (1929) found that animals in Australia which had recovered from natural or experimental infection were thereafter refractory to challenge with homologous or any Australian strain of virus. Manley (1934) although recording a considerable degree of protection following experimental infection, was unable to obtain a solid immunity, and Boughton and Hardy (1934) concluded that while artificial infection protected most animals against the naturally occurring disease, some were not immune to experimental challenge.

Aynaud (1921) claimed that experimental infection of sheep conferred an immunity which lasted at least nine months, and subsequently (Aynaud 1923) described the development of this immunity. He found that absolute protection against reinfection did not occur until the 20th day after inoculation, and that it was possible to reinfect animals up to the 15th day. However, these superinfections resulted in rapidly developing lesions which could heal within ten days. He demonstrated that although not manifest until the 20th day, immunity was acquired early in the evolution of the lesion; amputation of a lesion on the 5th day did not interfere with the subsequent development of immunity. Glover (1928) repeated much of Aynaud's work on immunity and came to similar conclusions, although he found that absolute protection had developed by the 15th day of infection and lasted for at least eight months. He also observed that immunity was not confined to the site of the lesion, inoculation of a thigh resulting in protection of the lips or opposite thigh. Seddon and Belschner (1929) confirmed this, reporting that vaccination of the inguinal area resulted in protection at the muzzle, and Boughton and Hardy (1934) felt that immunity spread gradually through the skin from the site of inoculation.

The results of several workers indicate that immunity in a previously infected animal does not necessarily preclude the development of lesions following subsequent challenge. Boughton and Hardy (1934) reported that although vaccinated animals were protected against the natural disease, some could be infected by rubbing the virus into scarified skin. They found however that such lesions progressed to the pustular stage only, and healed within eight or nine days. Hart and others (1949) observed that

challenge of vaccinated animals resulted in lesions which healed in ten to thirteen days, and Nisbet (1954) noted an accelerated reaction in some animals after rechallenge. Osman (1976) compared the development of lesions in susceptible and previously infected sheep and found that 234 out of 247 sheep (94.7%) displayed what he termed an accelerated response in which lesions had resolved within two weeks. The remaining 13 sheep (5.3%) were refractory to reinfection. The accelerated response was associated with viral multiplication and not thought to be simply a manifestation of delayed-type hypersensitivity, as had been reported in smallpox. Maeda (1979) found that animals recovered from orf infection reacted to challenge despite the presence of specific antibody.

It is probable therefore, that solid protection against reinfection is rare, and that in the majority of recovered animals, immunity is manifested in lesions which develop and heal more rapidly.

4.1.2) Vaccination

Aynaud (1923) investigated the use of vaccination to control the natural disease. He used a 1% emulsion of scab material in 50% glycerine in saline, which he rubbed into scarifications made in the skin of the medial thigh, and found that vaccinal lesions were always mild and showed no tendency to generalise. Although his study appears to have been poorly controlled, he concluded that vaccinated animals were well protected against natural challenge, and further observed that vaccination of a flock which was already infected could reduce the duration of disease from five or six to three weeks. Moussu (1923) also favoured vaccination in the face

of an outbreak, and Carre (1932) felt that the primary indication for vaccination was in lambs before they moved to other holdings for fattening.

Despite the caution of Howarth (1929) against the use of vaccination in a disease which was only seen in a mild form in the USA, Boughton and Hardy (1934) described experiments with vaccination which began in the spring of 1932. They confirmed the observation of Aynaud (1923) that secondary experimental inoculations made during the development of the primary infection resulted in mild lesions of short duration. They further observed that the second infection reduced the duration of the disease resulting from the first inoculation, and suggested that vaccination of infected animals might have some therapeutic benefit. Only a mild form of disease was seen to occur in 30 (0.38%) of 7,884 vaccinated lambs, while 6,667 (65.53%) of 10,173 control lambs developed severe infection. The following year (Boughton and Hardy, 1935) they claimed the successful vaccination of over two and a half million lambs and kids, and suggested that since infectious scab is shed into the environment by vaccinated animals, vaccination should be confined to holdings where the disease was already present.

Glover (1935) confirmed the usefulness of vaccination as a means of protecting sheep in Britain, but experienced more disease in vaccinated animals than was reported by other workers, although lesions were less severe than in controls. He advised that the use of an unattenuated vaccine in very young lambs should be avoided due to the possibility of severe lesions, although Aynaud (1923) had vaccinated newborn lambs without ill effect. Since then, Kerry

and Powell (1971) have reported on trials which indicate that lambs can be vaccinated effectively as early as 24 hours after birth.

Hart and others (1949) describe the use of vaccinations in Australia using a 0.1% emulsion of scabs in 50% glycerine in saline, and in over 50,000 vaccinations achieved results that were "all that could be desired".

Glover (1935) found that attempts to inactivate the vaccine using heat, formalin, irradiation or neutralisation with serum rendered it useless, and the vaccines used today are unattenuated and produced from sheep lesions in essentially the same manner as that described by Aynaud in 1923 (Association of the British Pharmaceutical Industry, 1985). Trials with alternative vaccines using virus derived from tissue culture have been successful, but at present their cost of production cannot compete with the traditionally prepared vaccine (Robinson, 1982).

4.2) Passive Immunity

Opinion has been divided as to whether lambs derive any maternal immunity of orf virus infection from their dams. Boughton and Hardy (1934) concluded that the lamb receives no protection from an immune mother, either transplacentally or in colostrum. Glover (1935) found that lambs born to immunised ewes were fully susceptible to experimental infection, but reported that the progeny of ewes vaccinated during pregnancy developed less severe natural lesions than lambs from unprotected dams. In contrast, Romero-Mercado (1969) demonstrated precipitating and complement fixing antibodies in colostrum from four out of six ewes, and

Poulain, Gourreau and Dautigny (1972) demonstrated significant titres of neutralising antibody in serum from lambs which had received colostrum from immunised ewes. Similarly Le Jan, L'Haridon, Madelaine, Cornu and Asso (1978) found a correlation between the neutralising antibody level in the serum of ewes and that of their lambs, but none of these authors provide evidence that these colostral antibodies confer protection against natural or experimental infection.

4.3) Mechanisms of Immunity

4.3.1) Humoral Responses

Aynaud (1923), using a crude neutralisation assay assessed by animal inoculation, was unable to demonstrate any specific activity in serum from immune animals, and concluded that protection was due to a tissue rather than humoral immunity. However, Glover (1933) using a similar assay, succeeded in demonstrating neutralising activity in serum from hyperimmune lambs, and to a lesser extent in convalescent sera. He found that the type of antigen preparation employed in the test was important and used an autolysate of scabs. He also demonstrated complement-fixing activity, but failed to detect specific antibody using the precipitin test. Manley (1934) also failed to detect precipitating antibodies, and found the complement fixation test (CFT) unsatisfactory, but demonstrated neutralising activity in both hyperimmune and convalescent sera. Rottgardt, Aramburu and Pirazzi (1949) reported detectable levels of complement-fixing antibodies in sera from infected sheep, and MacDonald (1951) found the test useful in the diagnosis of human infection. Nisbet (1954) found that sera taken from lambs between 15 and 60 days after experimental infection contained negligible

amounts of complement-fixing antibody, but Abdussalam (1958) demonstrated fixation with immune and convalescent sera from sheep, rabbits and man. He also detected neutralising and flocculating antibodies but considered the agglutination test to be the most appropriate serological diagnostic tool, since agglutinating antibodies were present to moderately high titres in sera from both immune and recovered sheep rabbits and humans.

Following the successful cultivation by Greig (1957) of orf virus in tissue culture, Plowright, Witcomb and Ferris (1959) reported variable results using a neutralisation test assayed in vitro, and Nagington and Whittle (1961) found neutralising activity in convalescent serum from sheep and humans. Since then, in vitro neutralisation assays have been used by Trueblood, Chow and Griner (1963), Poulain and others (1972), Precausta and Stellman (1973), Osman (1976) and Le Jan and others (1978).

Trueblood and others (1963) failed to detect antibodies in sera from convalescent sheep using the Ouchterlony double diffusion and tube precipitin tests, and the CFT. However, Romero-Mercado (1969) was able to demonstrate the presence of precipitating and complement fixing antibodies in convalescent and post-vaccinal sera; the majority of animals were producing antibodies detectable using both methods by the fourth week after infection. Complement-fixing antibodies persisted longer than precipitating antibodies, which had disappeared by the sixteenth week. Only one line of precipitation was seen following primary infection, but sera from some animals showed an additional line following secondary challenge. Frerichs (1980) found the double diffusion test of limited use for diagnostic purposes, and reports inconsistent results using an in vitro serum neutralisation assay.

DeMartini, Pearson and Fiscus (1978) described a ^{51}Chromium release assay for complement-mediated cytotoxicity in orf infected cell cultures as a method for the detection of virus specific serum antibody, and found that it was sensitive provided that its many inherent variables were controlled. Koptopoulos, Reid and Pow (1982) used a similar assay but could detect specific antibody only in experimentally infected animals. They also used the CFT, enzyme-linked immunosorbent assay (ELISA), and an indirect immunofluorescent antibody assay, and found the latter the most sensitive of all four methods.

Despite the fact that virus specific antibodies are detectable by a variety of methods in the serum of previously infected animals, there is evidence which suggests that they may not be entirely protective. Aynaud (1923) failed to protect a lamb against challenge 24 hours after intravenous administration of 240 ml of serum from two immune animals. Trueblood and others (1963) found that virus-antiserum mixtures were infective for lambs even though they did not produce cytopathic effect in tissue culture, and Osman (1976) failed to affect the subsequent development of an orf lesion in a lamb by the intravenous administration of hyperimmune serum immediately before inoculation.

4.3.2) Cellular Responses

The hypothesis of Aynaud (1923) that protection against re-exposure to orf infection is due to a tissue immunity rather than a humoral one, was largely ignored until Osman (1976) attempted to induce an accelerated response in two susceptible lambs by the inoculation of spleen and thymus cells from recently recovered animals. One lamb received 22×10^7 spleen cells

intravenously and $11x10^7$ intraperitoneally, while the other received similar quantities of thymus cells. The lambs were then challenged by scarification, but produced lesions which were typical and no different from those of controls. Maeda (1979), using indirect macrophage migration inhibition tests, direct leucocyte migration inhibition tests and lymphocyte transformation tests, demonstrated the involvement of cell mediated immune responses in sheep exposed to orf virus, and claims to have transferred a degree of immunity to susceptible lambs using sensitised lymphocytes. The results however were not conclusive, and are open to criticism on the grounds of histocompatability.

5) THE AGENT

5.1) Structure

Early observers of orf in sheep believed that the agent responsible was the "bacillus of necrosis". However, Aynaud (1921) attributed the disease to a specific virus, which he later demonstrated to be filterable (Aynaud, 1923). Filterability of the agent had also been reported by Zeller (1920) and Blanc and others (1922), and was subseqently confirmed by Jacotot (1926), Glover (1928), Howarth (1929) and Newsom and Cross (1934b). The use of the electron microscope in later years allowed more precise assessment of the size of orf virus. Abdussalam (1953) found monomorphic short rods with rounded ends and a mean size of 251.8nm x 158.18nm, while Ishii, Kawakami and Fukuhara (1953) reported elipsoidal particles of diameter 200-250nm. Nisbet (1954) described the virus as coccobacillary in shape with a mean size of 270nm x 163nm, and Abdussalam and Cosslett (1957) demonstrated by means of frequency distributions of dimensional measurements that

the virus was monomorphic and showed no evidence of division by simple fission.

It was not however until the application of negative staining techniques by Nagington and Horne (1962) that a clear impression of the structure of orf virus could be attained by electron microscopy. They described two forms of the virus, one having a woven pattern formed by diagonally running threads or tubules, and the other being an incomplete form without tubules. Average measurements of complete particles were 263.1nm x 157.4nm, and were in agreement with those of Abdussalam and Cosslett (1957). Subsequent reports established that the woven pattern was due to the superimposition of the images on the upper and lower surfaces of the virus of a spirally wound thread, which was probably protein in nature (Nagington, Newton and Horne, 1964; Buttner, Geise, Muller and Peters 1964). Incomplete forms were not described in these reports but different images of the virus could be produced by altering the pH conditions during negative staining. Acid pH was associated with the characteristic image with woven pattern, while alkaline conditions allowed penetration of the stain to reveal an internal structure composed of a triplet of strands surrounded by a heavily contrasted matrix zone. This structure was believed to contain the nucleic acid of the virus, which was DNA and calculated by chemical analysis to be present in quantities of about 2.85×10^{-16}g per particle, equivalent to a molecular weight of 171×10^{6}.

Mitchiner (1969) studied the structure of orf virus using cytochemical degradation methods and concluded that the internal structure was surrounded by an inner envelope consisting of protein

subunits covered by lipid and phospholipid. This was in turn covered by a lipid-protein external envelope, the residual space being occupied by cholesterol.

Robinson, Ellis and Balassu (1982) analysed the nucleic acid of orf virus using bacterial restriction endonucleases, and concluded that the DNA in virions was a linear molecule with a molecular weight of 88.8×10^6 and a guanine-cytosine (G + C) content of 64 percent.

5.2) Classification

Aynaud (1923) concluded that the agent responsible for orf infections had many biological characteristics in common with vaccinia virus. Walley (1888) had earlier likened the mammary lesions of orf in ewes to those of sheep-pox, and Theiler (1928), Seddon and Belschner (1929), Glover (1930), and Boughton and Hardy (1934) all classified the disease in the vaccinia-variola group. Morphological studies by electronmicroscopy supported an association with pox viruses (Abdussalam and Cosslett, 1957) and Fenner and Woodroofe (1960) demonstrated the ability of orf virus to reactivate heat inactivated members of the pox virus group, and considered that this characteristic justified its inclusion in the group.

Nagington, Plowright and Horne (1962) observed that particles of bovine papular stomatitis virus (BPS) were morphologically extremely similar to those of orf, and Friedman-Kien, Rowe and Banfield (1963) reported that the causal agent of milker's nodules in man was a poxvirus of similar structure to orf and BPS viruses. Peters, Muller and Buttner (1964) were unable to distinguish

between BPS and orf viruses on morphological grounds, and proposed that they be classified along with the virus of milker's nodules as "paravaccinia viruses". However, Nagington, Tee and Smith (1965) considered this inappropriate and proposed the term "orf group", which would also encompass the virus of chamois contagious ecthyma.

Although Huck (1966), Papadopoulos, Dawson, Huck and Stuart (1968) and even Nagington (1968) referred to the group as paravaccinia viruses, the International Committee on Taxonomy of Viruses now describe the viruses of orf, BPS, chamois contagious ecthyma and milker's node as the orf subgroup of the poxvirus group, under the international heading of "parapox virus" (Matthews, 1982). Parapox virus infections have also been described in pinnipeds (Wilson and Poglayen-Neuwall, 1971) and red squirrels (Sands, Scott and Harkness, 1984).

5.3) Resistance

Early work on the nature of orf virus established its marked resistance to dessication (Aynaud, 1923; Glover, 1928; Theiler, 1928), and subsequent reports have confirmed its considerable durability. Hart and others (1949) demonstrated that dried scabs retained their infectivity after storage for over 15 years at room temperature in the laboratory, and Livingston and Hardy (1960) produced typical lesions on lambs using an inoculum prepared from scab material which had been stored at 45°F for almost 23 years. However, the ability of the virus to survive outdoors may be somewhat less. Manley (1934) found that crusts exposed on the ground in Cyprus for 10 days during May lost their infectivity, although after exposure for 12 days in the shade of a large bush, similar material was still infectious. He concluded that sunlight

was the inactivating factor. Boughton and Hardy (1935) reported that virulent scab exposed to the conditions of the Texas summer lost its potency between 30 and 60 days despite being shaded from direct sunlight for most of the day. Winter conditions were not as virucidal however, and scab exposed from September to April retained their infectivity.

Several workers have investigated the effects of heat on infectivity. Aynaud (1923) considered that the critical temperature for the virus was 60°C although Manley (1934) found exposure to 54°C for 15 minutes to be lethal. Boughton and Hardy (1935) and Sawhney (1972) inactivated the virus by heating in a waterbath at 60°C for 30 minutes, and it is this value which has come to be accepted as the thermal death point of the virus (Buxton and Fraser, 1977). However, repeated freezing and thawing of orf virus suspensions do not seem to reduce their infectivity (Plowright, Witcomb and Ferris, 1959; Sawhney, 1972) and titres would appear to be similarly unaffected by ultrasonic treatment (Sawhney, 1972).

Reports on the susceptibility of orf virus to ether and chloroform have not been consistent. Aynaud (1923) found that viral infectivity was maintained after many hours contact with these agents, while Glover (1928) states that they both have a slow inactivating effect on the virus. Seddon and Belschner (1929) claim to have destroyed infectivity with chloroform and ether, but Trueblood and Chow (1963) reported that exposure to a 20% concentration of ether for 4 hours at 4°C did not affect the titre of a suspension of orf scab material. Precausta and Stellmann (1973) found that the strains of orf virus which they studied were

extremely sensitive to chloroform while only partially sensitive to ether, and this probably reflects the true situation; Buxton and Fraser (1977), indicate that the virus is inactivated by chloroform, and is only slightly sensitive to ether. The inconstancy of these reports can probably be attributed to variation in the test conditions employed by different workers.

A preoccupation of many workers with the effects of glycerin on the infectivity of orf virus has produced equally variable results. Although Theiler (1928) felt that the virus could be conserved for months in 50% glycerin, Glover (1928) and Manley (1934) recorded that this treatment resulted in a rapid loss of potency. In contrast, Boughton and Hardy (1934) considered that scabs emulsified in a 50% solution of glycerin in normal saline remained virulent for at least one year at 14°C. In the light of these equivocal results, it is interesting to note that the British Pharmacopoeia (Veterinary, 1985) recommends that the orf vaccine diluent should contain 25% of glycerol.

5.4) Strain Variation

Early investigations using cross-immunity experiments on isolates of orf virus from sheep from differed parts of Britain suggested that only one strain was involved (Glover, 1928) and subsequent studies suggested that strains from America and France (Glover, 1930) and Australia (Seddon and McGrath, 1931) were indistinguishable from the British virus. Field observations by Schmidt and Hardy (1932) suggested the existence of different strains affecting sheep and goats, and although Glover (1933) considered a caprine strain from Tanganyika to conform to the British type, he was unsure as to the relationships of a goat

isolate from Cyprus. Horgan and Haseeb (1947) carried out a series of cross-immunity tests using ovine and caprine strains from England and Cyprus, and concluded that there existed several strains of orf virus, some of which were immunologically identical and others which were closely related. They found no evidence of a relationship between strain difference and animal host, and felt that variation was most likely to be due to differences in quantity of one or more antigens. Sawhney (1966) concluded from his experiments with cross-immunity and serum neutralisation tests that isolates from England and Bulgaria were antigenically distinct from Rumanian and Czeckoslovakian strains, and Precausta and Stellman (1973) demonstrated that five isolates of the virus had similar resistance to various physicochemical agents but could be divided on the basis of serum neutralisation into two antigenic subtypes.

Using restriction endonuclease analysis to examine the DNA of six BPS and three orf isolates, Wittek, Herlyn, Shumperli, Bachmann, Mayr and Wyler (1980) found that the restriction profiles of the orf isolates were clearly distinct from those of BPS, but were themselves surprisingly heterogenous. Robinson, Ellis and Balassu (1981) used the same technique to examine 36 orf isolates in New Zealand, finding 22 different restriction patterns, and suggested that this genomic variation might be associated with antigenic differences.

Buddle (1981) failed to group orf isolates using serum neutralisation tests, but in an analysis of structural polypetides using gel electrophoresis he found that 11 isolates could be classified into 4 groups on the basis of differences in polypeptide profiles. In addition, he identified the polypeptides which

differed among groups as constituents of the surface component of the virion.

Evidence for the existence of strains of orf virus which differ at a genetic and structural level is therefore strong, and this heterogeneity appears to be reflected in antigenic differences. However, although Hardy (1964), and Beck and Taylor (1974), attributed vaccine breakdowns to the challenge with novel strains, the epidemiological significance of such variation has yet to be assessed.

5.5) Immunlogical Relationships

In order to resolve the question of whether orf merely represented an adaptation of vaccinia virus to affect goats and sheep, Aynaud (1923) carried out cross-immunity tests on three sheep and a calf, which suggested that the two viruses were unrelated. Similar tests carried out by Blanc and others (1922), Lanfranchi (1925), Jacotot (1926), and Glover (1930) all reinforced the belief that the viruses, although related, were immunlogically distinct. MacDonald (1951), using the CFT was unable to demonstrate a serological relationship between orf and vaccinia viruses, and Abdussalam (1958) reported similar results, adding that orf antiserum fails to inhibit vaccinia haemagglutination. In contrast, Webster (1958) demonstrated that orf virus shares complement-fixing antigens with vaccinia and also reported neutralising and precipitating antigens in common. However, Huck (1966), and Papadopoulos and others (1968) failed to detect a common antigenicity using gel diffusion tests.

There has been considerable interest in the relationship between orf virus and the viruses of sheep-pox and goat-pox. Blanc and

others (1922) were satisfied on the basis of cross-immunity studies that orf and sheep-pox were caused by different agents, and Theiler (1928) was similarly convinced. Glover (1930) was unsure whether such a distinction could be drawn between the viruses of orf and goat-pox, but Manley (1934) found no evidence of cross-immunity between the two viruses, and in addition failed to neutralise orf virus with goat-pox immune serum. However, cross-immunity tests carried out by Bennett and others (1944) on experimental goats indicated that goat-pox conferred protection against orf infection, but the reverse was not true. They concluded that the two viruses were immunlogically related, but that the goat-pox agent possessed at least one more antigenic component than orf.

Although Sharma and Dhanda (1971) were unable to demonstrate cross immunity, and failed to find cross-reactivity using gel precipitation tests, they concluded that orf virus shares complement-fixing antigens with sheep-pox and goat-pox. In a more recent study however (Renshaw and Dodd, 1978) the results of serum neutralisation tests and cross immunity studies indicate that isolates of goat-pox and orf from the USA are antigenically distinct. These authors attribute the divergence of reports on antigenic relationships between the two viruses to the existence of a multiplicity of strains of each agent. The viruses of sheep-and goat-pox and that of orf are presently classified as two separate genera of the family Poxviridae, Capripoxvirus and Parapoxvirus (Matthews, 1982).

In view of morphological similarities of the viruses of orf, BPS, milker's nodule and pseudocowpox (Nagington and others 1962; Friedman-Kien and others, 1963; Peters and others, 1964), it is not

surprising that close immunological relationships have been demonstrated between the members of the orf subgroup of the Poxvirus family. Huck (1966) reported that a pseudocowpox isolate was neutralised by orf antisera in tissue culture, and in gel diffusion tests identified an antigen common to the two viruses. Papadopoulos and others (1968) confirmed the existence of this common antigen and concluded that it was specific to the paravaccinia subgroup. Wittek and others (1980) carried out cross neutralisation tests on six BPS and three orf virus strains, and report extensive serological cross reactivity between all strains.

The agent responsible for ulcerative dermatosis of sheep would also appear to be closely related, if not identical to orf virus. The term was orginally proposed by Tunnicliffe (1949) to cover a group of skin diseases previously described under various names such as lip and leg ulceration, posthitis, balanoposthitis and ulcerative vulvitis, and a filterable virus has been demonstrated to be responsible (Tunnicliffe and Matisheck, 1941). Crossimmunity studies carried out by Trueblood and others (1963) indicated that the two viruses were immunologically distinct, but a study of their physicochemical properties which included filtration, ether sensitivity, susceptibility of experimental animals and behaviour in tissue culture failed to differentiate between the two agents (Trueblood and Chow, 1963). Subsequent investigations (Trueblood, 1966) revealed that each of the viruses was neutralised <u>in vitro</u> by homologous or heterologous rabbit sera, although ovine immune serum did not neutralise infectivity for tissue culture. A relationship was also indicated by the agar precipitin test although the orf virus isolate was seen to contain an antigenic component not present in the agent of ulcerative dermatosis. The author

concluded that the two conditions might be caused by different antigenic variants of the same virus. However, the controversy concerning these two agents remains unresolved, and Andrewes, Pereira and Wildy (1978) list the agent of balanoposthitis of sheep as a separate parapoxvirus.

The foregoing account describes the published literature relating to the topics under study at the time of the initiation of this project.

CHAPTER TWO

LYMPHATIC CANNULATION STUDIES

INTRODUCTION

Aynaud (1923), having failed to demonstrate neutralising activity in sera from immunised animals, concluded that a tissue immunity rather than a humoral one was responsible for recovery and protection from orf virus infections in sheep. However, most subsequent investigations of immunity in orf have been directed at the measurement of specific antibody responses (Robinson and Balassu, 1981), although Maeda (1979) provided some evidence for the involvement of cell-mediated immune mechanisms.

Orf virus is highly epitheliotropic (Aynaud, 1923) and experimental lesions remain localised at the site of inoculation (Aynaud, 1923; Glover, 1928; Schmidt and Hardy, 1932; Nisbet, 1954). There is no evidence of a naturally occurring viraemia (Glover, 1928; Morin and Baas, 1981; Bostedt, 1985), although the production of experimental orf lesions by scarifying the skin after intravenous inoculation of a viral suspension has been reported (Maeda, 1979). It is surprising therefore that current knowledge of immunity in orf virus infection of the sheep is confined to systemic responses.

After gaining access to the body, foreign antigens or microorganisms rapidly enter the lymph stream along a pressure gradient which exists across the walls of lymphatic vessels, and because of this, the majority of naturally occurring immune responses are initiated in the lymph node which drains the entry point of the invading agent (Morris, 1972). The events surrounding the initiation of nodal responses in sheep may be analysed using the lymphatic cannulation techniques described by Lascelles and Morris (1961), and such methods have been used by a number of workers to study the responses of several lymph nodes to a variety of antigens (Table 2.1).

Table 2.1. Previously described responses in antigen-stimulated ovine lymph nodes.

Immunising Agent	Cannulated Lymph Node	Reference
Dinitrofluorobenzene (DNFB)	Popliteal	Hall and Smith, 1971
Oxazalone	Prefemoral, Popliteal	Hall, Hopkins and Reynolds, 1980
Dinitrophenylated bovine serum albumin (DNP-BSA)	Popliteal	English, Morris and Adams, 1977
Ovalbumin	Popliteal, Hepatic, Coeliac, Intestinal	Beh and Lascelles, 1981
Human Serum Globulin	Popliteal	Hall and Morris, 1963
Swine Influenza Virus	Hepatic	Smith and Morris, 1970
"	Popliteal	English, Morris and Adams, 1977
Salmonella typhi (Killed)	Popliteal	Hall and Morris, 1963
"	Popliteal, Lumbar, Prefemoral	Hall, Morris, Moreno and Bessis, 1967
Salmonella Lipopolysaccharide	Popliteal	English, Morris and Adams, 1976
Ostertagia circumcincta	Gastric	Smith, Jackson, Jackson and Dawson, 1981
Chicken Red Blood Cells	Popliteal	Hall and Morris 1963
Human Red Blood Cells	Popliteal, Lumbar Prefemoral	Hall, Morris, Moreno and Bessis, 1967
Renal Homografts	Cervical	Pederson and Morris 1970
Skin Homografts	Prefemoral	Hall, 1967b
Ovine Squamous-Cell Carcinoma Cells	Popliteal Cervical	Al-Yaman and Willenberg 1985

Issekutz (1984) studied lymphocytes in efferent lymph from the popliteal nodes of sheep after the injection of live vaccinia virus into the drainage area, and demonstrated their ability to lyse homologous skin fibroblasts infected with vaccinia virus. This activity peaked on the 7th day after inoculation, and because it was allogeneically restricted, was attributed to cytotoxic T lymphocytes. Pearson, DeMartini and Fiscus (1979) investigated the alterations in cell populations and antibody titres which occurred in efferent popliteal lymph following the inoculation of orf and milker's nodule viruses into the drainage area, and noted a 15-25 fold increase in blastic lymphocytes by 72-168 hours after inoculation, and a specific antibody response which peaked after 120 hours. It is not clear however whether this brief report deals with the nodal response to orf virus infection of its drainage area, or merely to the presence of non-replicating viral antigens.

The cells of afferent lymph are of great interest in the study of the initiation of immune responses, since they are the cells which first come into contact with foreign antigen (Issekutz, Chin and Hay, 1980). Afferent lymphatic cannulation techiques were first used in the analysis of cell output from inflamed tissue by Smith, McIntosh and Morris (1970b), who recorded an increase in lymphocyte output following the induction of granulomata by the subcutaneous injection of antigens with Freund's adjuvant. Since then, it has been established that this increased output is due to the preferential migration of lymphocytes into inflammatory sites, rather than to local proliferation (Issekutz, Chin and Hay, 1980, 1981) and that this migratory discretion is largely confined to small thymus derived (T) lymphocytes (Issekutz, Chin and Hay, 1982).

In view of the of volume work which has been carried out on immune mechanisms in sheep using lymphatic cannulation techniques (Trnka and Cahill, 1980), it is surprising that there are no published reports on their application to the elucidation of the mechanisms involved in the immune response of the sheep to an orf lesion. It was therefore considered appropriate to employ these techniques to characterise the changes occurring in lymph which is entering and leaving a peripheral node during infection of its drainage area with orf virus.

MATERIALS AND METHODS

Animals

Initial cannulation experiments were performed on Scottish Blackface lambs aged between 6 months and one year. These animals proved unsatisfactory, in that they did not react well to confinement in metabolism crates, and tended by struggling to avulse their cannulas. Acclimatisation periods of up to 10 days in the crates did not alter this behaviour. All subsequent experiments were carried out on adult female sheep of various breeds (summarised in Table 2.2). Animals were confined indoors in metabolism crates for one week before surgery, and thereafter until the experiment was ended, and were fed a maintenance ruminant ration supplemented with concentrates. Water was available *ad libitum*. Food was withheld for 12 hours before surgery.

Table 2.2. Experimental sheep employed in lymphatic cannulation studies.

Sheep No.	Breed.
129	Merino
131	Merino
138	Merino
139	Merino
161	Suffolk
443	Dorset
764	Dorset
1269	Dorset
1272	Greyface
1310	Greyface
1313	Greyface

Anaesthesia

Anaesthesia was induced by inhalation of a gas mixture of 4% Halothane $N_2O:O_2$ (1:1) using a rubber face mask. As soon as the animals were sufficiently relaxed, an endotracheal tube was passed, and anaesthesia was maintained with 2% halothane using a Magill attachment. Before surgery, the operative site was clipped, thoroughly scrubbed, and treated with a proprietary chlorhexidine solution.

Efferent lymph preparations

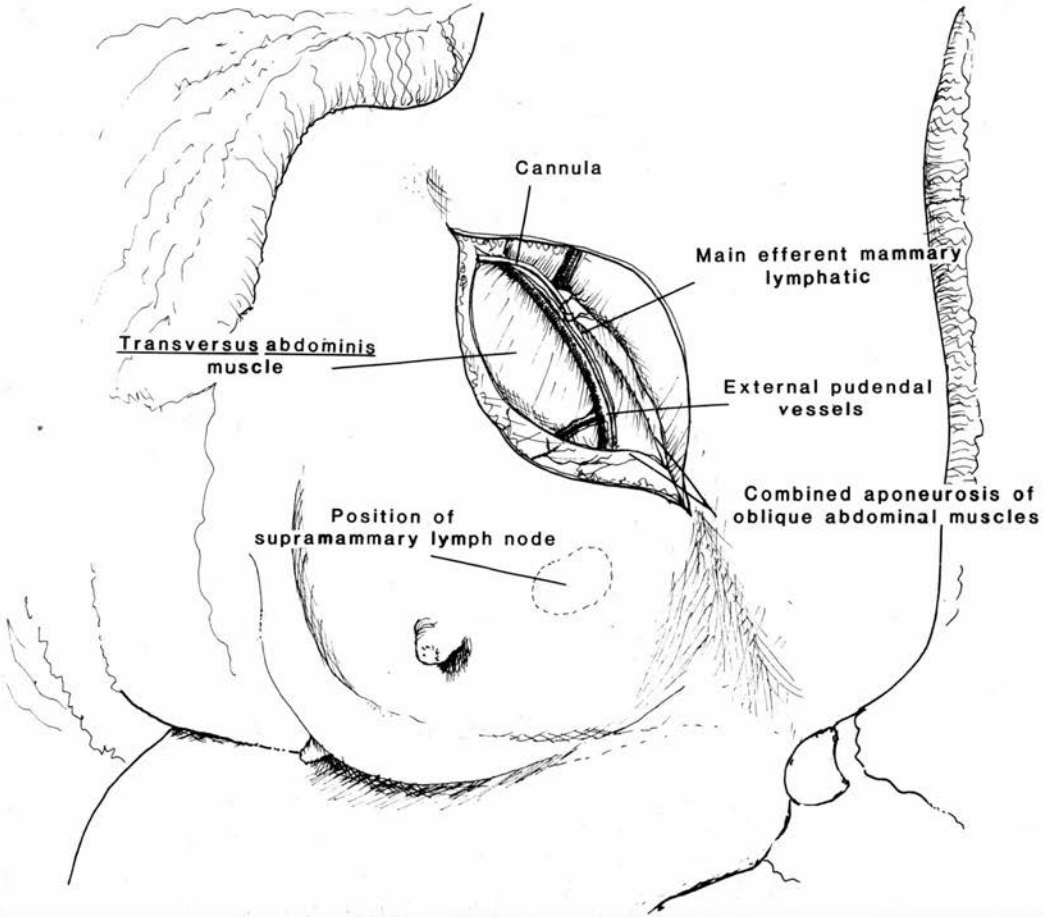
Efferent Popliteal Lymphatic Cannulation

Early attempts using the efferent popliteal lymphatic cannulation technique described by Hall and Morris (1962) proved unsatisfactory for reasons which are discussed later. The surgical technique will therefore not be described.

Efferent Supramammary Lymphatic Cannulations (Fig. 2.1)

Mammary lymph duct fistulae were created as described by Lascelles and Morris (1961). The animal was held in lateral recumbancy with its uppermost hind limb abducted. An incision approximately 12cm in length was made in the skin over the inguinal canal, and the loose connective tissue which lies between the abdominal wall and the medial aspect of the thigh was separated by blunt dissection. The pudendal blood vessels could then be seen through the combined aponeurosis of the external and internal oblique abdominal muscles, passing cranially towards the deep inguinal ring. An incision of about 4cm was made in the aponeurosis over the pudendal vessels, and the main mammary lymph

Fig. 2.1. Surgical approach to the efferent supramammary lymphatic duct of the sheep.


Cannula
Main efferent mammary lymphatic
Transversus abdominis muscle
External pudendal vessels
Combined aponeurosis of oblique abdominal muscles
Position of supramammary lymph node

duct could then be seen running in association with them. Additional smaller lymphatics were sometimes present along the course of these vessels, and occasionaly a further duct was seen running independently along the dorso-lateral aspect of the mammary gland. The main lymphatic was dissected free and ligated with silk, and any other ducts which were present were tied off.

Lengths of medical grade polyvinyl chloride (PVC) tubing (Dural Plastics Engineering Ltd., New South Wales, Australia.) of external diameter 0.80-0.96mm were employed as cannulas. Before use, these were sterilised by immersion in a proprietary chlorhexidine solution, and rinsed and repeatedly flushed with sterile physiological saline (0.9% NaCl in distilled water). The last flush which contained 100 international units (IU) heparin per ml, was held in the cannula by clamping one end with an artery forceps, and the free end was threaded subcutaneously to the operation site from a stab wound just ventral to the tuber coxae using a 15cm surgical steel needle.

A second ligature was laid around the duct 1cm distal to the first, and using iris scissors, a transverse incision was made in the vessel between these ligatures. The cannula was inserted into the duct through this incision for a distance of 2-3mm (against the flow of lymph), and the distal ligature was tightened around it to hold it firmly in place. The clamp was then released from the opposite end of the cannula so that lymph flow could be verified, and the free ends of the proximal ligature were used to further secure it in position. No further stay sutures were required. After the establishment of lymph flow, 0.25ml of 0.1% Evans blue dye in Ca^{++} free phosphate buffered saline (PBS) was injected

subcutaneously at the proposed site of infection on the skin of the mammary gland, to ensure that this site was indeed drained by the supramammary lymph node.

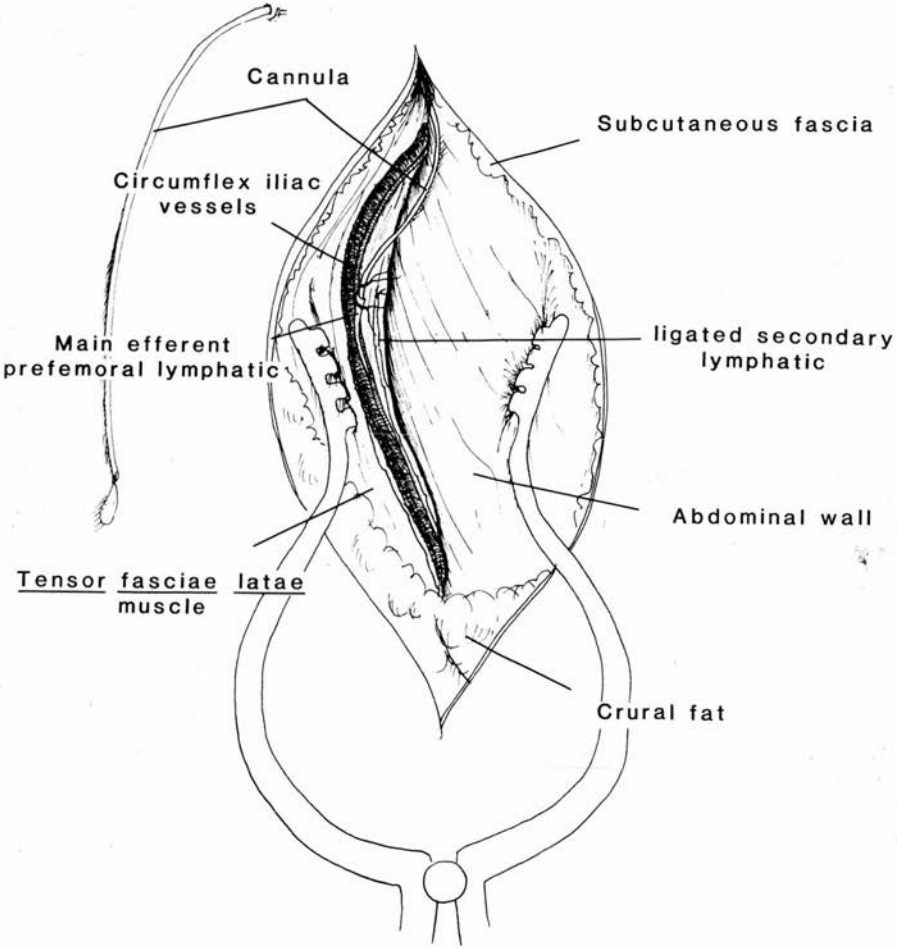
The combined aponeurosis of the internal and external oblique abdominal muscles was repaired with interrupted horizontal mattress sutures of 2-0 catgut, and the skin and subcuticulum were closed using vertical mattress sutures of 2-0 nylon. Collection bottles in these preparations were held in pockets of fabric surcingles secured around the abdomen.

Afferent Lymph

Afferent lymph for this study was derived from cannulations of the efferent prefemoral lymphatic duct performed 8 weeks after surgical removal of the node. The extraction of the node, which lies superficially in a subcutaneous fat pad on the cranial border of the tensor fasciae latae muscle, was a simple procedure performed under general anaesthesia and will not be described.

Cannulation of the efferent prefemoral lymphatic was performed as described by Hall (1967a). With the animal in lateral recumbancy, an incision 12cm in length was made through the skin and subcutaneous muscle along the anterior border of the thigh, starting just below the tuber coxae. The tensor fasciae latae muscle was exposed by blunt dissection of the subcutaneous fat, and retracted upwards and backwards to reveal its inner surface. With the muscle held in this position by means of self-retaining retractors, the circumflex iliac blood vessels could be identified on its deep surface, and associated with them, the efferent prefemoral lymphatic duct (Fig. 2.2). Smaller accessory ducts were

Fig. 2.2. Surgical approach to the efferent prefemoral lymphatic duct of the sheep. Prior removal of the lymph node renders this vessel a source of afferent lymph.


Cannula
Subcutaneous fascia
Circumflex iliac vessels
Main efferent prefemoral lymphatic
ligated secondary lymphatic
Abdominal wall
Tensor fasciae latae muscle
Crural fat

sometimes present, and these were tied off before cannulating the main duct. As with the supramammary preparation, the cannula was led to the operation site via a stab wound just below the tuber coxae, and the skin and subcutaneous tissue were repaired as described for the efferent supramammary preparation. Collection bottles were held in the pockets of fabric surcingles secured around the abdomen.

Experimental Protocol

Lymph was collected into polythene bottles containing powdered heparin, penicillin and streptomycin. Short (2-3hr) daily collections and overnight collections were made, and at each interval the volume of lymph was recorded and the flow rate calculated. Lymph collected overnight was used for total cell counts and for the detection of antibody, while the daytime collection was used for cell counts and the preparation of cytocentrifuge smears; cell counts and flow rates were used to calculate hourly cell output. Collections were made from animals with efferent lymphatic cannulations for 2 days before and up to 20 days after infection with orf virus. All afferent lymph samples were, however, collected after inoculation.

Challenge Virus

Animals were infected using an inoculum prepared from a pool of scab material harvested from lambs with experimentally induced orf lesions, ground in sterile sand and stored at -20°C. A 20% suspension of this was prepared in PBS containing 100 IU penicillin and 0.1mg streptomycin per ml, using a mortar and pestle. The suspension was clarified by centrifugation at 1200xg for 30 minutes

at 4°C, and the supernatant fluid was stored at -80°C in 100µl aliquots. Infections were established by application of this inoculum to skin sites which had been abraded with sharp plastic.

Cell Counts

A model ZB1 Coulter counter (Coulter Electronics Ltd., Luton, Bedfordshire UK.) fitted with a 100µ aperture tube was used. The instrument had been calibrated against a suspension of latex beads 12.78µ in diameter, and set so that particles with volumes greater than 45 and 220 fl (μ^3) were counted for determining "total" and "large" cells respectively. Samples were diluted 1:500 in a particle-free balanced electrolyte solution (Isoton II - Coulter, Coulter Electronics Ltd, Luton, Bedfordshire, England) and duplicate readings were taken for "total cells" and "large cells". Counts were taken for both overnight and short daytime collections.

Preparation of Cytocentrifuge Smears

Lymph samples from the daytime collection were diluted in Hanks balanced salt solution containing 1% foetal bovine serum (HFBS) to a concentration of 1 x 10^6 cells/ml. Cells were washed twice in HFBS at 180xg for 6 minutes, and 150µl of cell suspension were loaded into each sample chamber of a cytocentrifuge (Shandon Southern Instruments Ltd., Cheshire, UK.). Smears were prepared at 800 RPM for 5 minutes. For each sample one smear was stained with Leishman's stain, and in the case of efferent lymph, four replicates were fixed for 20 minutes in PBS containing 2% paraformaldehyde, for later assessment of cellular immunoglobulin content.

Preparation of Immunoglobulin Staining Reagents

These reagents were kindly prepared by Mr. A. Dawson, of the Moredun Research Institute. Isotype-specific pig hyperimmune sera were raised by repeated inoculation of purified ovine IgG and IgM. The IgG-specific serum was purified over ovine $F'ab_2$ immobilised on Sepharose 4B (Pharmacia, Milton Keynes, UK.) and immunoelectrophoresis of whole sheep serum with the purified reagent revealed precipitin arcs corresponding to IgG1 and IgG2, but no reaction associated with IgA or IgM. The IgM-specific serum was passed first over ovine IgG immobilised on a Sepharose 4B column, and then over a similar column with immobilised foetal lamb serum. Immunoelectrophoresis of whole sheep serum with this reagent revealed a single precipitin arc corresponding to IgM. Both antisera were then conjugated with horse-radish peroxidase using the method of Wilson and Nakane (1978).

Immunoglobulin Staining of Lymphocytes

Following fixation, smears were washed twice and stored in Tris-salt buffer (0.025M Tris, 0.14M NaCl, pH 7.5, with 0.002% sodium azide) for at least 24 hr. Before staining, endogenous peroxidase activity was removed by treatment with periodic acid/sodium borohydride. Smears were incubated for 1½ hr at room temperature with pig anti-sheep IgG or IgM conjugated with horseradish peroxidase. After washing twice in Tris-salt buffer, peroxidase activity was developed using 4% diaminobenzidine in Tris salt buffer (without sodium azide). Smears were then washed in tap-water and counter-stained in Meyer's haematoxylin before being dehydrated, cleared and mounted. At least 500 cells were counted on each smear and the percentage of stained lymphocytes was

recorded. In the case of Leishman-stained smears, the percentage of lymphoblasts (ie. large cells with basophilic staining cytoplasm) was recorded in efferent lymph preparations, while complete differential cell counts were performed on smears of afferent lymph cells.

Staining of T-Lymphocytes

T-lymphocytes in efferent lymph were identified using the monoclonal antibody T-80 (Miyasaka, Heron, Dudler, Cahill, Forni, Knaak and Truka, 1983), the gift of Dr. M. Miyasaka of the Basel Institute for Immunology, Switzerland. Lymph cells (2×10^6 in 1ml) were washed three times in cold HFBS. The supernatant was carefully removed and the cells resuspended in 100µl T-80 mouse ascites fluid, at a previously determined optimal dilution of 1:2,000 in cold HFBS. This suspension was incubated at 0°C for 30 minutes agitating occasionally. After three further washes in cold HFBS, cells were resuspended in 100µl sheep anti-mouse IgM peroxidase conjugate (Miles Scientific, Slough, England) diluted 1:20 in PBS with 1% bovine serum albumin (BSA) and incubated for a further 30 minutes at 0°C with occasional mixing. Cells were then washed twice in cold HFBS and cytocentrifuge smears prepared from 200µl aliquots. After air drying for 5 minutes smears were fixed for 15 minutes in PBS containing 4% paraformaldehyde and 1% glutaraldehyde. After two 5 minute rinses in PBS, peroxidase activity was developed using 3-amino-9-ethyl-carbazole (Graham, Lundholm and Karnowsky, 1965). At least 500 cells were counted in each smear and the percentage of stained cells was recorded.

Quantification of Virus-Specific Immunoglobulin

Titres of anti-viral immunoglobulin in efferent lymph were measured by an indirect enzyme-linked immunosorbent assay (ELISA) which is described in detail in Chapter 3.

Culture of Cells from Ovine Mammary Lymph

Efferent mammary lymphocytes from four sheep were cultured in Iscoves medium (Gibco Ltd., Paisley, Scotland) supplemented with 10% foetal bovine serum (FBS). Briefly, cells were washed twice in Iscoves with 10% FBS, and then 10ml of a suspension containing $2x10^7$ cells were inoculated into 25cm² tissue culture flasks (Nunclon, Nunc Inter Med, Denmark.). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Daily, 2ml samples of culture medium were taken and replaced with equal volumes of fresh medium. Cell viability was assessed by Trypan blue exclusion and the clarified supernatant fluids were stored at -20°C for later determination of specific antibody.

RESULTS

Lymphatic Cannulations

Efferent Cannulations

Initial experiments with efferent popliteal lymphatic cannulations proved unsatisfactory, and their failure can be attributed partly to the fractious nature of the Scottish Blackface lambs which resulted in avulsion of cannulas, and also to the unsuitability for this preparation which is inherent in British breeds of sheep (Hall, 1967a). Several attempts were made using aged Merino ewes which were available at the Institute, but these efforts met with equal failure, with spontaneous cessation of lymph flow occurring at variable intervals after cannulation. This is perhaps to be expected, in view of the report of Hall and Morris (1962) that lymph flow is less brisk in older animals due to the presence of smaller, more numerous ducts.

It was decided therefore to locate an alternative source of efferent lymph, and satisfactory results were obtained using the efferent supramammary model of Lascelles and Morris (1961), which has the additional advantage of draining an area which is frequently subject to natural infections (see Robinson and Balassu, 1981). Seven successful supramammary cannulations were performed, and in six animals, lymph flow was maintained for a sufficient period to allow monitoring of the entire response to infection (Tables A2-A8, Appendix One); that of the remaining sheep (1310) stopped spontaneously seven days after surgery. Lesions typical of orf virus infection developed in all animals with the exception of one (1269), which failed to show a lesion after seven days. This

animal, however, responded typically following rechallenge at that time. Despite some variation in the extent of lesions, all showed the same pattern of development: erythema which persisted for 3-5 days after challenge was followed by the appearance of white vesicles approximately 2mm in diameter on the abraded site; these gradually became pustules, and the vesiculo-pustular stage of the lesion lasted between 3 and 8 days, after which a scab developed; scabs became thicker with time and persisted until the end of the experiment.

Afferent Cannulations

Great difficulty was experienced in accumulating data on the afferent lymphatic response to orf virus infection, due to an apparent tendency of lymph draining a developing lesion to clot, which resulted in numerous cessations of lymph flow. Some of these intra-cannular clots could be removed using a probe of monofilament nylon nicked with a scalpel blade to form a barb at one end, but in other cases, only recannulation of the vessel could restore the flow of lymph. Intractable clots which formed thereafter resulted in the abandonment of the experiment. For this reason, with a given animal, it was only possible to monitor the changes occurring in lymph draining a lesion for part of the course of infection, and because of the interrupted flow, no real assessment could be made of flow rate and hourly cell output.

Consecutive daily samples of afferent lymph were collected from four adult Merino ewes for varying periods after challenge of the skin on the lateral aspect of the thigh with viral inoculum. Two of these animals were challenged on the day after surgery, and yielded lymph samples only until the fifth day of infection.

Consequently, the two remaining ewes were not cannulated until the fourth day after infection, and an intermittent flow was maintained for four days. The events of these afferent lymphatic cannulation experiments are summarised in Table A1, Appendix One, from which it can be seen it that was possible to perform differential counts on the cell populations of afferent lymph sampled from different sheep during days 0 to 8 of an orf virus infection of the drainage area.

The peripheral lymph which was collected by cannulating the efferent prefemoral lymphatic duct after removal of the lymph node was consistent in all respects with previous descriptions of afferent lymph, and in view of the absence of any appreciable blast cell response by day 6 which was characteristic of efferent lymph responses it is probable that all of the prefemoral lymph node tissue had been removed prior to the cannulation.

Cellular Changes

Efferent Lymph

Smears of efferent lymph collected before infection and stained with Leishman's stain showed a predominance of small and medium lymphocytes (Fig. 2.3a), as described by Hall and Morris (1963), and from 0.2-4.6% large cells with basophilic cytoplasm. Neutrophil polymorphs were seen only for the first 2-3 days after surgery, while eosinophil polymorphs were seen throughout the collection period, although rarely at levels above 1%. Erythrocytes appeared sporadically in some animals, but their presence seemed to bear no relation to the progress of infection.

Cellular responses were recorded in each of the animals with the exception of one (161). These responses varied considerably in

magnitude but were qualitatively similar (Fig. A1-A12, Tables A2-A8, Appendix One). In all animals a transient rise in cell output followed surgery and in some cases persisted into the first day after infection. Despite this, for 5-7 days after challenge no changes in the proportions of different cell populations were detected in the lymph. After this period, and coinciding roughly with the development of vesicles on the lesion, the daily total cell output began to increase, peaking between days 6 and 10, and declining to baseline levels between days 11 and 20. This rise in total cell output was accompanied by an increase in the proportion of lymphoblasts (Fig. 2.3b), ranging from 8.1% to 36% at the height of the response. Most of these lymphoblasts contained IgG (Fig. 2.4), while very few IgM-containing cells were found during any of the responses (<2.0%). In two of the sheep (443, 1313) cells from lymph taken at 2 and 11 days after infection were stained using a T cell-specific monoclonal antibody, T-80 (Fig. 2.5). In both, the proportion of T-80 staining cells fell as the response developed (Fig. A13, Appendix one; Table 2.3), although some large cells were expressing this antigen during the lymphoblast responses.

Table 2.3. Assessment of regional supramammary lymph T-cell responses during orf virus infection, using the monoclonal antibody T-80.

Sheep No.	Days after Infection	Cell Output ($x10^6$/hr)	% Blast	% T-80+
443	2	66.2	2.7	62.4
"	11	202.0	36.0	45.0
1313	2	155.8	1.4	73.4
"	11	106.5	14.7	52.6

Afferent Lymph

White cell counts for afferent lymph ranged from $0.6x10^6$ to $3.1x10^6$ cells/ml, and were always lower than those seen in efferent

lymph, which varied between $2.8x10^7$ and $16x10^7$ at the time of challenge. Results of the differential cell counts performed on Leishman's stained cytocentrifuge smears of afferent lymph collected from the four Merino ewes during the development of orf lesions, are summarised in Table A1, Appendix One, along with estimations of flow rate and cell output which were possible. The total and differential cell counts fluctuated continuously in each of the preparations, and allow only a general description of the cell populations which were seen during the cannulation periods. Small and medium lymphocytes represented the greatest proportion of cells in each sample of afferent lymph taken during the study (Fig. 2.3c), the remainder of the cell populations consisting of variable proportions of macrophages, neutrophil polymorphs and lymphoblasts. In contrast to the cell profiles seen in the efferent lymphatic experiment, the latter type of cell was seen only in small numbers in afferent lymph, not exceeding 1% until the fifth day after infection. Even after this time, no convincing lymphoblast response was recorded. Neutrophil polymorphs were most evident for the first two days after surgery, after which their numbers declined to low levels which were apparently unaffected by the course of infection.

The term "macrophage" is used for the purpose of this report as defined by Hall (1967b), and bears no relation to biological function. These cells were characterised by multilobed and sometimes horseshoe shaped nuclei, and abundant cytoplasm which had irregular borders. Their numbers varied with each preparation and fluctuated greatly from day to day, but did not increase or decrease in response to infection. At no time did they represent more than 9.2% of the afferent lymph cell population.

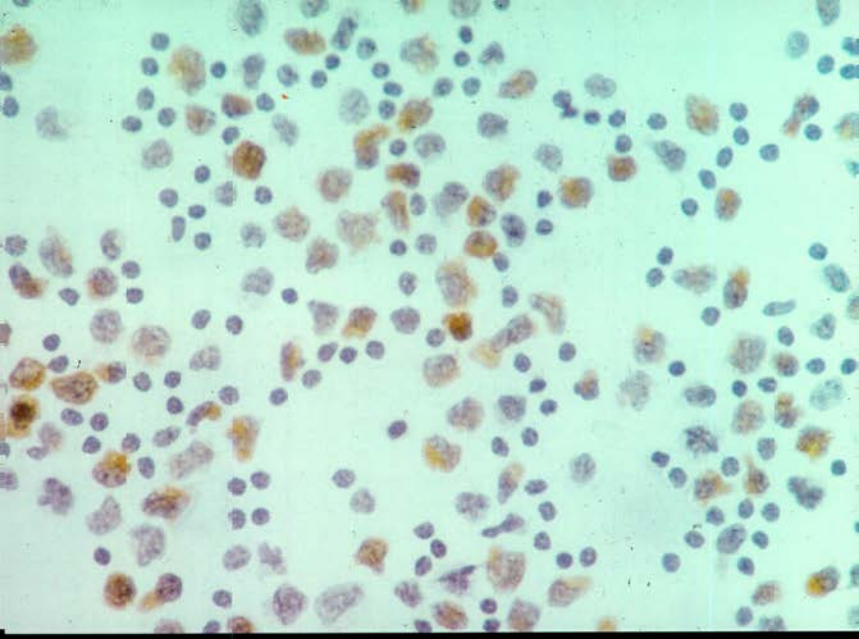
Fig. 2.3a. Cytocentrifuge smear of pre-response efferent lymph cells (Leishman's stain) showing a predominance of small to medium lymphocytes and occasional lymphoblast cells (arrowed) (sheep no. 443, day 2 after infection).

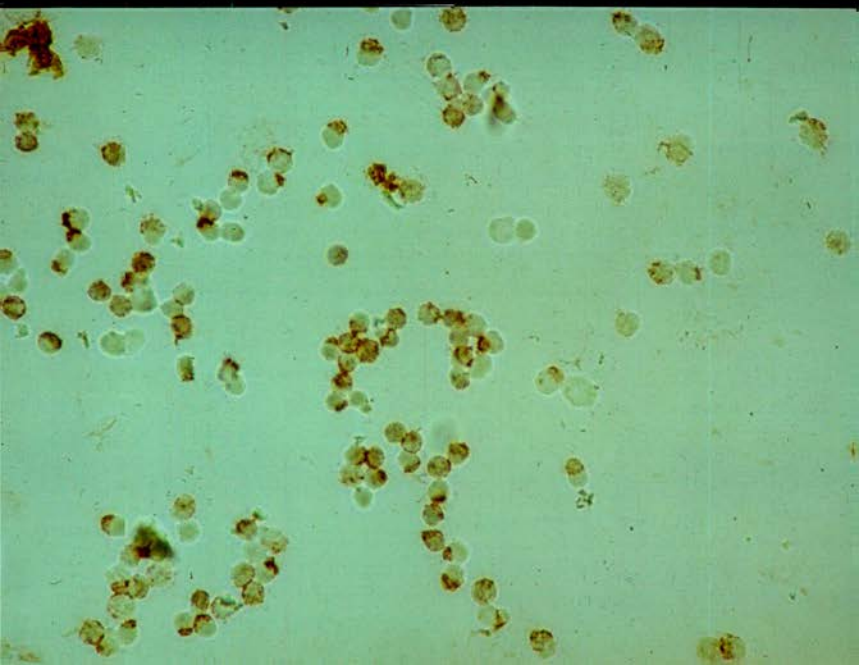
Fig. 2.3b. Cytocentrifuge smear of efferent lymph cells (Leishman's stain) during the response to experimental orf virus infection of the drainage area. Note increased proportions of lymphoblast cells (sheep no. 443, day 10 after infection).

Fig. 2.3c. Cytocentrifuge smear of afferent lymph cells sampled during experimental orf virus infection of the drainage area. Macrophages are present among a predominance of small to medium lymphocytes (sheep no. 129, day 7 after infection)

Fig. 2.4. Cytocentrifuge smear of efferent lymph cells sampled during the response to experimental orf virus infection of the drainage area. Cells have been stained with horseradish peroxidase-conjugated pig anti-sheep IgG (sheep no. 443, day 10 after infection).

Fig. 2.5. Cytocentrifuge smear of efferent lymph cells sampled during the response to experimental orf virus infection of the drainage area. Cells have been stained with the T-cell-specific monoclonal antibody T-80 (sheep no. 1313, day 2 after infection.





Eosinophil polymorphs were occasionally present in afferent lymph cytocentrifuge smears, but their levels never exceeded 0.4%. However, erythrocytes were frequently present in large numbers, but their occurrence seemed to be random and did not appear to be associated either with the surgical interference or the progress of infection.

Detection of Specific Antibody

When efferent lymph samples were tested for antibody using the ELISA assay, all, including preinfection samples, were found to be positive (Fig. A14, Appendix One). Two animals (764, 1272) made no apparent response, while one (1313) showed a marked increase in titre followng infection.

Supernatant fluids removed from efferent mammary lymphocyte cultures of animals 161, 443, 764 and 1313 all contained low titres of virus specific antibody, with cells from animal 1313 producing the largest amount (Table 2.4).

Table 2.4. Antibody titres detected by ELISA in supernatant fluids from cultured supramammary lymph cells after infection of the drainage area with orf virus.

Sheep No.	Days After Infection of Lymph Collection	Peak Titre (Reciprocal)
161	11	6
443	9	5
764	12	8
1313	9	150

DISCUSSION

Although no striking cellular changes were recorded in peripheral lymph travelling from an orf lesion to the draining lymph node, the analysis of lymph derived from the efferent lymphatic cannulations has provided a valuable insight into the immune events which follow challenge of the sheep with orf virus.

The problems which were encountered with the afferent lymphatic cannulation preparations, and the relative stability of the cell populations in peripheral lymph during antigenic challenge which was apparent from these experiments, were not at variance with the reports of other workers. Hall and Morris (1963) found that total and differential cell counts in afferent lymph taken from a given animal fluctuated continuously from day to day, and consequently were only able to describe in general terms the cell populations before and after subcutaneous injection of human serum globulin. Hall (1967b) noted that in his experiments with afferent popliteal cannulations, lymph flow was continually interrupted by blockage of the cannula. In addition, because of the tendancy of the cells in afferent lymph to adhere to each other, he placed little faith in differential cell counts, particularly in view of the low flow rates and total cell counts which he found were characteristic of these preparations. He reported that the grafting of skin from other sheep produced only trivial changes in the cell populations of the drainage lymph, which was in contrast to a dramatic response in the regional node.

Morris, Mereno and Bessis (1968) recorded similar results after the subcutaneous injection of human erythrocytes into an area drained by a cannulated afferent lymphatic. No major change in

cell populations of the peripheral lymph were evident except in animals which had been previously immunised, and even here the reaction was weak, cell output being only slightly elevated. They concluded that the transport of antigen to the regional lymph node was necessary for amplification of the immune response. Hall and Smith (1971) elicited somewhat more dramatic responses in peripheral lymph by painting the contact sensitising agent dinitrofluorobenzene (DNFB) onto the skin surface. However, these changes were dominated by large numbers of neutrophil polymorphs and were attributed to acute inflammatory events.

Morris and others (1968) noted the transport of human erythrocytes to the regional lymph node within afferent lymph macrophages, and Smith, McIntosh and Morris (1970a), found that colloidal carbon injected subcutaneously in the lower part of the hind limb appeared in macrophages of afferent popliteal lymph. However, Hall and Smith (1971) reported that minimal amounts of DNFB were transported from the skin to the regional node in association with cells and that most were transported in combination with protein. In view of the responses which were detected in efferent lymph sampled during these experiments from lymph nodes which were draining areas of infected skin, it is quite clear that viral antigen is transported to the regional lymph node. Unfortunately, it was not possible to establish by what mechanism this occurs, due to the lack of available specific markers for the virus. Early efforts to identify viral antigen in acetone fixed cytocentrifuge smears of afferent lymph cells using fluorescene isothiocyanate (FITC)-labelled antibody derived from a lamb which had been hyperimmunised with orf virus proved unsuccessful due to non-specific binding. Similar results were obtained with the

$F'ab^2$ fraction of this serum, directly labelled with FITC. Since any staining of afferent lymph smears using polyclonal sera can be questioned regarding specificity, in the absence of a monospecific serum, no further attempts were made to identify viral antigen in afferent lymph.

In contrast with the apparent feeble responses to antigenic challenge which have been reported for afferent lymph cell populations, dramatic cellular changes have been recorded by many workers in lymph flowing from ovine peripheral lymph nodes which have been stimulated with a variety of antigens (Hall and Morris, 1963; Morris, 1972; Trnka and Cahill, 1980). These changes have been well characterised, and involve an initial sudden and transient decrease in cell output followed by an increase in cells leaving the node by days 2 and 3 after antigenic challenge. About this time large lymphoblasts appear in the lymph reaching a peak by day 4 or 5, and remaining for 7 to 8 days. Responses in efferent lymph from nodes draining skin which has been painted with contact sensitising agents such as DNFB (Hall and Smith, 1971) and oxazalone (Hall, Hopkins and Reynolds, 1980) were similar. Pearson, DeMartini and Fiscus (1979) described the changes in efferent popliteal lymph following inoculation of orf virus and MNV into the drainage area, and again the results were in keeping with those found using conventional antigens.

The responses which occurred in efferent lymph derived from the sheep in this study following challenge with orf virus differed from those seen with previous antigenic models in that there was a considerable delay before the appearance of increased numbers of lymphoblasts in efferent lymph. A similar although shorter lag

occurred in the response of the popliteal node to DNFB (Hall and Smith, 1971) and was attributed to the time taken for significant amounts of the chemical to penetrate the skin, combine with proteins, enter the afferent lymph and reach the node. The dynamics of orf virus infection are presumably even more complicated. Animals were abraded using a procedure which had been shown in pilot experiments not to break the epidermis. It is probable therefore that appreciable amounts of antigen did not reach the node until several cycles of virus replication had occurred within the epidermis. The initiation of the cellular response coincided approximately with the development of the vesicle stage of the lesion. Wheeler and Cawley (1956) attributed the formation of vesicles to ballooning of infected cells followed by reticular degeneration, and Kluge and others (1972), in an ultrastructural study of orf infection in sheep found the highest concentration of virions in ballooned keratinocytes, between 72 and 143 hr after infection. Indeed, even at the height of infection, the predominant direction of antigen traffic is likely to be away from the node due to proliferative changes in the basal layers below the lesion.

Hall and Morris (1962) reported considerable variation in cell output between different popliteal cannulations prior to antigenic challenge. Similar variation was experienced in the present study using the supramammary cannulation model. Cell output at the time of challenge ranged from 2.8 to 16×10^7 cells per hour and might be attributed to variation in age, breed and stage of lactation of the sheep employed. Such individual variation may to some extent explain the diversity which was evident in the responses of these sheep. However, the complexity of the events which surround the

initiation and development of orf lesions in ovine skin may also be a contributing factor. The local cellular infiltration and epidermal hyperplasia which are histopathological features of orf lesions (Aynaud, 1923; Glover, 1928; Nisbet, 1954; Wheeler and Cawley, 1956) may in some cases result in only limited amounts of viral antigen reaching the node.

Hall and Morris (1962) also described a recovery period of 24hr following surgery, during which cell output was depressed. Following this period output reached a basal level which was maintained with minor fluctuations until the node was stimulated. The transient increase in cell output which followed surgery in the present study lasted longer, and seemed to exceed basal levels. This may have been associated with involution in the mammary gland. Indeed it is difficult to compare these results with those achieved by other workers using other efferent lymphatic cannulations, since the cellular changes in mammary lymph following stimulation with antigen have not been previously characterised. The actual extent to which these data were influenced by the fact that the node was draining an involuting gland remains unclear, but was probably not marked. Watson and Lascelles (1973) found that levels of immunoglobulin in lymph from mammary glands which had been immunised with Brucella abortus did not alter signficantly during involution except those of IgA, which showed a dramatic increase 7-10 days after involution had commenced, and returned to preinvolution levels 7-10 days later. This effect was attributed to the continued local production of IgA while the secretory activity of the gland was decreasing. Since no antigenic challenges at mammary mucosal surfaces were made during these experiments, the role in this response of IgA, which is associated

with mammary and other mucosal secretions of sheep (Lascelles and McDowell, 1970), was not investigated. However cells containing this isotype, along with T lymphoblasts, may have contributed to the population of lymphoblasts which did not stain for IgG or IgM.

The fact that all animals had specific antibody in their lymph prior to infection, coupled with the predominance of IgG-containing cells over those staining for IgM strongly suggests that all animals had experienced previous orf virus infection. Furthermore, the proportions of lymphoblasts present in lymph at the peak of the response are in line with those reported by Hall and Morris (1963) and English and others (1976) for secondary responses to other antigens. The reasons for the apparent lack of a specific antibody response in the lymph from two of the sheep (1272, 764) however are not clear. Antibody containing lymphoblast cells with specificities unrelated to those of orf viral antigens may have contributed to the plasmablast responses which were recorded in this experiment. Poskitt and others (1977) investigated the specificities of immunoglobulin containing cells in efferent popliteal lymph after antigenic stimulation of the node, and concluded that in addition to stimulating antigenic clones, the microenvironment within a lymph node responding to antigen induces the proliferation of lymphocytes of specificities unrelated to the immunising antigen. They were unsure whether this represented activation of memory cells specific for previously encountered antigens, or simply the indescriminate stimulation of bystander B-Lymphocytes.

Of equal interest is the striking specific antibody response which was detected during infection in lymph collected from animal 161 despite the apparent absence of a cellular response within the

node. Because the cannulated lymphatic in this ewe did not take up Evans blue dye injected during surgery at two sites on the skin of the udder, she was infected at a site behind the gland from which the dye was shown to reach the duct. This site was close to the midline and may have resulted in preferential stimulation of the contralateral node during infection. It is possible then that the specific antibody which was recorded in the lymph from this ewe was a reflection of a serum response induced in the contralateral lymph node, although it would appear that cells derived from the cannulated node were capable of producing virus specific antibody in culture. In any event it would appear that circulating antibody specific for orf virus provides only limited protection against reinfection. Initial challenge failed only in animal 1269, which admittedly had extremely high titres of lymph antibody at the time. However, infection did establish in this animal at the second attempt, when its lymph antibody titre was in excess of 1:3000.

Although it is possible that the site had not been sufficiently abraded before the application of the viral inoculum, the precise reasons behind the failure of initial challenge in this animal are not clear. The fall in lymph antibody titre which followed this unsuccessful challenge is equally intriguing. Hall and Morris (1963) noted a similar reduction in lymph antibody during the early stages of the secondary response of a sheep to human serum globulin, and attributed it to the binding by antigen of any antibody which was present in the lymph. It is unlikely that such an effect occurred in this animal, since in the absence of the development of a lesion, little if any viral antigen would have reached the node. It may be that the declining titres of lymph antibody were simply a reflection of recent exposure to orf virus

through a natural infection. Whatever the reasons behind the failure of this animal to develop a lesion following initial challenge, the data collected between this and the second successful attempt at infection are useful for control purposes, and suggest that abrasion of the skin per se does not give rise to marked cellular changes in the draining lymph node.

Although the proportion of cells staining with the T-80 monoclonal antibody in efferent lymph decreased during the progress of the response, the role of the T cell in immunity to orf virus infection should not be dismissed. Robinson and Balassu (1981) suggested by analogy with orthopox viruses that cellular immunity was probably the major factor in recovery and protection from orf virus infection. Issekutz (1984) detected cytotoxic T lymphocyte (CTL) activity in efferent lymph following subcutaneous injection of live vaccinia virus into the drainage area (although it is not clear if infection was established). He was unsure at that time whether the CTL and CTL precursors in lymph were large lymphoblasts or small lymphocytes, but later, (Issekutz,1985) associated cytotoxicity with the lymphoblast fraction. Buddle and Pulford (1984) recorded delayed-type hypersensitivity reactions following orf virus infections, and these findings have been borne out by experiments undertaken during this project (see Chapter Six, Part Two). Thus, the reduction seen in the proportions of cells staining for the T-80 antigen could be more a reflection of a vigorous plasmablast response than a weak T cell response. Furthermore, T cell counts performed using the T-80 monoclonal antibody must be interpreted in the light of the observation of Miyasaka and others (1983) that a certain population of lymphocytes in peripheral blood, while not recognised by T-80, still displays T

cell characteristics. Recruitment of cells from this population by the node would not have been detected by the methods reported here.

In summary, it appears that in previously infected sheep, the major element of the nodal response to subsequent challenge with orf virus is the production of large numbers of plasmablast cells most of which contain immunoglobulin of the IgG class, and which produce orf virus-specific antibody when cultured. The role of the T cell in the response has not been determined, although a proportion of circulating T cells are not detected by the methods used in this study. An initial lag in the onset of response is attributed to the time taken for sufficient antigen to be produced by viral replication and to travel to the node. It has not been possible, however, to establish the mechanisms by which viral antigen is transported in afferent lymph to initiate nodal responses.

CHAPTER THREE

QUANTITATIVE STUDY OF OVINE HUMORAL RESPONSES TO ORF VIRUS INFECTION

INTRODUCTION

The efferent lymphatic cannulation studies described in the previous chapter indicate that in the sheep, the major element of the response of a lymph node to secondary infection of its drainage area with orf virus is the production of large numbers of lymphoblasts with the characteristics of B cells. This suggests that the predominant effort of the ovine immune system in response to reinfection with orf virus is directed at the synthesis of virus specific antibody, which is somewhat surprising in view of some reports that humoral mechanisms do not provide complete protection against reinfection with the virus (Aynaud, 1923; Trueblood and others, 1963; Osman, 1976).

The characterisation of antibody responses in sheep infected with orf virus has occupied many investigators of the disease, but despite their efforts, which are reviewed in Chapter One, the nature of these responses remains unclear. This is partly attributable to the wide range of serological tests which have been employed, and also to the divergent results that have been achieved by different workers with any one assay. Robinson and Balassu (1981) concluded that no reliable serological test had emerged which could be used to assess the immune status of individual animals against the virus.

In order that the specificity of antibody responses in efferent lymph could be assessed, and so that the development and magnitude of humoral responses to orf virus infection in naturally and experimentally infected animals could be characterised, it seemed a priority to develop a sensitive and reproducible assay for the detection of orf virus specific antibody in ovine serum.

Over the years, many techniques have emerged for the measurement of antibody-antigen reactions. Minden, Anthony and Farr (1969), classified the events which follow the primary interaction of antigen and antibody as secondary or tertiary. Secondary events, such as precipitation, agglutination and fixation of complement occur in vitro, whereas tertiary events are manifested in vivo, and are represented by such reactions as passive cutaneous anaphylaxis, and the Prausnitz-Kustner and Arthus reactions. It has been established that serological assays which measure secondary and tertiary manifestations of antigen-antibody interactions often fail to detect significant amounts of antibody which are demonstrable by assays of primary binding (Minden and others, 1969). Since the majority of techniques which have been applied to the detection of orf virus specific antibody in test sera are dependant on the occurrence of events subsequent to the interaction of antigen and antibody (see Chapter One), considerable improvements in sensitivity might be achieved by the application of a primary binding assay.

Another factor which is important when measuring serological responses is the affinity of the antibodies concerned. Antibody affinity is a measure of the strength of the interaction between an antibody and its target antigenic determinant, and represents the summation of the attractive and repulsive forces between the two (see Roitt, Brostoff and Male, 1985). All antibody assays are to some extent influenced by antibody affinity, although some are less so than others. Peterfy, Kumsela and Makela (1983) found that the solid phase assays radioimmunoassay (RIA) and ELISA were the least affinity dependent of six antibody assays which they tested.

The ELISA test, originally described by Engvall and Perlmann (1972), is a primary binding assay of antibody which relies on the immobilisation of antigen on a solid phase (such as polystyrene) and the subsequent addition of test serum followed by an enzyme-conjugated anti-immunoglobulin. Unbound reagent is removed after each step by washing with an appropriate buffer, and the amount of enzyme which is bound to the solid phase is assessed by the addition of substrate. The test is of the same order of sensitivity as the corresponding radioimmunoassay, and has the advantage of more stable reagents and fewer specialised handling requirements (Engvall and Perlmann, 1972).

A micro-ELISA test which used 96-well polystyrene plates as the solid phase was described for Rubella serology by Voller and Bidwell (1975), and it is in this form that ELISA is now usually employed. Marennikova, Malceva and Habahpaseva (1981) investigated the use of a micro-ELISA to detect serum antibodies to vaccinia, monkey-pox and whitepox viruses in serum from hyperimmunised rabbits, and compared it with haemagglutination inhibition, indirect haemagglutination, precipitin, indirect fluorescent antibody and virus neutralisation tests. They were satisfied that ELISA represented the most sensitive assay and that it could be used to differentiate sera specific for closely related pox viruses.

The only available report on the use of ELISA in orf serology at the outset of this study was that of Koptopoulos and others (1982), who found the test rather insensitive and incapable of detecting antibody in serum from naturally infected animals. However, the sensitising antigen which they employed in their test was rather

crude, and it was considered that significant benefit could be gained from a further exploration of the use of ELISA in the quantification of ovine humoral responses to orf infection.

The following account describes the development of a micro-ELISA test for the detection of orf virus specific antibody in ovine serum, and its application to the characterisation of humoral responses in naturally and experimentally infected animals.

MATERIALS AND METHODS

Sera

Test sera were derived from three groups of animals. Group A was made up of thirteen lambs selected from a commercial flock which was undergoing an outbreak of orf at the Edinburgh University veterinary field station. Six Scottish Blackface lambs with no history of previous orf virus infection were chosen from the Moredun Institute flock to form Group B, while Group C consisted of two specific pathogen free (SPF) lambs (caesarian-derived colostrum-deprived lambs which had been kept in isolation and fed sterilised cow's milk).

Group A was maintained with the rest of the flock at pasture, and assembled weekly for blood sampling and grading of lesions (Table 3.1.), while Groups B and C were held in loose boxes for the duration of the experiment. Blood sampling of Group A commenced one week after the onset of the outbreak, and continued at weekly intervals until all lesions were healed. Groups B and C were bled once before inoculation and thereafter at 2-3 day intervals until lesions had resolved.

Table 3.1. Scoring of lesions in Group A lambs.

Severity of Lesion	Score
No lesion present	0
±	1
+	2
++	3
+++	4

Blood samples were collected in sterile vacutainers (Becton Dickinson, Rutherford, New Jersey.) and were held at 4°C for approximately 24 hours before being centrifuged at 2000xg for 30 minutes to remove the clotted fraction. The supernatant serum was then collected and stored in bijoux bottles at -20°C until it could be assayed for the presence of antibody.

Challenge virus

Groups B and C were experimentally infected with orf virus using the inoculum described in Chapter Two. Approximately 100µl of the virus suspension were applied to sites on the skin of the medial aspect of the thigh which had been lightly abraded with sterile sandpaper.

Preparation of ELISA antigen

Antigen for sensitisation of the solid phase of the test was prepared from purified orf virus derived from infected scabs. The latter had been collected from a persistently infected Friesland ram (U93, see Chapter Six) and stored at 4°C. Viral purification for all aspects of this project was carried out using a modification of the method described by Robinson and others (1982). Approximately 4g of scab was ground in 20ml of PBS using a mortar and pestle. The resulting suspension was clarified by centrifugation at 1200xg for 30 minutes at 4°C, layered on to two 3ml cushions of 36% sucrose (w/w in PBS) and centrifuged at 45,000xg for 30 minutes at 4°C using a Beckman SW40ti rotor in a Beckman L2-62B ultracentrifuge (Beckman RIIC Ltd., High Wycombe, Buckinghamshire, UK.). The pellets were resuspended in PBS with the aid of a glass homogeniser (Uniform, Jencons Scientific Ltd., Leighton Buzzard, Bedfordshire. UK.), layered on to two 7.5ml

continuous gradients (20-60% w/w in PBS) of Nycodenz (Nygaard and Co., Oslo, Norway) and ultracentrifuged under the same conditions. The viral bands, which settled at the level of the middle of the tube, were collected from below using a bent needle and syringe and each resuspended in 12ml PBS before being recovered by ultracentrifugation as above. The pellets of purified virus were then combined, resuspended in 500µl PBS, and a 3µl aliquot was examined for purity by electron microscopy as described in Chapter Six. Small amounts of granular material were usually seen to have co-purified with the virus, and although this could probably have been removed by an additional banding in Nycodenz, no further purification steps were performed for the purposes of this study.

These preparations of purified virus showed a noticable tendency to aggregate, and when used to sensitise polystyrene plates in initial ELISA trials, gave rise to unacceptably divergent results for duplicate samples of test sera which was attributed to uneven distribution of aggregated particles among the wells of the plates. This problem was remedied by the preparation of a solubilised ELISA sensitising antigen for use in all subsequent tests. Purified U93 virus in 500µl PBS was diluted 1:10 in carbonate buffer (0.05M, pH 9.6) containing 1% sodium docecyl sulphate (SDS), and incubated for 30 minutes at 37°C. To improve solubilisation, the mixture was then sonicated for 60 seconds using an ultrasonic disintegrator (MSE, Crawley, Sussex, UK.) at an amplitude of 14µ. The SDS was allowed to precipitate by chilling at 4°C, and removed by centrifugation at 500xg for 10 minutes at 4°C.

An optimal working dilution for this antigen of 1:20,000 was determined by titrating it against a standard serum taken from a

lamb which had been hyperimmunised with orf virus. Using the Bio-Rad protein estimation assay described in Chapter Four (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK.), this dilution was found to contain 0.54µg protein per ml. Sensitising antigen was stored in 100µl aliquots of a 1:100 dilution in carbonate buffer, at -20°C.

Preparation of standard antiserum (D573)

A lamb (D573) which had been experimentally infected with orf virus was inoculated intramuscularly at 16 and 18 weeks after infection with autogenous scab material in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Michigan, USA.). It was bled 10 days after the second inoculation, and the serum stored at -20°C.

ELISA Test

The test procedure and calculation of results were performed as described by Donachie and Jones (1982) with some modifications. A reagent volume of 200µl/well was used throughout the test, and all sera were diluted in PBS with 0.05% Tween 20 (PBS/Tween) containing 0.02% sodium azide (serum diluent). Test sera were diluted 1:200, and the standard antiserum was made up in eight two fold dilutions ranging from 1:200 to 1:25,600. Eight wells of SPF lamb serum diluted 1:200 were included in each test as a negative control. The layout of sera in test plates is illustrated in Fig 3.1.

The test consisted of four incubation stages representing successive applications of sensitising antigen, test serum, enzyme conjugated anti-immunoglobulin and substrate. After each incubation, unbound reagent was removed from the wells by three

rinses in PBS/Tween, shaking out excess fluid between each rinse. The procedure was as follows:

Step I. Polystyrene microtitre plates (type M129A, Dynatech Laboratories Ltd, Billinghurst, Sussex, UK.) were coated by an 18 hour incubation at 4°C with sensitising antigen diluted 1:20,000 in carbonate buffer.

Step II. Plates were washed, and incubated for 3 hours at room temperature with test and standard antiserum dilutions.

Step III. After a further wash, rabbit anti-sheep whole immunoglobulin (Dakopatts, Copenhagen, Denmark.) which had been conjugated with alkaline phosphatase by Mr. A. Dawson of the Moredun Institute using the method of Engvall and Perlmann (1972),was added to each well at a previously determined optimal dilution of 1:200 in serum diluent. The plates were then incubated for 2 hr at room temperature.

Step IV. Plates were again washed, and volumes of enzyme substrate (p-nitrophenylphosphate, Sigma Chemical Company, Poole, Dorset, UK.) at a concentration of 1mg/ml in a buffer (pH 9.8) containing 10% diethanolamine, 0.5mM magnesium chloride and 0.02% sodium azide were added to the wells. After an incubation of 1hr at room temperature, the enzyme reaction was stopped by the addition to each well of 50µl of 3M NaoH.

The results were recorded as light absorbance values at a wavelength of 405nm using an automatic multichannel spectrophotometer (Titertek Multiskan, Flow Laboratories, Irvine, Ayrshire, UK.). The data was also translated in digital form to a punch tape in order to allow computer analysis.

Fig. 3.1. Microtitre plate distribution of test and standard serum wells for orf virus specific ELISA.

Reference Serum Dilutions

TEST SERA

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	1:200	1:400	1:800	1	5	9	13	17	21	25	29
B												
C					2	6	10	14	18	22	26	30
D												
E					3	7	11	15	19	23	27	31
F												
G	1:1600	1:3200	1:6400	1:12800	4	8	12	16	20	24	28	32
H	1:25600			NEG. CONTROL								

DYNATECH MICROELISA®

Calculations

Results were expressed in relation to a computer linear regression derived from the absorbance values of the eight standard serum dilutions included in each test. Regression analyses were performed by the computer on the relationship between absorbance values and $\log_{10}$ serum dilution, $\log_{10}$ absorbance values and serum dilution, and $\log_{10}$ absorbance values and $\log_{10}$ serum dilution, and that with the best correlation coefficient (ie closest to -1.0) was selected for calculation of test serum titres. The negative exclusion point for the regression line was set at the mean absorbance value of the eight SPF negative control serum wells, and the titre of the reference antiserum was designated to be the dilution with this value. The titres of the test sera were then calculated by the computer using the formula:

$$T_t = \frac{D_t}{D_r} \times T_r$$

Where T_t = Titre of test serum

D_t = Reciprocal of test serum dilution (200)

D_r = Reciprocal dilution of reference serum giving same absorbance value as test serum

T_r = Titre of reference serum

RESULTS

Lesions

All of the animals of Group A had developed lesions by the second week of the outbreak, and these persisted for varying periods until all had healed by the eighth week. Although the majority of infections were mild and confined to the lips and nares, three animals developed severe labial lesions, and in one of these, secondary spread to the medial thigh was observed.

In both experimentally infected groups, lesions progressed following inoculation through macule, papule, vesicle pustule and scab stages as described by Aynaud (1923). Scabs formed from day 10 to day 15 after infection, and separated between days 20 to 25. There was no difference in severity between the groups. One of the lambs of Group C was euthanised on day 20 after inoculation following the development of a urethral blockage due to urinary calculi. By this time however, the animal's scabs had been shed.

Serological Responses

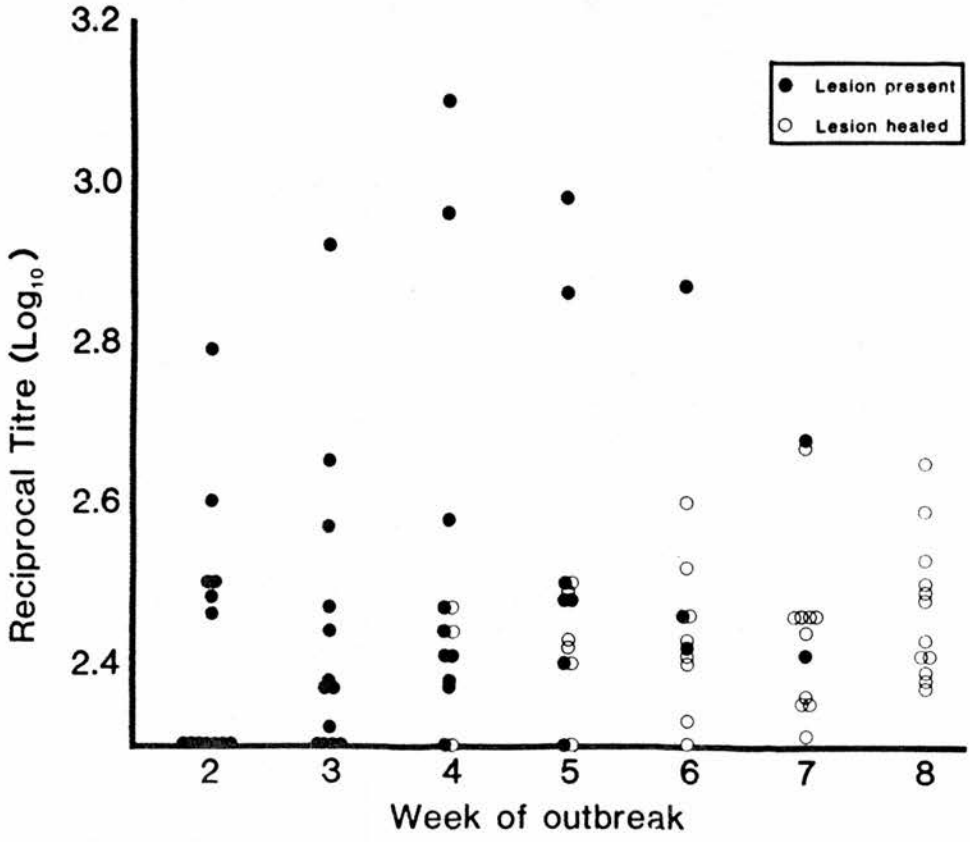
Specific antibody responses to infection were detected in all three groups (Table 3.2, 3.3, 3.4) and are illustrated graphically in Fig. 3.2, 3.3, 3.4. The responses in Group A sera showed great variation in magnitude which did not seem to be closely associated with the severity of lesions or the duration of infection (Fig. 3.5, Table 3.6), but in general, titres of antibody in sera from this group remained low throughout the response, as evidenced by the plot of its median values (Fig 3.2b).

The response in Group B showed a more obvious trend than that of the naturally infected group (Table 3.3, Fig 3.3). Only one animal was seronegative at infection, and although this individual made a sluggish response with no detectable antibody until day 12 after infection, the remaining lambs responded rapidly within the first week of inoculation, four of them with titres far in excess of those detected in Group A sera. Again, there was a noticable variation in the extent of these responses, but in each animal, antibody activity had not returned to preinoculation levels by the 38th day of the experiment.

The two lambs of Group C made low level antibody responses (Table 3.4, Fig. 3.4) one commencing at day 6 and the other at day 12 of infection. After the initial lag period, reponses were characterised by rapid increases in antibody titre, followed after two days by a gradual decline. The response of the surviving animal of this group was lower than that of its companion, and relatively brief when compared with Group A and B responses. It was also noted that lesions persisted longest in this lamb, having resolved in the other at the time of its euthanisation.

Fig. 3.2(a). Specific ELISA serum antibody titres for the lambs of group A during natural infection with orf virus.

Fig. 3.2(b). Specific ELISA serum antibody titres for the lambs of group A during natural infection with orf virus, median values.


Lesion present
Lesion healed
Reciprocal Titre (Log10)
3.2
3.0
2.8
2.6
2.4
2
3
4
5
6
7
8
Week of outbreak

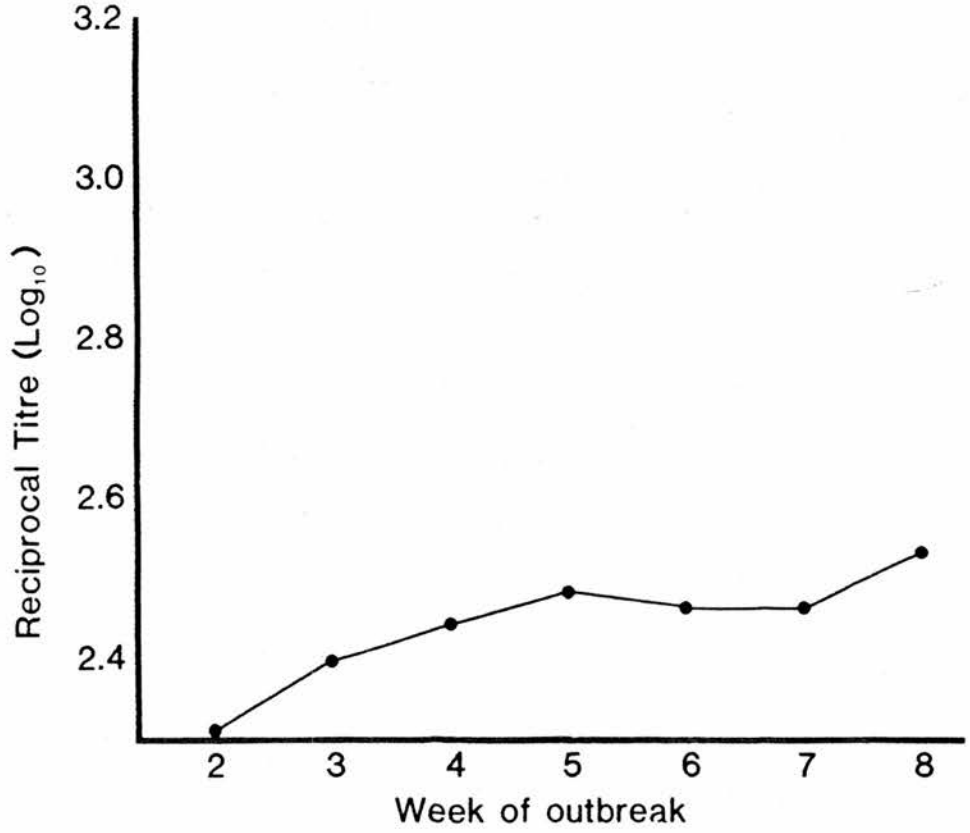

Reciprocal Titre (Log10)
3.2
3.0
2.8
2.6
2.4
2
3
4
5
6
7
8
Week of outbreak

Table 3.2. Specific ELISA serum antibody titres (log_{10} reciprocal) and lesion scores for the lambs of Group A during natural infection with orf virus.

Lamb No.	Week of Outbreak 1	2	3	4	5	6	7	8	Lesion* Score
461	ND	-	-	-	-	-	2.31	2.41	
	+	+	+	-ve	-ve	-ve	-ve	-ve	6
488	ND	2.79	2.92	2.96	2.98	2.87	2.67	2.59	
	-ve	+++	+++	+++	+++	±	-ve	-ve	17
726	ND	-	2.47	2.44	2.49	2.52	2.46	2.53	
	-ve	+	+	-ve	-ve	-ve	-ve	-ve	4
727	ND	2.50	2.44	2.47	2.50	2.40	2.35	2.43	
	+	+	+	-ve	-ve	-ve	-ve	-ve	6
729	ND	2.48	2.37	2.37	2.4	2.46	2.41	2.39	
	+	+	+	±	+	+	±	-ve	12
730	ND	2.60	2.57	2.58	2.50	2.60	2.46	2.49	
	-ve	+	+	+	+	-ve	-ve	-ve	8
735	ND	-	2.32	2.41	2.48	2.42	2.36	2.37	
	-ve	+	++	++	++	+	-ve	-ve	13
746	ND	2.50	2.65	3.10	2.86	ND	2.68	2.65	
	++	++	++	+	+	+	+	-ve	17
798	ND	2.46	2.38	2.44	2.40	ND	ND	ND	
	+	+	+	±	-ve				7
846	ND	-	2.37	2.47	2.48	2.41	2.46	2.50	
	+	+	+	+	+	-ve	-ve	-ve	10
867	ND	-	-	2.41	2.43	2.46	2.46	2.48	
	-ve	+	+	+	-ve	-ve	-ve	-ve	6
881	ND	-	-	-	-	2.33	2.35	2.38	
	-ve	±	+	+	+	-ve	-ve	-ve	7
885	ND	-	-	2.38	2.42	2.43	2.44	2.41	
	-ve	+	+	+	-ve	-ve	-ve	-ve	6

* see Table 3.1 ; ND = not done.

Fig. 3.3. Specific ELISA serum antibody titres for the lambs of group B during experimental infection with orf virus.

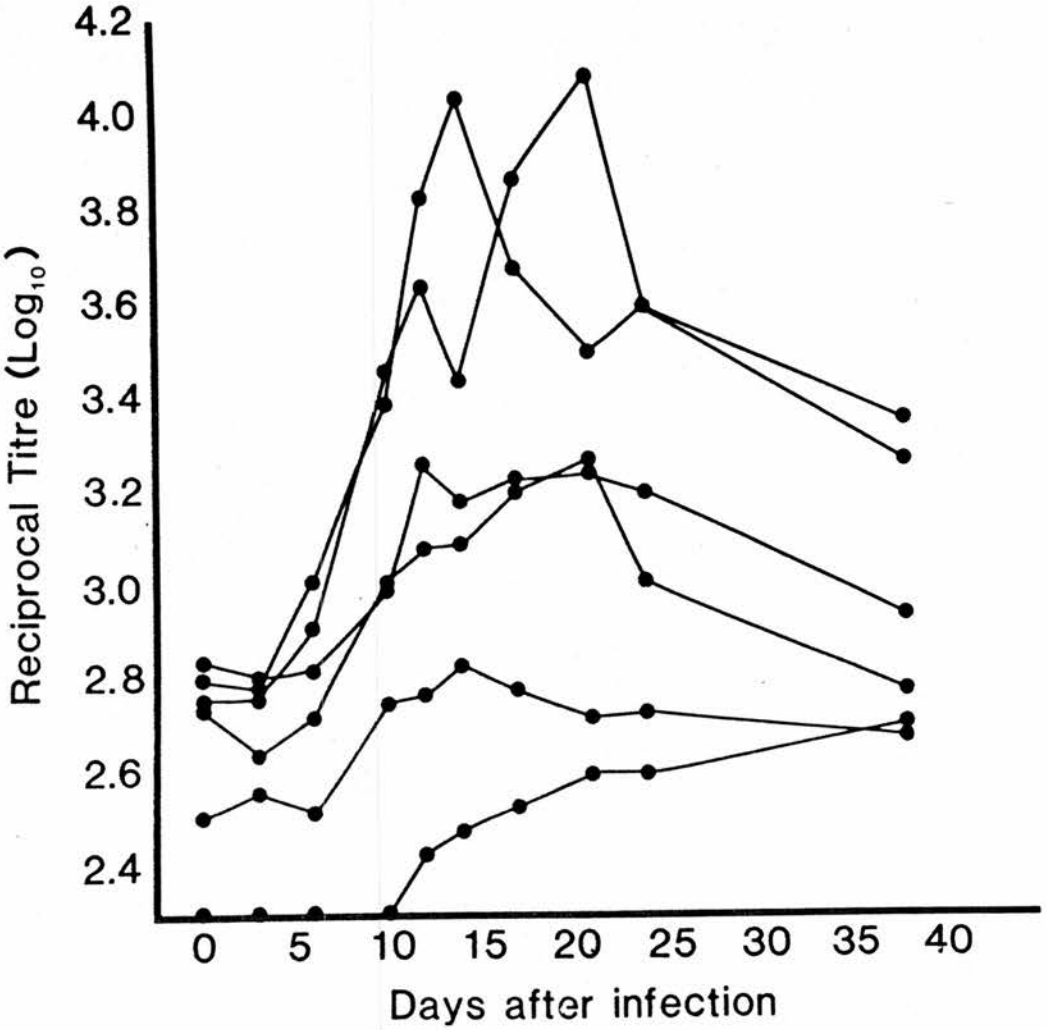

4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
Reciprocal Titre (Log10)
0
5
10
15
20
25
30
35
40
Days after infection

Table 3.3. Specific ELISA serum antibody titres (log_{10} reciprocal) for the lambs of Group B during experimental orf virus infection.

Days after infection	Lamb No. 1	2	3	4	5	6
0	2.73	2.50	2.83	2.79	2.75	-
3	2.63	2.55	2.79	2.78	2.75	-
6	2.71	2.51	2.81	3.00	2.90	-
10	3.00	2.74	2.98	3.38	3.45	-
12	3.07	2.76	3.25	3.82	3.63	2.42
14	3.08	2.82	3.17	4.03	3.43	2.47
17	3.19	2.77	3.21	3.67	3.86	2.52
21	3.26	2.71	3.23	3.49	4.07	2.58
24	2.99	2.72	3.19	3.60	3.60	2.59
38	2.77	2.67	2.93	3.35	3.26	2.70

Fig. 3.4. Specific ELISA serum antibody titres for the lambs of group C during experimental infection with orf virus.

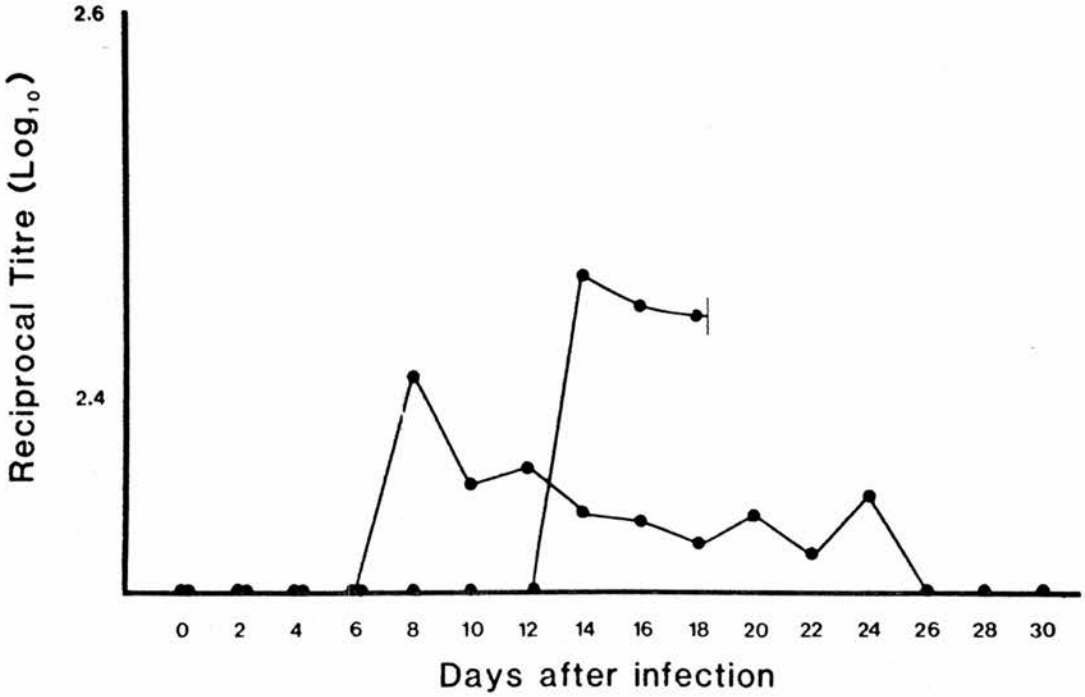

2.6
2.4
Reciprocal Titre (Log_{10})
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days after infection

Table 3.4. Specific ELISA serum antibody titres (log_{10} reciprocal) for the lambs of Group C during experimental orf virus infection.

Days After Infection	Lamb No. 1	Lamb No. 2
0	-	-
2	-	-
4	-	-
6	-	-
8	-	2.41
10	-	2.35
12	-	2.36
14	-	2.34
16	2.46	2.34
18	2.45	2.32
20	2.44	2.34
22	ND	2.32
24	ND	2.35
26	ND	-
28	ND	-
30	ND	-
32	ND	-

ND = not done.

DISCUSSION

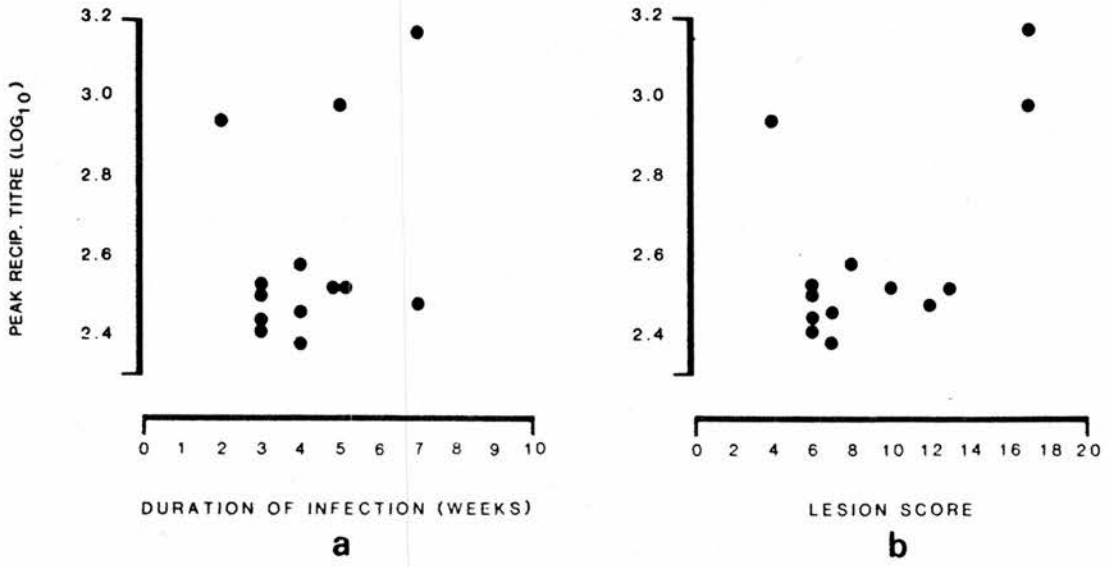
The micro-ELISA assay described in this report represents a reliable and sensitive method for the detection of orf virus specific antibody in ovine serum. Perhaps the most striking feature of the results of these experiments was the extent of individual variation which is apparent in antibody responses of lambs undergoing orf virus infection. Variation seen in the responses of the group A lambs was almost certainly exaggerated by the fact that these animals were not undergoing synchronous infections, but even the animals of Group B displayed a broad range of responses despite receiving identical challenges and reacting similarly. Since there was, in addition, no close association between the severity of lesions in the group A animals and their peak titres (Table 3.5, Fig. 3.5), it is unlikely that these differences were due to the extent of clinical disease.

Table 3.5. Relationship between specific antibody titre and severity of disease in Group A lambs during natural orf virus infection.

Peak* Titre	Total Lesion Score	Duration of Infection(wks)
2.38	7	4
2.41	6	3
2.44	6	3
2.46	7	4
2.48	12	7
2.50	6	3
2.52	10	5
2.52	13	5
2.53	7	4
2.58	8	4
2.94	4	2
2.98	17	5
3.16	17	7

* $\log_{10}$ reciprocal

Fig. 3.5(a),(b). Relationship between peak titre and lesion score (a, r=+0.386, P>0.05); and peak titre and duration of infection (b, r=+0.249, P>0.05) for the lambs of group A during natural infection with orf virus.


PEAK RECIP. TITRE (LOG_{10})
3.2
3.0
2.8
2.6
2.4
0 1 2 3 4 5 6 7 8 9 10
DURATION OF INFECTION (WEEKS)
a
3.2
3.0
2.8
2.6
2.4
0 2 4 6 8 10 12 14 16 18 20
LESION SCORE
b

However, the extent of humoral responses may be influenced by other factors, such as the existence of concomitant disease (Whitelaw, Scott, Reid, Holmes, Jennings and Urquhart, 1979; Reid, Buxton, Gardiner, Pow, Finlayson and Maclean, 1982), or environmental conditions and nutritional status (Webster, 1970; Newberne and Williams, 1970). Genetic factors may also be important. Nguyen (1984) found a genetic influence on the magnitude of the antibody response of experimental lambs to chicken red blood cells, and Biozzi, Sequeira, Stiffel, Ibanez, Mouton and Ferreira (1980), reporting on experiments on mice and guinea pigs concluded that all immunological functions are subject to quantitative regulation.

The rapid high responses of the five lambs in Group B which had pre-existing titres of virus specific antibody were probably anamnestic, whereas the remaining lamb which was seronegative at challenge made a characteristic primary antibody response with a gradual rise in titre which peaked only after five weeks of infection. This group was selected from the institute flock as having been free of clinical orf, and highlights the possibility of infections which are not obvious to the stockman. In contrast with antibody titres recorded in Group B sera, those of Group A remained comparatively low throughout the response, with only two of the lambs attaining values in excess of 2.6 ($\log_{10}$ reciprocal). These lambs had all been examined weekly from birth for the appearance of clinical orf and had been consistently negative prior to the onset of the outbreak. The response which was recorded in this group was therefore almost certainly a primary one.

In view of the fact that most of the Group A lambs were already infected at the time of first sampling and since only one of the animals in Group B had not previously experienced orf virus, the period required before the appearance of specific antibody in the serum after primary challenge could not be measured in these groups. The SPF lambs of Group C were therefore infected so that the initiation of serum antibody responses could be analysed. These animals showed lag periods of 6 and 12 days respectively, while no response was detected in the naive animal of Group B until 12 days after challenge (Fig 3.3, Table 3.3). Such delays are not surprising in the light of those which were recorded in Chapter Two before the appearance of immunoglobulin-containing lymphoblasts in the efferent lymph following inoculation of the nodal drainage area with orf virus. The appearance of detectable antibody in sera from all but three of the Group A lambs by the third week of the outbreak is also compatible with this order of lag period.

Several workers have demonstrated orf virus specific antibodies in colostrum from immune ewes and in serum from colostrum fed lambs (Romero-Mercado, 1969; Poulain and others, 1972; Le Jan and others, 1978), and it could therefore be argued that antibody detected early in the course of the disease in the group A lambs was maternal in origin, since none was older than 4 weeks when the outbreak was first noticed. No attempt was made during the course of this study to assess the immune status of the dams, or indeed to measure the levels or possible protective effects of colostral antibody in these lambs. However, a report by Buddle and Pulford (1984) since the completion of this experiment has confirmed the colostral transfer of orf virus specific antibody from ewes to their lambs, and indicates that maternally derived antibody does

not confer protection against challenge with orf virus at one month of age. The latter is perhaps to be expected in view of the report of Smith, Wells, Burrells and Dawson (1976), who calculated that maternally derived lgG, the predominant immunoglobulin of ovine colostrum, has a half life of 13.7 days in lambs serum. The similar value of 13.5 days reported by Reid and Boyce (1979) for colostrum derived louping-ill virus specific antibodies in lamb serum is a strong indication that only negligible amounts of maternal antibody would have been present in Group A sera at the commencement of sampling in the second week of the outbreak, when the lambs were 2-6 weeks old.

The ELISA system used in this study was more sensitive than that of Koptopoulos and others (1982), who used a crude sensitising antigen and were unable to detect antibody in naturally infected animals. Buddle and Pulford (1984) used an ELISA to investigate the colostral transfer of orf virus specific antibody from ewes to their lambs, and reported their results during the course of these studies. Their assay used untreated purified tissue culture-propagated virus as sensitising antigen and appears to be of the same order of sensitivity as that which is under discussion.

The test represents a considerable improvement on assays which have previously been employed in orf serology both in terms of practicality and sensitivity. The system is not however without its inherent weaknesses. Since ELISA was first described in 1972, no satisfactory method has been devised to convert light absorbance values into an estimate of antibody activity (Peterfy and others, 1983), and as a result, many workers have expressed the antibody content of a given serum as the absorbance value of a defined

dilution (Leinikki and Passila, 1977; Malvano, Boniolo, Dovis and Zannino, 1982). This methcd of data expression, which was employed by Koptopoulos and others (1982) to convey their results, can be misleading due to the non-linearity of the relationship between light absorbance and antibody activity, which follows a sigmoidal regression (Leinikki and Passila, 1976; Malvano and others, 1982).

An alternative method of using light absorbance values to assess serum antibody activity is to perform end-point titrations of e ach test serum sample. The titre of the serum can then be expressed as the last dilution which still yields a reaction above the chosen negative cut off level. Titres reported by Buddle and Pulford (1984) for serum and colostrum were derived in this manner, but because the absorbance/antibody-activity curve flattens out as it approaches its extinction value, minor variations in the test procedure can give rise to considerable variation in the end point, which presents difficulties when comparing results of different test runs (Leinikki and Passila, 1977, Malvano and others, 1982).

The use of a calibration curve derived from dilutions of reference serum to estimate the antibody activity of an ELISA test serum sample was originally described by Leinikki and Passila (1976), and such curves have also been used to express antibody levels in terms of micrograms per litre (Leinikki, Shekarehi, Dorsett and Sever, 1978). This method can be applied with only a single dilution of test serum, involves considerably less laboratory work (a potential source of error) than end point titrations, and results in substantial savings in reagents. However, the sigmoidal nature of the the relationship between light absorbance and antibody activity remains a potential source of error, particularly with test sera of low antibody activity.

For the purpose of this study, the titre of the reference serum was designated as that dilution which resulted in the same light absorbance value as the known negative (SPF) serum. This necessitates an extrapolation from the observed values, and since the curve actually levels off as it approaches its extinction point, this estimate is likely to be lower than the true titre. However, because test serum titres were expressed in this assay not as absolute values, but in terms of a ratio between their activity and that of the reference serum, the significance of this fact to the results reported here was regarded as minimal.

A source of error which is perhaps more significant to these calculations is the extent to which the reference serum dilution factors are correlated with their light absorbance values. It was for this reason that three different regressions of absorbance on serum dilution were analysed by the computer before choosing a standard curve. Computed correlations for the results reported here were as follows:

Group	Regression	Correlation
A	$\log_{10}$ Absorbance Vs dilution	- 0.9773
B	$\log_{10}$ Absorbance Vs dilution	- 0.9774
C	Absorbance Vs $\log_{10}$ dilution	- 0.9922

It would appear therefore that the assay described here provides an acceptably accurate assessment of antibody activity. The question remains whether this assessment is affected by the affinity of antibodies present in the test serum. Since the affinity of antibodies produced during an immune response increases

with time (Eisen and Siskind, 1964), it can be expected that a large proportion of the antibodies in the early serum samples of the first-time responders in these experiments were of low affinity, and the ability of the test to detect these low affinity antibodies is therefore an important consideration. Peterfy and others (1983) investigated the affinity dependence of six antibody assays (RIA, ELISA, Farr assay, haemagglutination, complement mediated haemolysis and precipitation) and found that the two solid-phase assays were least affected by antibody affinity. They observed that determination of antibody concentrations unaffected by affinity were best made using the lower, or "antigen excess" zone of the binding curve. Since most of the primary response sera tested by this assay produced absorbance values corresponding to the lower regions of the reference calibration curve, it is reasonable to assume that these results are truly quantitative, rather than measurements of antibody affinity.

The ELISA test described here has further potential which was not exploited during the course of these studies. A dissection of the humoral response in terms of antibody class specificity is a simple extension of the test accomplished by the use of class specific, enzyme-conjugated anti-immunoglobulin. In this way, the relative contributions of the different classes of antibody to the ovine humoral response in orf virus infection could be investigated.

CHAPTER FOUR

QUALITATIVE STUDY OF OVINE HUMORAL RESPONSES TO ORF VIRUS INFECTION

INTRODUCTION

As described in the preceding chapter, the use of ELISA in a quantitative assessment of the humoral response of the sheep to orf virus infection has revealed that these responses vary considerably in magnitude, particularly in animals which are experiencing infection for the first time. Although no clear association between antibody titre and the severity or duration of lesions was evident in the naturally infected group, the question of whether antibody responses influence the course of the disease remains unanswered in the absence of some estimation of the range of viral antigens against which individual animals deploy antibody.

Such a qualitative assessment of humoral responses is made possible by the Western Blotting method described by Burnette (1981), which combines the techniques of gel electrophoresis and solid phase immunoassay to allow a fine dissection of humoral responses to pathogenic agents. Electrophoresed polypeptides of the organism are transferred by a process of electroelution from the separating gel to a nitrocellulose membrane, where they become trapped by interactions which are poorly understood (Gershoni and Palade, 1983). Incubation of the membrane with test serum followed by an enzyme or radio-labelled anti-immunoglobulin provides an antigen-related profile of the antibody specificities present in the serum.

The excellent resolving powers of the method described by Laemmli (1970) for separating protein mixtures by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) have been applied to the analysis of poxvirus polypeptide structures (Turner and Baxby, 1979; Arita and Tagaya, 1980) and reveal complex patterns

with many polypeptides being shared by members of the orthopoxvirus genus. Differences within this genus are mainly confined to the 30-40 kiloDalton (kD) molecular weight range, although other genera within the group have clearly distinct polypeptide profiles (Arita and Tagaya, 1980). Furthermore, the analysis of orthopoxviruses by SDS-PAGE following controlled degradation by nonidet P 40 (NP40) and 2-mercaptoethanol (2ME) as described by Easterbrook (1966), has shown that the polypeptides which enable orthopoxviruses to be differentiated are located in surface and subsurface layers (Turner and Baxby, 1979). Using the same technique followed by a further fractionation of the surface components, Stern and Dales (1976) demonstrated that the surface tubules of vaccinia virus were composed of a single polypeptide with a molecular weight of 58kD, and could elicit antiviral neutralising activity when inoculated into rabbits.

Thomas, Flores and Holowczak, (1980) attempted to remove surface components from MNV using the method of Easterbrook (1966), and found that a sonication step was necessary in order to separate the surface tubules from this virus. However, after purification using the method described by Stern and Dales (1976), these tubules were shown by SDS-PAGE to be largely composed of a 42-45kD polypeptide.

Buddle, Dellers and Schurig (1984) analysed the structural polypeptides of orf virus preparations derived from 11 different sources using SDS-PAGE and found that differences were confined to the 30-40kD molecular weight range. From a further analysis of three of these strains using the methods described by Thomas and others (1980) they concluded that a major component of the orf virus surface tubules is represented in some strains by a 37kD polypeptide and in others by one with a molecular weight of 44kD.

It seemed likely therefore that the application of SDS-PAGE and the Western Blotting technique to the analysis of orf virus polypeptides would provide a valuable opportunity to examine the specificities of the antibody response which was suggested by the lymphatic cannulation studies of Chapter One to be a major element of the ovine immune response to orf virus infection. Moreover, it was anticipated that antibodies directed at viral surface epitopes could be detected by the analysis of virus which had been subjected to the controlled degradation technique described by Thomas and others (1980). The following account describes such an analysis using serum from the naturally and experimentally infected lambs which were the subject of ELISA studies discussed in the preceeding chapter.

MATERIALS AND METHODS

Sera

Sera for Western Blotting analysis were derived from each of the three animal groups described in the preceeding chapter. In addition, three sera which had previously been determined by ELISA to be of high titre were used as positive controls. Two of these (U93, U150) were derived from persistently infected Friesland rams (see Chapter Six), and the other (D573) was the reference serum described in the preceeding chapter, its source being a lamb which had been hyperimmunised with orf virus. Serum from an SPF lamb served as a negative control.

Virus

The majority of Western Blotting experiments were carried out using virus (U93) which had been purified from clinical material derived from a persistently infected Friesland ram. However, for comparative purposes, two additional viral preparations were also used (FS32/67, FS32/77), and these were derived from lambs which had been naturally infected during outbreaks at the Edinburgh University veterinary field station in the years 1967 and 1977 respectively.

Purification of Virus

Orf virus was purified from clinical material by banding in Nycodenz gradients, as described in Chapter Three. Viral bands were collected, diluted in PBS, and pelleted by ultracentrifugation at 45,000xg for 30 minutes at 4°C. The pellets of purified virus

were then combined and resuspended in 500µl PBS. An aliquot of 3µl was examined for purity by electron microscopy, as described in Chapter Six, and following an estimation of its protein content, was stored at -20°C until required.

Protein Estimation of Purified Virus

The protein content of purified viral preparations was assessed using the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK.). This is based on the method of Bradford (1976) which relies on the existence of two forms (red and blue) of Coomassie Brilliant Blue G-250. The red form is converted to the blue form upon binding of the dye to protein, which results in a shift in its light absorbance maximum from 465 to 595nm. This shift is proportional to the amount of protein present, allowing the construction of a standard curve from the absorbance values produced by dilutions of a reference protein. Test sample values can then be read off this curve to provide an estimate of their protein content.

Owing to the complex nature of orf virus particles, overnight NaoH digestions were performed on the purified virus suspensions (and consequently, on the reference protein dilutions) before each assay. A 5µl aliquot of the suspension was diluted 1:5 in PBS and digested overnight at room temperature in 200µl 0.2M NaoH. A similar digestion was carried out on duplicate 25µl aliquots of six doubling dilutions in PBS of BSA from 0.03125 mg/ml to 1.0 mg/ml. The digests were then neutralised with 200µl 0.2M Hcl and made up to 500µl by the addition of 75µl distilled water before the addition of an equal volume of the Bio-Rad dye reagent at a dilution of 1:2.5 in distilled water.

After 2 minutes, the absorbance at 595nm of the duplicate reference protein dilutions was measured against a reagent blank (prepared from 500µl buffer and 500µl dye reagent) with a spectrophotometer (Pye Unicam SP6-450UV/V15, Pye Unicam Ltd., Cambridge, UK.) using a 1cm light path, and the concentration of protein was plotted against corresponding absorbance values to produce a standard curve. The absorbance value of the test sample was then measured, and its protein concentration read off the curve. This value was then adjusted for dilution factor to give the approximate protein content of the orginal sample. Normally, three dilutions of the sample NaoH digest were made before the addition of dye reagent, in order to ensure that sample absorbtion values fell on the linear part of the standard curve. Each 1g of U93 clinical material consistently yielded a purified virus suspension containing approximately 1mg of protein.

Electrophoretic Separation of Viral Polypeptides

Viral antigens were separated by discontinuous SDS electrophoresis in polyacrylamide gels, and this was achieved using the gel compositions and buffer systems described by Laemmli (1970). Before electrophoresis, sample proteins were completely dissociated in the presence of SDS and 2-ME by immersion in boiling water for 90 seconds. Stacking and resolving gels contained 3% and 10% acrylamide respectively, and gel dimensions were 140mm x 160mm x 0.75 mm. Electrophoresis was carried under constant current conditions at a current density of 19mA/cm^2 until the tracking dye (bromophenol blue, 0.001%) included in the sample buffer had migrated approximately 8cm into the resolving gel (2 - 2½ hr).

Two alternative methods of loading gels with dissociated samples were employed. Where several antigen preparations were to be reacted with a single antiserum, a stacking gel with fifteen sample wells was used. For experiments where the reactions of several antisera with a single antigen preparation were to be investigated, samples were loaded into a single large well, resulting in a broad track which after transfer to nitrocellulose could be processed in strips. Gels were loaded with viral proteins at the rate of 5µg/mm² of sample well area, and a single track of molecular weight marker proteins was included in each run. The following molecular weight markers were used:

Equine cytochrome C (12.3kD))	
Equine myoglobin (17.2kD))	
Bovine chymotrypsinogen (25.7kD))	BDH Chemicals Ltd.,
Hen egg ovalbumen (45kD))	Poole, Dorset, UK.
Bovine serum albumin (66.25kD))	
Hen egg ovotransferrin (76-78kD))	
Phosphatase B (93kD))	Sigma Chemical Company,
Beta galactosidase (130kD))	St. Louis, Missouri, USA.

Electroblotting

Viral proteins in SDS polyacrylamide gels were transferred to nitrocellulose membrane using the method of Burnette (1981), as modified by Herring and Sharp (1984). Briefly, this involved the placing of the gel (with stacking gel removed) in a sandwich consisting of Whatmann 3mm filter paper (Whatman Ltd., Maidstone, Kent, UK.), an appropriately sized nitrocellulose sheet (pore size 0.2µ, Schleicher and Schull, Dassel, West Germany.), and a further sheet of filter paper. This sandwich was constructed most satisfactorily by prewetting the filter paper and nitrocellulose with electroblotting buffer (20mM Tris, 154mM glycine, 20% v/v methanol, pH 8.3), and laying the gel on the membrane with the

exclusion of air bubbles. Before completing the sandwich, the boundaries of the gel and the positions of the tracks and dye front were marked on the nitrocellulose membrane using a ball-point pen. The sandwich was then compressed in an electroblotting cassette (E-C Electroblot, E-C apparatus corporation, St. Petersburg, Florida, USA.) with the nitrocellulose towards the anode, and the cassette was placed in a tank containing electroblotting buffer. Buffer was continuously circulated across the cassette by means of a small pump. Transfer was allowed to proceed for 18 hr at a current density of 1.3mA/cm^2 and a voltage gradient of approximately 7.0 volts/cm.

After transfer, the membrane was trimmed and cut into appropriate strips. That strip which contained the molecular weight marker proteins was stained for 30 minutes in a solution of 30% methanol and 5% acetic acid containing 16µg/ml coomassie blue, and destained for a similar period in the same solution without the dye.

Reaction of Electroblots with Sera

Electroblots were probed with test sera using the protocol described by Herring and Sharp (1984), which involves three separate incubations and two washes, all of which are performed using a rocking platform.

In order to occupy non-specific binding sites on the membrane, strips were placed in horse serum diluted 1:1 in washing buffer (8.7mM sodium phosphate buffer pH 7.2 containing 500 mM NaCl, 0.5% Tween 80 and 1.0 mM EDTA), and incubated for 1 hr at 37°C. They were then transferred to the test serum (diluted 1:40 in washing

buffer containing 5% horse serum) and incubated for a further hour at room temperature. This incubation was followed by a thorough wash in washing buffer, which consisted of three rapid rinses, three 5 minute immersions and three more rapid rinses. Strips were then transferred to the detection serum, and incubated for 1 hr at room temperature. The detection serum was an affinity-column-purified rabbit IgG specific for ovine $F'ab_2$, which had been prepared by Mr A Dawson of the Moredun Insitute. This reagent had been labelled with ^{125}I using the chloramine T reaction (Hunter and Greenwood, 1962) by Mr N Inglis of the Moredun Institute, and was used at a previously determined optimal dilution of 1:800 in washing buffer containing 5% horse serum.

A further thorough wash was then performed, and the strips were dried under vacuum at room temperature and mounted on filter paper before being exposed to X-Ray film (Kodak X-Omat S, Kodak, Liverpool, UK.) in a radiographic cassette containing an intensifying screen.

Staining of Polyacrylamide Gels

Where necessary, viral polypeptides in polyacrylamide were stained with silver using the method of Morrissey (1981). Gels were prefixed in 50% methanol, 10% acetic acid for 20 minutes, followed by 5% methanol 7% acetic acid for 20 minutes. They were then fixed for 20 minutes in 10% glutaraldehyde, and rinsed repeatedly in distilled water for several hours. This was followed by immersion in 5µg/ml dithiothreitol for 20 minutes, before equilibrating in 0.1% silver nitrate for a further 20 minutes. Gels were then rinsed once rapidly in distilled water and twice rapidly in a small amount of developer solution before being soaked

in the latter (50µl of 37% formaldehyde in 100ml 3% sodium carbonate) until the desired level of staining had been achieved. Staining was stopped by the addition of 5ml of 2.3M citric acid per 100ml of developer solution used, followed by agitation for 10 minutes. This mixture was then discarded and gels were rinsed for approximately 30 minutes in several changes of distilled water before being photographed. For storage, gels were soaked for 10 minutes in 0.03% sodium carbonate to prevent bleaching, and sealed in heat sealable plastic bags. Gentle agitation throughout this procedure was achieved by the use of a rocking platform.

Controlled Degradation of Virus

A modification of the method described by Thomas and others (1980) was used. Approximately 400µg of purified orf virus were resuspended in 5ml of PBS containing 1% NP40 and 0.1% 2-ME and incubated in a waterbath at 37°C for 1 hr. The suspension was then sonicated intermittently on ice for 2 minutes using an ultrasonic disintegrator at an amplitude of 14µ, and the viral cores were pelleted by ultracentrifugation at 45,000xg for 30 minutes at 4°C. Viral components in the supernatant fraction were then precipitated by the addition of 2 volumes of chilled ethanol and incubation for 1 hr at 4°C, before being pelleted at 2,000xg for 10 minutes. This pellet and viral cores were each resuspended in 150µl PBS for later analysis by SDS-PAGE and Western Blotting.

Molecular Weight Estimations

Molecular weights of viral bands in gels and on Western Blots were calculated by reference to a standard curve derived from the plot of the relative mobilities of the reference proteins against the $\log_{10}$ of their molecular weights (Shapiro, Vinuela and Maizel, 1967).

RESULTS

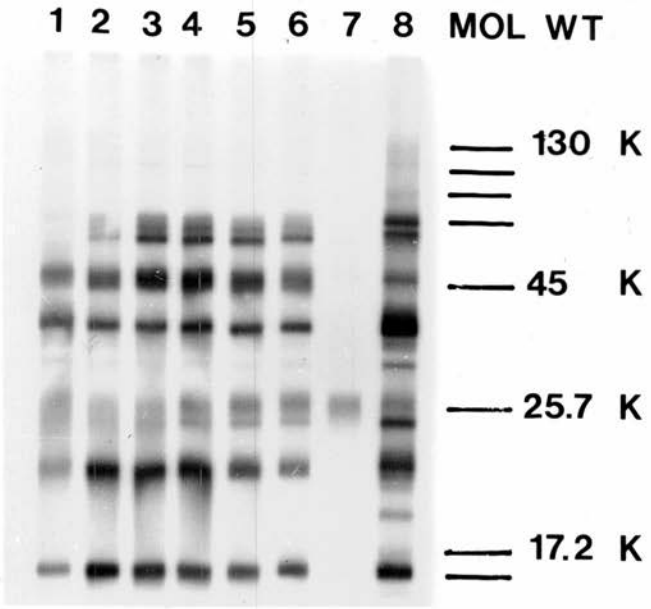
Western Blotting analysis of Sera from Naturally and Experimentally Infected lambs

Autoradiographs prepared using sera from groups A, B and C are presented in Fig. 4.1, 4.2, 4.3, 4.4. Two Western Blotting studies were performed on the group A sera. The first was a sequential analysis of the development of antigen recognition during the response of lamb 488, which achieved the highest ELISA titre. By the fifth week of the outbreak, this lamb was reacting with at least seven antigenic components of the virus (Table 4.1). Three weeks later, at the peak of its ELISA response, ten antigenic determinants were identified, and no further bands were detected by sera taken during the subsequent two weeks. A band with an approximate molecular weight of 29K is identifiable in all lanes (Fig 4.1) including that probed with SPF serum, and is probably the result of non-specific binding. There were obvious differences between the antigen recognition patterns shown by sera from lamb 488 and that of the hyperimmunised lamb D573 which was included as a positive control, in that the latter reacted with two determinants not recognised by 488 (77kD and 18kD).

Such diversity was also evident from the second Western Blotting analysis of group A sera, in which serum taken from each lamb on the fifth week of the outbreak was examined for its range of specificities (Fig 4.2). Although thirteen viral antigens were recognised by the group as a whole, only four of these were common to all of the sera (Table 4.2). An additional band (51kD) shared by the entire group was also present on an SPF serum-probed strip and was therefore of questionable significance. The most reactive

Fig. 4.1. Western Blot of U93 orf virus antigens probed with weekly serum samples from group A lamb 488 (lanes 1-6); SPF serum (lane 7), and serum from an orf virus-hyperimmunised lamb, D573 (lane 8). Bars represent approximate molecular weights (kD).

Fig. 4.2. Western Blot of U93 orf virus antigens probed with sera from the group A lambs during the fifth week of an orf outbreak (lanes 1-13); serum diluent alone (lane 14), and SPF serum (lane 15). Bars represent approximate molecular weights (kD).


1 2 3 4 5 6 7 8 MOL WT
130 K
45 K
25.7 K
17.2 K

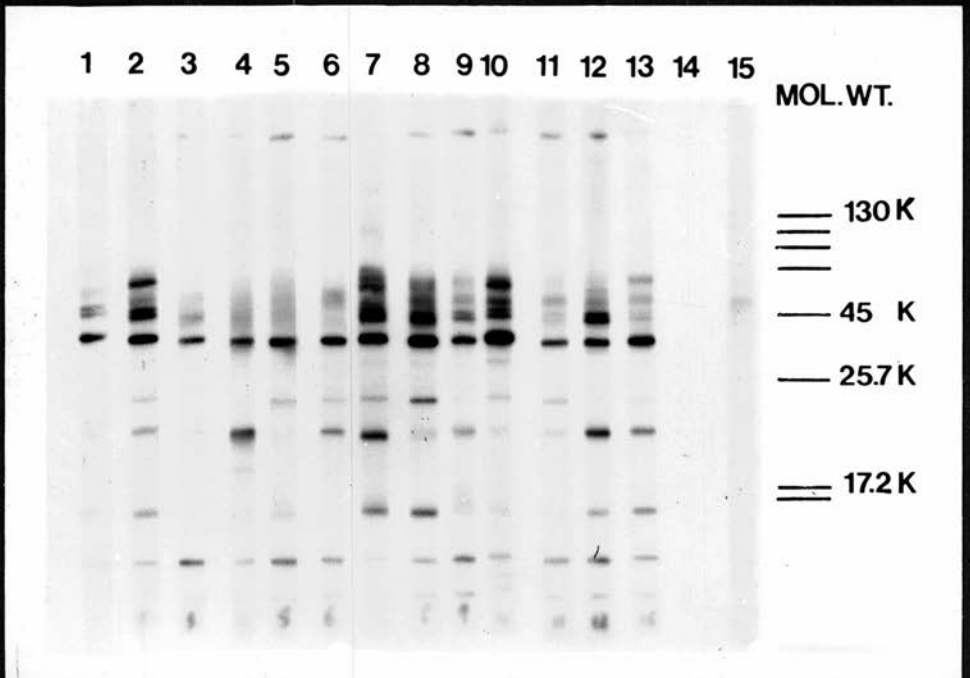

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
MOL.WT.
130 K
45 K
25.7 K
17.2 K

Table 4.1. Antigenic specificities present in sera from lamb 488 during the course of natural orf virus infection.

Molecular Weight(kD)	Week of Outbreak 2	3	4	5	6	7	Standard Sera D573	SPF
96	±	+	+	+	+	+	+	-
88	-	+	+	+	+	+	+	-
77	-	-	-	-	-	-	+	-
70	+	+	+	+	+	+	+	-
66	-	+	+	+	+	+	+	-
62	-	+	+	+	+	+	-	-
52	+	+	+	+	+	+	+	-
41	+	+	+	+	+	+	+	-
35	+	+	+	+	+	+	+	-
29	+	+	+	+	+	+	+	+
27	-	-	±	+	+	+	+	-
22	+	+	+	+	+	+	+	-
18	-	-	-	-	-	-	+	-
13	+	+	+	+	+	+	+	-

Table 4.2. Antigen specificities present in sera of Group A lambs five weeks after the onset of an outbreak of orf virus infection.

Molecular Weight(kD)	Lamb Number													SPF
	461	488	726	727	729	730	735	746	798	846	867	881	885	serum
87	-	+	-	-	-	-	-	-	-	-	-	-	-	-
63	+	+	-	-	+	-	-	+	+	-	-	+	-	-
59	-	+	+	+	+	+	+	+	-	+	-	+	+	-
57	-	+	+	-	+	-	+	+	-	-	-	-	-	-
51	+	+	+	+	+	+	+	+	+	+	+	+	+	+
47*	+	+	+	+	+	+	+	+	+	+	+	+	+	-
45*	+	+	+	+	+	+	+	+	+	+	+	+	+	-
40*	+	+	+	+	+	+	+	+	+	+	+	+	+	-
33	-	+	-	-	+	-	+	+	-	-	-	-	+	-
25	-	+	-	+	+	+	+	+	+	+	+	-	+	-
20	+	+	+	+	-	+	+	+	+	+	+	+	+	-
16	-	-	-	-	-	-	-	-	-	-	-	-	+	-
12	-	+	+	+	+	+	+	+	-	+	+	+	-	-
8*	+	+	+	+	+	+	+	+	+	+	+	+	+	-

* common to all members of the group

lamb was 488, whose serum identified 13 viral determinants, while the least reactive of the group was lamb 461, which produced detectable antibody against only 7 antigens. In terms of numbers of antigens detected, values for the group were normally distributed about a mean of 9.6±1.71 (standard deviation).

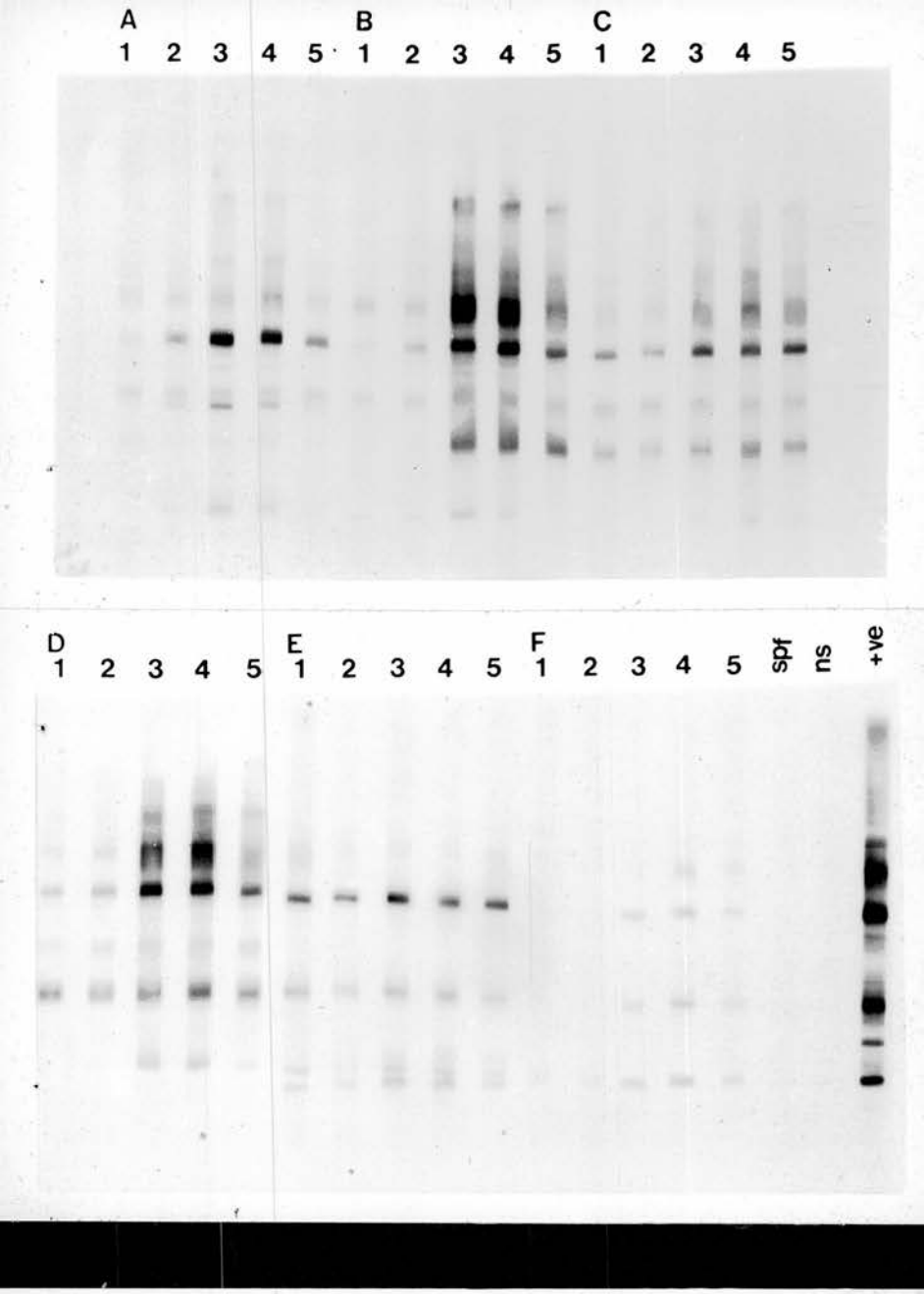
Analysis of group B sera revealed equally heterogenous Western Blotting profiles (Fig 4.3) and as expected, prechallenge sera from the five lambs which were seropositive before experimental inoculation could detect at least three viral antigens on nitrocellulose. Twelve different antibody specificities were evident in the group (Table 4.3), and the number of antigens identified by the different sera ranged from 4 in lamb 6, to 9 in lamb 1. With the exception of the latter animal, whose response was under way by day 6 after inoculation, additional specificities were not obvious in sera until the twelfth day of infection. However, one animal (lamb 3), whose prechallenge serum reacted with six viral determinants, showed no apparent extension of this specificity range throughout its response.

The absence of specific antibody detectable by ELISA in the serum of lamb 6 until day 12 after inoculation (see Chapter Three) was confirmed by analysis of its sera by Western Blotting, and the response of this animal was also the most restricted in range, with only 4 of the 12 antigens being detected. Only four antigen specificities were common to all members of the group (Table 4.3), with the remaining antigens apparently varying in their immunogenicity for the different animals.

The serological responses of the two SPF lambs of group C were limited in specificity to only three viral antigens (Fig 4.4, Table 4.4), and were not detectable by Western Blotting until the eighth

Fig. 4.3. Western Blot of U93 virus antigens probed with serum samples from the group B lambs at days 0,6,12,17 and 24 (lanes 1-5) after experimental infection with orf virus. Sections A,B,C,D,E and F refer to lambs 1,2,3,4,5 and 6; ns= serum diluent alone; +ve= serum from ram U93.

Fig. 4.4. Western Blot of U93 virus antigens probed with serum from group C lamb no.1 (lanes 1-6); lamb no.2 (lanes 7-15), and standard negative (SPF) serum (lane 16).


A
1 2 3 4 5
B
1 2 3 4 5
C
1 2 3 4 5

D
1 2 3 4 5
E
1 2 3 4 5
F
1 2 3 4 5
spf
ns
+ve

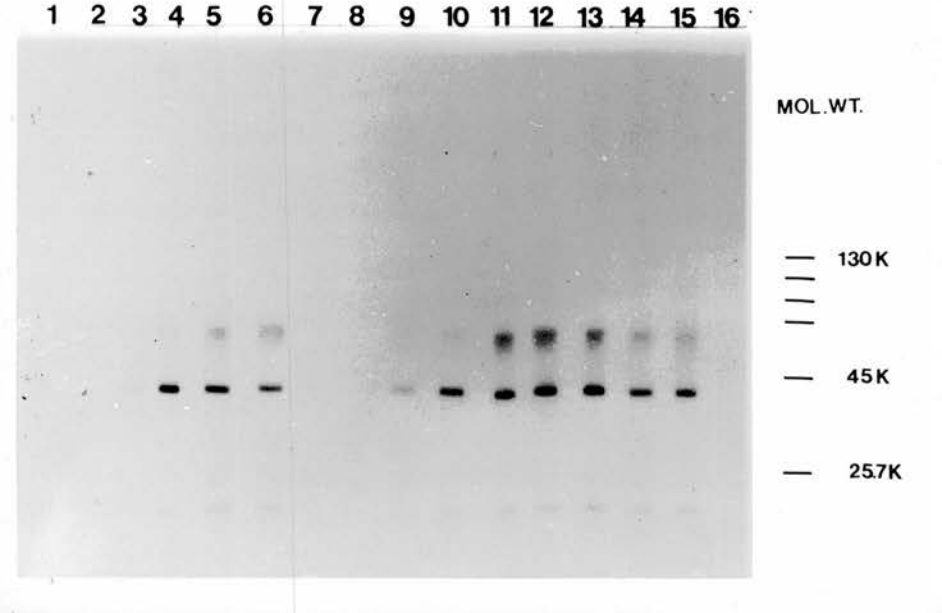

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
MOL.WT.
130K
45K
25.7K

Table 4.3. Antigen specificities present in sera from Group B lambs during experimental infection with orf virus.

Lamb No.	Days P.I.	Molecular Weight (kD)											
		96	72	63	51*	38*	31	27	24	20	16	12*	11
1	0	-	-	-	+	+	+	+	-	+	-	-	-
1	6	-	-	-	+	+	+	+	+	+	-	-	-
1	12	+	-	+	+	+	+	+	+	+	-	+	-
1	17	+	-	+	+	+	+	+	+	+	-	+	-
1	24	+	-	+	+	+	+	+	+	+	-	+	-
2	0	-	-	-	+	+	-	+	-	-	-	+	-
2	6	-	-	-	+	+	-	+	-	-	-	+	-
2	12	+	-	+	+	+	+	+	-	+	-	+	-
2	17	+	-	+	+	+	+	+	-	+	-	+	-
2	24	+	-	+	+	+	-	+	-	+	-	+	-
3	0	-	-	-	+	+	-	+	-	-	-	+	-
3	6	-	-	-	+	+	-	+	-	-	-	+	-
3	12	+	-	+	+	+	-	+	-	-	-	+	-
3	17	+	-	+	+	+	-	+	-	-	-	+	-
3	24	+	-	+	+	+	-	+	-	-	-	+	-
4	0	-	-	-	+	+	-	+	-	+	-	+	-
4	6	-	-	-	+	+	-	+	-	+	-	+	-
4	12	+	+	+	+	+	+	+	+	+	+	+	-
4	17	+	+	+	+	+	+	+	+	+	+	+	-
4	24	+	+	+	+	+	+	+	-	+	-	+	-
5	0	-	-	-	+	+	-	-	-	+	-	+	+
5	6	-	-	-	+	+	-	-	-	+	-	+	+
5	12	-	-	-	+	+	-	-	-	+	-	+	+
5	17	-	-	-	+	+	-	-	-	+	-	+	+
5	24	-	-	-	+	+	-	-	-	+	-	+	+
6	0	-	-	-	-	-	-	-	-	-	-	-	-
6	6	-	-	-	-	-	-	-	-	-	-	-	-
6	12	-	-	-	+	+	-	-	-	+	-	+	-
6	17	-	-	-	+	+	-	-	-	+	-	+	-
6	24	-	-	-	+	+	-	-	-	+	-	+	-

*common to all members of the group

day of infection. In both, initial antibody production was directed at a 38kD polypeptide, and by day 12 after inoculation the limits of the response was reached with the recognition of two futher antigens. It is possible however, that additional specificities may have been concealed in the low molecular weight band, which was not fully resolved.

Table 4.4. Antigen specificities present in sera from Group C lambs during experimental infection with orf virus.

Lamb No.	Molecular Weight(kD)	Days After Infection								
		0	4	8	12	16	20	24	28	32
1	51	-	-	-	+	+	+	ND	ND	ND
1	38	-	-	+	+	+	+	ND	ND	ND
1	22	-	-	-	+	+	+	ND	ND	ND
2	51	-	-	-	+	+	+	+	+	+
2	38	-	-	+	+	+	+	+	+	+
2	22	-	-	-	+	+	+	+	+	+

ND = not done

The results of SDS-PAGE analysis of the fractionated products of purified U93 virus after incubation in NP40 and 2ME followed by sonication are illustrated by the silver stained gel in Fig 4.5 which suggests that at least six viral components were released by the extraction. However, Western Blotting anaylsis of surface associated viral polypeptides using serum (D573) from an orf virus-hyperimmunised lamb revealed only one antigenic component in this fraction, with an approximate molecular weight of 38kD. This band was depleted in the blotting pattern of the viral cores when compared with that of the intact virus (Fig. 4.6).

Electron microscopic examination of U93 virus after incubation with NP40 and 2ME followed by sonication revealed that the majority

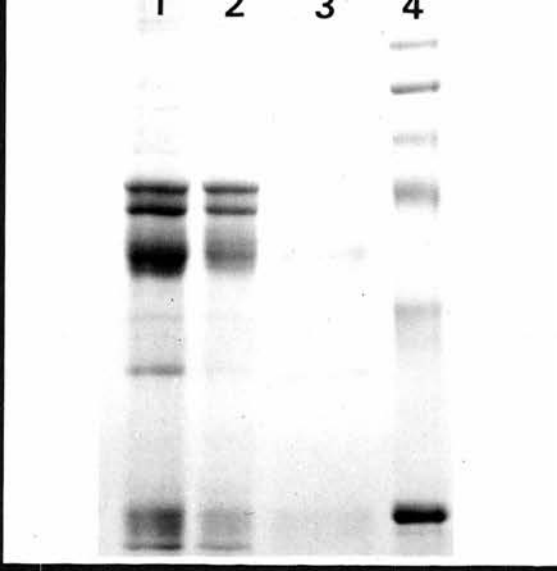
of viral particles had been stripped of their surface tubules and most were seen surrounded by disrupted tubular material (Fig 4.8,(a)). After fractionation, the pellet was seen to contain a predominance of naked viral cores (Fig 4.8,(b)) while the supernatant fraction contained organised material resembling the disrupted surface tubules seen in the whole preparation (Fig 4.8,(c)).

In order to investigate the dependence of the blotting patterns of a given serum on the strain of orf virus used as the source of nitrocellulose immobilised target antigen, three purified virus preparations (U150, FS32/67, FS32/77) derived from different outbreaks of orf were electrophoresed in adjacent wells of each half of an SDS polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was then divided in two, and one half was probed with serum from the hyperimmune lamb D573. The other half, which also contained molecular weight markers, was stained with coomassie blue as described above, and showed an obvious variation (Fig 4.7,(a)) in polypeptide profile between the three viral strains which is further discussed in Chapter Five. This heterogeneity was however considerably reduced in the Western Blotting patterns of D573 (Fig 4.7,(b)) serum with these strains, which are so similar that any differences could have been the result of an unavoidable reduced loading of the gel with FS32/77 due to the limited quantities of this strain which were available.

Fig. 4.5. Silver-stained SDS-PAGE gel of U93 virus preparations: intact (lane 1); pellet fraction, NP40/2ME extract (lane 2); ethanol precipitate of supernatant fraction, NP40/2ME extract (lane 3); size marker proteins (lane4).

Fig. 4.6. Western Blot of U93 orf virus preparations probed with serum from an orf virus-hyperimmunised lamb D573: intact (V); pellet fraction, NP40/2ME extract (VC); ethanol precipitate of supernatant fraction, NP40/2ME extract (SC). Bars represent positions of molecular weight marker proteins

Fig. 4.7. Western Blot of antigens of orf virus isolates U150 (lanel), FS32/67 (lane 2) and FS32/77 (lane 3) probed with serum from an orf virus-hyperimmunised lamb D573 (a), and stained with coomassie blue (b). S= molecular weight marker proteins.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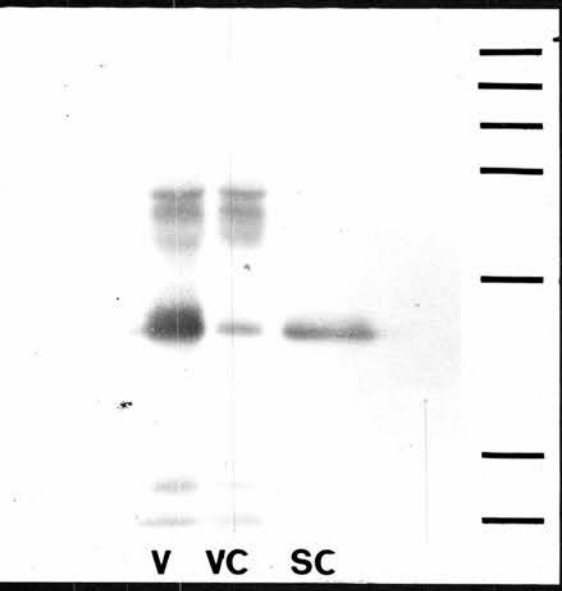
2
3
4


V
VC
SC

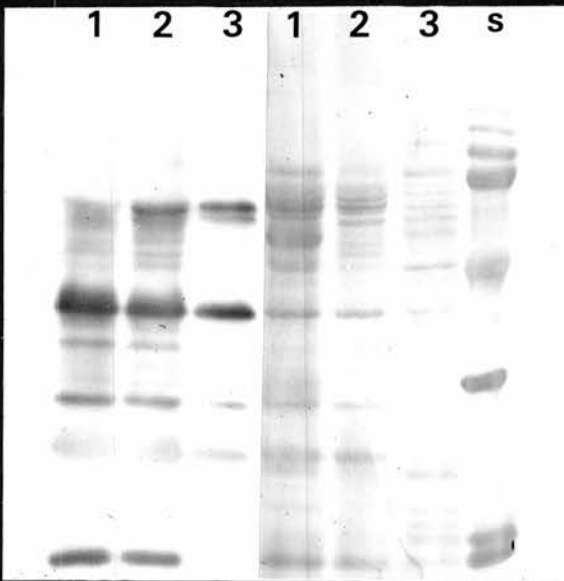
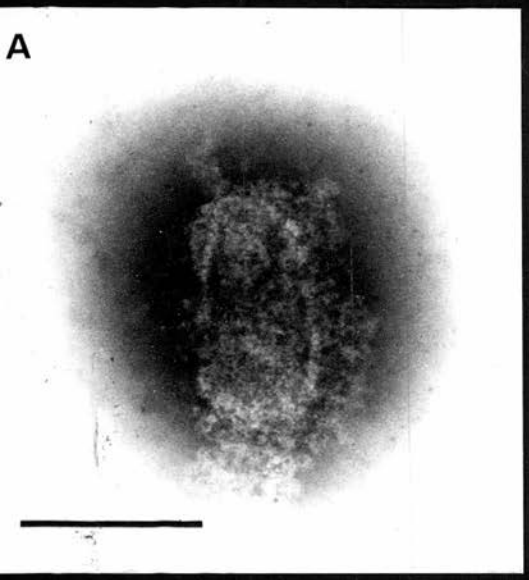

1
2
3
1
2
3
s

Fig. 4.8. Electron micrographs of orf virus U93 after treatment with NP40/2ME and staining with PTA. Sonicated whole preparation showing tubular material associated with viral cores (a); pellet fraction of sonicated preparation showing viral cores (b); supernatant fraction of sonicated preparation showing material resembling surface tubules (c). Bars represent 0.2μ.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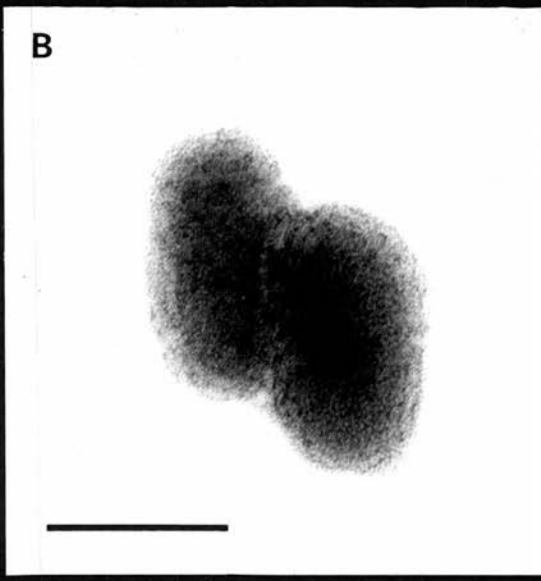
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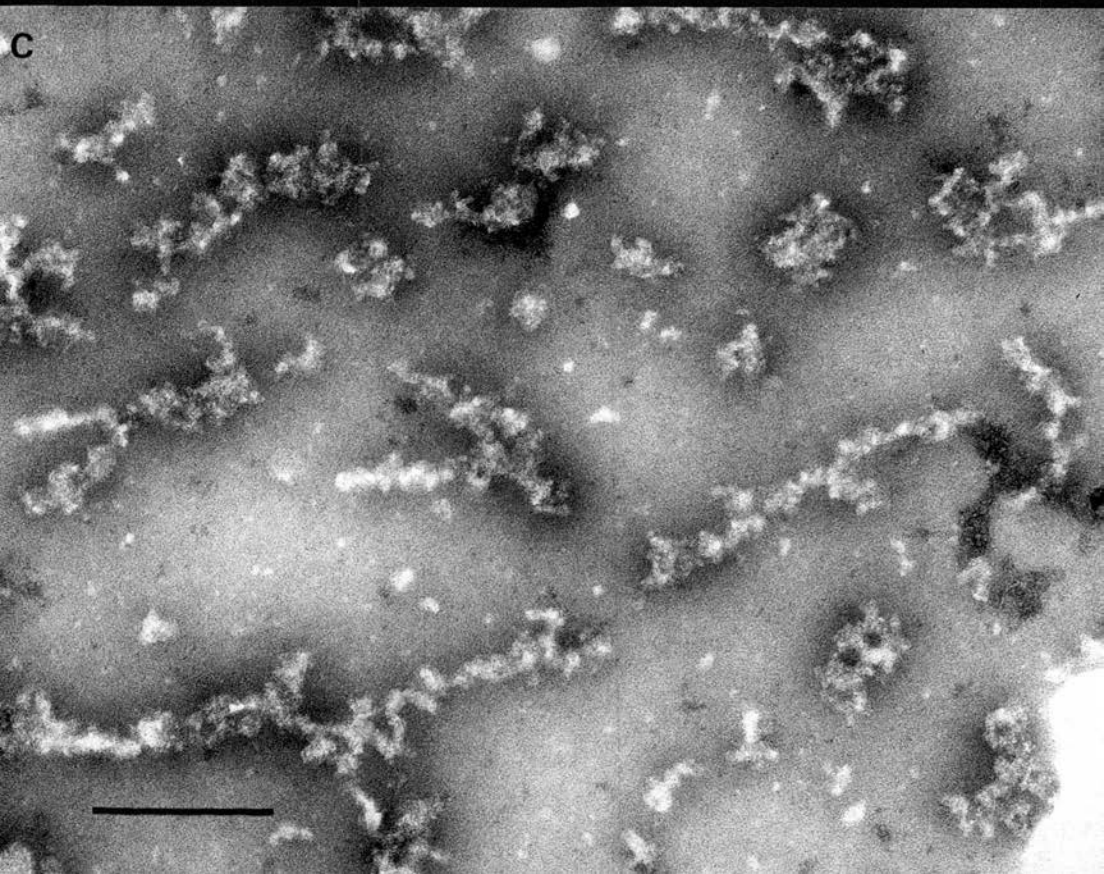

C

DISCUSSION

The antigenic valency of antibody responses in orf virus-infected sheep would appear from these results to be subject to the same individual variation which was evident from the ELISA studies reported in Chapter Three. In addition, the limited responses which were detected by ELISA in animals undergoing primary infections with the virus, were shown by the Western Blotting technique to be equally restricted in specificity.

In all, at least sixteen viral antigens were detected by sera from the three experimental groups, but although the 51kD antigen which appeared in the Western Blots of the group B and group C sera probably represents the 47kD, 45kD doublet in the group A blot, (Table 4.5), only one band (38-41kD) is clearly shared by the blotting patterns of all the sera tested. Because this 38-41kD viral component can be removed from the virion by sonication after treatment with nonionic detergent and a reducing agent, it is almost certainly surface associated (Thomas and others, 1980; Buddle and others, 1984), which suggests that a highly immunogenic surface antigen present in the U93 orf virus strain is shared both by the strain responsible for the Edinburgh University field station outbreak and that used for experimental infections at Moredun Institute.

However, data which is generated using the Western Blotting technique must be interpreted in the light of an important source of error which is inherent in the method. Prior to electrophoresis, virus samples for separation on SDS polyacrylamide gels are denatured by boiling in the presence of excess SDS and 2ME, and as a result, viral proteins are resolved in the gels as

linear polypeptides and transferred as such to nitrocellulose membranes. Therefore viral antigenic sites (epitopes) which result from structural folding of polypeptides, or the proximity of amino acid sequences from two or more polypeptide chains, are unlikely to be present on the membrane. It follows that serum antibodies specific for such epitopes may not be detected by the test, and therefore the complement of antibody specificities of orf virus antigens which was evident for the lambs in this study could in reality be greater.

In two of the blotting analyses described in this chapter, bands which were common to all of the test sera were also present in strips probed by SPF lamb serum (Fig 4.1, 4.2) indicating that these bands were the result of non-immune events. Non-specific binding such as this has been described by Herring and Sharp (1984) and attributed to mechanisms such as hydrophobic interactions between immunoglobulins and denatured proteins on the nitrocellulose membrane, or the binding of immunoglobulin to substances present in the mercaptoethanol of the sample denaturing buffer. These authors recommend the inclusion of control serum in the procedure so that artifactual bands may be detected.

Calculated molecular weights for the antigens which were detected by the various sera used in these studies did not correlate exactly between the different blots (Table 4.5) although in most cases were acceptably close. Molecular weight estimations were based on the linearity between $\log_{10}$ molecular weight and relative mobility of polypeptides in polyacrylamide gels (Shapiro and others, 1967) and were calculated by reference to a curve derived from the mobilities of eight standard proteins which were

electrophoresed at the edge of each gel. Since the linearity of this curve was restricted to a range of approximately 100kD and 16kD, any accuracy of these calculations is confined to these limits. Furthermore, the buffer front of these gels was invariably bowed rather that straight, giving rise to a distortion in mobility which increased with distance from the gel edge. This effect can be seen clearly in the strips (Fig. 4.3, E and F) probed with sera from the group B lambs 5 and 6. These strips were adjacent on the membrane, and the lower two bands can be seen to converge along the series of strips probed with sera from lamb 5, until only one is discernible in the strips probed with the lamb 6 sera. Molecular weight values which have been quoted for these antigens are therefore only approximate.

There are no published descriptions of the use of Western Blotting techniques in orf serology, with which these results may be compared. However, Thomas and others (1980) described the analysis by SDS-PAGE of MNV, a parapox virus closely related to orf virus. They reported a polypeptide profile which is distinct from but clearly as complex as that of vaccinia virus run under similar conditions, and found that 10 polypeptides could be released from the virion by treatment with NP40 and 2ME followed by sonication. When the characteristic surface tubules were partially purified from this surface associated fraction using the method of Stern and Dales (1976) the resulting preparation showed enrichment of a 45kD polypeptide and only traces of the other nine.

Buddle and others (1984) carried out a similar investigation with orf virus and compared the polypeptide profiles of 11 different strains. They reported differences in these profiles

Table 4.5. Orf viral antigens detected in all five Western Blotting experiments. The two specificities which were common to all sera tested are asterisked.

Antigen	Western Blot 1 (Fig 4.1)	2 (Fig 4.2)	3 (Fig 4.3)	4 (Fig 4.4)	5 (Fig 4.6)
1	96	-	96	-	-
2	88	87	-	-	-
3	77	-	72	-	-
4	70	63	63	-	-
5	66	59	-	-	-
6	62	57	-	-	-
7	52*	47*	51*	51*	-
8		45*			-
9	41*	40*	38*	38*	38*
10	35	33	31	-	-
11	-	-	27	-	-
12	27	25	24	-	-
13	22	20	20	22	-
14	18	16	16	-	-
15	13	12	12	-	-
16	-	8	11	-	-

which were confined to the 37kD to 44kD molecular weight region. Surface components of two of these isolates were removed using the method of Thomas and others (1980) and further fractionated as described by Stern and Dales (1976), and when examined by electron microscopy, revealed organised structures resembling the surface tubules described for vaccinia by Stern and Dales (1976) and for MNV by Thomas and others (1980). SDS-PAGE analysis of these preparations indicated that their main component was a 44kD polypeptide.

A direct comparison between the results of these workers and those reported here is not possible, since different electrophoretic systems were used, and even the source of the SDS used in discontinuous SDS PAGE has been shown to alter banding patterns (Swaney, Van DeWoude and Bachrach, 1974). However, it is likely that the 38-41kD polypeptide detected by the hyperimmune lamb serum in the blot of the surface associated fraction (Fig. 4.6) corresponds to the 44kD band described by Buddle and others (1984). No further fractionation of viral surface components was performed in this study in order to purify the surface tubules, but electron microscopic examination of the sonicated degradation mixture before fractionation revealed viral cores surrounded by disrupted surface tubular material. After fractionation, the surface associated fraction was seen to be composed mainly of this material while little was evident in the fraction containing the viral cores (Fig.4.8,(c)).

It would appear therefore that the 38-41kD polypeptide recognised by all the sera in this study is a major component of the surface tubules which are characteristic of orf virus. Stern

and Dales (1976) found that antiserum raised against surface tubules removed from vaccinia virus after treatment with detergent and 2ME had neutralising activity against the virus, which suggests that the neutralising activity which has been detected in convalescent orf sera by many investigators of the disease (reviewed in Chapter One) may be directed at surface tubule components.

Buddle and others (1984) found that the molecular weight of the surface tubule-associated polypeptide varied among the 11 isolates which they studied, and grouped these strains accordingly. However, polypeptide profiles prepared from six different orf strains during the course of this project have all featured a distinct band in the 38-41kD molecular weight range (see Fig. 5.7). Furthermore, the 38-41kD antigen of the orf virus strain (U93) used as test antigen for these Western Blotting studies, was universally recognised by all the sera which were assayed. This would suggest that either British strains of orf virus do not exhibit the same variation as North American strains, or that molecular weight differences in surface tubule polypeptides do not significantly alter their antigenicity.

Antigenic similarities between orf virus strains would appear not be confined to the 38-41kD surface tubule associated polypeptide. The seemingly identical patterns (Fig. 4.7) which resulted from the probing of electrophoresed polypeptides from orf virus preparations derived from three different sources suggest that the capacity of an orf virus-immune serum to react with viral antigen is less a function of the antigenic structure of the immunising virus than of variation in the immune responses of

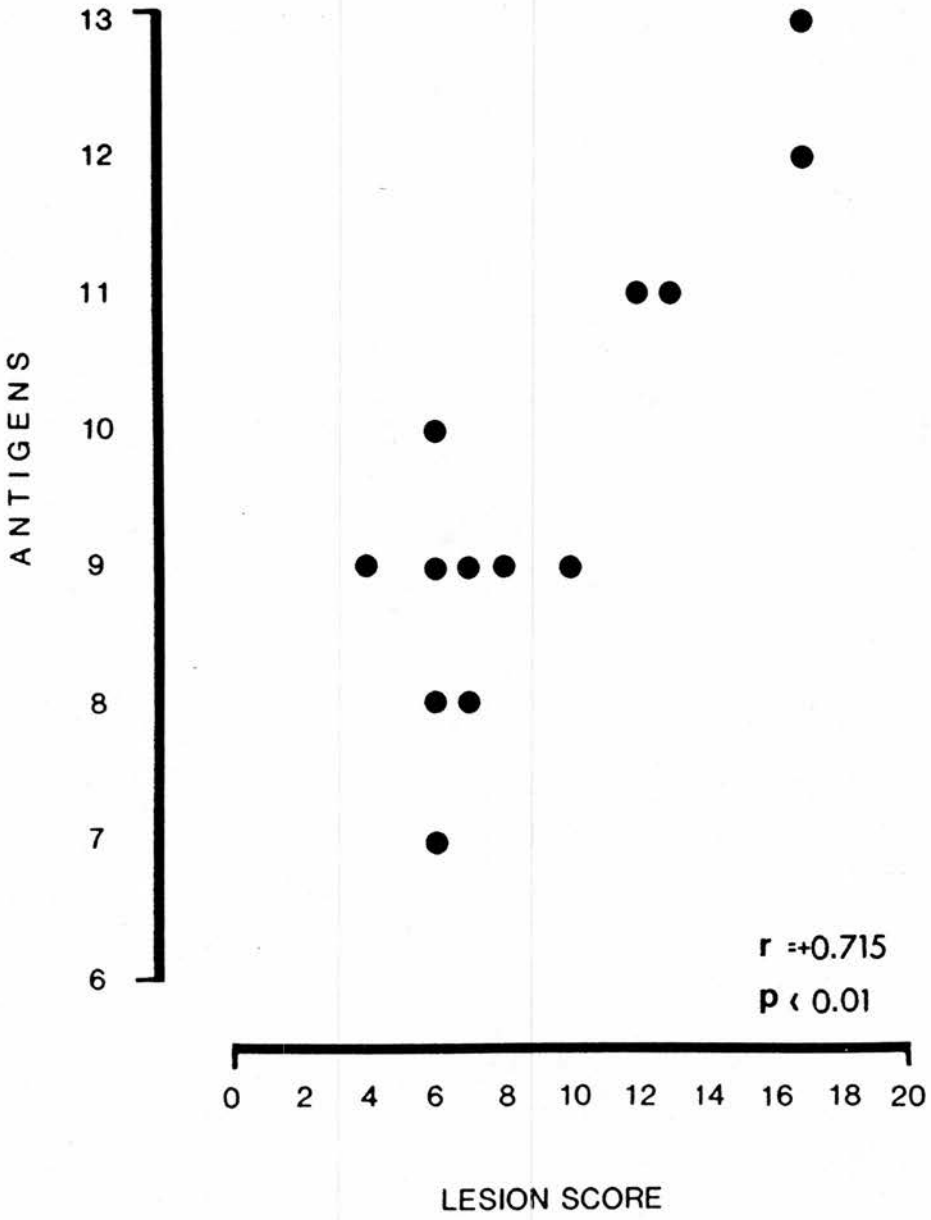
individual animals. This may furnish some explanation of the inconsistent results (reviewed in Chapter One) of attempts to demonstrate strain variation in orf virus using serological methods such as cross protection and cross neutralisation.

In any event, the amounts of orf viral antigenic specificities which are present in sera from infected animals would not appear to affect the outcome of the disease. A Spearman rank correlation analysis of the relationship between lesion score and serum antibody specificities among the group A lambs reveals that the number of viral antigens recognised by the animal is correlated ($r=+0.715$, $p<0.01$) with the severity of disease (Table 4.6, Fig. 4.9), which endorses the apparent ineffectiveness of humoral responses in orf virus infection which was suggested by the results of Chapter Three.

Table 4.6. Relationship between anti-viral serum antibody specificity and severity of disease in Group A lambs during natural orf virus infection.

Antigens	Total Lesion Score
7	6
8	6
8	7
9	4
9	6
9	7
9	8
9	10
10	6
11	12
11	13
12	17
13	17

Fig. 4.9. Relationship between anti-viral serum antibody specificity and severity of disease in the group A lambs during natural orf virus infection.


13
12
11
10
9
8
7
6
ANTIGENS
r =+0.715
p < 0.01
0 2 4 6 8 10 12 14 16 18 20
LESION SCORE

CHAPTER FIVE

ANALYSIS OF ORF VIRUS ISOLATES USING THE RESTRICTION ENZYME EcoRI

INTRODUCTION

Strain variation in any infectious agent can profoundly affect the epidemiology of the disease for which it is responsible, since the immune response produced by an animal during infection with one strain of a pathogenic organism may be insufficient to protect against challenge with a second strain. Hardy (1964) considered that breakdowns in orf-vaccination regimes in Texas were due to the presence of multiple strains of the virus, and indeed several workers have interpreted the results of cross-immunity studies as evidence for the existence of different immunological types of orf virus (Horgan and Haseeb, 1947; Sawhney, 1966; Precausta and Stellman, 1973).

Prior to the work of Gangemi and Sharp (1976), the use of bacterial restriction endonucleases for the characterisation of large DNA molecules such as those found in poxviruses, had not been attempted due to the excessive numbers of digestion fragments which were produced by most endonucleases. However these authors used the enzyme HindIII to analyse the genomes of three strains of vaccinia virus, and found that on the basis of a molecular weight difference in one of its fragments, one could be distinguished from the others.

Restriction endonucleases, which form part of a regulatory mechanism in the sexual cycles of some bacteria, have since been used by several workers to estimate heterogeneity among parapoxviruses. The initial studies of Wittek and others (1980) on six BPS and three orf strains, and Robinson and others (1982) on 36 New Zealand orf isolates, revealed a considerable degree of heterogeneity, which was in contrast with reports of similar

restriction patterns occurring within species (Gangemi and Sharp, 1976) and between species (Muller, Wittek, Schaffner, Schumperli, Menna and Wyler, 1978) of the orthopoxvirus genus. Since this heterogeneity precluded classification of the strains and species within the parapoxvirus family, Gassmann, Wyler and Wittek (1985) used DNA hybridisation techniques to assess the genetic relatedness of these viruses. By preparing restriction cleavage maps for 12 parapoxvirus isolates using the enzymes EcoRI and HindIII, they were able to select defined areas of the genome to use as hybridisation probes. They found that probes derived from the centre of the genomes showed extensive cross hydridisation within the genus, while terminal probes hybridised only within species. They concluded that the central region of the genome is highly conserved among parapoxviruses, while terminal sequences have evolved more rapidly and are only conserved within each species.

Rafii and Burger (1985) reported considerable heterogeneity between KpnI restriction profiles of 10 different strains of orf virus from the USA, which they had isolated in tissue culture, including a vaccine strain. They showed that the vaccine DNA hybridised with all of the isolates, and calculated that none of the isolates differed in sequence by more than 2.7% from the vaccine strain.

Heterogeneity among orf isolates has also been demonstrated at the structural level by Buddle and others (1984) who classified 11 isolates from the USA into four groups on the basis of their polypeptide profiles in polyacrylamide gels.

The purpose of this study was firstly to investigate the existence and extent of genetic heterogeneity among orf isolates in Britain, and secondly to assess its significance by comparison with phenotypic variation as assessed by analysis of polypeptide structure in polyacrylamide gels.

MATERIALS AND METHODS

Samples

Virus for DNA extraction was derived from natural outbreaks which occurred in the United Kingdom between 1967 and 1985. The origins of these samples are summarised in Table 5.1. The seven samples taken the University of Edinburgh veterinary field station had been stored at -20°C, while all other samples were stored at 4°C pending examination. One strain (ORF11) was studied after isolation in tissue culture, and was analysed following 22 passages in sheep thyroid (ST) cells and five in foetal lamb muscle (FLM) cells.

Purification of Virus

Virus was purified from scab material as described in Chapter Three. For DNA studies the final pellet of the purification procedure was suspended in 100µl of 10mM Tris with 1mM EDTA, pH 7.5 (TE buffer). The preparation of tissue culture passaged virus for nucleic acid analysis was carried out as follows:

Monolayers of $3x10^7$ FLM cells in two 375 cm^2 glass tissue culture flasks (Hamshire Glassware Ltd., Southhampton, UK.) were each inoculated with $1x10^7$ $TCID_{50}$ of the ORF11 strain of orf virus in 5ml of tissue culture medium. The virus was adsorbed for one hour at 37°C with occasional gentle shaking, before the addition to each flask of 200ml of 199 medium (Gibco Ltd., Paisley, UK.) supplemented with 5% FBS, and containing 100 IU penicillin and 0.1mg streptomycin per ml. After an incubation period of about 44 hours, when the cytopathic effect (CPE) was approximately 85%, the cells were removed from the flask

Table 5.1. Orf virus isolates used in restriction enzyme studies.

ISOLATE	SOURCE	YEAR
G2660	Natural outbreak investigated by Edinburgh Veterinary Investigation Centre (VIC)	1984
H1522	Natural outbreak investigated by Perth VIC	1985
H2708	Natural outbreak investigated by Perth VIC	1985
H2882	Natural outbreak investigated by Edinburgh VIC	1985
H2969	Natural outbreak investigated by Dumfries VIC	1985
U93) U150)	Chronically infected Friesland rams, Kent	1983
FS32/67)		1967
FS23/69)		1969
FS59/70)	Natural outbreaks, Edinburgh	1970
FS32/77)	University Veterinary Field Station	1977
FS57/84)		1984
FS162/85)		1985
FS273/85)		1985
HS1) HS2) HS3)	Natural outbreak, Midlothian	1985
ORF11	Tissue culture isolate, Moredun Research Institute (Sheep Thyroid: 22 passages Foetal Lamb Muscle: 5 passages)	1971

using a rubber policeman, and the contents of each flask were pooled, and clarified by centrifugation at 1200xg at 4°C. The supernatant fluid was collected and held at 4°C, and the pellet fraction was resuspended in 5ml PBS. The latter was then sonicated intermittently on ice for 2 minutes using an ultrasonic disintegrator (MSE, Crawley, Sussex, UK.) at an amplitude of 14μ, the cellular debris was removed by centrifugation as before, and the supernatant was collected and pooled with that of the previous clarification. Virus was pelleted from the pooled supernatant fractions by centrifugation at 30,000 xg for 30 minutes at 4°C using a high speed centrifuge (High Speed 18, MSE, Crawley, Sussex, UK.) and resuspended in PBS. This suspension was further purified by layering on to continuous gradients of Nycodenz as described in Chapter Three.

Extraction of DNA

Suspensions of purified virus in TE buffer were solubilised by the addition of one volume of 2% SDS in 50mM Tris, 100mM Nacl (SDS extraction buffer) and Proteinase K (Sigma Chemical Company, St. Louis, Missouri, USA.) to a final concentration of 150μg/ml, and incubated in a waterbath at 37°C for one hour. An equal volume of 3:2 (vol/vol) "phenol"-chloroform was added and the mixture was centrifuged for 10 minutes at 2,000xg ("phenol" consisted of a mixture of 500g of phenol, 70g of m-cresol and 200g of water containing 0.5g of 8-hydroxyquinoline as described by Herring, Inglis, Ojeh, Snodgrass and Menzies, (1982)). The resulting aqueous phase was carefully collected, and nucleic acid was precipitated from it by the addition of 1/20 volume of 5M Nacl and 2 volumes of ethanol. After freezing at -20°C for at least three

hours, the precipitate was collected by centrifugation at 2000xg for 10 minutes, and resuspended in TE buffer.

After a further cycle of ethanol precipitation and centrifugation, the precipitate was redissolved in 1ml TE buffer and the absorbance of this solution at 260nm was measured with a spectrophotometer (Pye Unicam SP6-450UV/VIS, Pye Unicam Ltd., Cambridge, UK.) using a 1cm light path. The quantity of DNA present was calculated using a conversion factor of 50µg/absorbance unit/ml (Maniatis, Fritsch and Sambrook, 1982), and the nucleic acid was reprecipitated and redissolved in TE buffer at an concentration of 0.3µg/µl. Samples containing an amount of DNA below the level of detection of the spectrophotometer were resuspended in 50µl buffer and their nucleic acid content estimated by comparison of fluorescence intensity following electrophoresis of a 2.5µl sample in a minaturised gel system ("Minigel", Bethseda Research Laboratories, Cambridge, UK.) together with a known quantity of bacteriophage lambda DNA restricted with HindIII. DNA samples were stored at -20°C until analysed using restriction enzymes.

Preparation of DNA from Scab Material for Hybridisation Studies

An alternative method used for the analysis of viral DNA, was the preparation of whole scab DNA by solubilisation in guanidinium isothiocyanate (GIT) and gradient centrifugation, followed by hybridisation with a radioactively labelled viral DNA probe.

Scab material was minced thoroughly with a scalpel blade, and suspended at a concentration of 0.5g per 10ml in a 5M solution of GIT in a buffer containing 50mM Tris and 50mM disodium EDTA. This mixture was incubated with constant agitation for 30 minutes at 37°C, and

clarified by centrifugation for 20 minutes at 2000xg. The cloudy supernatant fluid was collected and layered on to 2.5ml cushions of 40% (wt/wt) caesium chloride in the above buffer, and centrifuged for 18 hours at 100,000 xg, 4°C using an SW40ti rotor in a Beckman L5-65 ultra-centrifuge (Beckman RIIC Ltd., High Wycombe, Buckinghamshire, UK.). After careful aspiration of the supernatant fluid, the gelatinous pellet of nucleic acid was resuspended in TE buffer. This solution was made 0.15M Nacl, and contaminating RNA was digested by the addition of Ribonuclease A (Sigma Chemical Company, St. Louis, Missouri, USA.) to a concentration of 20µg/ml and incubation for 10 minutes at room temperature. A further incubation for one hour at 37°C was carried out following the addition of SDS to a concentration of 1% and Proteinase K to a concentration of 50µg/ml. DNA was then extracted by the addition of "phenol"-chloroform, and subsequent treatments were as described in the previous section. Samples were stored at -20°C pending hybridisation studies.

Restriction Endonuclease Treatment

The enzyme EcoRI was used throughout the study to produce restriction profiles of viral isolates. Derived from Escherichia coli, this enzyme cleaves DNA molecules within the palindromic hexanucleotide sequence

G/A A T T C

C T T A A/G

Normally, 1µg of DNA at a concentration of 25µg/ml was digested by 10 units of EcoRI (Bethseda Research Laboratories, Cambridge, UK.) in buffer conditions recommended by the manufacturer (100mM

Tris-Hcl, 50mM Nacl, 10mM $MgCl_2$, pH 7.5). Reactions were carried out for 1 hour at 37°C. For polyacrylamide gel electrophoresis, digestions were followed by extraction with an equal volume of 3:2 "phenol"-chloroform to remove protein, which would complicate the results of subsequent silver staining procedures. Restriction digests were prepared for electrophoresis by the addition of 5µl of 25% (wt/vol) sucrose containing 75mM EDTA and 0.1% bromophenol blue.

Gel Electrophoresis

For subsequent hybridisation studies, restricted DNA samples were electrophoresed on horizontal gels (20x25cm) of 0.8% agarose, in a buffer containing 36mM Tris, 30mM sodium dihydrogen orthophosphate and 1mM EDTA (Loening, 1969). Incorporation of 0.5µg/ml ethidium bromide into the gel allowed later visualisation of nucleic acid bands by UV light. Agarose was added to 350ml of buffer and heated in a microwave oven until dissolved. Gels were then poured on a horizontal gel apparatus (Bethseda Research Laboratories, Cambridge, UK.) and allowed to set with the sample-well comb in place. After removal of the comb, the gel tank was filled with the above buffer until the gel was just covered, and the restricted DNA samples were loaded into the wells. A HindIII digest of bacteriophage lambda DNA was included in each gel for sizing purposes, and electrophoresis was carried out at a voltage gradient of 0.7V/cm for 16 hours.

Later studies on orf DNA restriction profiles were performed using polyacrylamide gels under the conditions described by Laemmli (1970) as reported by McClenaghan, Herring and Aitken (1984). The resolving gel, whose dimensions were 16 x 32 x 0.15cm, contained

7.5% polyacrylamide and was overlaid with a 1.5cm deep stacking gel of 3% polyacrylamide. The running conditions were the same as those described in Chapter Four except that a current density of 9.5mA/cm was applied over 16 hours. A HindIII digest of bacteriophage lambda DNA and a HaeIII digest of bacteriophage ØX174 replicative form DNA were included in each gel as size markers.

Staining of Polyacrylamide Gels

Polyacrylamide gels were stained with silver using the modification of Sammons' method (Sammons, Adams and Nishizawa, 1981) described by Herring and others (1982). After removal of SDS and glycine by three washes in 10% ethanol with 0.5% acetic acid, gels were soaked in 0.011M silver nitrate for 30 minutes. They were then rinsed briefly in distilled water before the addition of a "developer" solution containing 0.75M NaOH and 0.1M formaldehyde, which had been degassed before use. Development was continued until the bands were clearly visible, for a maximum of 10 minutes, and was stopped by removal of the "developer" solution and the addition of 0.07M sodium carbonate. After several changes in this, gels were photographed and stored in heat-sealable plastic bags.

"Southern" Blotting of Agarose Gels

Transfer of DNA restriction fragments in agarose gels to nitrocellulose membranes was carried out using a modification of the method described by Southern (1975). Before transfer, DNA was denatured by soaking the gel for one hour at room temperature in several changes of 0.5M NaoH in 1.5M Nacl, and neutralised for a further hour at room temperature in several changes of 1M Tris in 1.5M Nacl, both with constant agitation.

The transfer apparatus consisted of a large plastic dish in which was placed a platform consisting of a stack of glass plates wrapped in Whatman 3MM paper. The latter served as a wick for the eluting buffer, which was a standard saline citrate buffer containing 0.3M sodium citrate in 3M Nacl, pH 7.0 (20xSSC). The dish was filled with 20xSSC almost to the top of the platform, and a piece of Whatman 3MM paper, larger than the gel in both dimensions was dampened in 2xSSC and placed on top of the platform. The gel, after a brief wash in 2xSSC was placed with its underside uppermost on the Whatman 3MM paper, and this in turn was covered by a slightly larger piece of nitrocellulose membrane (pore size 0.2μ, Schleicher and Schull, Dassel, West Germany.), similarly soaked in 2xSSC. Two pieces of 3MM paper, cut to the exact size of the gel were placed, dry, on top of the nitrocellulose membrane, and on top of these, a stack of paper towels cut just smaller than the 3MM paper. The stack was then compressed by a glass plate supporting a 500g weight.

Great care was taken to ensure that bubbles which formed between successive layers were excluded, as these interfere with the flow of eluting buffer from the reservoir. Transfer was allowed to proceed over 16 hours, after which the stack of towels was removed, and the gel and membrane were laid gel side up on a dry sheet of 3MM paper. After marking the positions of the sample wells on the membrane using a ball-point pen, the gel was peeled away and discarded. The nitrocellulose membrane was then soaked in 2xSSC for five minutes before being sandwiched between two sheets of 3MM paper and allowed to dry at room temperature. It was then baked at 80°C for two hours in a vacuum oven (Gallenkamp, Loughborough, Leicestershire, UK.), and stored at room temperature between two sheets of 3MM paper wrapped in foil.

Hybridisation of Southern Blots

Hydridisations were carried out as described by Manniatis, Fritsch and Sambrook (1982) with minor modifications. Blotted membranes were soaked in 6xSSC for 5 minutes before being placed in a heat sealable-plastic bag. Prehybridisation fluid, warmed to 68°C, was added in amounts of 0.2ml/cm² of membrane, and consisted of 20xSSC diluted 3.5:1 in 5x Denhardt's solution containing 100mg/ml denatured salmon sperm DNA. (5x Denhardt's solution is 0.1% Ficoll, 0.1% polyvinylpyrrolidine and 0.1% BSA in distilled water.) The bag was sealed after exclusion of as much air as possible, and submerged in a water bath at 68°C for 4 hours with occasional agitation to disturb bubbles.

After this, the bag was removed and opened with scissors to allow addition of the hybridisation probe. This was kindly prepared by Dr. A. J. Herring of the Moredun Institute, by labelling DNA prepared from purified (U93) virus with ^{32}P using the method of nick translation described by Rigby, Dieckmann, Rhodes and Berg (1977). The probe was denatured before use by boiling in the presence of 40% formamide, and 2 x 10^6 counts per minute in 100µl was added to the fluid in the bag. The bag was then itself double sealed, and sealed within a second bag before incubating overnight in an oven at 68°C.

After incubation, the bag was carefully opened with scissors, and the hybridised membrane was removed into a washing buffer composed of 0.1% SDS in 1xSSC. Three rapid rinses in this were followed by two 20 minute washes with constant agitation at room temperature. The membrane was then washed for 2 hours in two changes of 0.1% SDS in 0.1xSSC which had been preheated to 42°C.

These washes were performed with constant agitation in a water bath at 42°C, and subsequently, the membrane was dried at room temperature between two pieces of Whatman 3MM paper. It was then taped to a backing of 3MM paper, wrapped in clingfilm and exposed to X-Ray film (Kodak X-omat S, Kodak, Liverpool, UK.) overnight at -70°C, in a radiographic cassette containing an intensifying screen.

Polypeptide Profiles

In order that genotypic variation among isolates could be assessed in the light of an appraisal of phenotypic differences, polypeptide profiles were prepared from five of the isolates whose DNA restriction patterns were studies. Viral polypeptides were separated in 10% polyacrylamide gels and stained with silver as described in Chapter Four.

RESULTS

Recovery of Viral DNA

Retrieval of virus from clinical material was in most cases poor, and resulted in quantities of viral DNA which were not measurable by spectrophotometry. However isolates U93, U150 and G2660 yielded approximately 6µg of viral DNA per 1g of scab material, and 13.5µg of DNA was prepared from the viral harvest from two 375 cm^2 flasks of the tissue culture isolate. The viral DNA content of all other samples was approximated using minigels.

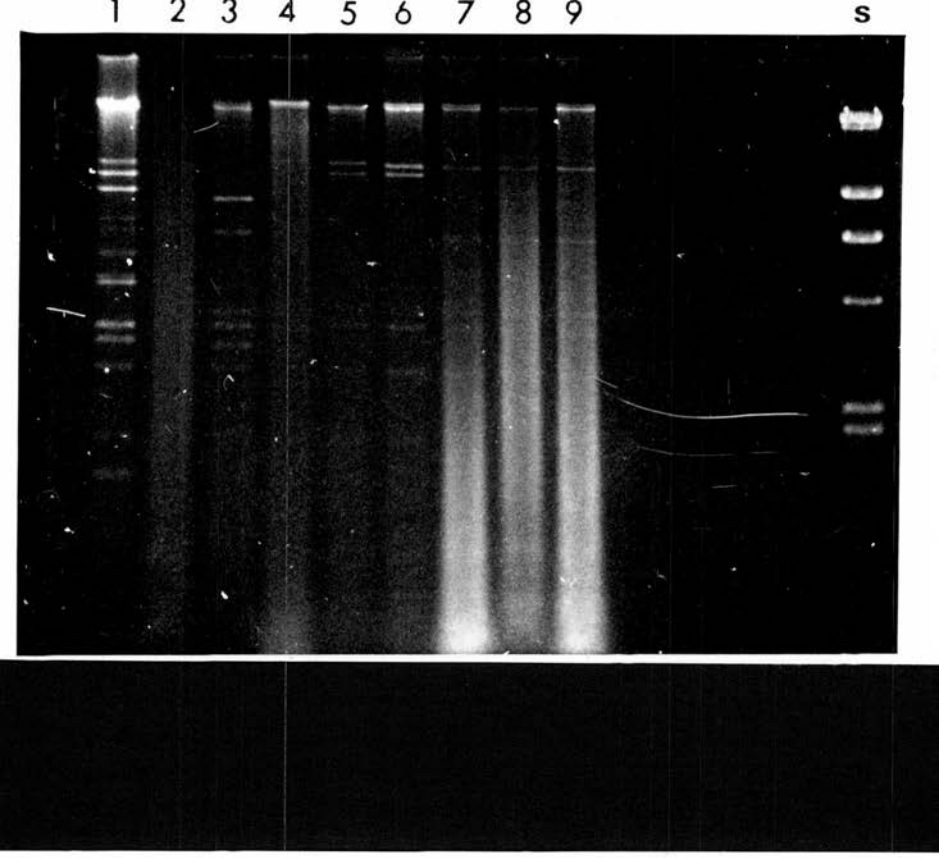
Agarose Gels

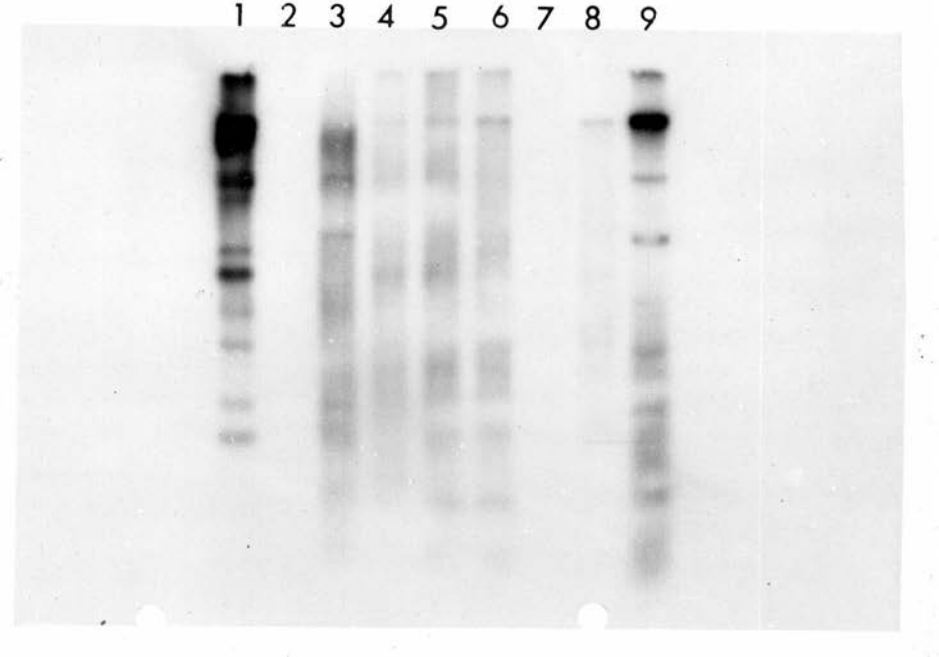
Direct visualisation or photography of ethidium bromide stained viral restriction patterns in agarose using a UV transilluminator (Ultra-Violet Products Inc., San Gabriel, California, USA.), were not sufficiently sensitive to detect the limited quantities of viral DNA which were retrieved from all but a few scab samples (Fig 5.1).

Poor results were also obtained using the ^{32}P labelled probe to detect viral nucleic acid in DNA extracts of whole scab. Although the probe hybridised satisfactorily to DNA prepared from purified homologous and heterologous virus, it failed to identify full restriction patterns of viral nucleic acid in restricted scab DNA and showed evidence of considerable non-specific hybridisation. An autoradiograph prepared from a hybridised blot is illustrated in Fig. 5.2.

Fig. 5.1. EcoRI restriction patterns of orf virus DNA in an agarose gel (0.8%) containing 0.5µg/ml ethidium bromide. Lane 1, U150; lane 3, ORF11; lane 4, H2882; lane 5, G2660; lane 6, H1552; lane 8, HS2; lane 9, HS3. S= bacteriophage lambda DNA digested with HindIII.

Fig. 5.2. Southern Blot of restriction patterns resulting from the digestion of orf virus DNA with EcoRI, after hybridisation with ^{32}P-labelled U93 virus DNA. Lane 1, DNA prepared from purified U93 virus; lanes 2-8, DNA prepared from GIT extracts of scabs derived from various outbreaks; lane 9, DNA prepared from purified HS1 virus.


1 2 3 4 5 6 7 8 9
S


1 2 3 4 5 6 7 8 9

Polyacrylamide Gels

Because it can be combined with an adaptation of the silver staining technique of Sammons and others (1981) which is considerably more sensitive than ethidium bromide fluorescence for the detection of resolved DNA bands (Nettleton Sharp, Herring and Herring, 1984), all further restriction studies on orf DNA were carried out using the polyacrylamide gel system.

EcoRI restriction profiles revealed complex patterns with varying numbers of restriction fragments, most of which were greater than 2.0 kilobases (kb) in size, and none of which migrated as far as the 1.358 kb HaeIII fragment of bacteriophage ϕX174 (figs.5.3,5.4,5.5). Most of the patterns contained some fragments which did not stain as intensely as the others, and the occasional presence of these bands in the high molecular weight regions of the gels suggests that they represent DNA which is present in submolar quantities.

Identical patterns were seen only in restriction profiles prepared from virus which was derived from animals undergoing infection on the same premises, either simultaneously (Fig. 5.3, lanes 1,2,3) or at different times (Fig 5.5, lanes 3,4,5,6). However animals undergoing concurrent infections on a given premises did not invariably yield matching patterns. Isolates U93 and U150 orginated from two rams which were part of an outbreak of orf characterised by persistent lesions on Friesland rams (see Chapter Six), but despite their common origin display markedly different EcoRI rstriction patterns (Fig.5.4, lanes 2,3 ; Fig.5.5, lanes 1,2)

Only one restriction fragment could be interpreted as being common to all the viral isolates studied, although several profiles had other bands in common. The conserved fragment was approximately 2.3kb in size (arrowed Figs.5.4, 5.5), although accurate sizing of bands in polyacrylamide gels is not possible. When compared with agarose gels (Fig. 5.1) resolution of high molecular weight fragments in the polyacrylamide system was poor, and indeed, the largest fragments migrated further in the latter system as assessed by comigration with bacteriophage lambda size markers. However, by plotting the $\log_{10}$ of the size marker fragment sizes against the distances of their migration, it can be seen that useful resolution was obtained in fragments of up to 9.4kb (Fig. 5.7)

Restriction patterns prepared from virus in scab material sampled during outbreaks at the University of Edinburgh veterinary field station in the years 1967 (Fig 5.4, lane 10), 1969, 1970, 1977 (Fig 5.5, lanes 3,4,5,6) and 1984 (Fig 5.4, lane 11) appear to be identical, and suggest that the same strain of virus persisted on the premises over this period. However the pattern obtained from virus sampled on the same premises in 1985 (Fig 5.5, lane 7), although sharing two fragments with those of previous years, is quite distinct, indicating the appearance of a new strain.

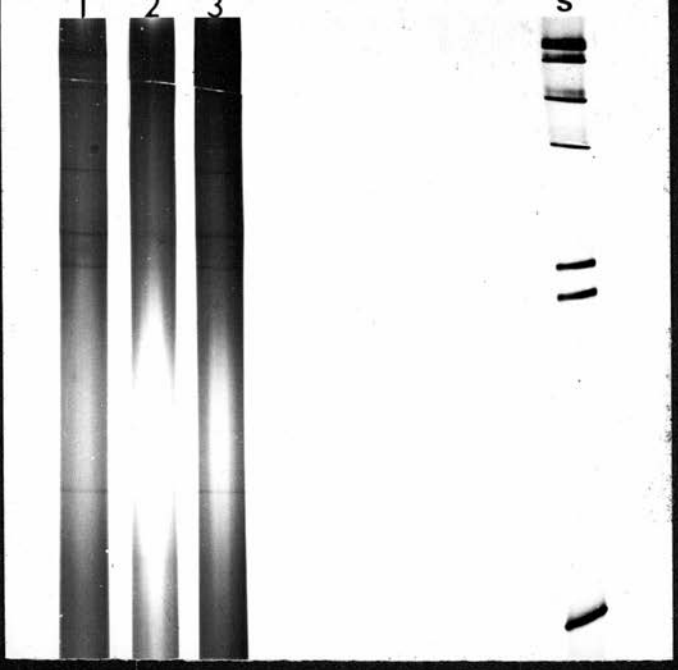
Polypeptide Profiles

Silver stained polypeptide profiles prepared from five of the isolates under study are shown in Fig 5.6, and these correspond to the restriction patterns in lanes 1-5 of Fig 5.5, Analysis of these suggests that despite the considerable genomic heterogeneity which

is apparent between isolates U93 and U150, their polypeptide profiles exhibit only quantitative differences. However, variable degrees of expression of certain polypeptides is also suggested by the polypeptide profiles of isolates FS23/69, FS59/70 and FS32/77, despite their apparently identical EcoRI restriction patterns. In addition, one of these isolates, FS32/77, lacks a 62kD polypeptide (arrowed, Fig. 5.7) which is expressed by the other two.

Fig. 5.3. EcoRI restriction patterns of orf virus DNA in an SDS-PAGE gel (7.5%) after staining with silver. Lane 1, HS1; lane 2, HS2; lane 3, HS3. S= bacteriophage lambda DNA digested with HindIII, phage ϕX174 DNA digested with HaeIII.

Fig. 5.4. EcoRI restriction patterns of orf virus DNA in an SDS-PAGE gel (7.5%) after staining with silver. Lane 1, ORF11; lane 2, U93; lane 3, U150; lane 4, H2962; lane 5, H2708; lane 6, H1552; lane 7, G2660; lane 8, H2882; lane 10, FS32/67; lane 11, FS57/84; lane 12, FS162/85. S= bacteriophage lambda DNA digested with HindIII, phage ϕX174 DNA digested with HaeIII.


1
2
3
S

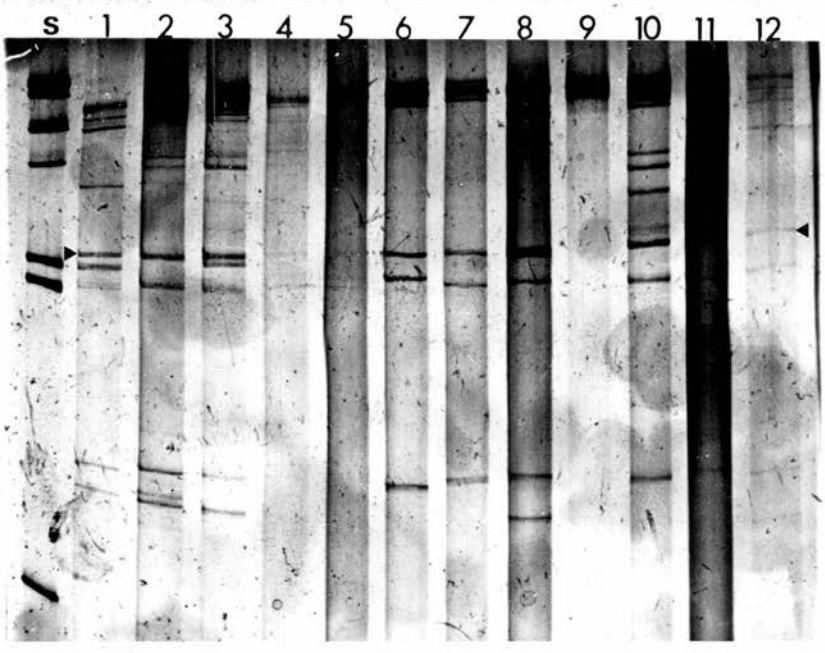

S
1
2
3
4
5
6
7
8
9
10
11
12

Fig. 5.5. EcoRI restriction patterns of orf virus DNA in an SDS-PAGE gel (7.5%) after staining with silver. Lane 1, U150; lane 2, U93; lane 3, FS23/69; lane 4, FS59/70; lane 5, FS32/77; lane 6, FS68/77; lane 7, FS273/85. S= bacteriophage lambda DNA digested with HindIII, phage ϕX174 DNA digested with HaeIII.

Fig. 5.6. Silver-stained SDS-PAGE gel (10%) containing polypeptide profiles of the five orf virus isolates featured in lanes 1-5 of Fig. 5.5. Lane 1, U150; lane 2, U93; lane 3, FS23/69; lane 4, FS59/70; lane 5, FS32/77; lane 6, size marker proteins. Arrows mark a 62kD polypeptide which is absent in FS32/77. lambda DNA digested with HindIII, phage ϕX174 DNA digested

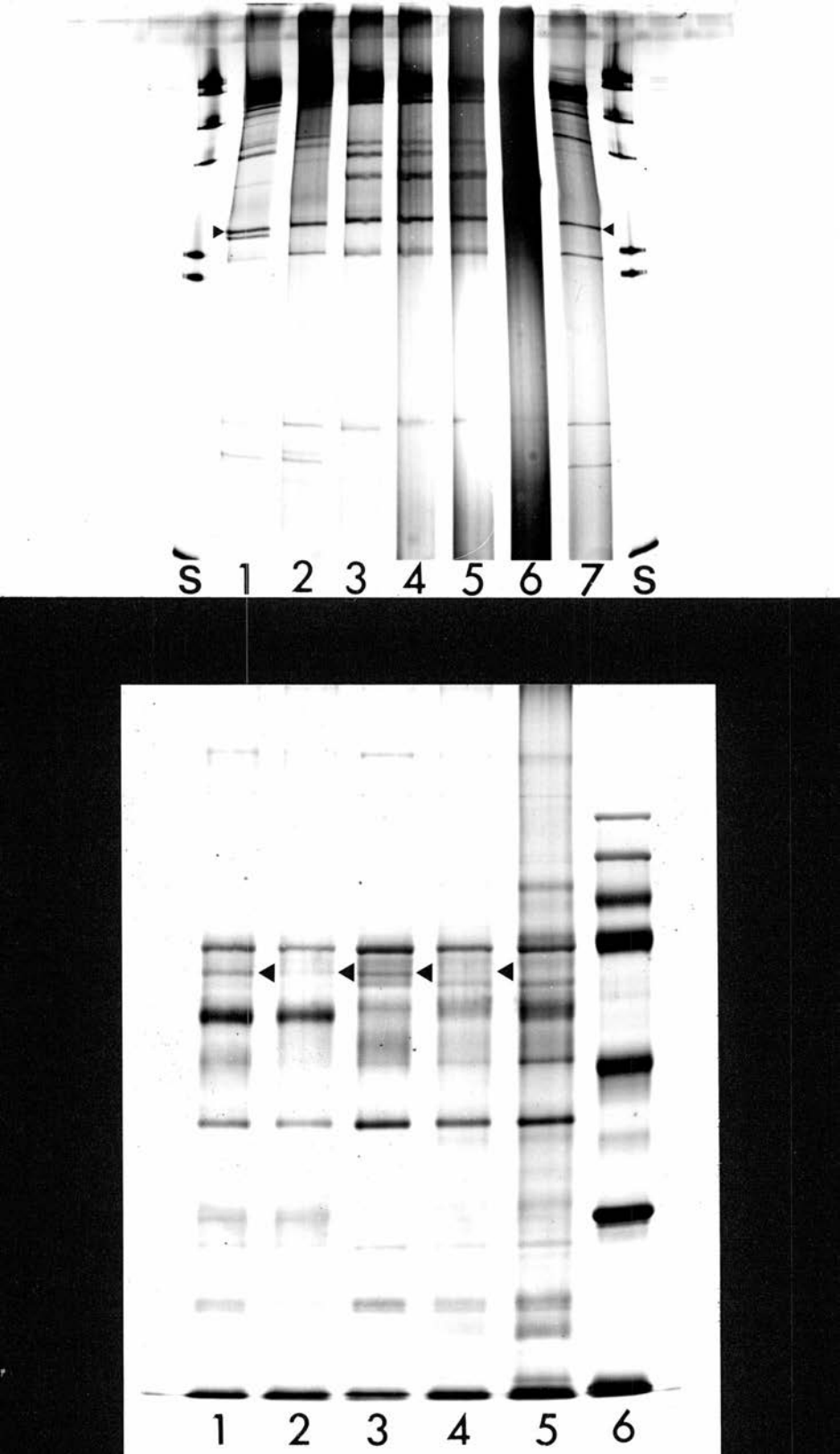
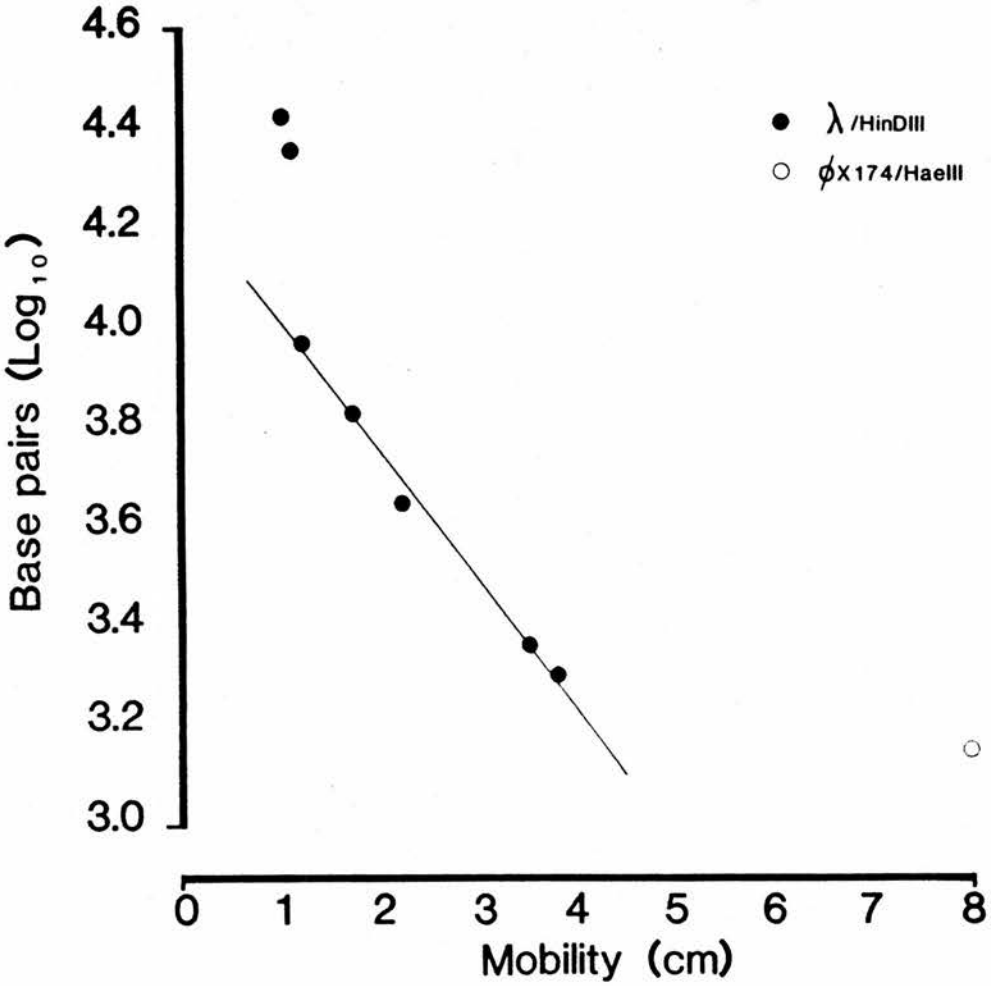

S 1 2 3 4 5 6 7 S
1 2 3 4 5 6

Fig. 5.7. Plot of relative mobility versus $\log_{10}$ fragment size (kb) of the bacteriophage lambda HindIII fragments from Fig. 5.4, showing useful resolution of fragments between 2.0 and 9.4kb in size.


4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
Base pairs (Log10)
λ /HinDIII
ϕX174/HaeIII
0
1
2
3
4
5
6
7
8
Mobility (cm)

DISCUSSION

This study has confirmed that British isolates of orf virus exhibit the same genomic heterogeneity reported for strains of orf from Europe (Wittek and others, 1980; Gassmann and others, 1985), New Zealand (Robinson and others, 1982) and the USA (Rafii and Burger, 1985). It further demonstrates that this variation can extend to isolates derived from animals undergoing synchronous infections on the same premises.

The amounts of orf virus which were recovered from the clinical material available for these experiments were disappointing, and compared only with the lowest values reported by Robinson and others (1982) who could recover up to 22.8µg of viral DNA from 1g of scab material. This discrepancy can be explained by the estabished variation which can occur in the viral content of different scab samples (Romero-Mercado and others, 1973) and the uneven distribution of virus within orf crusts (Harkness and others 1977). In addition, the quantities of clinical material available for preparation of virus were in many cases minimal. The combined effect of this restricted supply of clinical material and its low content of virus led to suboptimal loadings in agarose gels, which resulted in bands which were below the sensitivity limits of ethidium bromide staining. Expansion of each isolate by growth in tissue culture was not considered due to the danger of cross contamination in the limited facilities which were available for the handling of orf virus in culture.

In order to overcome this problem, a preliminary attempt was made to detect viral nucleic acid in extracts of DNA prepared from crude scab material using DNA-hybridisation techniques. It was

hoped that this technique would provide greatly increased sensitivity of detection, in addition to excluding the need for viral purification which inevitably involves losses. The results of this attempt, although promising, were not satisfactory due to a high background of non-specific hybridisation. This was attributed to contamination of the probe with host DNA, and although it is possible that some improvement could have been obtained by altering the stringency of the post-hybridisation washing conditions, it was considered that the technique was not worth pursuing. It was therefore decided to use polyacrylamide gels as the medium for electrophoresis of restriction digests, since they permit the use of a silver staining technique which is superior in sensitivity to the use of ethidium bromide (Herring and others, 1982).

Unfortunately, with the exception of isolate U150, it was not possible to compare EcoRI restriction patterns of the British strains in this study with those of foreign strains described previously (Wittek and others, 1980; Robinson and others 1982; Gassmann and others, 1985; Rafii and Burger, 1985), since all of these workers separated orf restriction fragments in agarose gels, which provide optimal resolution of the range of fragment sizes yielded by digestion of the orf virus genome with this enzyme. It would appear however that the EcoRI restriction pattern of isolate U150 in agarose is compatable with those reported by previous workers.

The disparity between restriction patterns produced in polyacrylamide gels and those prepared in agarose is demonstrated by a comparison between the EcoRI restriction profiles of isolate U93 in agarose (Fig. 5.1, lane 1) and in polyacrylamide (Fig.

5.4,lane 2; Fig. 5.5, lane 2). The patterns are obviously different, and indeed the HindIII fragments of bacteriophage lambda which were used in both as size markers, have produced equally distinct patterns. However, the largest restriction fragment of U93 can be seen to have a much greater mobility in polyacrylamide than in agarose, migrating to a position below the 9.4kb phage lambda fragment in the former, while in the latter it falls short of the 27kb fragment. This effect may be attributed to the fact that mobility of nucleic acid in polyacrylamide is not solely a function of its molecular weight, but is also directly related to its G+C content (Zeiger, Saloman, Dingman and Peacock, 1972). At an average value of 64% (Wittek, Keunzle and Wyler, 1979), the high G+C content of orf virus DNA can be expected to result in high mobility in polyacrylamide.

Despite this shortcoming, the polyacrylamide system used in this study was extremely sensitive and provided excellent resolution of fragments up to 5kb in length, resulting in distinct restriction profiles for each isolate. Other workers have reported similar resolving powers for polyacrylamide (Hansen, 1981; McClenaghan and others, 1984) although such gels have traditionally been used for fragments of DNA less than 1kb in size.

The reasons for the presence in most of the restriction profiles of fragments which appear to be sub-molar in quantity, are not clear. Robinson and others (1982) reported a similar effect, and attributed it to the presence of more than one strain of virus in the scab of origin. However, defective particles, which can be present in harvests of most DNA containing viruses (Joklik, 1982), could also produce this effect, since they contain only part of the virus genome.

The restriction profile heterogeneity does not appear to be reflected in marked variation at the protein level; only quantitative differences in polypeptide structure were detected between isolates U93 and U150 despite their dissimilar fragment patterns. In addition, isolate FS32/77, although lacking a 62kD polypeptide which is expressed by isolates FS23/69 and FS59/70, was indistinguishable from them on the basis of EcoRI restriction profiles. Wittek and others (1979) made similar observations when they reported close serological cross-reactivity in six BPS and three orf strains, despite extensive heterogeneity of restriction patterns after cleavage with six different enzymes. They calculated, using the "combinatorial" analysis of endonuclease restriction data described by Schumperli, Lagadec and Muller (1977), that approximately 80% sequence homology was required for any comigrating fragments between two molecules of DNA which have been cleaved with a restriction enzyme recognising a six nucleotide sequence. This would imply a considerable degree of DNA sequence homology between the isolates examined in this study. Rafii and Burger (1985) calculated that 10 isolates which they examined by restriction with the restriction enzyme KpnI had 97.3% sequence homology, despite considerable heterogeneity in restriction patterns. They concluded that differences in restriction profiles were due to minor sequence alterations throughout the DNA.

The recognition site of a given restriction enzyme can by definition be removed by the alteration of only one nucleotide in the recognition sequence. Such an alteration need not result in any change in ultimate product of the genetic code, since a given amino acid may be coded for by more than one nucleotide triplet or codon. Introns, or non-coding segments of DNA, may also contain

restriction enzyme recognition sites, and so affect fragment patterns. However, although such segments have been shown to occur in adenoviruses and the simian papovavirus, SV40 (Joklik, 1980), their presence in the orf genome has not been established, and is unlikely in view of the continuous coding sequences and tightly packed nature of genes in vaccinia virus genomes (Weir and Moss, 1985)

Although the information provided by restriction endonuclease analysis cannot be correlated with the biological activity, it can provide useful epidemiological data. Scab samples collected during orf outbreaks at the University of Edinburgh veterinary field station in the years 1967, 1969, 1970, 1977 and 1984 were shown by the technique to contain viruses which, if not identical, were very closely related, and probably represent recoveries of the same strain. In contrast, the 1985 outbreak was caused by an apparently different strain. In view of the results of the longevity study described in Chapter six, the persistence of a single strain of virus on this premises over such a long period is somewhat unexpected. A possible explanation could be that infectious virus survived the periods between outbreaks in the shelter of buildings or handling facilities, or indeed, the strain may have been maintained on the premises by means of sub-clinical infections in the older animals. However since the flock under study was not a closed one, it is surprising that newly acquired stock did not introduce different strains during these years, as presumably was the case with the strain responsible for the 1985 outbreak.

CHAPTER SIX

PART ONE: SURVIVAL OF ORF VIRUS UNDER BRITISH WINTER CONDITIONS

PART TWO: INVESTIGATION OF TWO CASES OF CHRONIC ORF

PART ONE

INTRODUCTION

Aynaud (1923) suggested that the recrudescence of orf virus infections in some flocks was due to persistence of the agent in scab material, and it is on this concept that the current understanding of the epidemiology of orf is based (see Robinson and Balassu, 1981). Overwintered virus in scab material shed by infected animals during the previous year is assumed to be the source of new outbreaks in the spring. Young lambs are thought to derive infection from this environmental pool of virus, with direct contact and the shedding of scabs by newly infected animals serving to amplify the outbreak.

The work of Romero-Mercado and others (1973) however appears to conflict with this traditional view. In an analysis of scabs taken from experimentally infected animals, they found that although large amounts of virus were detectable by CFT, immuno-precipitation and electron microscopy during the course of the disease, only negligible quantities were present in scabs collected within a few days of resolution of the lesion. Furthermore, although Livingston and Hardy (1960) reported that dried scab material was still infectious after storage for almost 23 years at 45°F, the survival time of the virus in suspension would appear to be markedly reduced (Plowright and others, 1959). In view of the rainfall which is characteristic of the British climate, it is difficult to imagine how scab material shed on pasture can remain dry. It was therefore decided to investigate the effect of British winter weather conditions on the infectivity of orf virus in scab material.

MATERIAL AND METHODS

Virus

The source of virus for this experiment was scab material which had been collected from a group of experimentally infected lambs. In order to minimise the effects of variation in particle numbers which occurs between different areas of an orf lesion (Harkness and others, 1977), the scabs were pooled, ground in sterile sand and stored at -20°C. This material was shown to contain orf virus particles on electron microscopic examination, and produced lesions typical of experimental orf infection when applied to the abraded skin of susceptible lambs. Five 1g aliquots of the preparation were measured, wrapped in muslin gauze and maintained for the period from November 19th 1984 to May 7th 1985 under contrived environmental conditions.

Experimental conditions

In order to simulate the shedding of scab material on to sheltered and exposed pasture, an aliquot was placed in each of two drained plastic pots which had been filled with earth and topped with fresh turf. One of these pots was sheltered from the rain by a small canopy of clear plastic, while the other received no shelter apart from a wire mesh cover to prevent interference by birds (Fig. 6.1). The three remaining aliquots were maintained in the laboratory as summarised in Table 6.1, one (aliquot A) being returned to -20°C. Meteorological records collected during the period at the Royal Observatory, Blackford Hill, Edinburgh (approximately 1.5 miles from the site of the experiment) were supplied by the Meteorological Office Climatological Services, Edinburgh.

Fig. 6.1. Equipment used to simulate exposure of orf scab material to outdoor winter conditions with and without rainfall.

Table 6.1 Summary of test conditions used during study period.

Aliquot	Exposure Conditions
A	-20°C
B	4.0±0.5°C
C	18±5°C
D	Outdoors/covered
E	Outdoors/no cover

After the period of exposure, 20% suspensions of each aliquot were made in PBS containing 100 IU penicillin and 0.1 mg/ml streptomycin using a mortar and pestel, and clarified by centrifugation at 1200xg for 30 minutes at 4°C. Six sites (4 cm^2) on the backs of each of three newly weaned lambs were lightly abraded with sharp plastic after removal of the wool using a proprietary depilating agent (Immac, Anne French, London UK.). A 100µl aliquot of each of the viral suspensions was applied to one site on each lamb, while the remaining site received an application of PBS alone. Six days after inoculation, punch biopsies were taken from all sites, and after processing in paraffin, were stained with haematoxylin and eosin for histological examination.

Electron Microscopy

The five test suspensions were examined by electron microscopy for the presence and appearance of orf virus particles. Pioloform/carbon-coated copper grids, after treatment with 0.1% bacitracin were loaded with 2µl of test suspension. After 35 seconds, grids were blotted with filter paper, washed twice in

distilled water and treated with 1.0% phosphotungstic acid (PTA), pH 7.0,for a further 35 seconds. They were then blotted dry and examined in a Siemens 1A electron microscope (Siemens, Sunbury-on-Thames, Middlesex, UK.) at an accelerating voltage of 80 kV, when a differential count was performed on a total of 100 particles from each grid.

RESULTS

Meteorological records for the period under study are summarised in Table 6.2, and when compared with mean figures which were available for these parameters suggest that the total rainfall, total sunshine and the number of days with snow lying were all slightly above average for the period.

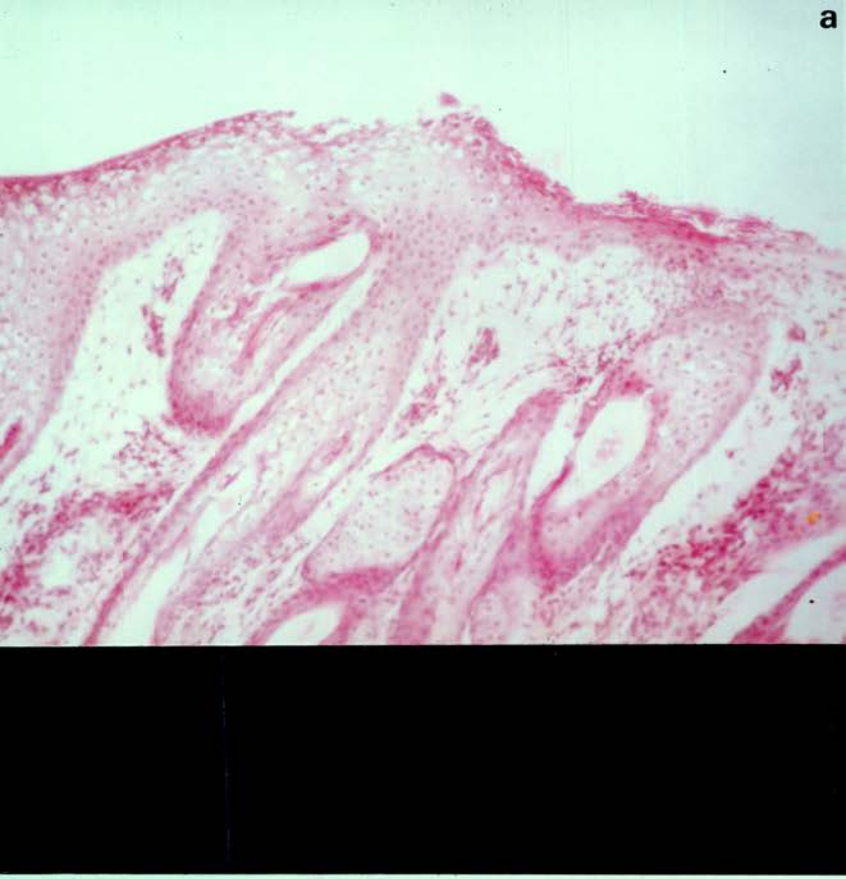
Table 6.2 Meteorological data (Royal Observatory, Blackford Hill, Edinburgh) for the period from Nov.17th., 1984 to May 7th, 1985. Figures in parentheses represent average figures for the years 1941-1970.

Weather Parameter	Nov (17-19)	Dec	Jan	Feb	Mar	Apr	May (1-7)
Max °C	10.9	12.3	10.0	11.2	12.1	16.2	15.5
	-	(6.5)	(5.3)	(5.7)	(8.0)	(10.9)	-
Min °C	2.3	-7.5	-6.5	-6.5	-3.1	-0.5	2.5
	-	(2.0)	(0.9)	(0.7)	(2.2)	(4.0)	-
Total Rain (mm)	41.6	45.3	37.9	10.4	86.0	70.0	1.5
	-	(54.0)	(54.0)	(41.0)	(36.0)	(38.0)	-
Total Sun (hours)	25.6	53.4	48.9	90.6	103.8	134.0	40.6
	-	(46.0)	(50.0)	(76.2)	(108.3)	(148.4)	-

The development of orf lesions at the test sites was difficult to assess macroscopically due to the rapid regrowth of wool. However, microscopic examination of the biopsy material revealed that histological lesions typical of orf virus infection had resulted from challenge with each of the aliquots except aliquot E (Fig.6.2, Table 6.3). Lesions were not detected at any of the control sites, and one of the lambs did not develop a lesion at the site challenged with aliquot D.

Electron microscopic examination indicated that four forms of orf virus particle were present on PTA stained grids prepared from the test suspensions (Fig.6.3, Table 6.4). Aliquots A-D contained a predominance of particles with the characteristic basket weave appearance which has been described for orf virus and referred to as type 1 (Peters and others, 1964) or Mulberry (M) type (Robinson and Balassu, 1981) particles. A proportion of particles had allowed penetration of stain to reveal their internal structure, corresponding to type 2 and 3 particles (Peters and others, 1964) and clear (C) forms (Robinson and Balassu, 1981). A further population were intermediate between these two types. While C forms were also present in material prepared from aliquot E, most of the particles in this preparation were rather amorphous, recognisable only by shape or size and somewhat similar to the Type 2b particles described by Nagington and Horne (1962). No type 1 forms could be found in this preparation.

Fig. 6.2. Histological section of test (a) and control (b) sites on an experimental lamb six days after challenge with a suspension prepared from aliquot A. (Haematoxylin and eosin, x40).


a

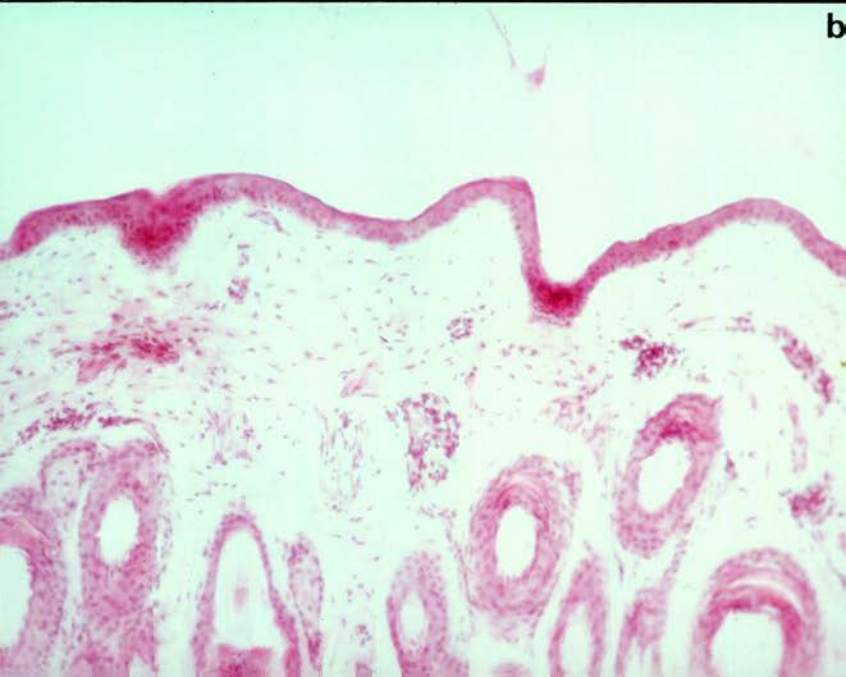

b

Fig. 6.3. Types of orf virus particle found on electron microscopic examination of suspensions prepared from aliquots A-E and stained with PTA. Type M (a); type C (b); intermediate (c); amorphous (d). Bars represent 0.2μ.

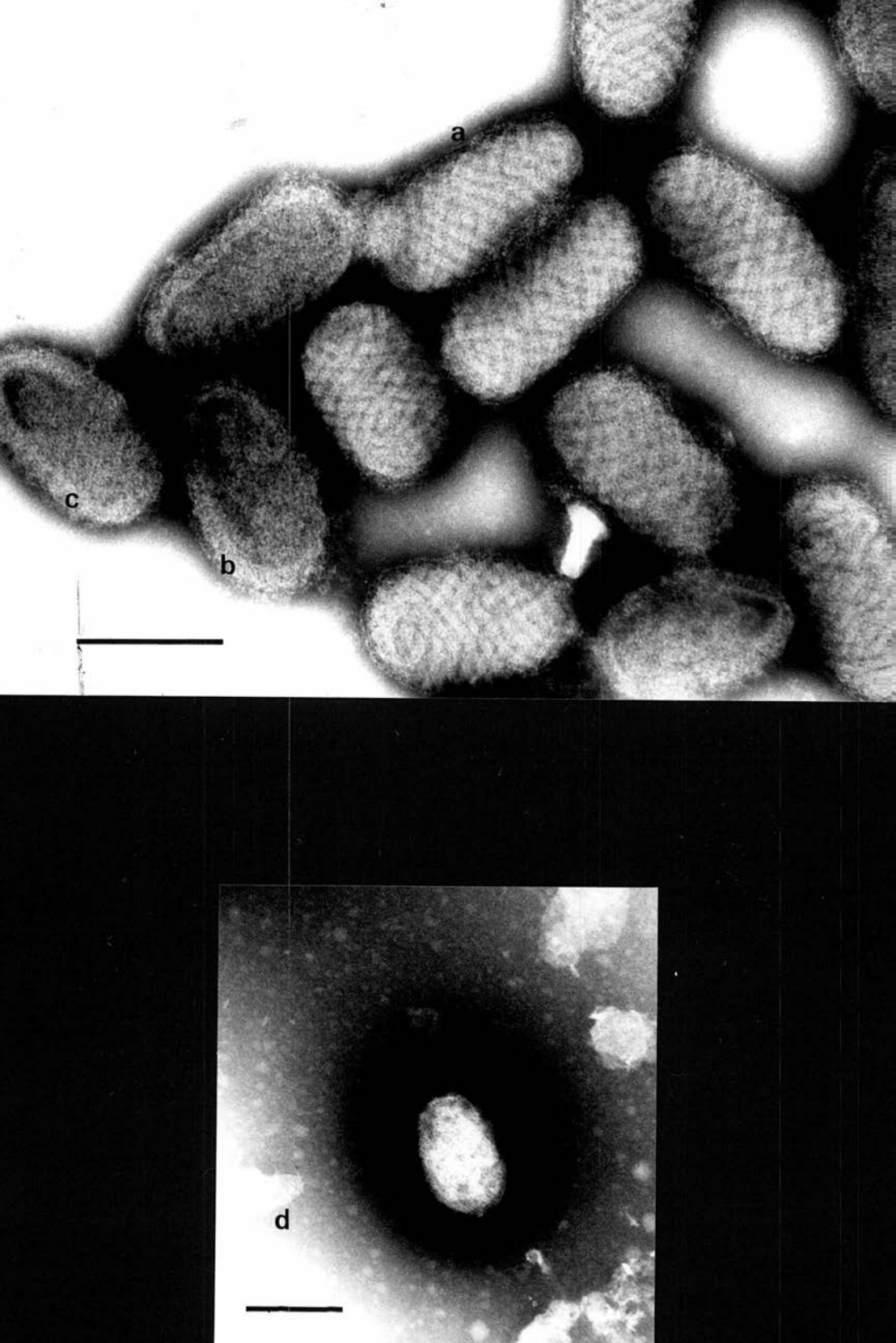

a
b
c
d

Table 6.3 Appearance of histological lesions typical of orf virus infection in three lambs after challenge with suspensions prepared from aliquots A-D.

Lamb	Aliquot					
No.	A	B	C	D	E	PBS
1	+	+	+	+	-	-
2	+	+	+	+	-	-
3	+	+	+	-	-	-

Table 6.4. Relative amounts of orf virus particle types seen on electron microscopic examination of suspensions prepared from infectious scab after exposure to defined experimental conditions.

	Particle Type (%)			
Aliquot	Type"M"	Type"C"	Intermediate	Amorphous
A	70	13	15	2
B	55	18	22	5
C	65	4	25	6
D	54	12	33	1
E	0	0	34	66

DISCUSSION

The results of this study indicate that although orf virus is capable of resisting the temperature fluctuations of a British winter, total exposure to the elements results in loss of infectivity. Manley (1934) concluded from his work on the viability of orf virus under natural conditions that direct sunlight was the principle inactivating factor, and Sawhney (1972) observed that the infectivity of orf virus in tissue culture is indeed susceptible to ultraviolet (UV) radiation.

Although both of the outdoor preparations in this study were exposed to a total of 496.9 hours of sunlight, it is likely that the clear plastic canopy used to shelter aliquot D from the rain afforded some protection against UV radiation. This would imply that either the latter or rainfall, or a combination of the two was responsible for the inactivation of virus in aliquot E. It is probable however that damage by UV radiation has little significance under natural circumstances, since it has poor penetrating power and is unlikely to reach virus within the substance of crusts lying on pasture.

This suggests that the major factor involved in the inactivation of virus in aliquot E was exposure to rainfall, of which 292.7 mm was recorded during the study period. This effect was more likely to be the result of hydration of the scab than elution of viral particles into the soil, since virions were present in the sample after exposure. Furthermore, Plowright and others (1959) have shown that despite its extreme resistance to desication, orf virus has a half-life of only 0.463 days when suspended in tissue culture maintenance medium. Hydrated scab material may constitute an even

less suitable environment since it would almost certainly contain adventitious bacteria. Rainfall is a prominent feature of the British climate, and it is therefore unlikely that scab material which is shed on exposed pasture will retain infectivity for extended periods. In addition, the work of Romero-Mercado and others (1973) indicates that only negligible amounts of orf virus are present in scabs by the time they are shed naturally from the animal.

These observations clearly call into question the significance of virus which has overwintered on pasture as a source of new infections in the spring, although the possible role of virus surviving in the shelter of farm buildings cannot be excluded. Only a proportion of outbreaks are however associated with housing, which suggests that viral persistence on animals, in the form of clinical or subclinical infections may be an important epidemiological factor in orf.

PART TWO

INTRODUCTION

At the outset of this project, there were no descriptions in the literature of chronic or persistent forms of orf virus infection in sheep, and it seemed generally accepted that uncomplicated cases of the disease were self limiting, with lesions resolving in 2-3 weeks (see Robinson and Balassu 1981). In October of 1983, two Friesland rams were referred by a practising veterinary surgeon to the Moredun Institute, with a history of chronic exfoliative lesions on the skin of the poll and ears. The condition had been a problem on the farm of origin since 1967 and had an incidence of 4-5% in rams of the Friesland breed. There was some evidence to suggest a predeliction for these rams since in-contact tups of other breeds (including Dorset Down, Oxford Down and Romney Marsh) consistently remained unaffected. Diseases was most evident during the tupping season, with an apparent partial regression occuring during the spring and summer months. The farmer was not aware of a problem with orf in his ewes and lambs, although the disease had made sporadic appearances on the premises in the past.

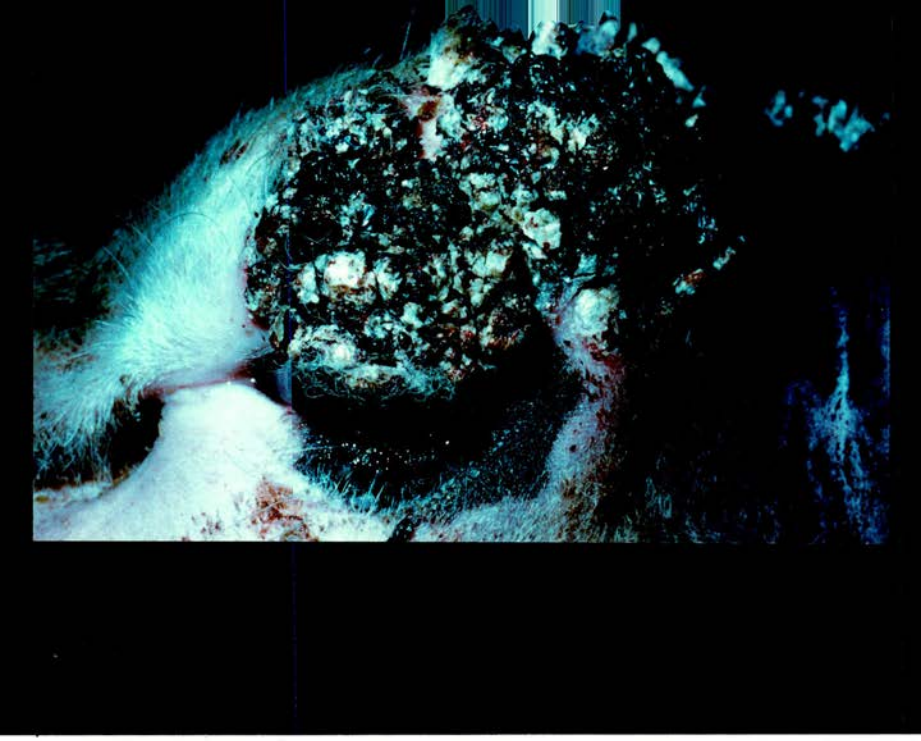
Clinical examination of both rams revealed extensive hyperkeratotic lesions of the poll which extended down to cover the greater part of both ears (Fig. 6.4). These lesions were 1-2 cm in thickness and were seen on close examination to be composed of large numbers of plug-like structures which were themselves composed of collections of keratinaceous fibres. Removal of these plugs was resented by the animals and exposed underlying papillae which bled profusely. The rams were otherwise in good health and showed no evidence of dullness, pyrexia or loss of appetite.

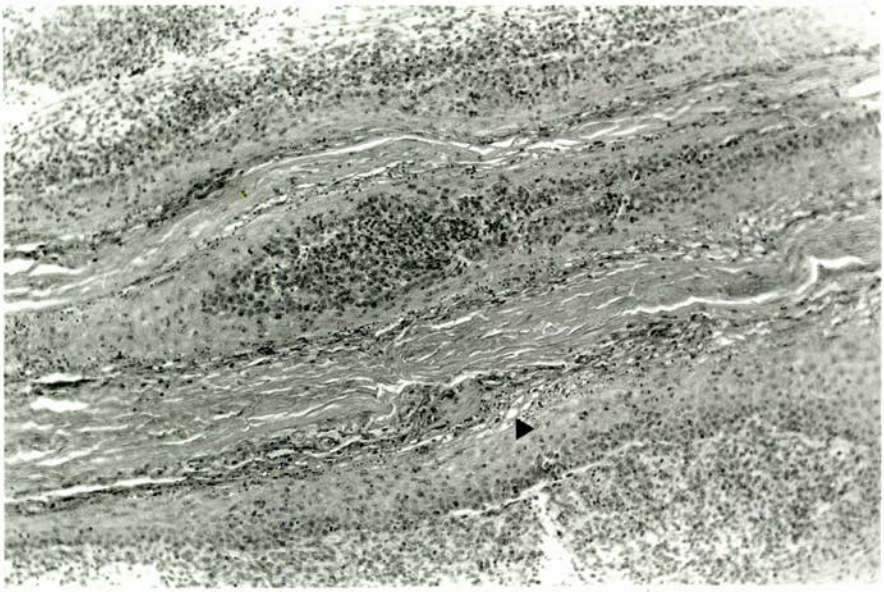
On histological examination of "punch-biopies" taken from the affected areas, considerable abnormality of epidermal growth was evident (Fig. 6.5). In addition to hyperkeratosis, marked hyperplasia of the epidermis was present giving rise to a papillomatous appearance. Many epidermal areas exhibited foci with the histological appearance of orf virus infection and the dermis was extensively infiltrated with monoculear cells and polymorphonuclear leucocytes. Examination of lesion material by electon microscopy as described in part one of this chapter revealed large numbers of orf virus particles.

The condition was accordingly diagnosed as orf virus infection, and represents an extraordinary manifestation of the disease, not only because of the nature of these lesions but also from the point of view of the persistence of virus in them over such a long period (Romero-Mercado and others, 1973). An investigation was therefore carried out to establish whether this syndrome was due to a novel strain of orf virus, or the result of a deficient immune response in these animals.

Fig. 6.4. Gross appearance of chronic orf lesions on the face of ram U93.

Fig. 6.5. Histological appearance of biopsy material derived from a chronic orf lesion on the poll of ram U93, showing epidermal hyperplasia and reticular degeneration (arrowed). (x40)





MATERIALS AND METHODS

Challenge of Susceptible Lambs

A 20% suspension of lesion material from ram U93 was prepared in PBS containing 100 IU penicillin and 0.1 mg streptomycin per ml, using a mortar and pestle, and clarified by centrifugation at 1200xg at 4°C. Two newly weaned lambs were lightly abraded on the medial surface of the right thigh using sterile sandpaper, and 100µl of the viral suspension was applied to each. The animals were then examined daily for the appearance and development of lesions.

Serology

Serum samples taken from both animals soon after arrival were analysed by ELISA and the Western Blotting technique, as described in Chapters Three and Four. Subsequent samples taken after treatment regimens were assessed by ELISA only (Table 6.5).

Treatment regimens

Since the veterinary surgeon who had referred these animals was anxious to establish a method by which further cases might be treated, two therapeutic measures were employed in an attempt to induce regression of lesions. These were inoculation with autogenous clinical material in CFA and the administration of the drug levamisole. Both CFA and levamisole have been shown to have immunopotentiating effects (see Werner, 1979) which are not completely understood. Ram U93 received three intramuscular injections of levamisole (Nilverm, Imperial Chemical Industries, Cheshire, UK.; 150 mg in 5 ml) at four day intervals, while ram

U150 received a single intramuscular injection (6 ml) of 50% CFA (Difco Laboratories, Rutherford, New Jersey, USA.) and 5% autogenous scab material in PBS.

Assessment of Cell-Mediated Immunity (CMI)

Orf virus specific CMI responses in these rams were assessed by means of an intradermal delayed-type hypersensitivity (DH) test using purified orf virus (see Materials and Methods, Chapter Three) derived from ram U93 as test antigen. The test was performed 18 months after the arrival of the rams at the institute, by which time their lesions had regressed. For comparative purposes, four Scottish Blackface lambs which had been experimentally infected with orf virus 8 weeks previously were also tested. Four sites on the medial surface of the left thigh of these animals were inoculated intradermally with 100µl of 30µg, 15µg, 7.5µg and 3.25µg respectively of purified orf virus diluted in PBS. A fifth site received a control inoculum consisting of 10% SPF lamb serum in PBS. In order to maintain the antigenic integrity of this preparation, no attempt was made to inactivate it; pilot experiments had shown that intra-dermal inoculation of live orf virus does not give rise to infections, although isolated pustules are occasionally seen to develop where the needle has penetrated the epidermis (McKeever, unpublished observations). Skin thickness at each site was measured before and 48 hours after inoculation using a micrometer calipers (Quicktest A02T, Carobronze Ltd., London, UK.).

In order that orf-specific CMI responses in these rams could be compared with CMI responses directed at other antigens, they were sensitised with the contact sensitising agent 2, 4-dinitro-1-fluorobenzene (DNFB) (Sigma Chemical Company, St. Louis, Missouri, USA.). This was achieved as described by McConnell, Lachmann and Hobart (1974) by painting the inner surface of both ears with 100µl of 5% DNFB in a 1:1 solution of acetone and mineral oil ("Miglyol", Dynamit Nobel, Witten, Ruhr, West Germany.), equivalent to approximately 10µg DNFB per animal. Six Scottish Blackface lambs were also sensitised as control animals. Eight weeks later the animals were challenged by the application of 100µl of 5 dilutions of DNFB (2%, 1%, 0.5%, 0.25%, 0.125%) in 4:1 acetone:Miglyol to sites on the medial surface of the right thigh. Skin thickness at each site was measured before and at 48 hours after challenge. Mean skin thickness increases in the rams were compared with those of control groups for both orf antigen and DNFB using the Student t test.

RESULTS

Challenge of susceptible lambs with a suspension of clinical material derived from ram U93 resulted in a development of lesions typical of experimental orf virus infection in both animals. The classical features of macule, papule, vesicle and pustule (see Robinson and Balassu 1981) were observed, with scabs developing on the lesions by the 10th day after infection. Lesions healed uneventfully within four weeks.

Serology

Analysis by ELISA of serum samples taken from both rams on arrival at the institute revealed high titres of orf virus specific antibody (Table 6.5) which on Western Blotting analysis were seen to be directed at a large complement of viral antigens (Fig. 6.6).

Therapeutic measures

A large indurated swelling was seen to develop on ram U150 at the inoculation site of autogenous scab in CFA, but no untoward effects were noted in ram U93 following the administration of levamisole. However, no beneficial effects were evident in either ram as a consequence of these measures, and lesions persisted essentially unchanged for a further 9 weeks, when a partial regression was apparent in February 1984. Analysis by ELISA of serum samples taken at 3 and 12 weeks after treatment revealed a total increase of 85% in the virus specific antibody titres of ram U93 during this period. In contrast, those of ram U150 had actually fallen by 65.6% after three weeks, and were only marginally recovered twelve weeks after inoculation (Table 6.5).

Fig. 6.6. Western Blot of U93 orf virus antigens probed with sera from rams U93 and U150 soon after their arrival at the Moredun Institute.

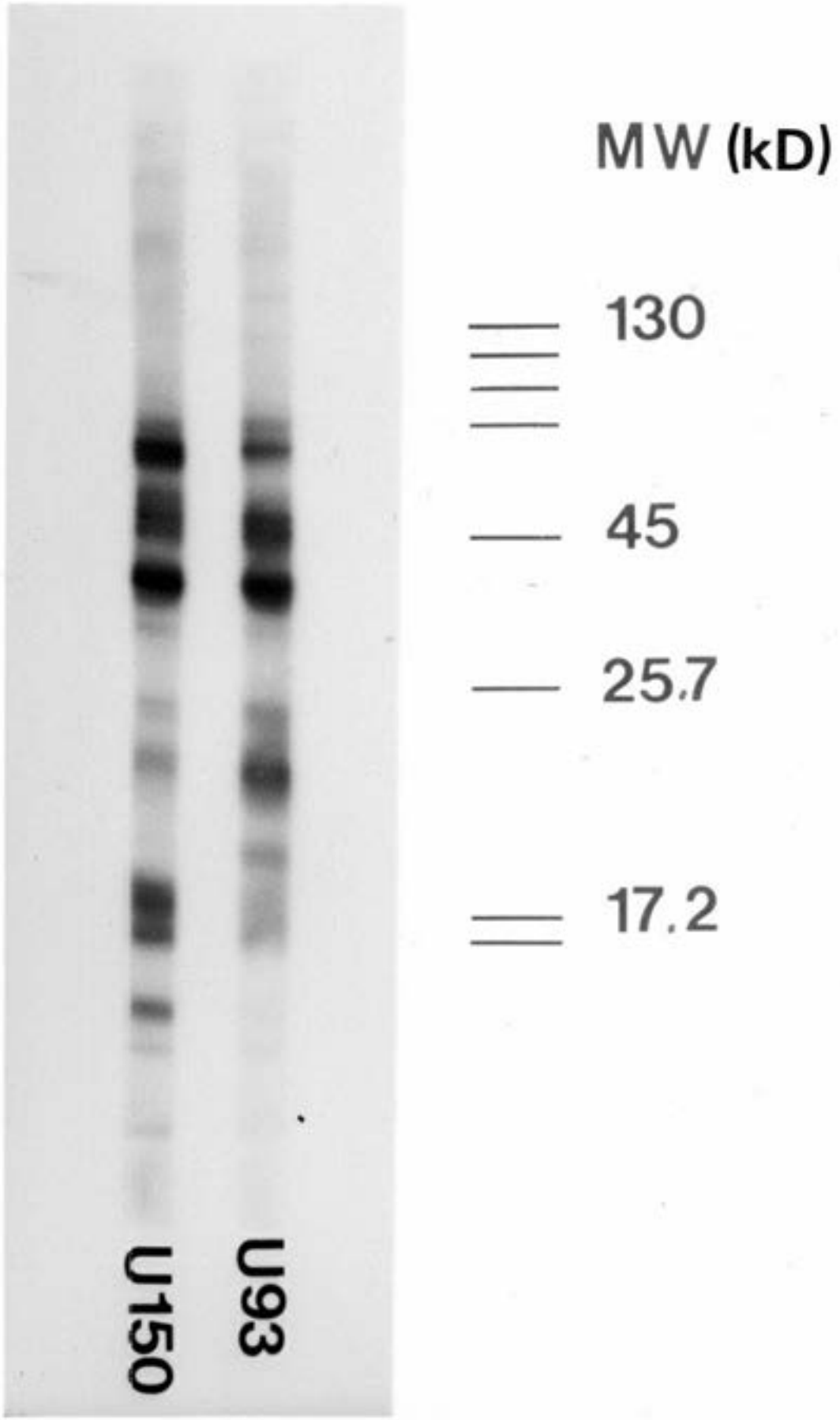

MW (kD)
130
45
25.7
17.2
U150
U93

Table 6.5. Orf virus specific ELISA antibody titres (log_{10}, reciprocal) in persistently infected Friesland rams before and after therapeutic measures.

Sheep No.	Weeks After Treatment	Reciprocal Titre (log_{10})
U93	-1	5.19
"	3	5.39
"	12	5.46
U150	-1	5.74
"	3	5.28
"	12	5.29

In view of the comprehensive range of antibody specificities for viral antigen which were already present in the sera of the rams on their arrival at the institute, no Western Blotting analyses were carried out on their sera after treatment.

Cell-Mediated Immune Responses

Skin thickness increments following intradermal inoculation with orf virus in the rams and control animals at 48 hours after challenge are presented in Table 6.6 and Fig. 6.7, and indicate that at all but the lowest level of challenge, DH responses to the virus in these chronically infected animals were significantly less marked than those in lambs which had made normal recoveries from the disease. In order to determine whether this relative anergy was confined to orf virus antigens, the rams and six further control animals were sensitised to DNFB, and challenged 60 days later by topical application of the substance in varying dilutions to the skin of the medial thigh. The increments in skin thickness which were recorded 48 hours after this challenge are represented

Fig. 6.7. Comparison of 48 hr skin thickness responses (mm) to intradermal inoculation of purified orf virus in rams U93 and U150 (0) following recovery from chronic orf, with those of four lambs which had recovered from experimentally induced disease (●). Bars represent standard deviations.

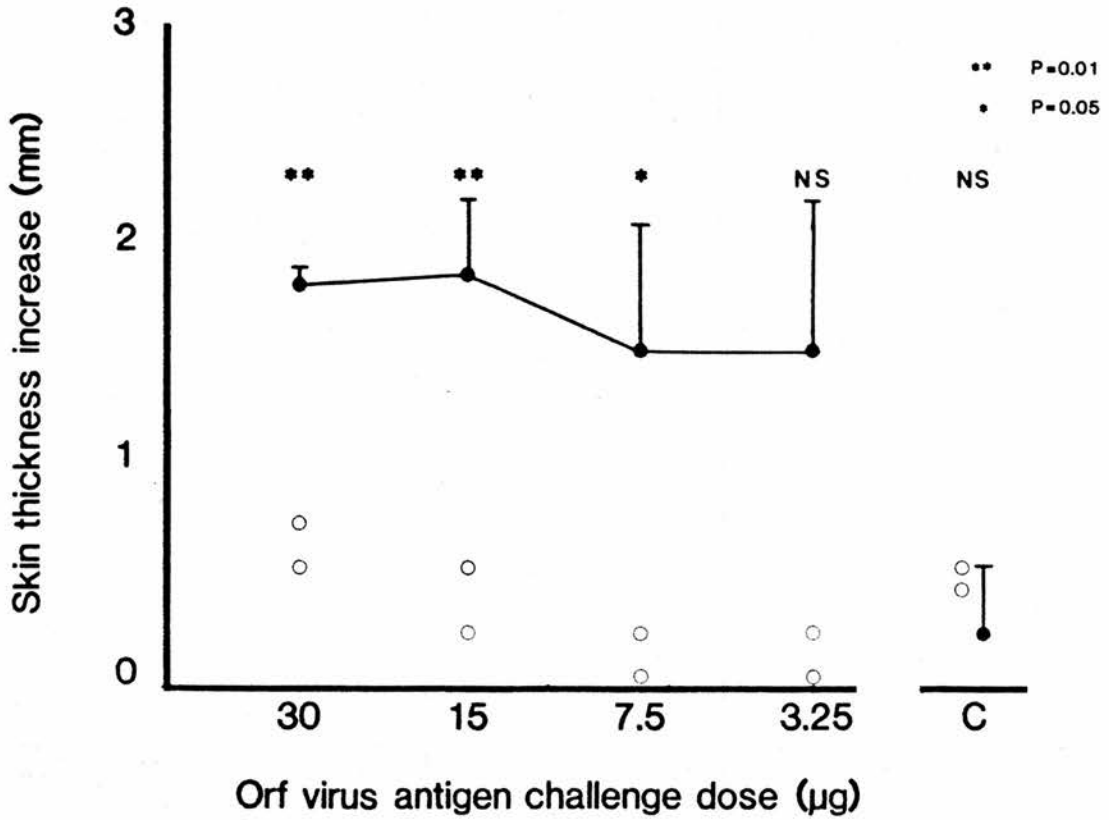

3
2
1
0
Skin thickness increase (mm)
** P=0.01
* P=0.05
**
**
*
NS
NS
30
15
7.5
3.25
C
Orf virus antigen challenge dose (µg)

Table 6.6. Skin thickness increases (mm) 48 hours following intradermal inoculation of purified orf virus after recovery from experimental (1-4) and chronic (U93,U150) infection with the agent.

Sheep No	ORF VIRUS CHALLENGE 30µg	15µg	7.5µg	3.25µg	Control
1	ND	2.1	1.0	1.8	0.1
2	1.8	1.5	1.0	0.5	0.0
3	1.7	1.6	2.1	2.0	0.7
4	1.9	2.2	1.9	1.9	0.3
Mean	1.8	1.85	1.5	1.55	0.28
±SEM	±0.06	±0.18	±0.29	±0.35	±0.15
U93	0.7	0.5	0.2	0.0	0.5
U150	0.5	0.2	0.0	0.2	0.4
Mean	0.6	0.35	0.1	0.1	0.45
±SEM	±0.1	±0.15	±0.1	±0.1	±0.05
*P	<0.01	<0.01	<0.01	>0.05	>0.05

ND: Measurements not taken due to the development of a pustule at the needle penetration site
SEM = standard error of the mean;

*as calculated by Student t test

Fig. 6.8. Comparison of 48 hr skin thickness responses (mm) to skin painting with DNFB in rams U93 and U150 (O) with those of six control lambs (●) eight weeks after sensitisation with the agent. Bars represent standard deviations.

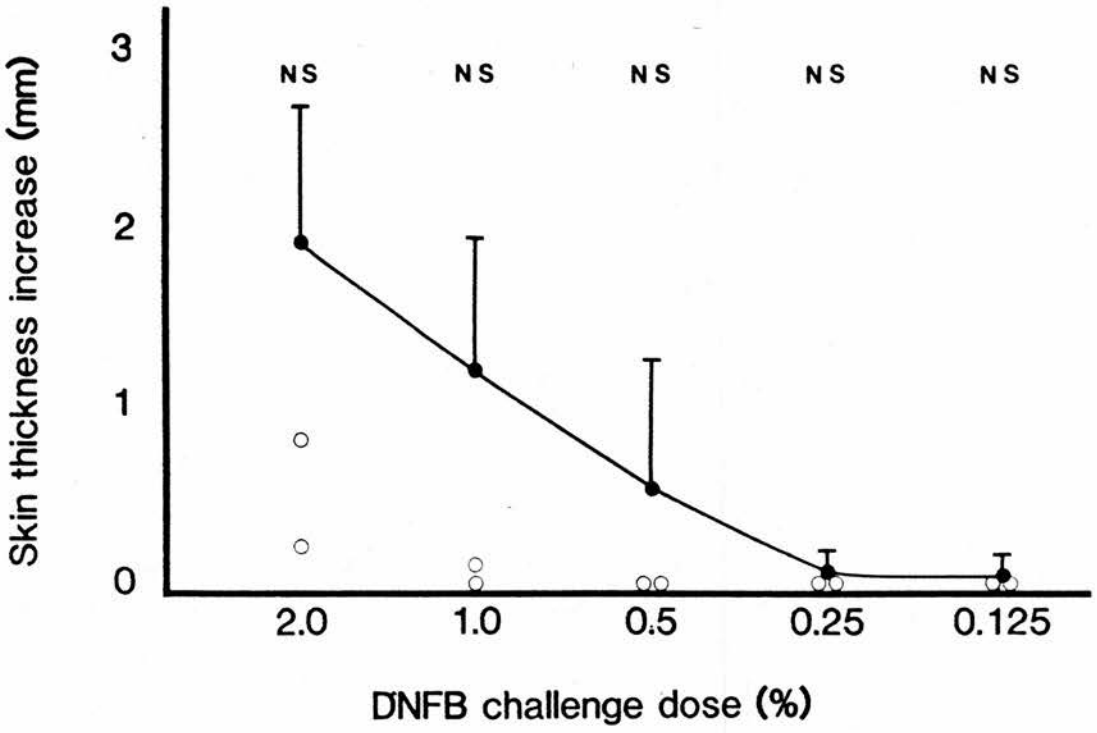

Skin thickness increase (mm)
3
2
1
0
NS
NS
NS
NS
NS
2.0
1.0
0.5
0.25
0.125
DNFB challenge dose (%)

Table 6.7. Skin thickness increases (mm) measured 48 hours following challenge with DNFB eight weeks after sensitisation.

Sheep No.	DNFB CONCENTRATION 2%	1%	0.5%	0.25%	0.125%
5	0.8	0.7	0.4	0.0	0.0
6	2.2	1.4	1.4	0.0	0.0
7	1.6	0.2	0.0	0.0	0.2
8	2.9	2.2	1.6	0.1	0.1
9	1.4	0.9	0.0	0.0	0.0
10	2.6	1.9	0.0	1.0	0.0
Mean ±SEM	1.97 ±0.32	1.22 ±0.31	0.57 ±0.30	0.18 ±0.16	0.05 ±0.03
U93	0.8	0.1	0.0	0.0	0.0
U150	0.2	0.0	0.0	0.0	0.0
Mean ±SEM	0.5 ±0.3	0.05 ±0.05	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
*P	>0.05	>0.05	-	-	-

* as calculated by Student t test

in Table 6.7 and Fig. 6.8, and indicate that although less marked, the DH responses of the two rams to DNFB were not significantly different from those of control animals.

Progress of lesions

Although the condition in both rams showed an incomplete regression during February 1984, lesions were still extensive in July 1984, and clinical material collected at this time was shown by electron microscopy to contain large numbers of orf virus particles. Regressions of lesions continued gradually over the following months with no apparent scarring, and in April 1985 only isolated focal areas of mild hyperkeratosis could be found on either ram. Since no viral particles were demonstrable by electron microscopy in material from these areas, it was assumed that the animals had now eliminated infection, and it was at this time that investigations into their CMI responses were initiated.

DISCUSSION

Whatever the reasons behind the persistent orf lesions which have been described in these rams, they illustrate the ability of orf virus to survive the periods between outbreaks on animals rather than in the environment. Although the epidemiological significance of this is not clear, it may be considerable in view of the data reported in Part One of this Chapter, and the evidence of Romero-Mercado and others (1973) that scab material shed naturally from orf-infected animals contain only negligible quantities of virus. Indeed, a brief report published during the course of this investigation (Grieg, Linklater and Clark, 1984) suggests that these rams are not unique in manifesting persistent orf lesions. These authors describe papillomatous lesions which persisted for eight months on the face, ears and legs of a newly purchased blue-faced Leicester ram. The condition was complicated by secondary infection with Corynebacterium pyogenes which necessitated the euthanisation of the animal, but samples of lesion material taken at autopsy had histological features consistent with orf virus infection, and contained large numbers of orf virus particles. It would appear that no further investigations were carried out regarding the factors which had given rise to these persistent lesions, but the possible alternatives of a novel strain of virus or an immune deficiency on the part of the animal are proposed.

Since challenge of two susceptible lambs with a suspension of clinical material collected from ram U93 resulted in the development experimental orf lesions which were characteristic in all respects, it is unlikely that the persistent nature of orf

lesions in these rams was the result of a viral strain with increased virulence. Indeed restriction-endonuclease analysis of orf virus DNA prepared from these animals (see Chapter Five) indicates that each ram was infected with different strain of the virus. It seems reasonable to conclude therefore that despite the extremely high antibody titres present in sera from both animals, neither was mounting an adequate immune response against the agent.

The failure of both immunopotentiating therapeutic measures to cause resolution of lesions is difficult to interpret, since the mechanisms through which their effects are mediated are poorly understood (see Werner, 1979). The administration of autogenous scab material in CFA had been used during this project in the production of orf virus-specific hyperimmune lamb serum (see Chapter Three) and so is clearly capable of stimulating the humoral wing of the ovine immune response to orf virus. However, virus-specific antibody titres in ram U150 actually fell following the administration of autogenous clinical material in CFA (Table 6.5) although levels remained high when compared with responses of conventional animals (Chapter Three). Possible explanations for this apparent paradox are feed-back inhibition of immunoglobulin synthesis by IgG antibody (Sinclair, 1969) or its suppression by immune complexes (see Theofilopoulos, 1980).

In contrast, orf virus-specific antibody titres in serum from ram U93 were seen to increase following the administration of levamisole (Table 6.3) although this was not reflected in an improvement in its condition. Rojas, Lezica, Cortada, Olivari, Feirstein and Lebenstein (1974) administered levamisole by a single intramuscular injection to 149 cattle and 26 goats suffering from

foot-and-mouth disease (FMD), at dose rates of 1-6 mg/kg and 2 mg/kg respectively. They report the disappearance of symptoms within 48 hours in all of the goats and all but one of the cattle. Epithelial lesions had healed in all animals by the 120th hour after treatment, and untreated animals continued to show clinical signs for at least 10 days. These authors speculate that the beneficial effect of levamisole in FMD is through the induction of interferon. However no beneficial effect was obvious following the administration of this substance to ram U93 in spite of a treatment regime which involved three successive intramuscular administrations of the drug at a rate of 3mg/kg. It is not possible to explain this observation in the absence of a clear understanding of the mechanisms responsible for the reported immunopotentiating effects of levamisole. In any event, neither of these attempted therapeutic measures can be construed as controlled experiments. Rather, they were carried out in an attempt to identify a possible means by which the referring veterinary surgeon might deal with further cases, and their results can only be interpreted as observations.

Perhaps the most enlightening of the experiments performed on these animals was the intradermal DH test. Although statistical analysis of such small groups is open to criticism, it would appear that skin reactions in the rams were significantly lower than in 4 control animals at all dose levels of orf antigen but the lowest, while no significant deficiencies were observed in their DH responses to DNFB.

The delayed-type hypersensitivity response was originally defined by Zinsser (1921) in the context of the tuberculin reaction

and involves the interaction of sensitised T-lymphocytes and a non-specific population of macrophages as effector cells (see Nash and Gell, 1981). Such T-cell-macrophage interactions have been shown to be important in the control of poxvirus infections (Blanden, 1971) and the demonstration by Maeda (1979) that peripheral blood lymphocytes of orf infected sheep inhibited the *in vitro* migration of guinea pig macrophages suggests that DH reactions may also be involved in the elimination of orf virus from infected animals.

Because the non-specific arming of macrophages by T-cells which characterises the DH reaction may result in host tissue damage, it is not surprisingly subject to tight regulation, which appears to be mediated mainly by antigen specific suppressor T-cells (see Liew, 1982). It is tempting to speculate that an imbalance in this homeostasis was responsible for the reduced DH reactions to orf virus which were observed in the two rams, and that this in turn led to the persistence of their orf lesions over such a long period. However, no firm conclusions can be made in the absence of further studies, which were precluded by the lack of further affected animals for study.

CHAPTER SEVEN

GENERAL DISCUSSION

In view of the vigorous blast cell-associated cytoxic T-lymphocyte (CTL) response which was recorded in ovine efferent lymph by Issekutz (1984, 1985) following the inoculation of vaccinia virus into the nodal drainage area, it was perhaps surprising to find (Chapter Two) that in six sheep, most of the lymphoblast response of the regional lymph node to orf virus infection could be accounted for by IgG or IgM producing cells. Nonetheless, the apparent lack of a response in the T-80 staining population of efferent lymphocytes which was observed in two of these sheep lends support to the conclusion that secondary orf virus infection in the sheep results in a stimulus to the regional lymph node which is mainly confined to the cortical or B-lymphocyte area.

However despite the fact that both of these investigations have involved the study of ovine responses to poxviruses, the nature of the nodal stimulus in each was probably quite different. Issekutz (1984, 1985) inoculated 10^7 plaque forming units (PFU) of the WR strain of vaccinia virus subcutaneously in the sheep which he studied, and since particulate antigens have been demonstrated in afferent lymph within a matter of hours following subcutaneous injection (Morris and others, 1968; Smith and others, 1970a) this would almost certainly have resulted in a rapid, massive antigenic challenge to the node. In contrast, challenge with orf virus in the present study was achieved by the epicutaneous application of a suspension of infective scab material following abrasion of the site with sharp plastic. This procedure would presumably have allowed only minimal amounts of viral antigen to reach the node until replication of virus had occurred within the epidermis, a conclusion which is borne out by the 5-7 day lag period which was

evident in the efferent lymph responses of all six animals. Indeed, the proliferative changes which are a characteristic feature of epidermal orf lesions (Glover, 1928; Wheeler and Cawley, 1956) may, by displacing infected cells towards the skin surface, ensure that only limited amounts of viral antigen actually reach the node.

The fact that six of the sheep involved in the efferent cannulation studies had moderate levels of antibody in their lymph at the time of infection may provide a further explanation for the apparent lack of detectable T lymphocyte responses in their lymph following infection. Mullbacher and Blanden (1979) demonstrated in mice that the inoculation of virus-immune serum before infection with ectromelia virus significantly reduced the capacity of the mouse to mount a primary CTL response. Blocking activity coincided with the IgG peak in fractionated serum, and they concluded that this effect was due to the occupation by the serum of antigenic patterns on the virus which are recognised by CTL's. In the presence of lymph antibody, it is likely that a high proportion of cell associated or free viral antigen would arrive at the regional node in the form of immune complexes, giving rise to less than optimal antigen presentation to CTL precursors.

A further consideration may also serve to reconcile the data reported here with those of Issekutz (1984, 1985). The latter worker did not attempt to define what proportion of the anti-vaccinia lymphoblast response was responsible for the observed virus specific cytotoxicity. Most of his assays were performed using an effector:target ratio of 100:1, which suggests that the numbers of CTL's may in fact have been small. It has been stated that in

two of the sheep described in Chapter Two, some large lymphocytes were seen to express the T-80 antigen at the height of the nodal response. It is possible therefore that demonstrable levels of CTL activity were in fact present in responding lymph from these sheep, mediated by a small population of T-lymphoblasts.

Although the majority of lymphoblasts elaborated by the regional lymph node in response to orf virus infection in all six sheep were observed to contain IgG, in two of the animals, no appreciable virus specific antibody response was detected in lymph. As discussed in Chapter Two, this discrepancy may partly be explained by the possibility of non-specific activation (Poskitt and others, 1977). However, the Western Blotting analyses described in Chapter Four clearly demonstrate that although many orf virus antigens are recognised by convalescent and hyperimmune sera, most of these are associated with the viral core, and only one is surface associated. In order to prevent aggregation, the sensitising antigen used in this ELISA was partially solubilised by incubation in SDS, followed by a brief sonication. Easterbrook (1966) found that even prolonged exposure to detergent resulted in no morphological changes in vaccinia virus, and sonication per se does not affect the infectivity of orf virus (Sawhney, 1972). It is likely therefore that the ELISA test used during this project preferentially detects antibodies which are directed at surface components of the virus, and as such may not have provided a measure of lymph antibody responses to internal orf virus antigens. Nonetheless, good serum antibody responses were detected by ELISA in five sero-positive lambs after experimental orf infection (Chapter Three), and the precise reasons behind the absence of an appreciable rise in antibody titre in lymph from two cannulated sheep during their responses remain unclear.

In any event, certain aspects of these experiments suggest that serum antibody responses may be of limited significance in overall recovery and protection from the disease. In no instance was an animal which was sero-positive by ELISA refractory to experimental infection, and furthermore, within the naturally infected group of lambs described in Chapters Three and Four, the highest antibody titres and most comprehensive recognition of antigen were associated not with rapidly resolving lesions, but with disease which was of greater severity and longer duration. It would appear therefore that the major effort of the lymph node which drains an orf lesion is essentially ineffective both in eliminating the virus and in preventing further infection.

However, the observations of several workers suggest that absolute protection against reinfection is not a consistent feature in lambs which have recovered from the disease, but that subsequent infections are less severe and of shorter duration (Boughton and Hardy, 1934; Hart and others, 1949; Nisbet, 1954). Indeed Osman (1976) found that only 13 out of 247 previously infected lambs were completely refractory to rechallenge with orf virus, although the successful challenges resulted in rapidly resolving lesions which allowed only limited viral replication. The interval between challenge and rechallenge did not alter this so-called "accelerated response". This is strong evidence to suggest that in the majority of sheep, immunity to orf virus infection is manifested by more rapid clearance of virus rather than absolute protection. The question arises then of how these accelerated responses are mediated, and how they may be reconciled with the above observations on regional lymph node responses in orf virus infection.

Similar accelerated reactions have long been recognised in human vaccinal lesions, and were described for smallpox by Jenner (1798) who wrote of the inoculation of variolous matter into the arm of a woman who had recovered from cowpox "... It is remarkable that variolous matter, when the system is disposed to reject it should excite inflammation on the part to which it is applied more speedily than when it produces the Small Pox". McKinnon and Defries (1931) noted the contribution of delayed hypersensitivity (DH) reactions to the accelerated response which is a feature of the revaccination of human subjects with vaccinia virus, and Pincus and Flick (1963a) proposed that in guinea pigs, even primary vaccinal lesions had a significant DH component. These authors found that parenteral inoculation of anti-mononuclear cell serum before intradermal challenge with live vaccinia virus prevented the development of a characteristic primary vaccinal lesion, and in addition prevented DH reactions to three previously inoculated antigens. There was evidence that viral replication took place however, in that all treated animals developed haemagglutination inhibition antibodies to the virus, which were equivalent to untreated control animals.

In a further study, the same authors (Flick and Pincus, 1963) demonstrated that rabbits which were neonatally tolerised to vaccinia virus by the intravenous inoculation of a large dose of purified, inactive particles, failed to develop characteristic lesions on subsequent intradermal challenge with live virus. Several of these rabbits had died by the 7th day of infection, and internal organs yielded vaccinia virus on tissue culture isolation, while none of the surviving animals showed DH reactions to intradermally inoculated inactive virus. This apparent correlation

between failure to develop a primary vaccinal lesion and inability to produce DH reactions to viral antigens led the authors to conclude that an allergic component is an important feature of the primary lesion, preventing appreciable amounts of virus from leaving the site of infection.

Pincus and Flick (1963b) also demonstrated that in vaccinia inoculated human subjects, DH reactions to inactivated virus are evident as early as the fourth day after primary vaccination, which suggests that DH also participates in the human primary vaccinal lesion.

In that orf is a skin-related pox virus infection of sheep, it is tempting to extrapolate from these findings and suggest that the accelerated response defined by Osman (1976) in previously infected sheep is also in part a manifestation of DH. This would appear to be borne out by the demonstration in Chapter Six, Part Two of DH responses in four lambs with known history of orf virus infection and the apparent absence of such reactions in the two persistently infected rams. In addition, Buddle and Pulford (1984) have reported that DH responses to viral antigens can be detected in lambs approximately three weeks after infection with orf virus at 1-4 days old, finding that this, rather than serum antibody was associated with protection (a state which they attributed to lambs which developed lesions less than 3mm in diameter on rechallenge). Maeda (1979) demonstrated that lymphocytes derived from orf virus infected sheep were capable of impairing the <u>in vitro</u> migration of guinea pig macrophages, a property which has been classically associated with DH, and attributed to the lymphokine, macrophage inhibition factor (MIF) (Bloom and Bennett, 1966).

It is currently conceived (Liew, 1982) that induction of DH responses is dependent on the recognition by specific T-lymphocytes of antigen in association with Class II major histocompatability (MHC) antigens on the surface of antigen presenting cells (APC). Such sensitised T-cells form a long lived recirculating pool which proliferate locally on further encounters with antigen. Soluble products (lymphokines) of these cells attract and activate non-specific macrophages which are the effector cells of the response. There is evidence (Miller and Butler, 1983) that DH responses are augmented by a population of antigen-specific helper Tlymphocytes, and modulated (Phanuphak, Moorhead and Claman, 1974) by specific suppressor T-cells.

Contact sensitivity is a DH reaction which occurs in the skin of sensitised subjects in response to certain simple chemicals (Eisen, 1959). Macher and Chase (1969a,b) provided strong evidence that elements within the skin play an important role in the induction of these responses. They showed that the development of contact sensitivity to picrylchloride and dinitrochlorobenzene (DNCB) in guinea pigs was dependant on an interaction between the hapten and the skin.

Thomas, Forni, Shevach and Green (1977) carried out <u>in vitro</u> studies which suggested a critical role for macrophages and Class II antigens in hapten specific activation of T lymphocytes. There is a population of cells within mammalian epidermis which resemble macrophages in several respects. Langerhans cells are bone marrow derived dendritic cells (Katz, Tamaki and Sachs, 1979) that bear receptors for complement components and the Fc portion of immunoglobulin, and express Class II MHC antigens (see Wolff and

Stingl, 1983). Toews, Bergstresser and Streilein (1980) have provided strong evidence that murine epidermal Langerhans cells process and present the hapten DNFB to immunocompetent lymphocytes. They showed that areas of skin depleted of Langerhans cells artificially (by exposure to UV light) or inherently (as is the case in tail skin) did not give rise to sensitisation following the application of DNFB in an immunogenic manner, and later demonstrated similar characteristics for Langerhans cells in Syrian hamsters (Streilein and Bergstresser, 1981). It is not clear however, if the antigen presentation event between Langerhans cells and specific T-lymphocytes occurs in the skin or within the draining lymph node (Streilein, 1983). Nagao, Inaba and Iijima (1976) have demonstrated that Langerhans cells take up vaccinia virus particles following inoculation in humans, which suggests a possible role for these cells in the presentation of viral antigens during epidermal infections.

There are indications then, that the induction of DH responses which are detectable in sheep following infection with orf virus, and which may be responsible for the accelerated reaction which is characteristic of secondary exposure to the agent, is dependant on the presentation of viral antigen to T-lymphocytes by epidermal Langerhans cells. Such a reaction need not be reflected in dramatic changes in draining lymph. Hall and Smith (1971) investigated the changes occurring in ovine lymph draining a contact sensitivity reaction to DNFB, and found only equivocal evidence in terms of blast cell formation of a cellular response in afferent lymph. However, changes in efferent lymph were very similar to the blast cell responses which were recorded by Hall and Morris (1963) following stimulation with heterologous serum

proteins or bacterial antigens, and involved elaboration of specific antibody. These authors did not attempt to establish whether the DNFB induced blast cells in efferent lymph were of the B- or T-lymphocyte lineage, but their observations do suggest that the development of a DH response within a secondary orf lesion is compatable with the cellular changes which were recorded during these studies both in efferent and afferent lymph.

Whether or not it was a major contributing factor to the long term persistence of orf virus infection in the two Friesland rams described in the preceding chapter, the apparent absence of orf virus specific DH reactivity in these animals is intriguing. It has already been suggested (Chapter Six, Part Two), that the reduced DH reactions which were encountered in these animals may have been the result of an imbalance in the mechanisms which control these responses. The poll area and much of the face of both these rams was hairless, with the skin having an obvious flushed appearance. It has been shown that exposure to ultraviolet (UV) radiation can result in dramatic reductions in the expression of class II MHC antigens by murine and human Langerhans cells (Aberer, Schuler, Stingl, Honigsman and Wolf, 1981), and that UV light induced perturbations in Langerhans cell populations can give rise to inefficient contact sensitisation in mice (Toews, Bergstresser and Streilein, 1980) and hamsters (Streilein and Bergstresser, 1981), and result in specific unresponsiveness. The results of Granstein, Lowy and Greene (1984) suggest that in mice this unresponsiveness is the result of the presentation of antigen to precursor suppressor T-cells by a UV resistant APC population within the epidermis. It is interesting to speculate that the poor DH responses which were evident in these rams following intradermal

challenge with orf virus were the result of UV light-induced changes in the epidermal Langerhans cell population at the site of infection. However, Langerhans cell distribution and function have not been characterised for sheep, and the validity of such an extrapolation is somewhat questionable.

Irrespective of its causes, the fact that these animals sustained an active infection with orf virus over such a long period assumes a high degree of significance in the light of the observed inability of virus in scab material to survive complete exposure to the elements during winter. A reappraisal of the epidemiology of orf virus infection in sheep would seem appropriate, with particular reference to the significance of inapparent or subclinical infections in the initiation of orf outbreaks. To this end serological surveys of unaffected flocks using ELISA might provide a useful indication of undetected or subclinical disease. Restriction enzyme analysis as described in Chapter Five would also prove useful in allowing an investigation of the patterns of spread of a particular virus strain within a given area. Where outbreaks are observed in vaccinated flocks (see Trueblood and others, 1963; Beck and Taylor, 1974; Buddle and others, 1984.) the technique could be used to establish whether the vaccine virus or an unrelated strain was responsible.

It is clear that further work is required to consolidate the conclusions which have been derived from these studies. While there was little detectable evidence of a marked T-cell response in lymph from the regional node during secondary orf virus infection, it is possible that secondary T-cell responses in orf take place at the site of infection, being mediated by long lived circulating

memory T-cells (see Liew, 1982). In contrast the presence of memory B-cells in the germinal centres of lymph nodes (Stein, Gerdes and Mason, 1982) is consistent with a secondary B-cell response within the regional node. It may be that T-cell responses to orf virus infection would be more clearly seen in lymph generated by a node which is responding to primary exposure to the virus. It would seem a priority therefore to carry out similar cannulation studies on animals which have not been exposed to orf virus.

Unfortunately, the identification of such animals which are also suitable for cannulation purposes may present problems. Screening by ELISA is a possible solution, but poor responders might escape detection. The use of SPF animals as an alternative must be assessed in the light of the considerable resources which would be required to maintain SPF conditions until lambs were sufficiently mature to allow cannulation, particularly in view of the potentially high failure rate which is inherent in this technique (see Chapter Two).

However, such problems are not insurmountable, and if a response was evident in the T-cell populations of lymph from regional nodes during primary infection, as assessed by T-80 staining, or the binding of lectins such as peanut agglutinin (Fahey, 1980), responding T-cells could be characterised by <u>in vitro</u> assays for cytotoxicity directed at virus infected cells and proliferation in the presence of viral antigen. If these cells could be maintained <u>in vitro</u> by repeated stimulation with viral antigen, it might be possible to derive clones whose specificities could be defined by blocking with monoclonal antibodies raised against orf virus or

infected cells. In this light it would be interesting to establish whether the 38-41kD orf virus surface antigen which appears to be immunogenic for B-cells is also recognised by T-cell subsets. Virus specific monoclonal antibodies might also allow the detection of viral antigen in or on the surface of afferent lymph cells, although antigen which has been processed by these cells may well be present on the surface in an altered form (see Grey and Chestnut, 1985).

Recent advances in molecular biology might allow the identification and cloning of the viral genes whose products stimulate T-cell responses in orf infected animals. In vitro expression systems could then yield large amounts of these antigens, which might form the basis of a novel orf vaccine.

In short, it is concluded that although the greater part of the regional lymph node's response to secondary infection with orf virus infection in sheep is humoral, the immune mechanisms involved in elimination of the virus at the site of infection are probably cellular in nature. There is obvious heterogeneity within the orf virus population in Britain, although this is not reflected in marked antigenic differences. In spite of its remarkable resistance, the virus is inactivated by prolonged exposure to rainfall, and may in part be maintained by subclinical or inapparent infections during the periods between outbreaks.

REFERENCES

ABDUSSALAM, M. (1953) Contagious pustular dermatitis of sheep. Proceedings, XVth International Veterinary Congress, Stockholm, 1953 part 1, volume 1, 387-393.

ABDUSSALAM, M. (1957a) Contagious pustular dermatitis II. Pathological histology. Journal of Comparative Pathology, 67: 217-222.

ABDUSSALAM, M. (1957b) Contagious pustular dermatitis III. Experimental infection. Journal of Comparative Pathology, 67: 305-319.

ABDUSSALAM, M. (1958) Contagious pustular dermattis IV. Immunological reactions. Journal of Comparative Pathology, 68: 23-35.

ABDUSSALAM, M. and COSSLETT, V.E. (1957) Contagious pustular dermatitis virus. I. Studies on morphology. Journal of Comparative Pathology, 67: 145-156.

ABERER, W.; SCHULER, G.; STINGL, G.; HONIGSMANN, H. and WOLFF, K. (1981) Ultraviolet light depletes surface markers of Langerhans cells. Journal of Investigative Dermatology, 76: 202-310

ALTARA, I. (1925) Immunita e vaccinazione nella stomatite pustolo-contagiosa degli ovini. Nuova Veterinaria, 3: 4. (Abstract, Bulletin de l'Institut Pasteur, 23: 341).

AL-YAMAN, F.M. and WILLENBORG, D.O. (1985) The immune reactivity of the regional and distant lymph nodes to autochthonous ovine squamous cell carcinomata. Veterinary Immunology and Immunopathology, 9: 261-272.

ANDREWES, C., PEREIRA, H.G. and WILDY, P. (1978) Viruses of Vertebrates, 4th edition. Balliere Tindall, London.

ANON. (1985a) Association of the British Pharmaceutical Industry, Compendium of Data Sheets for Veterinary Products 1985-86, pp. 130, 171. Datapharm Publications Ltd, London.

ANON. (1985b) British Parmacopoeia (Veterinary) p. 168. Her Majesty's Stationary Office, London (1985).

ARITA, M. and TAGAYA, I. (1980) Virion polypeptides of poxviruses. Archives of Virology, 63, 209-225.

AYNAUD, M. (1921) La stomatite pustuleuse contagieuse des ovins. Comptes Rendues Hebdomadaires des Seances de l'Academie des Sciences, Paris, 173: 950. (Abstract, Bulletin de l'Institut Pasteur, 20: 75.)

AYNAUD, M. (1923) La stomatite pustuleuse contagieuse des ovins (chancre du mouton) Annales de L' Institut Pasteur, 37: 498-527.

BECK, C.C. and TAYLOR, W.B. (1974) Orf: it's awrful! Veterinary Medicine and Small Animal Clinician, 69: 1413-1417.

BEH, K.J. (1985) The antibody-containing cell response of the lamina propria of the sheep and the uptake of soluble antigen into different ileal lymph following intraintestinal infusion of soluble or particulate antigen. Immunology, 54: 479-485.

BEH, K.J. and LASCELLES, A.K. (1981) The effect of route of administration of antigen on the antibody containing cell response in lymph of sheep. Immunology, 42: 577-582.

BELSCHNER, H.G. (1950) Sheep Management and Diseases pp. 417-424. Angus and Robertson, Sydney/London.

BENNETT, S.C.J., HORGAN, E.S. and HASEEB, M.A. (1944) The Pox diseases of sheep and goats. Journal of Comparative Pathology, 54: 131-160.

BERRY, A. (1901) Contagious pustular dermatitis of sheep. Journal of Comparative Pathology and Therapeutics, 14: 307-312.

BIOZZI, G.; SIQUEIRA, M.; STIFFEL, C.; IBANEZ, O.M.; MOUTON, D. and FERREIRA, V.C.A. (1980) Genetic selection for relevant immunological functions. In M. Fougerou and J. Dausser, Editors. Immunology 80, Progress in Immunology IV pp. 432-457. Academic Press, London.

BLANC, G., MÉLANIDI, C. and CAMINOPETROS, J. (1922) Recherches expérimentales sur une maladie éruptive de la chèvre observée en Grèce. Annales de l'Institut Pasteur, 36: 614-619.

BLANDEN, R.V. (1971) Mechanisms of recovery from a generalised viral infection: mouse pox. III. Regression of infectious foci. Journal of Experimental Medicine, 133: 1090-1104.

BLOOD, D.C., HENDERSON, J.A. and RADOSTITS, O.M. (1979) Veterinary Medicine - a textbook of the disease of cattle, sheep, pigs and horses. 5th Edition, pp 699-700. Balliere Tindall, London.

BLOOM, B.R. and BENNETT, B. (1966) Mechanisms of a reaction in vitro associated with delayed type hypersensitivity. Science, 153: 80-82

BOSTEDT, H. (1985) Contagious pustular dermatitis in sheep: clinical features and control. Pro Veterinario, 3: 10-11.

BOUGHTON, I.B. and HARDY, W.T. (1934) Contagious ecthyma (sore mouth) of sheep and goats. Journal of the American Veterinary Medical Association, 85: 150-178.

BOUGHTON, I.B. and HARDY, W.T. (1935) Immunisation of sheep and goats against sore mouth (contagious ecthyma). Bulletin of the Texas Agricultural Experimental Station, no. 504, pp 1-16.

BRADFORD, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

BUDDLE, B.M. (1981) Contagious ecthyma virus infection of sheep: Virologic and immunologic investigations. PhD thesis, Virginia State University and Polytechnic Institute.

BUDDLE, B.M., DELLERS, R.W. and SCHURIG, G.G. (1984) Heterogeneity of contagious ecthyma virus isolates. American Journal of Veterinary Research, 45: 75-79.

BUDDLE, B.M. and PULFORD, H.D. (1984) Effect of passively acquired antibodies and vaccination on the immune response to contagious ecthyma virus. Veterinary Microbiology, 9: 515-522.

BURNETTE, W.N. (1981) "Western blotting"; electrophoretic transfer of proteins from sodium dodecyl sulphate - polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated Protein A. Analytical Biochemistry, 112: 195-203.

BUTTNER, D.; GIESE, H.; MULLER, G. and PETERS, D. (1964) Die Feinstruktur riefer Elementarkorper des Ecthyma contagiousum und der stomatitis papulosa. Archiv fur die Gesamte Virusforschung, 14: 657-673.

BUXTON, A. and FRASER, G. (1977) Animal Microbiology Vol.2, pp. 690-691. Blackwell Scientific Publications, Oxford.

CARNE, H.R.; WICKHAM, N.; WHITTEN, W.K. and LOCKLEY, R.P. (1946) Infection of man by the virus of contagious pustular dermatitis of sheep. Australian Journal of Science, 9: 73-74.

CARRE, M. (1932) Quelques maladies peu connues du mouton. Recueil de Médecine Vétérinaire, 107: 852-873.

CONNELL, R. (1954) Contagious ecthyma in Rocky Mountain bighorn sheep. Canadian Journal of Comparative Medicine, 18: 59-60.

DEMARTINI, J., PEARSON, L. and FISCUS, S. (1978) Chromium-51-release assay of antibody and complement-mediated cytotoxicity for contagious ecthyma virus-infected cells. American Journal of Veterinary Research, 39: 1922-1926.

DIETERICH, R.A.; SPENCER, G.R.; BURGER, D.; GALLINA, A.M. and VANDERSCHALIE, J. (1981) Contagious ecthyma in Alaskan musk-ox and Dall sheep. Journal of the American Veterinary Medical Association, 179: 1140-1143.

DONACHIE, W. and JONES, G. (1982) The use of ELISA to detect antibodies to Pasteurella haemolytica A2 and Mycoplasma ovipneumoniae in sheep with experimental chronic pneumonia. In: The ELISA: Enzyme Linked Immunosorbent Assay in Veterinary Research and Diagnosis, R.C. Wardley and J.R. Crowther editors, pp 115-124. Martinus Nijhoff Publishers, The Hague/Boston/London.

EASTERBROOK, K.B. (1966) Controlled degradation of vaccinia virus in vitro: an electron microscopic study. Journal of Ultrastructure Research, 14: 484-496.

EISEN, H.N. (1959) Hypersensitivity to simple chemicals. In Cellular and Humoral Aspects of Hypersensitive States. H.S. Lawrence, editor. pp. 89-122. Cassell, London.

EISEN, H.N. and SISKIND, G.W. (1964) Variations in affinities of antibodies during the immune response. Biochemistry; 3: 996-1008.

ENGLISH, L.S., MORRIS, B. and ADAMS, E. (1976) The synthesis of immunoglobulins by lymphoid cells in the sheep. The contribution made by the lymphoblasts to the total immunoglobulin and antibody production by lymphoid cells of the popliteal node during a secondary response to bacterial lipopolysaccharide. Cellular Immunology, 27: 274-286.

ENGLISH, L.S., MORRIS, B. and ADAMS, E. (1977) The modulating effects of lymph on the synthesis of immunoglobulins by lymphoblasts. Cellular Immunology, 28: 90-100.

ENGVALL, E and PERLMANN, P. (1972) Enzyme-linked immunosorbent assay, ELISA III. Quantitation of specific antibodies by enzyme labelled anti-immunologobulin in antigen coated tubes. Journal of Immunology, 109: 129-135.

FAHEY, K.J. (1980). The binding of lectins to sheep tissues and circulating cells: peanut agglutinin, a marker for presumptive Tlymphocytes. Australian Journal of Experimental Biology and Medical Sciences, 58: 557-569.

FENNER, F. and WOODROOFE, G (1960) The reactivation of poxviruses II. The range of reactivating viruses. Virology, 11: 185-201.

FLICK, J.A. and PINCUS, W.B. (1963) Inhibition of the lesions of primary vaccinia and of delayed hypersensitivity through immunological tolerance in rabbits. Journal of Experimental Medicine, 177: 633-646.

FRERICHS, G.N. (1980) Studies on orf virus infections in sheep. PhD thesis, University of London.

FRIEDMANN, P.S. (1981) The Immunobiology of Langerhans cells. Immunology Today, 2: 124-128.

FRIEDMAN-KIEN, A.E., ROWE, W.P.; and BANFIELD, W.G. (1963) Milkers nodules: isolation of a poxvirus from a human case. Science, 140: 1335-1336.

GANGEMI, J.D. and SHARP, G. (1976) Use of a restriction endonuclease in analysing the genomes from two different strains of vaccinia virus. Journal of Virology, 20: 319-323.

GASSMANN, U., WYLER, R. and WITTEK, R. (1985) Analysis of parapoxvirus genomes. Archiv fur die Gesamte Virusforschung, 83: 17-31.

GERSHONI, J.M. and PALADE, G.E. (1983) Protein blotting: principles and applications. Analytical Biochemistry, 131: 1-15.

GLOVER, R.E. (1928) Contagious pustular dermatitis of the sheep. Journal of Comparative Pathology and Therapeutics, 41: 318-340.

GLOVER, R.E. (1930) Variola in domestic animals. Contagious pustular dermatitis of the sheep and goat. Proceedings 11th International Veterinary Congress, London, 1930. Vol III. 29 -43.

GLOVER, R.E. (1933) Contagious pustular dermatitis: A. Cross immunity experiments with various strains. B. Serological tests. Third Annual Report of the Director, Institute of Animal Pathology (Cambridge) 1932-1933, 1-13.

GLOVER, R.E. (1935) Contagious pustular dermatitis of sheep: Vaccination. Fourth Annual Report of the Director, Institute of Animal Pathology (Cambridge) 1934-1935, 1-15.

GRAHAM, R.C., LUNDHOLM, U. and KARNOWSKI, M. J. (1965) Cytochemical demonstration of peroxidase activity with 3-amino-9-ethyl-carbazole. Journal of Histochemistry and Cytochemistry, 13: 150-152.

GRANSTEIN, R.D., LOWY, A. and GREENE, M.I. (1984) Epidermal antigen presenting cells in activation of suppression: identification of a new functional type of ultraviolet radiation-resistant epidermal cell. Journal of Immunology, 132: 563-565.

GRAUSGRUBER, W. (1964) Lippengrind (Ecthyma Contagiosum) bei Gemsen. Zentralblatt fur Bakteriologie, Parasitenkunde & Infektionskrankheiten 1.(Orig.) 195: 175-181. (Abstract, Veterinary Bulletin, 35: 506).

GREIG, A.S. (1956a) Contagious ecthyma of sheep, I. Attempts to infect other hosts. Canadian Journal of Comparative Medicine, 20: 448-452.

GREIG, A.S. (1957) Contagious ecthyma of sheep, II. In vitro cultivation of the virus. Canadian Journal of Comparative Medicine, 21: 304-308.

GREIG, J.R. (1956b) The Shepherd's Guide to the Prevention and Control of the Disease of the Sheep, pp. 44-66. Her Majesty's Stationery Office, Edinburgh.

GREY, H. and CHESTNUT, R. (1985) Antigen processing and presentation to T cells. Immunology Today, 6: 101-106.

HALL, J.G. (1967a) A method for collecting lymph from the prefemoral lymph node of unanaesthatised sheep. Quarterly Journal of Experimental Physiology, 52: 200-205.

HALL, J.G. (1967b) Studies of the cells in the afferent and efferent lymph of lymph nodes draining the site of skin homografts. Journal of Experimental Medicine, 125: 737-754.

HALL, J.G., HOPKINS, J. and REYNOLDS, J. (1980) Studies on efferent lymph cells from nodes stimulated with oxazalone. Immunology, 39: 141-149.

HALL, J.G. and MORRIS, B. (1962) The output of cells in lymph from the popliteal node of sheep. Quarterly Journal of Experimental Physiology, 47: 360-369.

HALL, J.G. and MORRIS, B. (1963) The lymph-borne cells of the immune response. Quarterly Journal of Experimental Physiology, 48: 235-247.

HALL, J.G.; MORRIS, B.; MORENO, G.D. and BESSIS, M.C. (1967) The ultrastructure and function of the cells in lymph following antigenic stimulation. Journal of Experimental Medicine, 125: 91-109.

HALL, J.G. and SMITH, M.E. (1971) Studies on afferent and efferent lymph of lymph nodes draining the site of application of Fluorodinitrobenzene (FDNB). Immunology, 21: 69-79.

HANSEN, J.N. (1981) Use of solubilisable acrylamide disulphide gels for isolation of DNA fragments suitable for sequence analysis. Analytical Biochemistry, 116: 146-151.

HARDY, W.T. (1964) Contagious ecthyma in sheep and goats. Proceedings of the US Livestock Sanitation Association, 67: 293-299.

HARKNESS, J.W.; SCOTT, A.C. and HERBERT, C.N. (1977) Electron microscopy in the rapid diagnosis of orf. British Veterinary Journal, 133: 81-87.

HART, L., HAYSTON, J.T., and KEAST, J.C. (1949) Observations on contagious pustular dermatitis of sheep. Australian Veterinary Journal, 25: 40-45.

HATZIOLOS, M. (1930) Observations sur une épizootie d'ecthyma contagieux des lèvres. Revue Générale de Médecine Vétérinaire, 39: 263-267.

HEBERT, D.M., SAMUEL, W.M. and SMITH, G.W. (1977) Contagious ecthyma in a mountain goat of British Columbia. Journal of Wildlife Diseases, 13: 135-136.

HERRING, A.J.; INGLIS, N.F.; OJEH, C.; SNODGRASS, D.R. and MENZIES, J.D. (1982) Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver stained polyacrylamide gels. Journal of Clinical Microbiology, 16: 473-477.

HERRING, A.J. and SHARP, J.M. (1984) Protein blotting: the basic method and its role in viral diagnosis. In: Recent Advances in Virus Diagnosis, M. S. McNulty and J. B. McFerran editors, pp. 115-124. Martinus Nijhoff Publishers, The Hague/ Boston/ London.

HOARE, E.W. (1913) Contagious pustular dermatitis of sheep. In: A System of Veterinary Medicine, E.W. Hoare editor, pp. 300-309. Balliere, Tindall and Cox, London.

HORGAN, E.S. and HASEEB, M.A. (1947) The immunological relationships of strains of contagious pustular dermatitis virus. Journal of Comparative Pathology, 57: 1-7.

HOWARTH, J.A. (1929) Infectious pustular dermatitis of sheep and goats. Journal of the American Veterinary Medical Association, 75: 741-760.

HUCK, R.A. (1966) A paravaccinia virus isolated from cow's teats. Veterinary Record, 78: 503-504.

HUNTER, W.M. and GREENWOOD, F.C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature; 194: 495-496.

ISHII, S., KAWAKAMI, Y. and FUKUHARA, S. (1953) Pustular dermatitis of sheep and goat, I. The property of the causative agent. Experimental Report of the Government Experimental Station on Animal Hygiene, Japan, No. 27, pp. 141-150. (Abstract, Veterinary Bulletin, 25: 347).

ISSEKUTZ, T. B. (1984) Kinetics of cytotoxic lymphocytes in efferent lymph from single lymph nodes following immunisation with vaccinia virus. Clinical and Experimental Immunology, 56: 515-523.

ISSEKUTZ, T. B. (1985) Characteristics of lymphoblasts appearing in efferent lymph in response to immunisation with vaccinia virus. Immunology, 56: 23-31.

ISSEKUTZ, T.B., CHIN, G.W. and HAY, J.B. (1980) Lymphocyte traffic through granulomas: differences in the recovery of Indium-lll-labelled lymphocytes in afferent and efferent lymph. Cellular Immunology, 54: 79-86.

ISSEKUTZ, T.B., CHIN, G.W. and HAY, J.B. (1981) Lymphocyte traffic through chronic inflammatory lesions: differential migration versus differential retention. Clinical and Experimental Immunology, 45: 604-614.

ISSEKUTZ, T.B., CHIN, G.W. and HAY, J.B. (1982) the characterisation of lymphocytes migrating through chronically inflamed tissues. Immunology, 46: 59-66.

JACOTOT, H. (1926) L'ecthyma contagieux des levres en Annam (chancre des ovins) Annales de l'Institut Pasteur, 40: 49-61.

JENNER, E. (1798) An enquiry into the causes and effects of the variolae vaccinae, p. 13. Samson Low, London.

JENSEN, R. and SWIFT, B.L. (1982) Diseases of Sheep. 2nd Edition. Lea and Febiger, Philadelphia.

JOHANNESSEN, J.V.; KROGH, H.K.; SOLBERG, I.; DALEN, A.; VAN WIJNGAARDEN H. and JOHANSEN, B. (1975). Human orf. Journal of Cutaneous Pathology, 2: 265-283.

JOKLIK, W.K. (1980) Principles of Animal Virology. Appleton-Century-Crofts. New York.

JUBB, K.V. and KENNEDY, P.C. (1970) Pathology of Domestic Animals, Vol II. 2nd Edition, pp. 595-596. Academic Press, New York and London.

KATER, J.C. and HANSEN, N.F. (1962) Contagious ecthyma in wild thar in the South Island. New Zealand Veterinary Journal, 10: 116-117.

KATZ, S.I., TAMAKI, K. and SACHS, D.H. (1979) Epidermal Langerhans cells are derived from cells originating in bone marrow. Nature, 282: 324-326.

KERRY, J.B. and POWELL, D.G. (1971) The vaccination of young lambs against contagious pustular dermatitis (letter). Veterinary Record, 88: 671-672.

KLAUS, G.G.B. (1978) The generation of B-memory cells with preformed antigen-antibody complexes. Immunology, 34: 643.

KLUGE, J.P., CHEVILLE, N.F. and PEERY, T.M. (1972) Ultrastructural studies of contagious ecthyma in sheep. American Journal of Veterinary Research, 33: 1191-1200.

KOPTOPOULOS, G., REID, H.W. and POW, I. (1982) Cytotoxic antibodies in orf virus infections of sheep. Zentralblatt fur Veterinarmedizin, B, 29: 284-291.

KUMMENEJE, K. and KROGSRUD, J. (1978) Contagious ecthyma (orf) in the musk ox (Ovibos muschatos). Acta Veterinaria Scandinavica, 19: 461-462.

KUMMENEJE, K. and KROGSRUD, J. (1979) Contagious ecthyma (orf) in reindeer (Rangifer t. tarandus). Veterinary Record, 105: 60-61.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 227: 680-685.

LANCE, W., ADRIAN, W. and WIDHALM, B. (1981) An epizootic of contagious ecthyma in Rocky Mountain Bighorn sheep in Colorado. Journal of Wildlife Diseases, 17: 601-603.

LANCE, W.R., HIBLER, C. and DEMARTINI, J. (1983) Experimental contagious ecthyma in Mule deer, White tailed deer, Pronghorn and Wapiti. Journal of Wildlife Diseases, 19: 165-169.

LANFRANCHI, A. (1925) Di alcune richerche sperimentali su la stomatite pustulo-contagiosa degli ovini. Nuova Veterinaria, 3: 1-4. (Abstract, Bulletin de l'Institut Pasteur, 23: 341).

LASCELLES, A.K. and McDOWELL, G.H. (1970) Secretion of IgA in the sheep following local antigenic stimulation. Immunology, 19: 613-620.

LASCELLES, A.K. and MORRIS, B. (1961) Surgical techniques for the collection of lymph from unanaesthatised sheep. Quarterly Journal of Experimental Physiology, 46: 199-205.

LEINIKKI, P. and PASSILA, S. (1976) Solid phase antibody assay by means of enzyme conjugated to anti-immunoglobulin. Journal of Clinical Pathology, 29: 1116-1120.

LEINIKKI, P. and PASSILA, S. (1977) Quantitative semiautomated, enzyme linked immunosorbent assay for viral antibodies. Journal of Infectious diseases, 136: S294-S299.

LEINIKKI, P.O.; SHEKARCHI, I.; DORSETT, P. and SEVER, J.L. (1978) Enzyme linked immunosorbent assay determination of specific Rubella antibody levels in micrograms of immunglobulin G per milliliter of serum in clinical samples. Journal of Clinical Microbiology, 8: 419-423.

LeJAN, C.; L'HARIDON, R.; MADELAINE, M.F.; CORNU, C. and ASSO, J. (1978) Transfer of antibodies against the CPD virus through colostrum and milk. Annales de Recherche Veterinaire, 9: 343-346.

LIEW, F.Y. (1982) Regulation of delayed-type hypersensitivity to pathogens and alloantigens. Immunology Today, 3: 18-23.

LIVINGSTON, C.W. and HARDY, W.T. (1960) Longevity of contagious ecthyma virus. Journal of the Amercian Veterinary Medical Association, 137: 651.

LOENING, U.E. (1969) The determination of the molecular weight of ribonucleic acid by polyacrylamide-gel electrophoresis. Biochemical Journal, 113: 131-138.

McCLENAGHAN, M., HERRING, A.J. and AITKEN, I.A. (1984) Comparison of Chlamydia psittaci isolates by DNA restriction endonuclease analysis. Infection and Immunity, 45: 384-389.

McCONNELL, I., LACHMANN, P.J. and HOBART, M.J. (1974) Restoration of specific immunological virginity. Nature, 250: 113-116.

MACDONALD, A. (1951) Complement fixation in the diagnosis of contagious pustular dermatitis in man. Journal of Pathology and Bacteriology, 63: 758-761.

MACDONALD, A. and BELL, T.M. (1961) Growth of contagious pustular dermatitis virus in human tissue cultures. Nature, London, 192:91-92.

MACFADYEAN, J. (1901) Annual Report from the principal of the Royal Veterinary College to the Royal Agricultural Society. Journal of Comparative Pathology and Therapeutics, 14: 95-96.

MACHER, E. and CHASE, M.W. (1969a) Studies on the sensitisation of animals with simple chemical compounds. XI. The fate of labelled picrylchloride and dinitrochlorobenzene after sensitising injections. Journal of Experimental Medicine, 129: 81-102.

MACHER, E. and CHASE, M.W. (1969b) Studies on the sensitisation of animals with simple chemical compounds XII. The influence of excision of allergenic depots on onset of delayed hypersensitivity and tolerance. Journal of Experimental Medicine, 129: 103-121.

McKEEVER, D. (1984) Persistent orf . Veterinary Record, 115: 334-335. (letter)

McKINNON, N.E. and DEFRIES, R.D. (1931) Interpretation of reactions following revaccination. Canadian Journal of Public Health, 22: 33-39.

MAEDA, A.D. (1979) Observations on the Immune Responses of Sheep to Orf Virus. PhD Thesis, Edinburgh.

MALVANO, R.; BONIOLO, A.; DOVIS, M. and ZANNINO, M. (1982) ELISA for antibody measurement; aspects related to data expression. Journal of Immunological Methods, 48: 51-60.

MANIATIS, T., FRITSCH, E.T. and SAMBROOK, J. (1982) Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory, 1982.

MARENNIKOVA, S.S., MALCEVA, N.N. and HABAHPASEVA, N.A. (1981) ELISA - a simple test for detecting and differentiating antibodies to closely related orthopoxviruses. Bulletin of the World Health Organisation, 59: 365-369.

MATTHEWS, R.E. (1982) Classification and nomenclature of viruses. Fourth Report of the International Committee on the Taxonomy of Viruses. Intervirology, 17: 1-200.

MÉLANIDI, C. and STYLIANOPOULO, M. (1928) L'ecthyma contagieux du mouton en Grèce. Revue Générale de Médecine Vétérinaire, 37 : 559-562.

MILLER, S.D. and BUTLER, L.D. (1983) T cell responses induced by the parenteral injection of antigen-modified syngeneic cells. I. Induction, characterisation and regulation of antigen-specific T helper cells involved in delayed-type hypersensitivity responses. Journal of Immunology, 131: 77-85.

MINDEN, P., ANTHONY, B.F. and FARR, R.S. (1969) A comparison of seven procedures to detect the primary binding of antigen by antibody. Journal of Immunology, 102: 832-841.

MITCHINER, M.B. (1969) The envelope of vaccinia and orf viruses: an electron-cytochemical investigation. Journal of General Virology, 5: 211-220.

MIYASAKA, M.; HERON, I.; DUDLER, L.; CAHILL, R.; FORNI, L.; KNAAK, T. and TRUKA, Z. (1983) Studies on the differentiation of T lymphocytes in sheep. I. Recognition of a sheep T lymphocyte differentiation antigen by a monoclonal antibody T-80. Immunology, 49: 545-553.

MORIN, M.L. and BAAS, E.J. (1981) Current Veterinary Therapy. Food Animal Practice. J.L. Howard editor, pp. 628-630. W.B.Saunders Company, Philadelphia.

MORRIS, B. (1972) The cells of lymph, and their role in immunological reactions. Handbuch Der Allgemeinen Pathologie, 3: 405-456.

MORRIS, B., MORENO, G. and BESSIS, M. (1968) Ultrastructure des cellules de la lymph afférente aux ganglions périphériques avant et après stimulation antigènique. Nouvelle Revue Francaise d'Hématologie, 8: 145-154.

MORRISSEY, J.H. (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Analytical Biochemistry, 117: 307-310.

MOUSSU, G. (1923) Ecthyma contagieux des lèvres chez le mouton ou <<maladie du chancre>>. Recueil de Médecine Vétérinaire, 99: 5-13.

MULLBACHER, R. and BLANDEN, R.V. (1979) The effect of virus immune serum on anti-viral cytotoxic T cells in vivo and in vitro. Journal of General Virology, 45: 73-80.

MÜLLER, H.K.; WITTEK, R.; SCHNAFFER, W.; SCHÜMPERLI, D.; ,MENNA, A. and WYLER, R. (1978) Comparison of five poxvirus genomes by analysis with restriction endonucleases HindIII, BamI and EcoRI. Journal of General Virology, 38: 135-147.

NAGAO,S.; INABA, S. and IIJIMA, S. (1976) Langerhans cells at the sites of vaccinia virus inoculation. Archives for Dermatological Research, 256: 23-31.

NAGINGTON, J. (1968) The growth of paravaccinia viruses in tissue culture. Veterinary Record, 82: 477-482.

NAGINGTON, J. and HORNE, R.W. (1962) Morphological studies of orf and vaccinia viruses. Virology, 16: 248-260.

NAGINGTON, J., NEWTON, A.A. and HORNE, R.W. (1964) The structure of orf virus. Virology, 23: 461-472

NAGINGTON, J., PLOWRIGHT, W. and HORNE, R.W. (1962) The morphology of bovine papular stomatitis virus. Virology, 17: 361-362.

NAGINGTON, J., TEE, G.H. and SMITH, J.S. (1965) Milker's nodule virus infections in Dorset and their similarity to orf. Nature (London), 208: 505-507.

NAGINGTON, J. and WHITTLE, C.H. (1961) Human orf. isolation of the virus by tissue culture. British Medical Journal, 2: 1324-1327.

NASH, A.A. and GELL, P.G.H. (1981) The delayed hypersensitivity T cell and its interaction with other T-cells. Immunology Today, 2: 162-165.

NETTLETON, P.F.; SHARP, J.M.; HERRING, A.J. and HERRING, J.A. (1984) IBR virus excretion after vaccination, challenge, and immunosuppression. In: Latent Herpes Virus Infections in Veterinary Medicine, G. Wittmann, R.M. Gaskell and H.J. Rziha editors, pp. 191-209. Martinus Nijhoff Publishers. Boston /The Hague /Dordrecht/Lancaster.

NEWBERNE, P.M. and WILLIAMS, G. (1970) Nutritional influences on the course of infections. In: Resistance to infectious disease. R.H. Dunlop and H.W. Moon editors, Saskatoon Modern press, Saskatchewan.

NEWSOM, I.E. and CROSS, F. (1934a) Soremouth in sheep transmissible to man. Journal of the American Veterinary Medical Association, 84: 799-802.

NEWSOM, I.E. and CROSS, F. (1934b) Soremouth in feeder lambs due to a filtrable virus. Journal of the American Veterinary Medical Association, 84: 233-247.

NGUYEN, T.C. (1984) The immune response in sheep: analysis of age, sex and genetic effects on the quantitative antibody response to chicken red blood cells. Veterinary Immunology and Immunophathology, 5: 237-245.

NISBET, D.I. (1954) An Investigation into the Pathology and Immunology of a Dermatitis of Sheep Common in the South-East of Scotland, and its Comparison with Similar Conditions Elsewhere. PhD Thesis, Edinburgh.

NÜRNBERG, H. (1942) Uber den austeckenden Maulgrind der Schafe und Ziegen. Doctoral thesis, Institute für Veterinär-Pathologie der Universität, Berlin, pp. 5-47. Cited in Johannessen, Krogh, Solberg, Dalen van Wijngaarden and Johansen (1975) Journal of Cutaneous Pathology, 2: 265-283.

OKADA, H.M.; OKADA, K.; NUMAKUNAI, S and OHSHIMA, K. (1984) Histopathologic studies on mucosal and cutaneous lesions in contagious papular dermatitis of Japanese serow (Capricornis crispus). Japanese Journal of Veterinary Science, 46: 257-264.

OSMAN, O.A.H. (1976) Experimental Orf in Susceptible and Previously Infected Sheep. PhD Thesis, Edinburgh.

PAPADOPOULOS, O.A.; DAWSON, P.S.; HUCK, R.A. and STUART, P. (1968) Agar gel diffusion studies of paravaccinia viruses. Journal of Comparative Pathology, 78: 219-225.

PEARSON, L., DEMARTINI, J. and FISCUS, S. (1979) Cellular responses in efferent lymph during parapox virus infections of sheep. Federation Proceedings, 38: 1158.

PEDERSON, N.C. and MORRIS, B. (1970) The role of the lymphatic system in the rejection of homografts; a study of lymph from renal transplants. Journal of Experimental Medicine, 131: 936-969.

PETERFY, F., KUNSELA, P. and MAKELA, O. (1983) Affinity requirements for antibody assays mapped by monoclonal antibodies. Journal of Immunology, 130: 1809-1813.

PETERS, D., MÜLLER, G. and BUTTNER, D. (1964) The fine structure of paravaccinia viruses. Virology, 23: 609-611.

PHANUPHAK, P., MOORHEAD, J.W. and CLAMAN, H.N. (1974) Tolerance and contact sensitivity to DNFB in mice. III. Transfer of tolerance with "suppressor cells". Journal of Immunology, 113: 1230-1236.

PINCUS, W.B. and FLICK, J.A. (1963a) Inhibition of the primary vaccinal lesion and of delayed hypersensitivity by an anti-mononuclear serum. Journal of Infectious Diseases, 113: 15-21.

PINCUS, W.B. and FLICK, J.A (1963b) The role of hypersensitivity in the pathogenesis of vaccinia virus infection in humans. Journal of Paediatrics, 62: 57-62

PLOWRIGHT, W., WITCOMB, M.A and FERRIS, R.D. (1959) Studies with a strain of contagious pustular dermatitis virus in tissue culture. Archiv für die Gesamte Virusforschung, 9: 214-231.

POSKITT, D.C.; FROST, H.; CAHILL, R.N.P. and TRNKA, Z. (1977) The appearance of non-specific antibody-forming cells in the efferent lymph draining antigen-stimulated single lymph nodes. Immunology, 33: 81-89.

POSPISCHIL, A. and BACHMANN, P.A. (1980) Nuclear changes in cells infected with parapoxviruses stomatitis papulosa and orf: an in vivo and in vitro ultrastructural study. Journal of General Virology, 47: 113-121.

POULAIN, J., GOURREAU, J.M. and DAUTIGNY, A. (1972) Ecthyma contagieux du mouton: anticorps sériques neutralisants. Annales de Recherche Vétérinaires, 3: 571-579.

PRECAUSTA, P. and STELLMANN, C. (1973) Isolation and comparative study in vitro of five strains of contagious ecthyma of sheep. Zentralblatt fur Veterinarmedizin B, 20: 340-355.

RAFII, F. and BURGER, D. (1985) comparison of contagious ecthyma virus genomes by restriction endonucleases. Archives of Virology, 84: 283-289.

REID, H.W. and BOYCE, J.B. (1979) The effect of colostrum-derived antibody on louping-ill virus infection in lambs. Journal of Hygiene, Cambridge, 77: 349-354.

REID, H.W.; BUXTON, D.; GARDINER, A.C.; POW, I.; FINLAYSON, J. and MACLEAN, M.J. (1982) Immuno-suppression in toxoplasmosis: studies in lambs and sheep infected with louping-ill virus. Journal of Comparative Pathology, 92: 181-189.

RENSHAW, H.W. and DODD, A.G. (1978) Serologic and cross-immunity studies with contagious ecthyma and goat-pox virus isolates from the western United States. Archives of Virology, 56: 201-210.

RIGBY, P.W.J.; DIECKMANN, M.; RHODES, C. and BERG, P. (1977) Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. Journal of Molecular Biology, 113: 237-251.

ROBERTSON, A. (1976) Handbook on Animal Diseases in the Tropics. 3rd edition. A. Robertson editor, pp. 9-11. British Veterinary Association, London.

ROBINSON, A.J. (1982) Scabby Mouth Vaccine. Proceedings of the Sheep and Beef Cattle Society of the New Zealand Veterinary Association's 12th Seminar, 1982.

ROBINSON, A.J. (1983) Prevalence of contagious pustular dermatitis (orf) in six million lambs at slaughter: a three year study. New Zealand Veterinary Journal, 31: 161-163.

ROBINSON, A.J. and BALASSU, T.C. (1981) Contagious pustular dermatitis (orf). Veterinary Bulletin, 51: 771-782.

ROBINSON, A.J., ELLIS, G. and BALASSU, T. (1982) The genome of orf virus: restriction endonuclease analysis of viral DNA isolated from lesions of orf in sheep. Archives of Virology, 71: 43-55.

ROITT, I.M., BROSTOFF, J. and MALE, D. (1985) Immunology, Gower Medical Publishing, London/New York.

ROJAS, A.; LEZICA, B.; CORTADA DE LA PERA, N.; OLIVARI, A.; FEIRSTEIN, J. and LEBENSTEIN, M. (1974) Levamisol: Accion en Fiebre Aftose. Revista de Medicina Veterinaria (Buenos Aries), 55: 263.

ROMERO-MERCADO, C.H. (1969) Virus Particles, Antigens and Antibodies in Sheep Infected with Orf, with Special Reference to Diagnosis. M.Sc. Thesis, Edinburgh University.

ROMERO-MERCADO, C.H.; MCPHERSON, E.A.; LAING, A.H.; LAWSON, J.B. and SCOTT, G.R. (1973) Virus particles and antigens in experimental orf scabs. Archiv fur die Gesamte Virusforschung, 40: 152-158.

ROTTGARDT, A.A., ARAMBURU, H.G. and PIRAZZI, A.J.G. (1949) Complement fixation test in contagious ecthyma (correspondence). Nature (London), 163: 219-220.

SAMMONS, D.W., ADAMS, L.D. and NISHIZAWA, E.E. (1981) Ultrasensitive silver based color staining of polypeptides in polyacrylamide gels. Electrophoresis, 2: 135-141.

SAMUEL, W.M.; CHALMERS, G.A.; STELFOX, J.G.; LOEWEN, A. and THOMSEN, J.J. (1975) Contagious ecthyma in bighorn sheep and mountain goat in western Canada. Journal of Wildlife Diseases, 11: 26-31.

SANDS, J.J.; SCOTT, A.C. and HARKNESS, J.W. (1984) Isolation in cell culture of a poxvirus from the red squirrel. Veterinary Record, 114: 117-118.

SAWHNEY, A.N. (1966) Studies on the virus of Ecthyma Contagiousum III. Multiplicity of virus strains. Bulletin de 1' Institute de Microbiologie, Sofia, 18: 179-183.

SAWHNEY, A.N. (1972) Studies on the virus of contagious pustular dermatitis: physico-chemical properties. Indian Veterinary Journal, 49: 14-19.

SCHMIDT, H. and HARDY, W.T. (1932) Soremouth (contagious ecthyma) in sheep and goats. Texas Agricultural Experiment Station, Bulletin No. 457.

SCHMIDT, H. and TUNNICLIFFE, E. (1929) Annual Report of the Texas Agricultural Experiment Station, pp. 12 and 140.

SCHÜMPERLI, D., LAGADEC, R. and MÜLLER, H.K. (1977) DNA sequence homology estimation by combinatorial analysis of endonuclease restriction data. Journal of General Virology, 38: 161-166.

SEDDON, H.R. and BELSCHNER, H.G. (1929) Infectious labial dermatitis or "scabby mouth" in sheep and goats. Veterinary Research Report, Department of Agriculture, New South Wales, 5: 36-44.

SEDDON, H.R. and McGRATH, T.T. (1931) Cross immunity tests with virus of infectious labial dermatitis of sheep. Veterinary Research Report, Department of Agriculture, New South Wales, 6: 109-110.

SELBIE, F.R. (1944) Experiments on the transmission to the rabbit of the virus of contagious pustular dermatitis of the sheep. Journal of Comparative Pathology, 54: 161-167.

SHAPIRO, A.L., VINUELA, E. and MAIZEL, J. (1967) Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochemical and Biophysical Research Communications, 28: 815-820.

SHARMA, S.N. and DHANDA, M.R. (1971) Studies on sheep and goat-pox viruses: relationship with contagious pustular dermatitis and vaccinia viruses. Indian Journal of Animal Sciences, 41: 864-867.

SINCLAIR, N.R. St.C. (1969) Regulation of the Immune Response I. Reduction in ability of specific antibody to inhibit long lasting IgG immunological priming after removal of the Fc fragment. Journal of Experimental Medicine, 129: 1183-1201.

SMITH, J.B., McINTOSH, G.H. and MORRIS, B. (1970a) The traffic of cells through tissues; a study of peripheral lymph in sheep. Journal of Anatomy, 107: 87-100.

SMITH, J.B., MCINTOSH, G.H. and MORRIS, B. (1970b) The migration of cells through chronically inflamed tissues. Journal of Pathology, 100: 21-29.

SMITH, J.B. and MORRIS, B. (1970) The response of the hepatic lymph node of the sheep to swine influenza virus. Australian Journal of Experimental Biology and Medical Science, 48: 47-55.

SMITH, T.C., HEINER, W.E. and FOREYT, W.J. (1982) Contagious ecthyma in an adult Dall sheep (Ovis dalli dalli) in Alaska. Journal of Wildlife Diseases, 18: 111-112.

SMITH, W.D.; JACKSON, F.; JACKSON, E.; DAWSON, A. McL. and BURRELLS, C. (1981) Changes in the flow and composition of gastric lymph in sheep repeatedly infected with Ostertagia circumcincta. Journal of Comparative Pathology, 91: 553-564.

SMITH, W.D.; WELLS, P.W.; BURRELLS, C. and DAWSON, A. McL. (1976) Maternal immunoglobulins and Parainfluenza 3 inhibitors in the serum and nasal secretions of newborn lambs. Clinical and Experimental Immunology, 23: 544-553.

SOUTHERN, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology, 98: 503-517.

STEIN, H., GERDES, J. and MASON, D. (1982) The normal and malignant germinal centre. Clinics in Haematology, 11: 531-559.

STERN, W. and DALES, S. (1976) Biogenesis of vaccinia: isolation and characterisation of a surface component that elicits antibody suppressing infectivity and cell-cell fusion. Virology, 75: 232-241.

STREILEIN, J.W. (1983) Skin-associated lymphoid tissues (SALT): origins and functions. Journal of Investigative Dermatology, 80: (Supplement) 12-16.

STREILEIN, J.W. and BERGSTRESSER, P.R. (1981) Langerhans cell function dictates induction of contact hypersensitivity or unresponsiveness to DNFB in Syrian hamsters. Journal of Investigative Dermatology, 77: 272-277.

SWANEY, J.B., VAN DE WOUDE, G.F. and BACHRACH, H.L. (1974) Sodium dodecylsulphate dependant anomalies in gel electrophoresis: alterations in the banding patterns of foot-and-mouth disease virus polypeptides. Analytical Biochemistry, 58: 337-346.

THEILER, A. (1928) Ecthyma contagiousum of sheep and goats. 13th and 14th Annual Reports of the Director of Veterinary Education and Research, Department of Agriculture, Union of South Africa. Part 1: 9-15.

THEOFILOPOULOS, A. (1980) Immune complexes in humoral immune responses: suppressive and enhancing effects. Immunology Today, 1: 1-3.

THOMAS, D.; FORNI, G.; SHEVACH, E. and GREENE, I. (1977) The role of the stimulator cell in contact sensitivity. Journal of Immunology, 118: 1677-1681.

THOMAS, V.; FLORES, L. and HOLOWCZAK, J.A. (1980) Biochemical and electron microscopic studies on the replication and composition of milker's node virus. Journal of Virology, 34: 244-255.

TOEWS, G.B., BERGSTRESSER, P.R. and STREILEIN, J.W. (1980) Langerhans cells: sentinels of skin associated lymphoid tissue. Journal of Investigative Dermatology, 75: 78-82.

TRNKA, Z. and CAHILL, R.N.P. (1980) Aspects of the immune response in single lymph nodes. Monographs in Allergy, 16: 245-259.

TRUEBLOOD, M.S. (1966) Relationships of ovine contagious ecthyma and ulcerative dermatosis. Cornell Veterinarian, 56: 521-526.

TRUEBLOOD, M.S. and CHOW, T.L. (1963) Characterisation of the agents of ulcerative dermatosis and contagious ecthyma. American Journal of Veterinary Research, 24: 47-51.

TRUEBLOOD, M.S., CHOW, T.L. and GRINER, L.A. (1963) An immunologic study of ulcerative dermatosis and contagious ecthyma. American Journal of Veterinary Research, 24: 42-46.

TUNNICLIFFE, E.A. (1949) Ulcerative dermatosis of sheep. American Journal of Veterinary Research, 10: 240-249.

TUNNICLIFFE, E.A. and MATISHECK, P.H. (1941) A filterable virus demonstrated to be the infective agent in ovine balanoposthitis. Science, 94: 283-284.

TURNER, A. and BAXBY, D. (1979) Structural polypeptides of orthopox virus: their distribution in various members and location within the virion. Journal of General Virology, 45: 537-545.

VOLLER, A. and BIDWELL, D.E. (1975) A simple method for detecting antibodies to rubella. British Journal of Experimental Pathology, 56: 338-339.

WALLEY, T. (1888) Malignant Aphtha in sheep. Journal of Comparative Pathology and Therapeutics, 1: 118-123.

WALLEY, T. (1890) Contagious dermatitis: "orf" in sheep. Journal of Comparative Pathology and Therapeutics, 3: 357-360.

WATSON, D.L. and LASCELLES, A.K. (1973) Mechanisms of transfer of immunoglobulins into mammary secretion of ewes. Australian Journal of Experimental Biology and Medical Science, 51: 247-254.

WATT, J.A. (1971) The Shepherd's Guide, pp. 50-52. Her Majesty's Stationery Office, Edinburgh.

WATT, J.A. (1982) Contagious pustular dermatitis. In Diseases of Sheep. W.B. Martin editor, pp. 185-188. Blackwell Scientific Publications, Oxford/London/Edinburgh/Boston/Melbourne.

WEBSTER, A.J.F. (1970) Environmental and physiological interactions influencing resistance to infectious diseases. In: Resistance to infectious diseases. R.H. Dunlop and W.H. Moon Editors, Saskatoon Modern Press, Saskatchewan.

WEBSTER, R.G. (1958) The immunological relations of contagious pustular dermatitis to the mammalian pox group. Australian Journal of Experimental Biology and Medical Science, 36: 267-274.

WEIR, J.P. and MOSS, B.M. (1985) Use of a bacterial expression vector to identify the gene encoding a major core protein of vaccinia virus. Journal of Virology, 56: 534-540.

WERNER, G. (1979) Immunopotentiating substances with antiviral activity. Pharmacology and Therapeutics, 6: 235-273.

WHEELER, C.E. and CAWLEY, E.P. (1956) the microscopic appearance of ecthyma contagiosum (orf) in sheep, rabbits and man. American Journal of Pathology, 32: 535-545.

WHITELAW, D.D.; SCOTT, J.M.; REID, H.W.; HOLMES, P.H.; JENNINGS, F.W. and URQUHART, G.M. (1979) Immuno-suppression in bovine trypanasomiasis: studies with louping-ill vaccine. Research in Veterinary Science, 26: 102-107.

WILKINSON, G.T. and PRYDIE, J. (1970) Possible orf (contagious pustular dermatitis, contagious ecthyma of sheep) infection of the dog. Veterinary Record, 87: 766-777.

WILSON, M.B. and NAKANE, D.K. (1978) Immunofluorescence and related straining techniques, W. Knapp, K. Holubar and G. Wick, editors. Elsevier/North Holland Biomedical Press.

WILSON, T.M. and POGLAYEN-NEUWALL (1971) Pox in South American sealions (Otaria byronia). Canadian Journal of Comparative Medicine, 35: 174-177.

WITTEK, R.; HERLYN, M.; SCHUMPERLI, D.; BACHMANN, P.; MAYR, A. and WYLER, R. (1980) Genetic and antigenic heterogeneity of different parapoxvirus strains. Intervirology, 13: 33-41.

WITTEK, R., KUENZLE, C.C. and WYLER, R. (1979) High C+G content in parapoxvirus DNA. Journal of General Virology, 43: 231-234.

WOLFF, K. and STINGL, G. (1983) The Langerhans cell. Journal of Investigative Dermatology, 80: (Supplement) 17-21.

YOUATT, W.(1837) Sheep: Their Breeds, Management and Diseases (To which is added the mountain shepherd's manual), p.414. Baldwin and Craddock, London.

ZARNKE, R.L.; DIETERICH, R.A.; NEILAND, K.A. and RANGLACK, G. (1983) Serologic and experimental investigations of contagious ecthyma in Alaska. Journal of Wildlife Diseases, 19: 170-174.

ZEIGER, R.S.; SALOMON, R.; DINGMAN, C.W. and PEACOCK, A.C. (1972) Role of base composition in the electrophoresis of microbial and crab DNA in polyacrylamide gels. Nature New Biology, 238: 65-69.

ZELLER, H. (1920) Uber Pocken bei Ziegen Sudwest Africa. Arbeiten aus dem Reichsgesundheitsamte, 25: 501. Cited in Theiler (1928) 13th and 14th Annual Reports of the Director of Veterinary Education and Research, Department of Agriculture, Union of South Africa. Part 1, 9-15.

ZINSSER, H. (1921) Studies on the tuberculin reaction and on specific hypersensitivites in bacterial infections. Journal of Experimental Medicine, 34: 495-524.

APPENDIX ONE

LYMPHATIC CANNULATION DATA

Table A1. Afferent lymph responses in four sheep after infection of the drainage area with orf virus.

Days after Infection	Flow Rate	Hourly Cell Output(x106)	Differential Leucocyte Count %SL	%LL	%N	%M	%E
		Sheep No. 131					
0	ND	ND	98.6	0.2	0	1.2	0
1	6.3	13.9	84.5	0.7	5.6	9.2	0
2	8.2	11.1	85.6	0.8	5.6	8.0	0
3	8.9	8.4	89.8	0.2	1.3	8.7	0
4	ND	ND	92.3	0.4	0.6	6.7	0
5	ND	ND	86.6	3.4	2.4	7.6	0
		Sheep No. 138					
0	ND	ND	86.5	0.3	4.5	7.9	0.3
1	9.8	10.1	89.6	1.0	5.6	3.4	0.4
2	7.9	9.5	91.1	0.6	1.4	6.8	0
3	6.9	7.6	95.7	0.7	0.9	2.7	0
		Sheep No. 129					
5	6.1	5.7	72.0	2.2	20.4	4.5	0
6	2.5	5.1	91.1	3.0	0.8	5.1	0
7	ND	ND	91.0	4.2	0	4.8	0
8	2.5	5.6	91.0	2.8	0.3	5.6	0.2
9	ND	ND	89.0	0.6	0.2	9.2	0.4
		Sheep No. 139					
4	ND	ND	84.3	0.2	5.7	9.2	0.2
5	1.0	5.7	84.5	1.0	5.4	9.2	0
6	0.6	3.7	95.2	0.6	0.6	3.6	0
7	4.8	7.2	89.3	1.6	0.6	7.9	0
8	4.8	8.9	87.2	6.4	0.6	5.8	0

SL = small lymphocyte; LL = large lymphocyte;
N = neutrophil; M = macrophage; E = eosinophil
ND = not done.

Fig. A1. The output of cells in lymph from the supramammary lymph node of sheep no. 161 following infection of the drainage area with orf virus. The unhatched area represents the total cell output, the blocked area the output of blast cells.

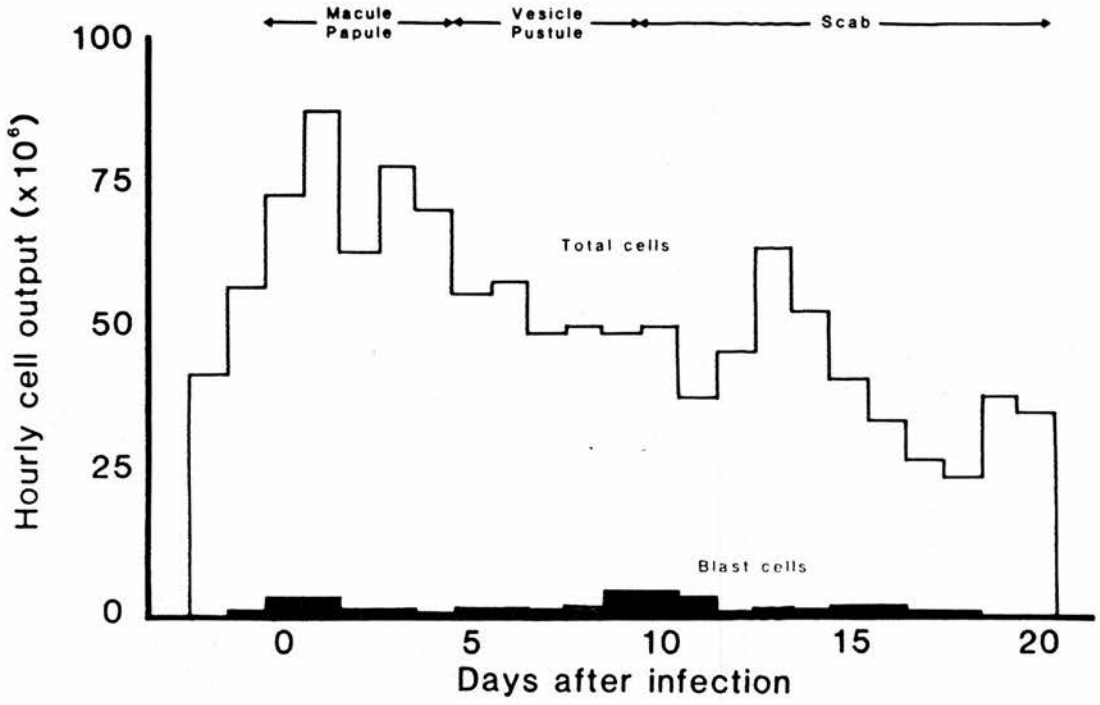

Macule
Papule
Vesicle
Pustule
Scab
100
75
50
25
0
Hourly cell output (x10⁶)
Total cells
Blast cells
0
5
10
15
20
Days after infection

Table A2. Data recorded from efferent supramammary lymph derived from sheep no. 161 during experimental infection of the drainage area with orf virus.

Sheep No.161

Days After Infection	Flow Rate (ml/hr)	Hourly Cell Output ($x10^6$) Total	Blast	IgG+	IgM+	Lymph Antibody Titre (Log_{10} Reciprocal)
-2	3.70	41.0	0.01	ND	ND	ND
-1	4.99	56.9	0.57	ND	ND	ND
0	9.28	72.9	3.20	ND	ND	2.66
1	12.52	88.4	2.83	ND	ND	ND
2	12.35	63.3	1.01	ND	ND	2.69
3	13.23	78.6	0.90	ND	ND	ND
4	10.59	70.2	0.56	ND	ND	2.63
5	8.26	55.6	1.45	ND	ND	ND
6	7.82	58.3	1.52	ND	ND	2.50
7	6.45	49.1	1.08	ND	ND	ND
8	8.62	50.3	1.60	0.40	0	2.45
9	6.49	49.5	3.90	0.79	0	ND
10	6.45	50.0	4.00	1.80	0	2.59
11	5.56	38.2	3.10	0.60	0	ND
12	6.36	45.9	0.93	0.55	0	2.98
13	6.14	64.6	1.68	0.78	0	ND
14	4.56	53.1	1.33	0.74	0	3.05
15	5.14	41.3	1.82	1.07	0	ND
16	4.60	34.1	1.98	1.50	0	3.08
17	4.76	27.4	0.60	0.49	0	ND
18	4.41	24.4	0.68	0.44	0	3.30
19	4.63	38.3	1.38	0.31	0	ND
20	3.03	35.1	1.54	0.35	0	3.19

ND = not done

Fig. A2. The output of cells in lymph from the supramammary lymph node of sheep no. 443 following infection of the drainage area with orf virus. The unhatched area represents the total cell output, the blocked area the output of blast cells.

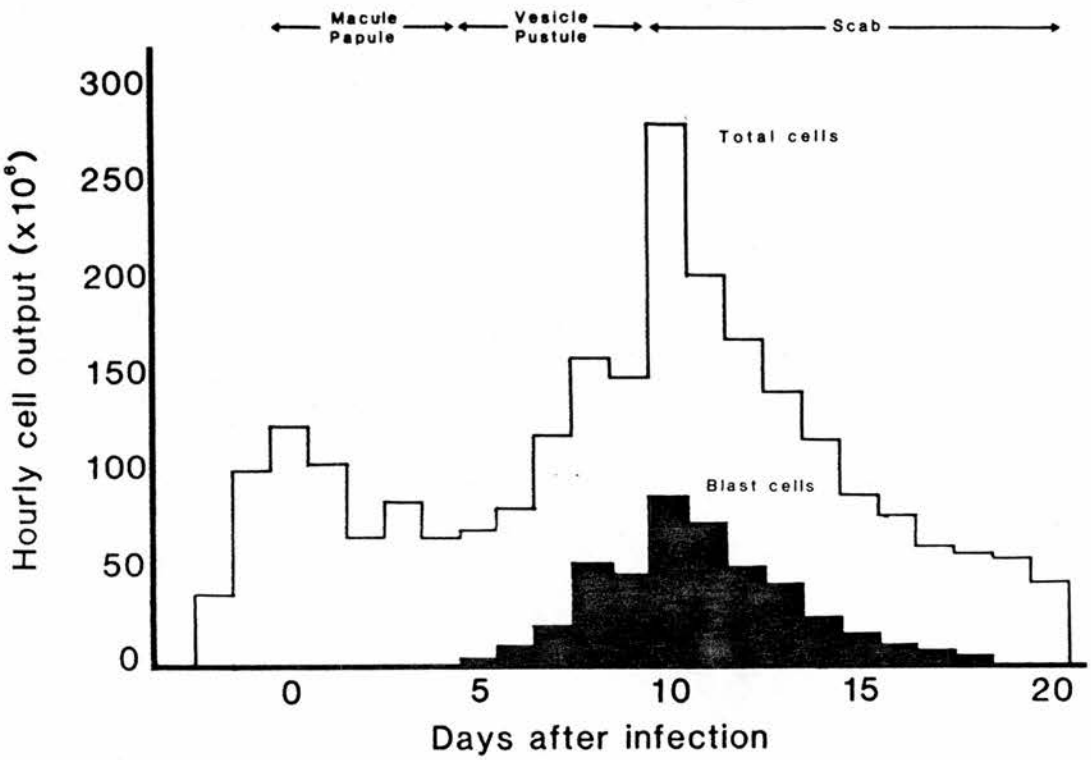

Macule
Papule
Vesicle
Pustule
Scab
Total cells
Blast cells
Hourly cell output (x10⁶)
300
250
200
150
100
50
0
0
5
10
15
20
Days after infection

Fig. A3. The output of immunoglobulin-containing cells in lymph from the supramammary lymph node of sheep no. 443 following infection of the drainage area with orf virus. The unhatched area represents the total blast cell output, the hatched area the output of IgG containing cells and the blocked area the output of IgM containing cells.

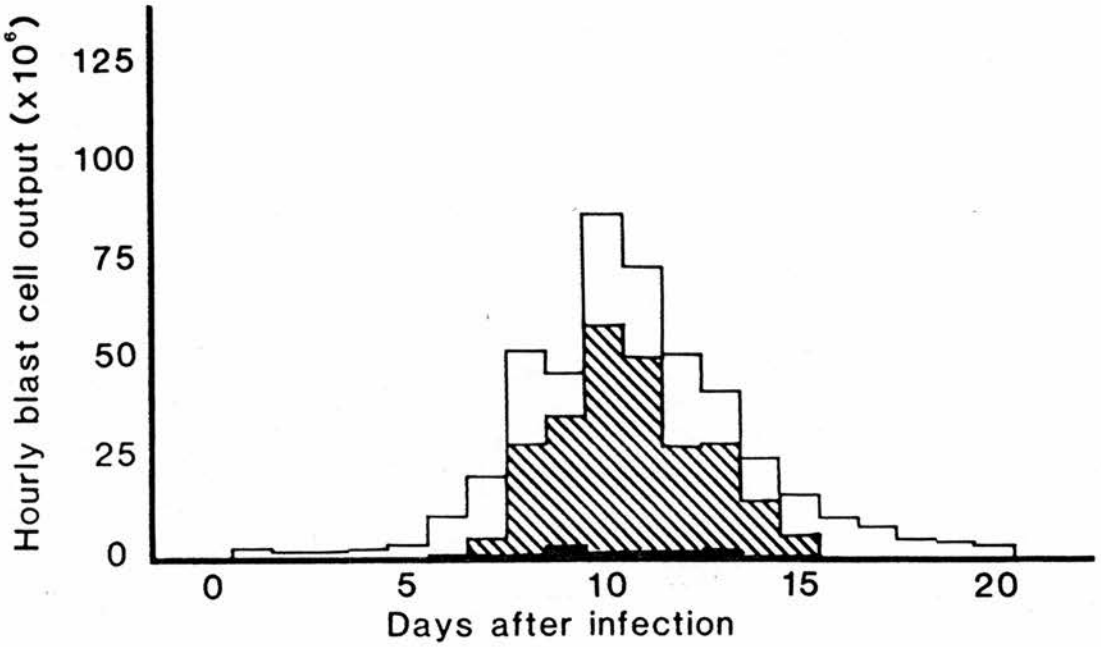

Hourly blast cell output (x10⁶)
125
100
75
50
25
0
0
5
10
15
20
Days after infection

Table A3. Data recorded from efferent supramammary lymph derived from sheep no. 443 during experimental infection of the drainage area with orf virus.

Sheep No. 443

Days After Infection	Flow Rate (ml/hr)	Hourly Cell Output ($x10^6$) Total	Blast	IgG+	IgM+	Lymph Antibody Titre (Log_{10} Reciprocal)
-2	10.67	35.7	ND	ND	ND	ND
-1	11.40	99.8	ND	ND	ND	2.57
0	9.54	122.1	ND	ND	ND	2.49
1	7.72	103.8	2.18	ND	ND	ND
2	7.18	66.2	1.79	ND	ND	2.64
3	7.90	84.0	1.43	ND	ND	ND
4	8.46	64.8	1.81	ND	ND	2.56
5	7.16	69.2	2.97	1.25	0.28	ND
6	5.54	80.0	9.68	0.96	0.32	2.48
7	7.57	118.3	20.00	4.73	0	ND
8	7.67	158.9	51.64	27.91	0.64	2.55
9	6.48	148.6	46.08	35.38	2.08	ND
10	7.13	280.4	86.92	58.32	1.12	2.68
11	6.82	202.0	72.71	50.69	1.21	ND
12	8.79	168.0	50.57	27.22	1.01	3.09
13	8.07	140.0	41.44	28.00	1.40	ND
14	8.29	116.6	24.48	13.75	0	2.91
15	6.98	87.8	15.37	5.30	0	ND
16	7.21	76.2	9.83	ND	ND	2.76
17	5.18	60.6	ND	ND	ND	ND
18	4.55	56.5	4.35	ND	ND	2.64
19	7.29	54.5	3.38	ND	ND	ND
20	6.79	41.0	2.70	ND	ND	ND

ND = not done

Fig. A4. The output of cells in lymph from the supramammary lymph node of sheep no. 764 following infection of the drainage area with orf virus. The unhatched area represents the total cell output, the blocked area the output of blast cells.

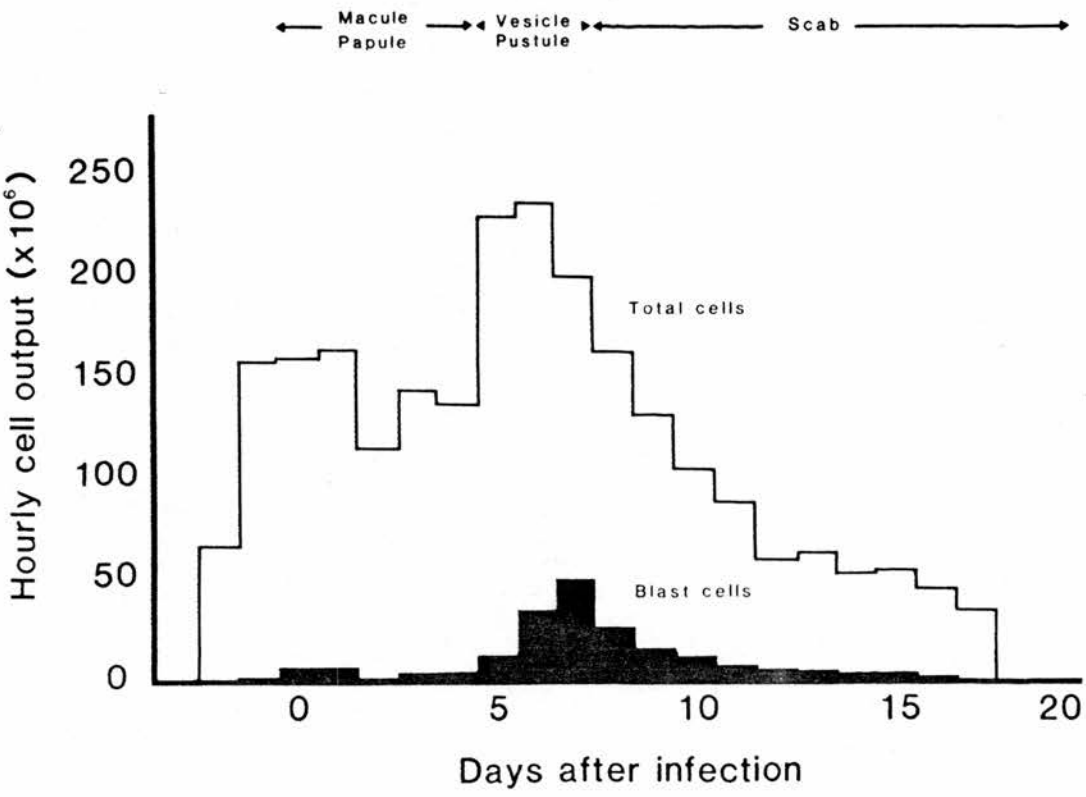

Macule
Papule
Vesicle
Pustule
Scab
Hourly cell output (x10⁶)
250
200
150
100
50
0
Total cells
Blast cells
0
5
10
15
20
Days after infection

Fig. A5. The output of immunoglobulin-containing cells in lymph from the supramammary lymph node of sheep no. 764 following infection of the drainage area with orf virus. Counts were not made until the eighth day of infection; the unhatched area represents the total blast cell output, the hatched area the output of IgG containing cells and the blocked area the output of IgM containing cells.

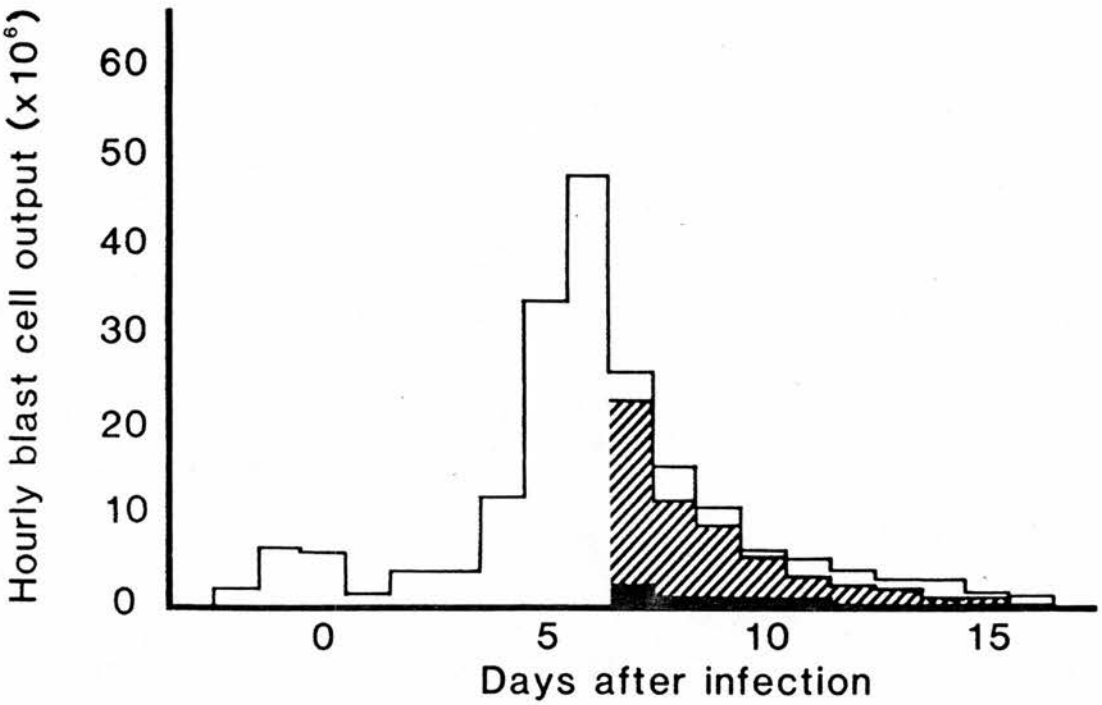

Hourly blast cell output (x10⁶)
60
50
40
30
20
10
0
0
5
10
15
Days after infection

Table A4. Data recorded from efferent supramammary lymph derived from sheep no. 764 during experimental infection of the drainage area with orf virus.

Sheep No. 764

Days After Infection	Flow Rate (ml/hr)	Hourly Cell Output ($x10^6$) Total	Blast	IgG+	IgM+	Lymph Antibody Titre (Log_{10} Reciprocal)
-2	5.22	66.2	0	ND	ND	ND
-1	6.94	157.9	1.74	ND	ND	ND
0	6.90	159.9	6.24	ND	ND	2.47
1	6.35	163.2	5.87	ND	ND	ND
2	4.49	114.3	1.14	ND	ND	2.62
3	8.48	143.4	3.73	ND	ND	ND
4	10.85	136.5	3.82	ND	ND	2.45
5	14.17	228.5	11.88	ND	ND	ND
6	14.25	235.0	33.84	ND	ND	2.49
7	10.97	198.3	47.59	ND	ND	ND
8	7.84	161.8	25.89	22.98	2.27	2.61
9	6.98	130.1	15.10	10.15	0.78	ND
10	5.34	104.7	10.88	8.79	0.84	2.60
11	4.41	81.0	6.32	5.18	0.80	ND
12	4.19	59.7	5.14	3.46	0.72	2.48
13	4.85	62.4	3.99	2.37	0.12	ND
14	5.97	52.6	2.84	1.79	0.32	2.45
15	5.43	53.5	2.78	1.07	0.32	ND
16	4.15	43.6	1.40	1.13	0.34	2.56
17	3.68	33.4	0.94	0.33	0.27	ND

ND = not done

Fig. A6. The output of cells in lymph from the supramammary lymph node of sheep no. 1269 following infection of the drainage area with orf virus. The unhatched area represents the total cell output, the blocked area the output of blast cells.

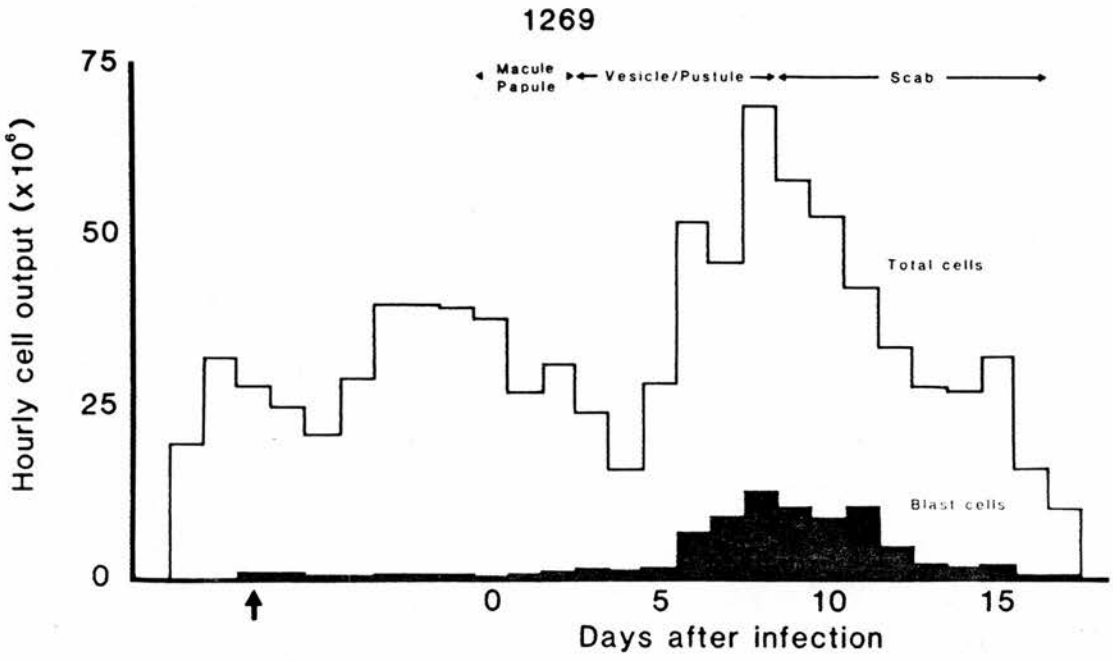

1269
Macule
Papule
Vesicle/Pustule
Scab
Total cells
Blast cells
Hourly cell output (x10⁶)
75
50
25
0
0
5
10
15
Days after infection

Fig. A7. The output of immunoglobulin-containing cells in lymph from the supramammary lymph node of sheep no. 1269 following infection of the drainage area with orf virus. The unhatched area represents the total blast cell output, the hatched area the output of IgG containing cells and the blocked area the output of IgM containing cells.

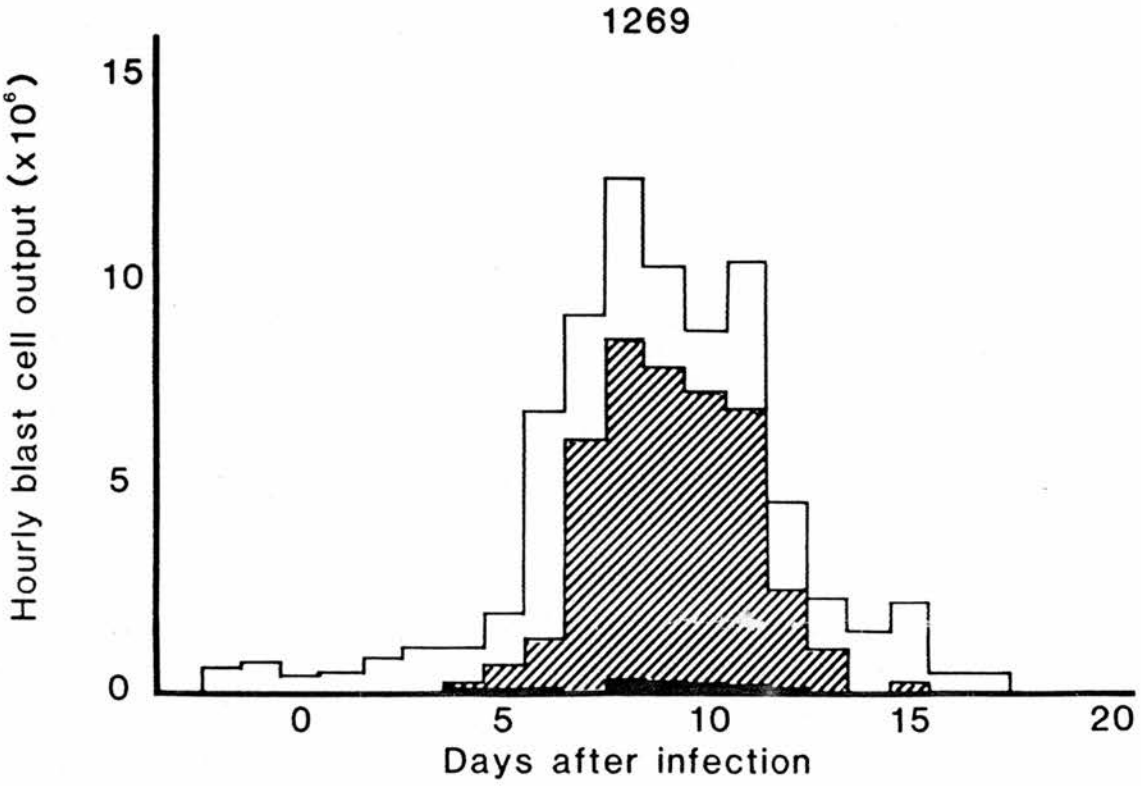

1269
Hourly blast cell output (x10⁶)
15
10
5
0
0
5
10
15
20
Days after infection

Table A5. Data recorded from efferent supramammary lymph derived from sheep no. 1269 during experimental infection of the drainage area with orf virus.

Sheep No.1269

Days After Infection	Flow Rate (ml/hr)	Hourly Cell Output ($x10^6$) Total	Blast	IgG+	IgM+	Lymph Antibody Titre (Log_{10} Reciprocal)
-2	1.35	19.4	ND	ND	ND	ND
-1	2.48	31.7	ND	ND	ND	ND
0	4.18	28.2	0.70	ND	ND	4.32
1	7.00	24.7	0.78	ND	ND	ND
2	8.19	20.7	0.33	ND	ND	4.08
3	10.42	29.0	0.62	ND	ND	ND
4	12.57	39.8	0.56	ND	ND	3.75
-2	8.75	39.7	0.56	ND	ND	ND
-1	8.13	39.5	0.70	ND	ND	4.01
0	6.77	37.9	0.38	ND	ND	3.52
1	4.59	27.1	0.49	ND	ND	ND
2	4.44	31.0	0.78	ND	ND	3.56
3	3.32	24.3	1.04	ND	ND	ND
4	2.81	15.9	1.03	0.19	0.10	3.87
5	4.40	28.4	1.85	0.63	0.06	ND
6	6.19	51.7	6.72	1.24	0.01	3.60
7	5.65	45.8	9.11	6.05	0	ND
8	6.09	68.9	12.45	8.54	0.28	3.63
9	6.09	57.8	10.29	7.86	0.23	ND
10	6.84	52.8	8.75	7.28	0.21	3.77
11	4.51	42.5	10.46	6.80	0.17	ND
12	4.84	33.7	4.52	2.43	0.07	4.24
13	5.16	28.2	2.22	1.01	0	ND
14	6.86	22.5	1.46	0	0	4.26
15	6.49	32.4	2.10	0.19	0	ND
16	1.72	15.9	0.49	0	0	4.07
17	2.18	9.6	0.46	ND	ND	ND

ND = not done

Fig. A8. The output of cells in lymph from the supramammary lymph node of sheep no. 1272 following infection of the drainage area with orf virus. The unhatched area represents the total cell output, the blocked area the output of blast cells.

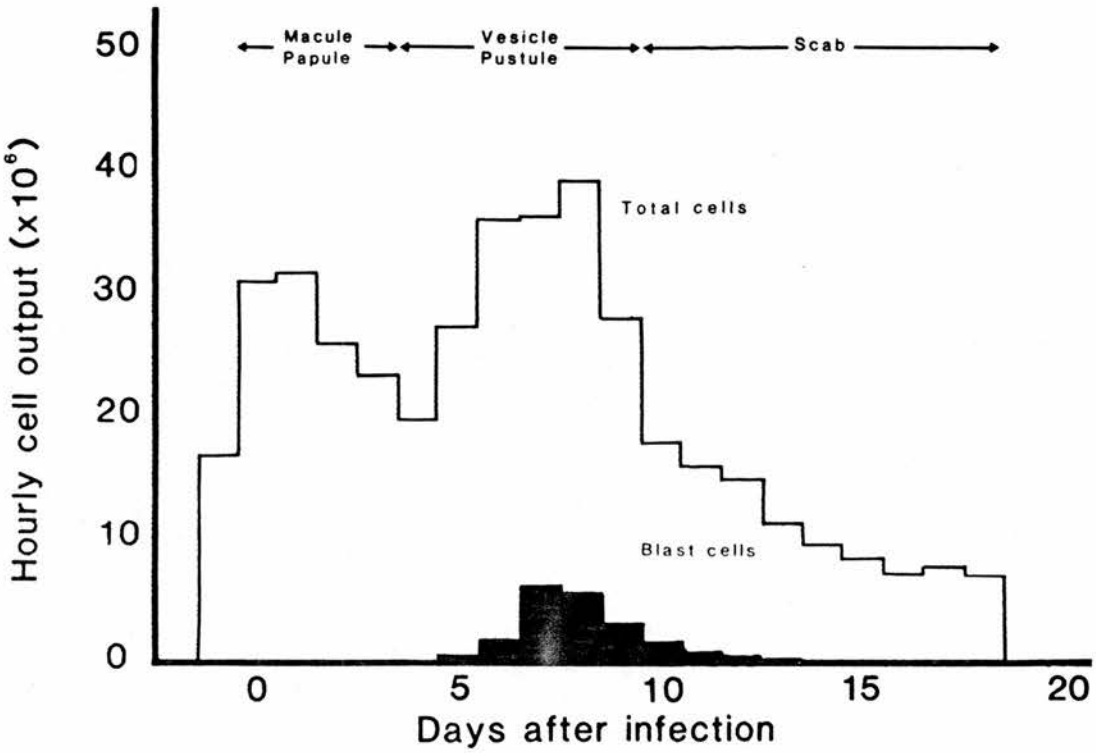

Macule
Papule
Vesicle
Pustule
Scab
Total cells
Blast cells
Hourly cell output (x10⁶)
50
40
30
20
10
0
0
5
10
15
20
Days after infection

Fig. A9. The output of immunoglobulin-containing cells in lymph from the supramammary lymph node of sheep no. 1272 following infection of the drainage area with orf virus. The unhatched area represents the total blast cell output, the hatched area the output of IgG containing cells and the blocked area the output of IgM containing cells.

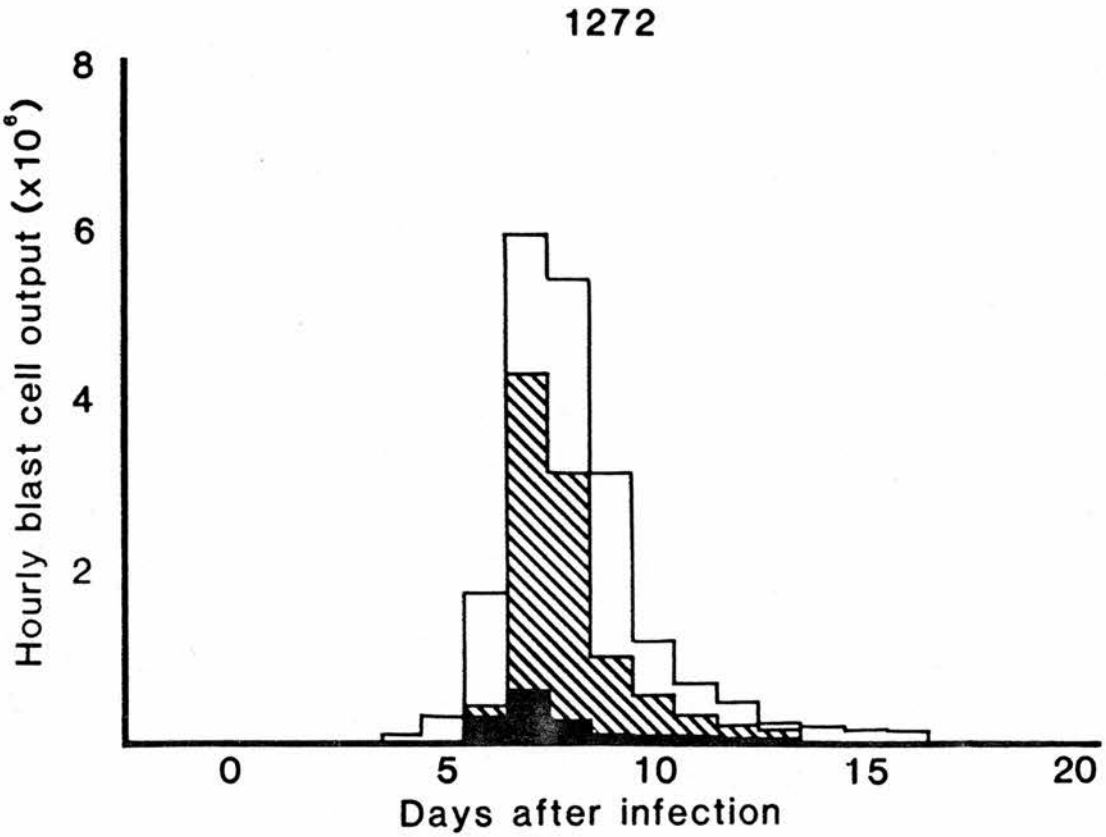

1272
Hourly blast cell output (x10⁶)
8
6
4
2
0
5
10
15
20
Days after infection

Table A6. Data recorded from efferent supramammary lymph derived from sheep no. 1272 during experimental infection of the drainage area with orf virus.

Sheep No. 1272

Days After Infection	Flow Rate (ml/hr)	Hourly Cell Output ($x10^6$) Total	Blast	IgG+	IgM+	Lymph Antibody Titre (Log_{10} Reciprocal)
-1	2.04	16.8	ND	ND	ND	2.45
0	3.75	30.9	ND	ND	ND	2.48
1	4.80	31.4	ND	ND	ND	ND
2	4.50	25.7	ND	ND	ND	2.51
3	3.97	23.3	ND	ND	ND	ND
4	5.13	19.7	0.10	ND	ND	ND
5	5.99	27.1	0.27	ND	ND	ND
6	7.51	36.0	1.69	0.65	0.29	2.45
7	6.50	36.2	5.90	4.27	0.58	ND
8	4.39	39.1	5.43	3.13	0.23	2.62
9	3.40	27.2	3.13	0.98	0.05	ND
10	2.84	17.7	1.24	0.53	0.04	2.48
11	2.75	15.7	0.67	0.30	0.06	ND
12	2.65	14.9	0.45	0.18	0.03	ND
13	2.27	10.9	0.22	0.14	0	ND
14	2.40	9.4	0.17	0	0	2.36
15	2.40	8.0	0.14	0	0	ND
16	1.87	7.0	0.13	0	0	ND
17	1.84	7.6	0.14	0	0	ND

ND = not done

Fig. A10. (a) The output of cells in lymph from the supramammary lymph node of sheep no. 1310 following infection of the drainage area with orf virus. The unhatched area represents the total cell output, the blocked area the output of blast cells. (b) The output of immunoglobulin-containing cells in the lymph during the same period. The unhatched area represents the total blast cell output, while the hatched area represents the output of IgG containing cells

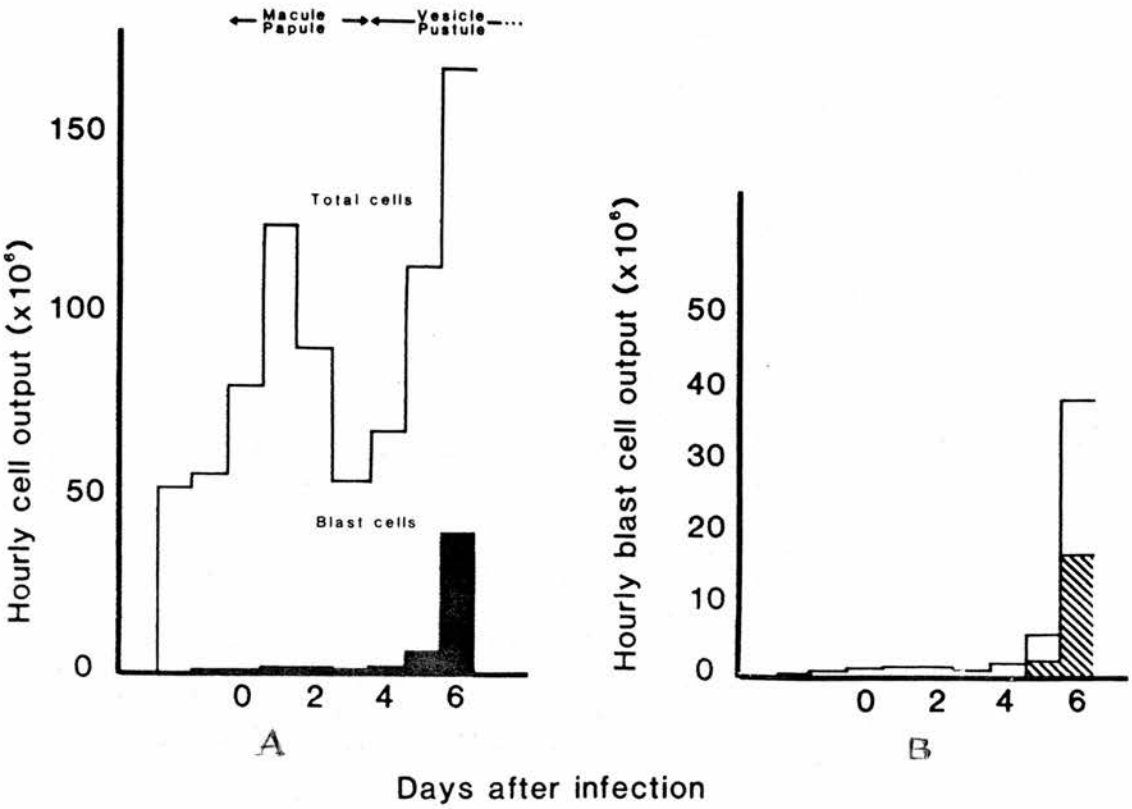

Macule
Papule
Vesicle
Pustule
Hourly cell output (x10⁶)
150
100
50
0
Total cells
Blast cells
0 2 4 6
A
Hourly blast cell output (x10⁶)
50
40
30
20
10
0
0 2 4 6
B
Days after infection

Table A7. Data recorded from efferent supramammary lymph derived from sheep no. 1310 during experimental infection of the drainage area with orf virus.

Sheep No. 1310

Days After Infection	Flow Rate (ml/hr)	Hourly Cell Output ($x10^6$) Total	Blast	IgG+	IgM+	Lymph Antibody Titre (Log_{10} Reciprocal)
-2	6.62	51.2	0.15	ND	ND	ND
-1	3.51	54.8	0.77	ND	ND	ND
0	5.69	79.8	0.95	ND	ND	ND
1	11.47	124.9	1.25	ND	ND	ND
2	11.53	90.4	1.18	ND	ND	ND
3	7.29	53.3	0.59	ND	ND	ND
4	7.86	66.9	1.87	ND	ND	ND
5	10.10	113.0	5.88	2.03	0	ND
6	15.37	168.4	38.74	16.84	0	ND

ND = not done

Fig. All. The output of cells in lymph from the supramammary lymph node of sheep no. 1313 following infection of the drainage area with orf virus. The unhatched area represents the total cell output, the blocked area the output of blast cells.

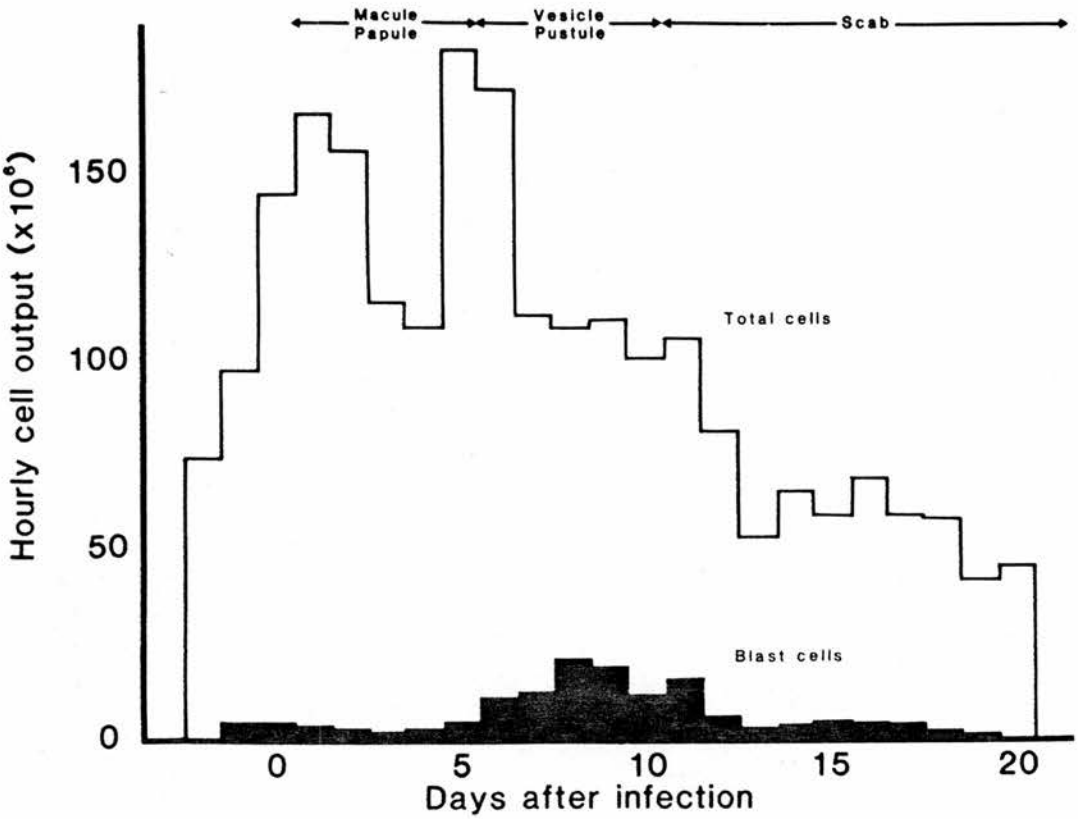

Macule
Papule
Vesicle
Pustule
Scab
Hourly cell output (x10⁶)
150
100
50
0
Total cells
Blast cells
0
5
10
15
20
Days after infection

Fig. A12. The output of immunoglobulin-containing cells in lymph from the supramammary lymph node of sheep no. 1313 following infection of the drainage area with orf virus. The unhatched area represents the total blast cell output, the hatched area the output of IgG containing cells and the blocked area the output of IgM containing cells.

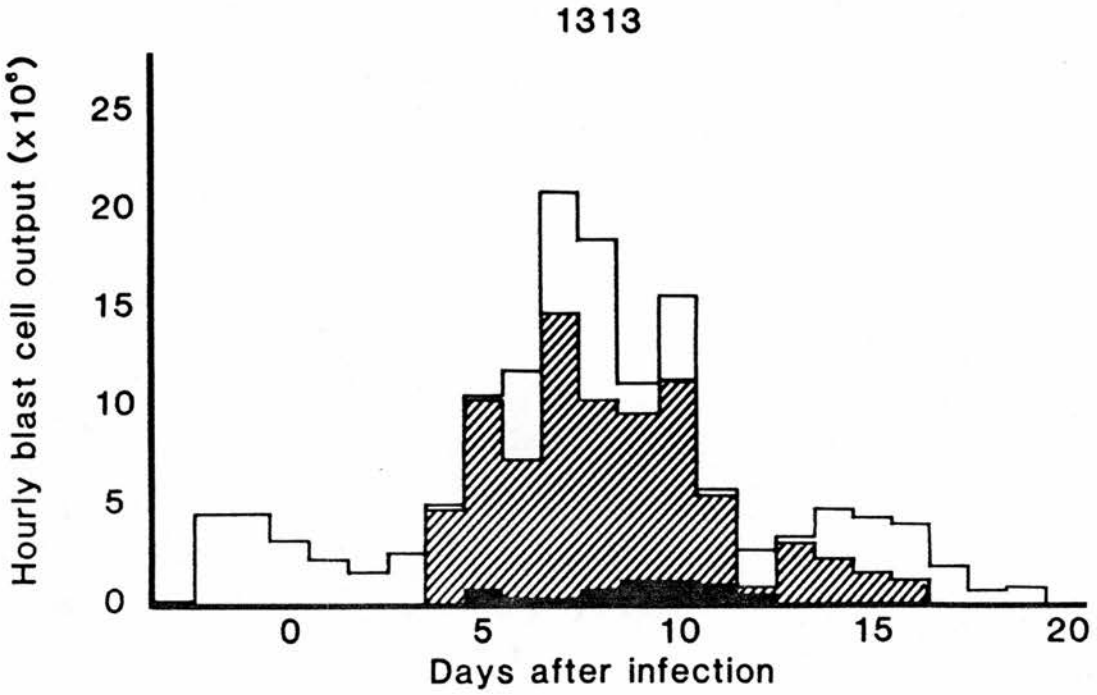

1313
Hourly blast cell output (x10⁶)
25
20
15
10
5
0
0
5
10
15
20
Days after infection

Table A8. Data recorded from efferent supramammary lymph derived from sheep no. 1313 during experimental infection of the drainage area with orf virus.

Sheep No.1313

Days After Infection	Flow Rate (ml/hr)	Hourly Cell Output ($x10^6$) Total	Blast	IgG+	IgM+	Lymph Antibody Titre (Log_{10} Reciprocal)
-2	7.64	74.0	0.15	ND	ND	ND
-1	4.83	98.0	4.51	ND	ND	3.04
0	6.01	145.0	4.64	ND	ND	2.98
1	8.95	166.2	3.16	ND	ND	ND
2	10.47	155.8	2.18	ND	ND	2.98
3	10.41	116.0	1.62	ND	ND	ND
4	12.77	109.6	2.52	ND	ND	2.94
5	15.04	183.6	4.96	4.77	0	ND
6	11.02	172.0	10.49	10.32	0.69	2.83
7	9.48	112.4	11.80	7.19	0.22	ND
8	7.50	110.0	20.78	14.74	0.22	3.28
9	6.37	111.2	18.46	10.34	0.67	ND
10	6.67	101.8	11.20	9.56	1.02	4.77
11	5.30	106.5	15.66	11.30	1.07	ND
12	6.23	80.8	5.66	5.50	0.97	5.14
13	5.05	53.4	2.66	0.96	0.42	ND
14	5.03	65.3	3.33	3.13	0	5.12
15	7.68	59.2	4.80	2.25	0	ND
16	5.75	69.2	4.30	1.50	0	4.76
17	5.30	59.9	4.07	1.20	0	ND
18	6.24	58.2	1.86	ND	ND	4.9

ND = not done

Fig. A13. The output of cells in lymph from the supramammary lymph nodes of two sheep (443, 1313) at days 2 and 11 after infection of the drainage area with orf virus. Total cells (□); T-80 staining cells (▨); blast cells (■). The high total cell output seen at day 2 in animal 1313 is a reflection of a transient rise in cell output which occurred in response to surgery and which was less marked in other animals.

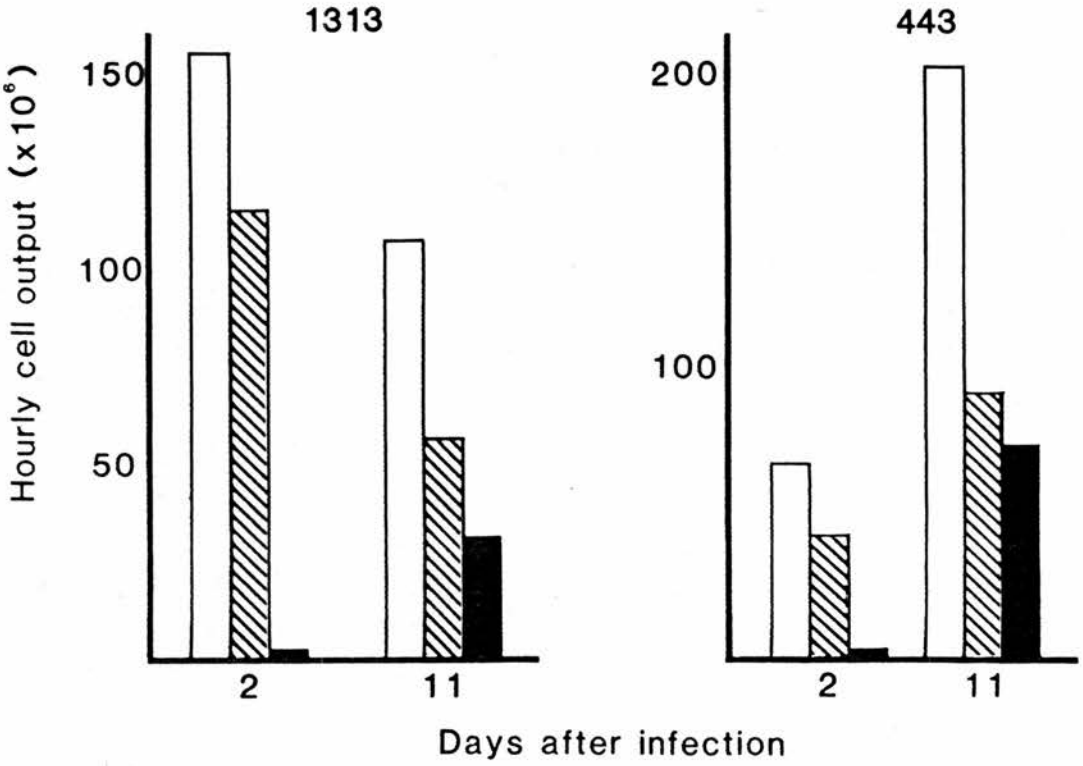
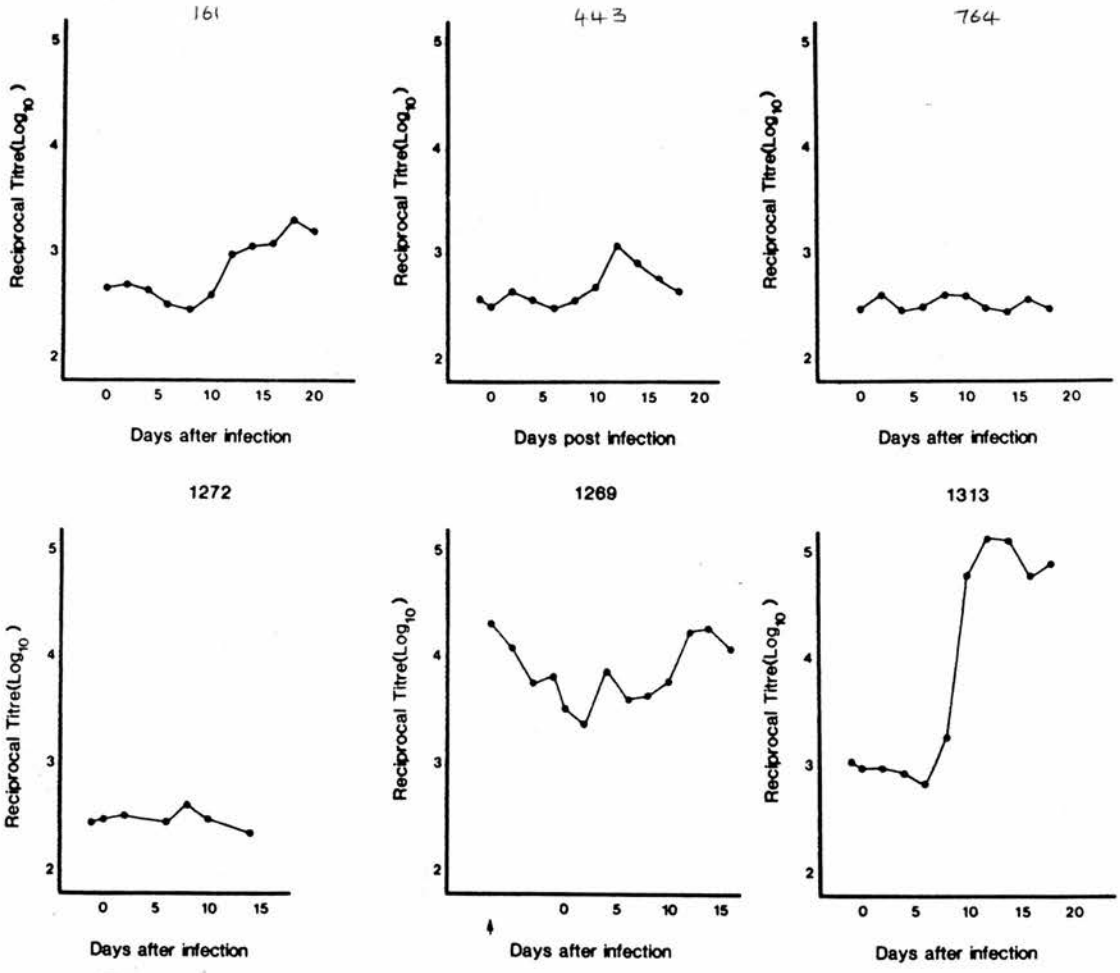

1313
443
Hourly cell output (x10⁶)
150
100
50
200
100
2
11
2
11
Days after infection

Fig. A14. Virus-specific antibody titres ($\log_{10}$ reciprocal) detected by ELISA in efferent supramammary lymph of six sheep after infection of the drainage area with orf virus.


161
443
764
1272
1269
1313
Reciprocal Titre(Log10)
Days after infection
Days post infection

APPENDIX TWO

REAGENTS AND SUPPLIERS

Acetic acid: May and Baker, Dagenham, Essex, UK.

Acetone: May and Baker, Dagenham, Essex, UK.

Acrylamide, bis-acrylamide: BDH Chemicals Ltd., Poole, Dorset, UK.

Bacitracin: Sigma Chemical Company, St. Louis, Missouri, USA.

Bio-Rad protein assay kit: Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK.

Bovine serum albumin: Miles Scientific, Slough, UK.

Carbazole (3-amino-9-ethylcarbazole): Sigma Chemical Company, St. Louis, Missouri, USA.

Caesium chloride: Bethseda Research Laboratories, Cambridge, UK.

Chloroform: Koch Light Ltd., Haverhill, Suffolk, UK.

Citric acid: Koch Light Ltd., Haverhill, Suffolk, UK.

Complete Freund's adjuvant: Difco Ltd., Rutherford, New Jersey, USA.

Coomassie Brilliant Blue: Sigma Chemical Company, St. Louis, Missouri, USA.

Diaminobenzidene: Sigma Chemical Company, St, Louis, Missouri, USA.

Diethanolamine: BDH Chemicals Ltd., Poole, Dorset, UK.

Dithiothreitol: Sigma Chemical Company, St. Louis, Missouri, USA.

DNFB (2,4-dinitro-1-fluorobenzene):Sigma Chemical Company, St. Louis, Missouri, USA.

EcoRI: Bethseda Research Laboratories, Cambridge, UK.

Ethanol: James Burrough plc., London, UK.

Ethidium bromide: Sigma Chemical Company, St. Louis, Missouri, USA.

Ethylenediaminetetracetate (EDTA); diand trisodium salts: Sigma Chemical Company, St. Louis, Missouri, USA.

Ficoll: Pharmacia, Milton Keynes, UK.

Formaldehyde: BDH Chemicals Ltd., Poole, Dorset, UK.

Formamide: BDH Chemicals Ltd., Poole, Dorset, UK. Bedfordshire, UK.

Glutaraldehyde: BDH Chemicals Ltd., Poole, Dorset, UK.

Guanidinium isothiocyanate: Bethseda Research Laboratories, Cambridge, UK.

"Halothane": May and Baker Ltd., Dagenham, Essex UK.

Heparin: Sigma Chemical Company, St. Louis, Missouri, USA.

"Hibitane": Imperial Chemical Industries, Cheshire, UK.

8-Hydroxyquinoline: BDH Chemicals Ltd., Poole, Dorset, UK.

"Immac": Anne French, London, UK.

Iscoves Medium: Gibco, Paisley, UK.

Isoton II: Coulter Electronics Ltd., Luton, Bedfordshire, UK.

Levamisole: Nilverm, Imperial Chemical Industries, Cheshire, UK.

m-Cresol: BDH Chemicals Ltd., Poole, Dorset, UK.

Medium 199: Gibco, Paisley, UK.

2-mercaptoethanol: Sigma Chemical Company, St. Louis, Missouri, USA.

Methanol: FSA Laboratory Supplies, Loughborough, Leicestershire, UK.

Miglyol 812: Dynamit Nobel, Witten, Ruhr, West Germany.

Mycostatin: E. R. Squibb and Sons, Wirral, Merseyside, UK.

Nonidet P40: Sigma Chemical Company, St. Louis, Missouri, USA.

Nycodenz: Nygaard and Company, Oslo, Norway.

Paraformaldehyde: Koch Light Ltd. Haverhill, Suffolk, UK.

Penicillin, sodium benzyl: Crystapen, Glaxo Laboratories, Greenford, Middlesex, UK.

Periodic Acid: BDH Chemicals Ltd., Poole, Dorset, UK.

Phage Lambda: Sigma Chemical Company, St. Louis, Missouri, USA.

Phage 0X174: Bethseda Research Laboratories, Cambridge, UK.

Phenol: BDH Chemicals Ltd., Poole, Dorset, UK.

Phosphotungstic acid: BDH Chemicals Ltd., Poole, Dorset, UK.

p-Nitrophenyl phosphate: Sigma Chemical Company, St. Louis, Missouri, USA.

Polyvinylpyrollidine: Sigma Chemical Company, St. Louis, Missouri, USA.

Proteinase K: Sigma Chemical Company, St. Louis, Missouri, USA.

Rabbit anti-mouse IgM (peroxidase conjugated): Miles Scientific, Slough, UK.

Rabbit IgG anti-sheep whole immunoglobulin: Dakopatts, Copenhagen, Denmark.

Ribonuclease A: Sigma Chemical Company, St. Louis, Missouri, USA.

Salmon sperm: Sigma Chemical Company, St. Louis, Missouri, USA.

Sepharose 4B: Pharmacia, Milton Keynes, UK.

Silver nitrate: BDH Chemicals Ltd., Poole, Dorset, UK.

Size marker proteins: BDH Chemicals Ltd., Poole, Dorset, UK. (betagalactosidase and phosphorylase A: Sigma Chemical Company, St. Louis, Missouri, USA.)

Sodium azide: BDH Chemicals Ltd., Poole, Dorset, UK.

Sodium borohydride: Sigma Chemical Company, St. Louis, Missouri, USA.

Sodium carbonate: BDH Chemicals Ltd., Poole, Dorset, UK.

Sodium chloride: Koch Light Ltd., Haverhill, Suffolk, UK.

Sodium dihydrogen orthophosphate: BDH Chemicals Ltd., Poole, Dorset, UK.

Sodium dodecyl sulphate (SDS): BDH Chemicals Ltd., Poole, Dorset, UK.

Sucrose: Koch Light Ltd., Haverhill, Suffolk, UK.

Streptomycin sulphate: Evans Medical Ltd., Greenford, Middlesex, UK.

Tris 7-9: Sigma Chemical Company, St. Louis, Missouri, USA.

Tween 20: Sigma Chemical Company, St. Louis, Missouri, USA.

Tween 80: Sigma Chemical Company, St. Louis, Missouri, USA.

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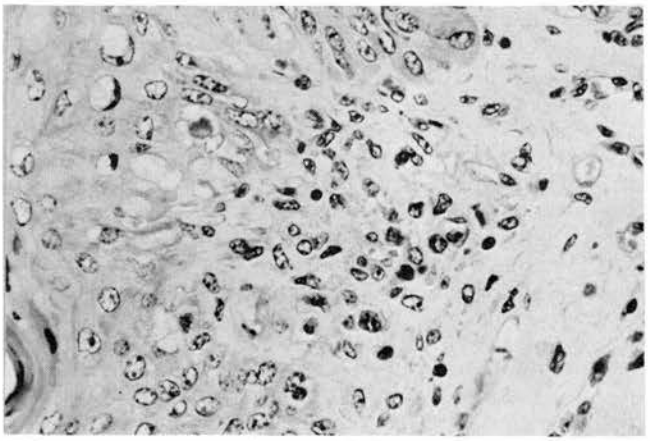

FIG 1: Vacuolisation of the epithelium and mononuclear cell infiltration in the reticulum. Haematoxylin and eosin × 400

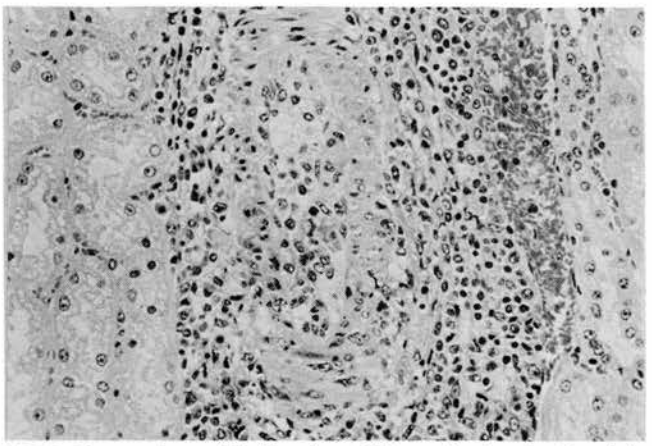

FIG 2: Mononuclear cell infiltration with karyorhexis and pyknosis in an artery of the kidney. Haematoxylin and eosin × 200

Microscopic examinations revealed systemic mononuclear cell infiltrations in epithelial tissues and blood vessels with degeneration or necrosis. Epithelial tissues of the urinary bladder, the upper alimentary and respiratory tracts were affected (Fig 1) and epithelium was occasionally eroded to induce haemorrhages.

Vascular lesions were observed in the kidney, spleen, brain, aorta, lymph nodes and near the epithelial degenerations. The vascular walls, especially in the adventitia, were infiltrated with mononuclear cells. Karyorhexis and pyknosis were scattered in the walls, while fibrinoid deposition was minimal (Fig 2).

In the other viscera, mononuclear cell infiltrations were perivascular. The lymph nodes showed mild lymphoid hyperplasia with occasional focal necrosis.

Most of the large mononuclear cells were immature and their cytoplasm stained basophilic with May-Giemsa's and dark pink with pyronin. Remaining mononuclear cells resembled macrophages or lymphocytes. After trypsinisation of paraffin sections (Huang and others 1976), only a few of the mononuclear cells in the inflammatory foci stained by fluorescein isothiocyanate-labelled antiserum to bovine IgG (Miles, USA), while lymphocytes in the lymph nodes and spleen stained positively. No inclusion bodies or syncytial giant cells were detected in any tissues.

The clinical manifestations, histopathological lesions and contact of the affected cattle with lambing sheep suggested that the present disease in Japan was sheep-associated malignant catarrhal fever and that it showed an enzootic character.

It has been proposed that the nature of malignant catarrhal fever is a cell-mediated immune response to virally infected tissues. Evidence in support of this view includes the nature of the cell response; the fact that the mononuclear cells show the morphological character of lymphoid cells; the absence of an acute host response to viral antigen such as inclusion body formation in the epithelium, and lesions which resemble those in graft-versus-host reactions (Liggitt and DeMartini 1980a,b).

Another proposal is that wildebeest-associated malignant catarrhal fever is a lymphoproliferative disease because of its similarities to herpesvirus induced lymphomas such as herpes saimiri or Epstein-Barr virus infection (Hunt and Billups 1979). However, no confirmation of these proposals is forthcoming as yet.

Recently, a cell line derived from sheep-associated malignant catarrhal fever affected animals demonstrated large granular lymphocytes or T-lymphocytes, which carry the agent of sheep-associated malignant catarrhal fever into rabbits and ruminants (Reid and others 1984). On the other hand, the mononuclear cells of malignant catarrhal fever has been regarded as reticulo-endothelial type cells in Malaysia and Japan (Oshima and others 1977, Vanselow 1980). Since the mononuclear cells composing the cellular exudate in the present cases were not shown to be IgG-bearing cells, but their cytoplasm stained for RNA, it is suggested that they are of the lymphoid series.

References

CASTRO, A. E., DALEY, G. G., ZIMMER, M. A., WHITENACK, D. L. & JENSEN, J. (1982) *American Journal of Veterinary Research* **43**, 5

FUJIMOTO, Y., SATOH, H., USHIJIMA, J. & YAMASHITA, S. (1958) *Japanese Journal of Veterinary Research* **6**, 93

HUANG, S. N., MINASSIAN, H. & MORE, J. D. (1976) *Laboratory Investigation* **35**, 383

HUNT, R. D. & BILLUPS, L. H. (1979) *Comparative Immunology, Microbiology and Infectious Diseases* **2**, 275

LIGGITT, H. D. & DeMARTINI, J. C. (1980a) *Veterinary Pathology* **17**, 58

LIGGITT, H. D. & DeMARTINI, J. C. (1980b) *Veterinary Pathology* **17**, 73

MUSHI, E. Z. & RURANGIRWA, F. R. (1981) *Veterinary Research Communications* **5**, 127

OSHIMA, K., MIURA, S., NUMAKUNAI, S. & SATO, T. (1977) *Japanese Journal of Veterinary Science* **39**, 293

REID, H. W., BUXTON, D., BERRIE, E., POW, I. & FINLAYSON, J. (1984) *Veterinary Record* **114**, 582

STORZ, J., OKUNA, N. McCHESNEY, A. E. & PIERSON, R. E. (1976) *American Journal of Veterinary Research* **37**, 875

VANSELOW, B. A. (1980) *Veterinary Record* **107**, 15

Survival of orf virus under British winter conditions

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THE current concept of the epidemiology of orf or contagious pustular dermatitis in sheep is that young susceptible lambs derive infection from virus in scab shed by the previous year's infected animals (Robinson and Balassu 1981).

Virus in dry scabs is extremely stable (Glover 1930, Livingston and Hardy 1960) although its survival in suspension is markedly reduced (Plowright and others 1959). In view of the rainfall which is characteristic of the British winter, it is unlikely that scab shed on pasture can remain dry. It was therefore decided to investigate the effect of British winter weather conditions on the infectivity of virus in scab.

Scabs collected from lambs with experimentally induced orf lesions were used as a source of virus for the study. In order to avoid the effects of variation in particle numbers which occurs between different areas of an orf lesion (Harkness and

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TABLE 1: Summary of test conditions used during the study period

Aliquot	Exposure conditions
A	−20°C
B	4 ± 0·5°C
C	18 ± 5°C
D	Outdoors/covered
E	Outdoors/no cover

TABLE 2: Meteorological data* for the period from mid November 1984 to May 1985

Maximum air temperature (°C)	16·2
Minimum air temperature (°C)	−7·5
Total rainfall (mm)	292 (287)
Days with snow lying	18 (16·2)
Total sunshine (hours)	496·9 (486·3)

* Meteorological data from the Royal Observatory, Blackford Mill, Edinburgh Average figures are shown in brackets

others 1977) the scabs were pooled, ground in sterile sand and stored at −20°C. Five 1 g aliquots of this preparation were wrapped in muslin gauze and maintained from November 1984 to May 1985 under the conditions summarised in Table 1. Aliquots D and E were fixed to fresh turf in drained plastic pots and one of these pots was sheltered from rain by a small canopy of clear plastic sheeting, the other receiving no shelter other than a wire mesh cover to prevent interference by birds.

Meteorological records collected during the period at a station 1·5 miles from the site of the experiment, were supplied by the meteorological office climatological services and are summarised in Table 2 along with average figures for the same period.

After the period of exposure, 20 per cent suspensions of each aliquot were made in phosphate buffered saline containing penicillin and streptomycin and cleared by centrifugation at 1200 *g* for 30 minutes at 4°C. Six sites (2 cm × 2 cm) on the backs of each of three lambs were lightly abraded using sharp plastic after removal of the wool using a proprietary depilating agent (Immac; Anne French). Each of the viral suspensions was inoculated on to one site of each lamb, while the remaining site received an application of phosphate buffered saline alone. Only a single dilution of inoculum was used since there is no practical method of titrating the infectivity of orf virus in scabs. Six days after inoculation, punch biopsies were taken from all sites for histological examination.

Histological lesions typical of orf (Aynaud 1923, Glover 1928) were produced by each of the suspensions with the exception of that prepared from aliquot E (Table 1). Lesions were not detected at any of the control sites.

Electron microscopy of the suspensions made from aliquots A to D were seen to contain a predominance of particles showing the characteristic basket weave appearance described by Nagington and Horne (1962). In material prepared from aliquot E, most of the particles were rather amorphous, recognisable only by shape and size.

The results of this study indicate that although orf virus is capable of resisting the temperature fluctuations of a British winter, total exposure to the elements results in loss of infectivity. Manley (1934) concluded from his work on the viability of orf virus under natural conditions that direct sunlight is the principal inactivation factor and Sawhney (1972) observed that the infectivity of virus in maintenance medium is indeed susceptible to ultraviolet radiation. It is probable, however, that damage by ultraviolet radiation has little significance under natural circumstances, since it has poor penetrating power and is unlikely to reach virus within the substance of crusts lying on pasture.

It would appear that the major factor involved in the inactivation of virus in aliquot E was exposure to rain. This effect was more likely to be the result of hydration of the scab than elution of viral particles into the soil, since virions, although degenerate, were present in the sample after exposure. Rainfall is a prominent feature of the British climate and it is therefore unlikely, in view of the results of this study, that scab shed in exposed areas can retain infectivity for long periods.

This raises questions regarding the significance of virus overwintered on pasture as a source of new infections in the spring. Alternative sources are virus which has survived in the shelter of buildings and that which has persisted on animals, in the form of clinical or subclinical infection.

Only a proportion of outbreaks are, however, associated with housing and two recent reports indicate that chronic infection may occur. Greig and others (1984) described a prolonged course of disease in a shearling blue faced Leicester ram, in which papillomatous lesions persisted for eight months and electron microscopy revealed large numbers of orf particles in autopsy samples.

McKeever (1984) reported two similar cases in Friesland rams which had papillomatous lesions containing copious quantities of orf virus particles for over a year. Serological studies on the latter animals using an enzyme-linked immunosorbent assay (ELISA) detected high titres of antibody and since then it has been demonstrated using the western blotting technique that they both showed comprehensive recognition of viral antigens (D. J. McKeever, unpublished data).

Whatever the reasons behind these persistent orf lesions, it is apparent that orf virus can survive from one year to the next on chronically infected animals. In view of the lability of orf virus in scabs exposed to the elements which this study has suggested, it is possible that persistent infections contribute to the survival of virus between epizootics.

References

AYNAUD, M. (1923) *Annals de L'Institut Pasteur* **37**, 498

GLOVER, R. E. (1928) *Journal of Comparative Pathology and Therapeutics* **41**, 318

GLOVER, R. E. (1930) First Annual Report, Director, Institute of Animal Pathology, Cambridge University, p17

GREIG, A., LINKLATER, K. A. & CLARK, W. A. (1984) *Veterinary Record* **115**, 149

HARKNESS, J. W., SCOTT, A. C. & HERBERT, C. N. (1977) *British Veterinary Journal* **133**, 81

LIVINGSTON, C. W. & HARDY, W. T. (1960) *Journal of the American Veterinary Medical Association* **137**, 651

McKEEVER, D. J. (1984) *Veterinary Record* **115**, 334

MANLEY, F. H. (1934) *Veterinary Journal* **90**, 80

NAGINGTON, J. & HORNE, R. W. (1962) *Virology* **16**, 248

PLOWRIGHT, W., WHITCOMB, M. A. & FERRIS, R. D. (1959) *Archiv für die gesamte Virusforschung* **9**, 214

ROBINSON, A. J. & BALASSU, T. C. (1981) *Veterinary Bulletin* **51**, 771

SAWHNEY, A. N. (1972) *Indian Veterinary Journal* **49**, 14

Timber treatment and bats

CONTROLLED laboratory experiments established that timber treatments, used to manufacturer's specifications, containing γ-HCH or pentachlorophenol caused the death of pipistrelle bats (*Pipistrellus pipistrellus*) roosting in contact with treated timber. The bats died within seven days when the timber had been treated six weeks previously, and within 23 days when it had been treated 14 months previously. If the bats were prevented from grooming they took longer to die. Acrylic resin used as a sealant reduced the lethal effect but polyurethane varnish did not. No obvious harm was caused to bats roosting in contact with timber treated with the synthetic pyrethroids permethrin, cypermethrin and deltamethrin at concentrations effective against wood-boring beetles.

P. A. RACEY & S. M. SWIFT (1986) *Biological Conservation* **35**, 205