THE DEVELOPMENT OF, AND HOST RESPONSE TO, OVINE CONTAGIOUS PUSTULAR DERMATITIS

by

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A thesis submitted to the Faculty of Science of the University of New England, Armidale, N.S.W., in partial fulfilment of the requirements of the degree of Bachelor of Science with Honours

November 1983

ACKNOWLEDGEMENTS

There are many people I would like to thank for their support during 1983. Unfortunately neither time nor space permit me to mention them all.

I would particularly like to thank my supervisor, Dr R.C. Chubb, for his suggestions and encouragement throughout the year and for his valuable criticism in the preparation of this dissertation.

My wife Cheryl has been supportive well beyond the call of duty and I would like to express my sincere appreciation for all her encouragement and support, without which it would have been very difficult to manage.

Three people who have been very helpful to me during the course of this work are Mrs Heather Elworthy who painstakingly read my hand-writing and turned it into print, Miss Carol Quilkey who gave much valuable technical assistance, and Mrs Robyn Bradley who, as well as encouraging me, always had time to explain techniques and methods.

I would especially like to thank my parents who have enabled me to pursue my chosen studies and have always been supportive and helpful in the extreme.

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ABSTRACT

Several aspects of contagious pustular dermatitis were studied. One of these was the histology of lesion development with particular reference to sites of viral replication, using indirect fluorescent antibody staining and characteristic inclusion bodies. The immunological responses of the sheep including hyperplasia, hyperkeratinization and cellular infiltration were noted.

A fluorescent antibody staining technique was used to assess the humoral response to CPD infection. It was found that the humoral response to CPD infection was slight. The eliciting of a cutaneous delayed type hypersensitivity response implicated the cell mediated immune responses in recovery and subsequent protection from CPD.

Material from scabs was examined using the electron microscope. Two forms of CPD virus particle, the 'M' and 'C' forms were found. The morphologies of both these types of particles were identical to those forms described elsewhere by various authors.

Artificially infected sheep were examined for signs of viremia. No evidence for the presence of viremia was found, however, there was spread of virus in the area adjacent to the site of inoculation.

It was shown by challenging, that some animals, although susceptible to infection at sites remote from the initial infection, were resistant to infection at the site of initial infection, thus raising the question of "local immunity".

CHAPTER 1

LITERATURE REVIEW

Contagious pustular dermatitis is an exanthematous disease of sheep and goats, caused by a virus of the family Poxviridae. It has been recorded since the late 1800's and has been reported from most sheep or goat raising areas including those in Europe, the Middle East, the United States, Africa, Asia, Alaska, South America, Canada, New Zealand and Australia (FAO/WHO/OIE 1972).

Contagious pustular dermatitis has been described under a large number of synonyms. Contagious ecthyma, contagious pustular stomatitis, infectious labial dermatitis, infectious pustular dermatitis, sore mouth, orf, vesiculo-pustular stomatitis or dermatitis, malignant aphtha, vuilbek and scabby mouth have all been used to name the disease at some time. Some confusion still exists as to the correct nomenclature, so in the remainder of this work, as in most of the recent work in this field, contagious pustular dermatitis (CPD) shall refer to the disease in animals while orf shall be used to describe the disease in humans.

A comprehensive review of the literature concerning CPD was published in 1981 by Robinson and Balassu, and apart from some specific quotes, this work will not be cited further in this review. Due acknowledgement is hereby given.

1.1 THE DISEASE AND HOST

1.1.1 The Hosts

Primarily, CPD is a disease of sheep and goats although it has been reported as a natural disease in the following: man (Carne *et al* 1946; Lloyd *et al* 1951; Pask *et al* 1951; Purdy 1955; Fastier 1957; Lang 1961; Hunter 1964; Leavell, 1968; Liebermann 1977; Kim and Tarrier 1977; Sweeney and Woolfson 1978; Loubet *et al* 1980; Peterson 1980; Johannesson *et al* 1980; Ganske 1981; Sinn 1982), steenbok and alpacas (Robertson 1976), chamois and thar (Daniel and Christie 1963), reindeer (Kummenje and Krogsrod 1979), musk ox (Kummenje and Krogsrod 1979; Dietrich *et al* 1981), the dog (Wilkinson and Prydie 1970), mountain goat (Connel 1954; Samuel *et al* 1975), bighorn sheep (Lance 1981; Lance *et al* 1981; Turner 1982), dall sheep (Dietrich *et al* 1981; Smith *et al* 1982), and the red squirrel (Scott *et al* 1981).

There is much conflict in the literature as to which animals can or cannot be infected in the laboratory with CPD virus. Aynaud (1923) could not infect mice, rats, guinea pigs, cats, dogs, pigs, chickens, pigeons or frogs. Howarth (1929) could not infect cattle and many workers including Howarth (1929), Boughton and Hardy (1935) and Plowright *et al* (1954) could not infect rabbits. However cattle have been infected (Bennett 1944; Huck 1966) and experimental work has been done using infected rabbits (Abdussalam 1957; Darbyshire 1961; Selbie 1944; Bennett *et al* 1944). Abu-Samra and Walton (1981) could only infect rabbits in the foot pad when initiating a simultaneous infection of *Dermatophilus congolensis* and CPD.

It is generally agreed that, unlike many other pox viruses, CPD virus is unable to propagate in the chick embryo although there are three reports of limited multiplication on the chorioallantoic membrane of the chick embryo at low passage level (Abdussalam 1957; Sawhney 1966; Rao and Singh 1981).

1.1.2 Clinical Signs and Appearance

In sheep and goats the lesions mostly appear on or near the hairline and elsewhere on the lips and muzzle. In some cases the lesions appear on and in the nostrils, around the eyes, on the thigh, coronet, vulva, udder and axilla (BVA 1955; Walker 1964; Robinson *et al* 1981). In rare cases, mostly involving young lambs, lesions are found on the tongue, gums, roof of the mouth and the oesophagus. It has also been reported a number of times to cause lesions in the rumen (Darbyshire 1961; Morales *et al* 1971; Thiel and Rudolph 1981), sometimes with a concurrent phycomycosis (Morales *et al* 1971).

In a report by Darbyshire (1961) it was shown that a severe form of CPD virus caused an outbreak involving the gastrointestinal tract, lungs, heart, as well as the buccal cavity, cheeks, tongue and lips. Another severe case was reported by Valder *et al* (1979) who found pharyngitis, genital lesions and infection of the hooves which led to lameness and, in some cases, sloughing of the hoof.

The lesion has been described by a number of workers including Aynaud (1927), Abdussalam (1957), Darbyshire (1971), and Jubb and Kennedy (1968). The self limiting lesions begin as a mild erythema around the point of inoculation or infection, between two and four days post infection. This erythema increases over the next few days as papules begin to form. Vesicles first appear as part of the papule until the vesicle expands and becomes

pustular over the next two days. The pustule is enclosed under a thin, tightly stretched layer of the epidermis which is easily broken until the formation of a scab which covers the infected area. The scab formation is complete somewhere between 8 and 12 days post infection. Occasionally earlier stages of the infection in the form of discrete papules, vesicles and pustules appear on the border of the primary lesion. However the whole infection does not spread further than 10-20 mm, illustrating the self-limiting nature of the infection.

In some cases pustules give way to ulcers and occasionally these become verrucose masses resembling papillomata (Glover 1928; Selbie 1944; BVA 1955). A malignant form has also been described which invades the buccal cavity and produces papillomatous outgrowths on the tongue, palate and inside the lips (BVA 1955; Valder *et al* 1979).

An unusual outbreak of CPD was reported by Gardiner *et al* (1967) who describe oedema of the lips and intermandibular spaces, exudative and proliferative dermatitis of the lips, associated commisures and on the rest of the muzzle and face. Further to this, irregular linear erosions 0.5 to 1.0 cm long by 0.2 to 0.5 cm wide, appeared on the gingival mucosae and dental pads and raised confluent greyish yellow lesions on the dorsum of the tongue and on the hard palate are also described.

This outbreak is similar to an outbreak in the United Kingdom described by Darbyshire (1961) which, as well as the above signs, showed a mucopurulent nasal discharge, cyanosis of the tongue, congestion of the turbinate bones, buccal ulceration and erosive stomatitis.

Although there are a number of different appearances of CPD only rarely do the clinical signs differ appreciably from the more common form showing erythema, papule, vesicle, pustule and scab.

1.1.3 Morbidity and Mortality

In sheep and goats morbidity varies from low (10%) to as high as 100% (Walker 1964; Gardiner *et al* 1967). The level of morbidity in the flock depends on the age of the animals, time of year, breed of sheep or goat, the strain of the virus and the condition of the immediate environs (Schmidt and Hardy 1932). High morbidity is more common in areas where prickly bushes and/or other plants or material which may damage the epithelial layer abound.

Mortality is generally low, about 1% to 2% (Schmidt and Hardy 1932; BVA 1955; Walker 1964). However, mortality rates as high as 7%, 15%, 20%, 23% and 78% have been reported by Darbyshire (1961) who attributed these deaths solely to CPD virus. Reports of high mortality are, in general, the result of secondary infection.

Many complicating secondary infections often leading to septicemia, have been described, the most serious and well-known of these are the *Fusobacterium necrophorum* (Newson and Cross 1931, Marsh and Turncliff 1937), *Dermatophilus congolensis* (Cooper *et al* 1970; Munz 1976; Abu-Samra and Walton 1981), and Streptothricosis (Munz 1976). Other complications which may also be fatal include larvae of blow fly and screw worm fly infestations. These complications may result in a mortality of between 20% and 50% (Robinson and Balassu 1981).

1.1.4 Epidemiology

Currently there are two views on the source of primary infection of CPD outbreaks. The most commonly held view is that virus is shed into the environment with the scab where it can remain viable for a long time. Like other poxviruses CPD virus is known to be very stable and it has been shown that it can remain viable in the scab for two years in the shade (Boughton and Hardy 1935), and for several months in full sun (Boughton and Hardy 1935). It has also been shown that dried ground scab stored at 7°C still produced typical lesions of CPD when inoculated onto susceptible lambs after 22 years and 8 months (Livingstone and Hardy 1960).

Once in the environment it is thought that one or two susceptible animals become infected and act as the foci of infection. The lesions in these animals progress and eventually scabs containing virus are shed. From this point the infection rapidly spreads to the remainder of the flock.

It is generally thought that the virus gains entrance to the host following a trauma which breaches the integrity of the epidermis. Such a breach is often due to thistles, stubble, thorns and other sharp or abrasive material in the environment.

The less well accepted view of infection stems from the observations of Romero-Mercado *et al* who failed to find virus particles in the late scabs which dropped off infected animals. In 1976 Robertson extrapolated these results stating that the source of new outbreaks is latently infected carrier animals.

This theory has never been well supported and Harkness (1977) described a technique to obtain maximum numbers of viral particles in scabs. Harkness (1977) and others have since found viral particles in late scabs which rests as evidence against Robertson's (1976) theory of latently infected animals. However, Buddle (1981) found that corticosteroid treatment of vaccinated animals induced recrudescence at sites of previous infection but could not isolate virus from the scabs thus leaving unconfirmed the possibility of the existence of latent infection. Buddle (1981) suggests that latent infection is important in the initiation of CPD outbreaks. Perhaps then both latently infected carriers and scab material in the environment are responsible for the perseverence of the disease.

Suckling lambs which are infected with CPD are able to infect the udder of their dam, and ewes infected around the coronet or vulva, at the time of lambing can infect the lamb during parturition.

1.1.5 Diagnosis

Diagnosis of CPD is generally based on the clinical signs. In areas where an outbreak of foot and mouth and/or bluetongue is either a concern or a possibility, it is important to be able to rapidly distinguish between CPD and these diseases.

A number of methods can be used to confirm an initial diagnosis or make a diagnosis in its own right, however few are in regular use. The most rapid and probably the most frequently used is electron microscopy (EM). Electron microscopy diagnosis of CPD has been described by a number of workers including Spradbrow and Francis (1969), Gerstl (1973), Ebermann (1977), Harkness *et al* (1977) and Martin and Palmer (1982). Diagnosis by EM involves the examination of ground-up scab material or vesicle fluid which has been cleared by centrifugation and stained with 2% or 3% phosphotungstinic acid. The ease and rapidity of this method is, no doubt, assisted by the large particle size and the characteristic morphology of the CPD virion.

Diagnosis of CPD using an agar gel diffusion-precipitation test has been described by Papadopoulos *et al* (1967), Sharma and Dhanda (1971), and Sawhney *et al* (1973). However, Sawhney *et al* (1973) found that this technique was not sensitive enough to detect antibodies in the sera of convalescent animals. They conclude that positive diagnosis is best made using ground scabs, from the animal to be tested, as antigen against known positive sera and not using standard known positive antigen against unknown sera. As the scabs are a late stage in the lesion of CPD it would be a significant advantage, by saving time, if vesicle fluid could be shown to be as good an antigen source as scab

material, however this would not then be suitable for avesicular forms of the disease.

A complement fixation technique for the diagnosis of CPD has been described by Abdussalam (1958) and Romero-Mercado *et al* (1973). These authors found it to be a satisfactory technique. It has not, however, featured prominantly in the literature.

A capillary agglutination test has been reported but was not found to be a particularly successful technique (Abdussalam 1958; Sawhney *et al* 1972). Histopathology has been used as a diagnostic technique, however it is an impractical method. It is most commonly used for orf in humans, when medical practitioners, encountering orf for the first time, remove the lesion and accept the histopathologist's and electron microscopist's report.

A fluorescent antibody technique has been used to diagnose orf in human patients. Erickson *et al* (1975) found that this was a useful and accurate test, performed using CPD virus cultured in human cell culture.

The older, well tried method of inoculating known susceptible animals and immune animals has also been widely used. It is however, giving way to the more modern, more effective and cheaper techniques.

1.1.6 Treatment, Prevention and Control

Treatment of CPD, in common with other viral diseases, is seldom effective, practical or economic. The best that can usually be done is to prevent secondary infection.

If the lesions adversely affect feeding it may be useful to remove the scabs and apply an oily disinfectant dressing although this will delay healing (Walker 1964). Other topical treatments which have been described include 5% aqueous copper sulphate (Robinson and Balassu 1981), creosote, 3% phenol solution, and 7% iodine (Beck and Taylor 1974). Some of these treatments are likely to be worse than the disease. Sanderson (1976) recommends intramuscular injection of lithium antimony thiomalate in water, 1 ml for lambs and 3 ml for adult sheep, repeated at six-day intervals. This was found to be a successful treatment.

Should secondary bacterial infection be a problem, suitable antibiotics may be employed to good use according to Walker (1964) who also recommends the application of an insecticide repellent if screw-worm fly or blow fly strike is likely to be a problem.

Prevention of the disease, on a CPD-free holding is best done by ensuring that all new or travelling stock are CPD-free and perhaps holding new animals in a quarantine paddock for one to two weeks before moving them to the remainder of the property. It is also important to keep fencing in good order to prevent stock from neighbouring properties, which may be infected, from entering the property and infecting naive stock.

Prevention and control of CPD on properties with a history of CPD is best accomplished with a yearly vaccination with one of the number of vaccines available. Should an outbreak occur, it is best checked by an immediate vaccination, which will limit the course of the disease to 3-4 weeks rather than the usual 8-10 weeks. The vaccines in production are reported to give a good immunity (Walker 1964; CSL 1980; Robinson and Balassu 1981). However, there are reports of vaccines either not promoting immunity or conferring only local immunity (Trueblood *et al* 1963; Schmidt 1967). In the case of local immunity vaccination is not likely to be useful as it is done on the thigh. Vaccination around the mouth with the autogenous vaccine would possibly be as serious as the disease itself.

Recurrence of the disease in vaccinated animals was reported by Trueblood *et al* (1963) and by many workers since then. Although this may be due to strain variation or poor vaccination technique, it is known that accelerated responses to challenge do sometimes occur in previously immunized animals (Schmidt 1967; Richter and Jansen 1968). It is possible that this apparent failing of vaccination may actually have been the observation of the earlier unknown 'accelerated response'.

The vaccines that are available are ground-up lesions from experimentally infected, otherwise healthy, lambs. The ground lesions are suspended in physiological saline or other buffer, some containing glycerin and/or chloroform as a preservative (CSL 1981).

The vaccine then, is a live, fully virulent virus. The disease condition is avoided solely due to the site of vaccination. Usually vaccination is on the inside of the hind leg on the bare skin in the thigh region, and is done by a light scarification and application of the viral suspension. Both Australian manufacturers of CPD vaccine enclose a scarifier/applicator stick which works in the same fashion as a nib pen, releasing viral suspension as the scarification is made. Alternative vaccination sites are occasionally used. Beck and Taylor (1974) report the bare skin of the underside of the tail or the axilla being used in the case of pregnant or lactating ewes to prevent infection of the udder. The CSL (1981) recommends vaccinating fat lambs

destined for market on the axilla or brisket because, in some cases, the lesion may cause a blemish in the choice-cut leg which must be dressed out.

As the vaccine is fully active CPD virus, it is unwise to vaccinate unless CPD is known to be on a property, as it is introducing the virus and disease to that property.

1.1.7 Histopathology

The histopathology of CPD virus infection was first studied by Aynaud in 1927, who described the histology of experimentally induced lesions as well as naturally occurring lesions on the tongue.

Infiltration with polymorphonuclear leucocytes, the vesico-papulo stage, multilocular pustular stages, proliferation of the cells of the rete malphigi and formation of the scab layer from cell debris, fibrin and other exudates has been described previously by a number of authors (Aynaud 1928; Glover 1928; Selbie 1944; Wheeler and Cawley 1956; Jubb and Kennedy 1963).

Ballooning degeneration of cells of the prickle cell layer has been described (Glover 1928; Selbie 1944; Wheeler and Cawley 1956; Kluge *et al* 1972). However, Abdussalam (1957) found no such ballooning degeneration but found reticular degeneration and states that "the opinion of some previous workers that ballooning occurred in CPD is not supported by their own descriptions or illustrations".

Intracytoplasmic inclusion bodies within enlarged keratinocytes have been described by Kluge *et al* (1972), Abdussalam (1957), Selbie (1944), and were found to be periodic acid schiff (PAS), positive and eosinophilic (Kluge *et al* 1972). These inclusions may be of varying sizes (Kluge *et al* 1972), but are generally between 4 and 8 microns in diameter (Abdussalam 1957). Kluge *et al* (1972) demonstrated that these inclusion bodies and other intracytoplasmic granules contained concentrated viral antigens by staining frozen sections with fluorescein conjugated anti-contagious ecthyma serum.

1.1.8 Immunity

Little work has been done on the immune responses to CPD. The work which has been done has generally concentrated on the antibody responses to CPD and length of immunity conferred by vaccination.

The antibody that has been found, whether complement fixing, agglutinating, precipitating or neutralizing, has been found in low levels (Abdussalam 1958; Buddle 1981). Antibody levels in previously infected animals have not been found to be high and have completely gone by 5 months PI (Robinson and Balassu 1981). Neutralizing antibody has been found by Jan *et al* (1978) but not by Trueblood *et al* (1963) or Schmidt (1967). De Martini *et al* (1978) and Koptopoulos *et al* (1982) detected cytotoxic antibodies to CPD using a ^{51}Cr release assay of CPD virus infected cells. Koptopoulos *et al* (1982) found little correlation between antibody levels detected by this method and antibody levels as detected by indirect immunofluorescence, enzyme linked immunosorbent assay and complement fixation tests.

Antibodies to CPD have been found in the colostrum of the ewe (Poulain *et al* 1972; Jan *et al* 1978) who also found that lambs born to immune ewes were resistant to infection. Boughton and Hardy (1935), Schmidt (1967), Richter and Jansen (1968) and Buddle (1981) found that such lambs were not resistant to infection. Jan *et al* (1978) found that the degree of protection conferred on a lamb was proportional to antibody levels in the sera of the lamb and the sera and colostrum of the ewe, suggesting that immunity may be conferred via the placenta or the colostrum or both. These findings are interesting when compared with those of Buddle (1981) who found that an animal's own antibody level could not be used to predict its own level of resistance to CPD.

There is much variability of antibody level to CPD but at least some of this is attributable to variability inherent in the technique used to measure the antibody level.

The only work on the cellular immune response of the host to CPD that has been reported is that of Buddle (1981) who implicated the cell mediated immune responses in recovery and subsequent protection from CPD by eliciting a cutaneous delayed type hypersensitivity reaction to CPD. However, Buddle (1981) also found that the degree of protective immunity could not be predicted using virus specific lymphocyte stimulation indices.

The duration of immunity of vaccinated animals is a much debated point. Challenge experiments have indicated no immunity (Wachendorfer *et al* 1980), immunity of 6-8 months (BVA 1955; Schmidt 1967), one year (Frericks 1982), 2-3 years (El Dahbaby *et al* 1980; CSL 1981) and some reports suggest that immunity may be life-long. Some of this variability may be the result of differences in the conditions in which the experimental animals are kept after

vaccination until challenge. Of particular importance is whether endemic virus is or is not present in the immediate environment, for it has been suggested that, in the field, lifelong immunity after vaccination is the result of continual challenge with endemic virus (Robinson and Balassu 1980).

1.2 THE VIRUS

1.2.1 Morphology

The first direct study of the infective agent of CPD was performed in 1957 by Abdussalam and Cosslett. Their study examined the staining characters and morphology under the light and electron microscope. It describes the basic size (252 nm x 158 nm) and shape as being short monomorphic rods.

Since this time, there has been much improvement in the techniques of electron microscopy and a number of studies have examined the morphology of CPD virus. Some of these workers include Nagington and Horne (1962), Nagington *et al* (1964), Peters *et al* (1964), Westwood *et al* (1964), Mitchner (1969), Spradbrow and Francis (1969), Kluge *et al* (1972), Pospischil (1981) and Saginova and Roslyakou (1982).

From the results of the above workers, many things have become clear about the structure of CPD virus. It is approximately 260 x 160 nm, giving it an axis ratio of 1.6. Two types of particle are found, the 'M', or mulberry form is well known for its basket weave appearance which results from the crossing over of tubular protein threads on the surface of the virion. The 'C' form or clear form has an electron dense centre with a thick well-defined envelope. By analogy with vaccinia virus, described by Westwood *et al* (1964), it is suggested that the 'C' forms are damaged 'M' form particles which allows penetration of the negative stain to the centre.

The core of the intact virion is often seen to be dumb-bell shaped due to the presence of large lateral bodies on either side of the nucleoid (Palmer and Martin 1982). The internal structure of the nucleoid itself consists of an 'S' shaped tubular structure (Nagington *et al* 1964).

1.2.2 Physico-Chemical Properties

The virus of CPD belongs to the genus parapoxvirus of the family Poxviridae. It possesses a similar antigen to the other known poxviruses

(Woodruff and Fenner 1962) which is probably a nucleoprotein (Webster 1958). The CPD virus is similar also in many other ways to other poxviruses.

Wittek *et al* (1979) report a guanine plus cytosine content of 63% and Robinson *et al* (1982) report that the DNA has a molecular weight of 88.8 x 10^6. Like other known poxviruses CPD is stable, perhaps it is the most stable of the poxviruses. It is highly thermostable and not inactivated until held at 60°C for 30 minutes (Sawhney 1972). Powdered dried scabs have been found to retain their infectivity after 22 years and 8 months when stored in the refrigerator. at 7°C (Livingston and Hardy 1960). Plowright (1959) found that freeze-thawing does not reduce infectivity but increases it and suggests that this is because intra cellular virus is released from the cells.

The CPD virus is resistant to glycerol and only slightly sensitive to ether, chloroform and toluene (Trueblood and Chow 1963).

1.2.3 Strain Variation

In the past it has been thought that there was no, or little difference, between the strains of CPD virus. Before 1947 it had been shown, with cross protection tests, that all tested strains were similar (Robinson and Balassu 1981).

Trueblood *et al* (1963) suggested that the problem of strain variation had not been fully studied and that because vaccinated animals did sometimes become infected, that strain variation was a possibility. Evidence has mounted for the possibility of strain variation. Difference in mortality and morbidity rates, and the appearance of severe forms of disease which showed different clinical signs from those clinical signs normally recorded, such as those reported by Valder *et al* in 1979, Darbyshire in 1961 and Gardiner *et al* in 1967, all suggest that there may be different strains. Trueblood and Chow (1963) found that CPD virus and the agent that causes ulcerative dermatitis were identical in morphology and showed some similarities on the basis of cross protection tests. Previously ulcerative dermatitis was thought to be a distinctly separate condition but this thought was reviewed and it is currently thought that ulcerative dermatitis is an atypical form of CPD.

On the basis of cross protection and neutralization tests, Sawhney (1966) concluded that there are many different strains of CPD virus, as did Wittek (1980) on the basis of restriction endonuclease analysis of the DNA of 36 strains of field isolates from New Zealand. Robinson *et al* (1982) showed that

the DNA patterns were not altered by 22 passages in sheep cell culture or by 2 passages in sheep. However, Wittek *et al* (1980) showed some difference in DNA patterns between the 8th and 137th passage in cell culture.

It seems then that strain variation does exist. Confirmation of this should be simple, based on the identification and analysis of a large number of isolates from different areas.

1.3 CONCLUSION

Contagious pustular dermatitis virus, endemic in a large part of the world, remains a problem in sheep raising areas and in the area of industrial relations. Although vaccination is carried out, it is a living vaccine which ensures continuance of endemic virus. A greater understanding of the immune response to CPD virus and the virus itself will lead to, indeed is leading to, the development of more efficacious, less troublesome vaccination programs which may eventually result in the eradication of the disease.

CHAPTER 2

INTRODUCTION

Contagious pustular dermatitis (CPD) is an exanthematous disease of sheep and goats which is highly epitheliotrophic. It is caused by a virus belonging to the family Poxviridae and is a member of the genus Parapoxvirus. The disease, which causes typical 'pock' type lesions, mainly around the mouth, has been reviewed fully in the previous chapter.

Although some recent work has been done on immune responses to CPD (Buddle 1981; Kaptopoulous *et al* 1982; Jan *et al* 1978), development of new vaccines (Mayr *et al* 1981) and vaccination programs (Blankevoort 1981; Buttner 1981), very little work has gone on in this field generally, and even less in Australia particularly.

There is increasing evidence concerning the importance of the cell mediated immune (CMI) responses to many other poxvirus infections (Morita 1973; Srivastava and Singh 1980) but little, if any, work has been published concerning the role of CMI in recovery and protection to CPD. Robinson and Balassu (1981) however report a personal communication with Dr L. Fastier (ICL Tasman Labs, Upper Hutt, New Zealand) who used an intradermal test of heat inactivated CPD virus antigen to determine the immune status of animals by the ensuing delayed type hypersensitivity (DTH) reaction. No comment on the success of this method is made other than to say that a DTH is seen in immune animals.

Cellular immunity is quoted as being of "vital importance" in the recovery and protection in sheep from sheep pox virus infection (Srivastava and Singh 1980). It is anticipated that CMI will be of similar importance in the recovery and protection from CPD virus infection. This probable importance of CMI to CPD infection is also reported by Robinson and Balassu (1981).

A review of the available literature indicates that the effect of the mitogen, phytohaemagglutinin (PHA), has not been examined in sheep. Although the immunological significance of the *in vivo* transformation of lymphocytes by PHA has not been clearly defined, it is known that PHA stimulates lymphocytes and there is conclusive evidence that the transformation of lymphocytes is not dependent on earlier sensitization to PHA itself (Graeves *et al* 1968; Stadecker and Leskowitz 1974). Stadecker and Leskowitz (1974) demonstrated a cutaneous response to a number of mitogens, including PHA, in the guinea pig, and Graeves

et al (1968) demonstrated a transformation of peripheral lymphocytes *in vitro* of the chicken.

A number of methods have been used to measure serum antibody levels to CPD. Sawhney *et al* (1973) report a successful agar gel diffusion precipitin test for CPD antibodies. Dubey and Sawhney (1979) report success with immunoelectrophoresis, serum neutralization and capillary tube agglutination. Abdussalam (1957) reports a complement fixation test to be successful, as well as the techniques previously mentioned. De Martini *et al* (1981) describe a successful chromium-81 release assay of antibody and complement mediated cytotoxicity. No reference however, is made to the use of a fluorescent antibody technique for estimating antibody levels to CPD in the sheep.

The histopathology of CPD virus infection in sheep has been studied previously (Glover 1928; Selbie 1944; Wheeler and Cawley 1956; Abdussalam 1957). Kluge *et al* in 1972 performed an ultrastructural study, but apart from this, there have been no recent studies of the histopathology of CPD virus infection in the sheep. No use has been made of fluorescent antibody staining in the study of histology previously except to show that cytoplasmic inclusions contained viral antigens.

The histopathology, antibody levels and immunity studies that have been carried out previously were invariably done on overseas strains of CPD virus. As there is substantial evidence that Australian strains differ from overseas strains in a number of ways (Robinson and Balassu 1981), including absolute inability to grow on eggs and the low virulence, it is significant that this study used, in all cases, Australian strains of CPD virus.

There is some evidence that a systemic viremia occurs following an infection with CPD. Viremia is thought to occur because in some cases lesions have been found on the heart and lungs (Darbyshire 1961), and it has been reported that lesions may occur on places other than the initial site of infection if the integrity of the dermis is compromised (Gardner *et al* 1967). However, no experimental work has been reported which was designed to establish the presence or absence of systemic or localized viremia.

Electron microscopy has been used to diagnose CPD in sheep (Spradbrow 1969; Harkness *et al* 1977). Electron microscopy is also useful in making observations of the size, shape and morphology of the CPD virion.

In the light of these findings it was decided that work should be done on the Australian CPD virus in the following areas:

1. The cell mediated immune responses, as manifested by DTH, to contagious pustular dermatitis.

2. The effect of the mitogen, phytohaemagglutinin on the *in vivo* stimulation of lymphocytes.

3. Fluorescent antibody staining (FAS) as a technique to measure serum antibody levels as well as the use of FAS combined with normal staining to study the histology of the development of the CPD lesion.

4. Experiments to verify or refute the presence of a systemic viremia following a CPD virus infection.

5. Electron microscopic observation of the CPD virion.

CHAPTER 3

MATERIALS AND METHODS

3.1 VIRUS

Six isolates of contagious pustular dermatitis (CPD) were used throughout these experiments. Three of these isolates were Commonwealth Serum Laboratories vaccine strains, batches 047-1, 046-51 and 045-12. The remaining three isolates were obtained from various properties in the New England region and were designated Kwat, Pea and Adgr, after the owner or the property name. All virus, either as scab material or in fluid, were stored at -70°C.

All experiments used the above isolates or virus derived from second or further passage in sheep. Isolate and passage level are indicated for each experiment.

Viral preparations, when made from scab material, were made by grinding in a 5 ml 'vitro' tissue grinder. The ground material was made up to approximately 10% w/v using phosphate buffered saline (PBS) containing 50 iu of penicillin (Crystapen) and 0.05 mg of Streptomycin per ml of diluent. The homogenous solution was left for 30 minutes at room temperature to enable these antibiotics to take effect.

3.2 NOMENCLATURE

To express the strain of virus used, the animal, and the day post-inoculation (PI), the following is an example of the convention used: CSL/9/24. CSL is the virus strain, 9 indicates the animal number, and 24 indicates the day PI. This applies for both sections and sera.

3.3 EXPERIMENTAL ANIMALS

Experimental animals (Merino/Border Leicester crossbred wethers) were obtained from the University of New England's farm, which has not previously recorded an outbreak of contagious pustular dermatitis. While infected, the experimental animals were housed in an isolation room of the University's small animal house. Each animal was individually caged (Figure 1) and fed a maintenance ration of oats and lucerne chaff or Fielders sheep nuts. Water was provided *ad libitum*.

Figure 1: Individually Caged Experimental Sheep.

3.4 BLOOD AND SERA COLLECTION

Blood of experimental animals was collected from the jugular vein, using a 10 or 20 ml 'Terumo' syringe and an 18 g x 30 mm needle. When whole blood was required, citrate phosphate dextrose (Gibson *et al* 1961) was the anti-coagulant of choice. It was used at 1 ml to 9 ml of blood.

When serum was required the blood was allowed to clot in the syringe, the clot loosened, and the sample left overnight. Sera was decanted into $\frac{1}{4}$ or $\frac{1}{2}$ oz Bijou bottles and stored at -20°C until use.

3.5 ELECTRON MICROSCOPY

Scab material was prepared for electron microscopy by grinding a small portion of tissue in a sterile Vitro (USA) 5 ml tissue grinder, with a few drops of distilled water. This solution was homogenized by vigorous grinding. The solution was used as is, and after clearing by centrifugation (10,000 revs for 2 minutes). A drop of each solution was placed onto 3 mm, G400 electron

microscope grids (Polaron Equipt, Watford) which had previously been prepared with Formvar®.

Preparation for electron microscopy was as the method described by Spradbrow (1969). A drop of 2% phosphotungstic acid buffered to pH 7.2 with potassium hydroxide was overlaid on the drop of material to be examined. This negative stain was allowed to act for 2 or 3 seconds, and the surplus was then removed using a clean filter paper. The grid was then examined using the University's Phillips EM 300 Transmission Electron Microscope, at 64 kv. The prepared grids were scanned at 17,000 x for a minimum of 5 minutes before being recorded as negative.

Electron micrographs were used to record the virus found in several of the preparations.

3.6 GROSS APPEARANCE AND HISTOPATHOLOGY

3.6.1 The Lesion

Discrete lesions were cultivated on the thigh and inquinal regions of susceptible animals, where wool was not normally present. To inoculate discrete sites the whole area was washed well with warm soapy water and swabbed with 70% ethyl alcohol. Scarifications were then made at discrete sites with the scarifier supplied with CSL Scabby Mouth vaccine, by the method suggested by CSL (1980). These scarifications were made so as to break the epidermis but not so as to draw blood. The inoculum was then rubbed into the site using a sterile swab.

3.6.2 Biopsy

The discrete lesions which resulted from inoculation were biopsied at daily intervals until Day 3, and from then until Day 25 at two-day intervals. Before excision, local anaesthesia was induced at the site by a series of subcutaneous injections, totalling 2 ml, of 2% lignocaine (Xylocaine®, Astra Pharmaceuticals, Sydney). Five to 10 minutes elapsed between anaesthetic injections and excision to allow the anaesthetic time to take effect. Excision was made using forceps and size 11 scalpel blades. The site was circumscribed and the dermal layer lifted. The dermis was then cut away from the subcutaneous tissue. After excision severed subcutaneous blood vessels were clamped with artery forceps and tied off using medium/hard, size 00, chromic catgut (Bell and Croyden, London). The wound was then sutured with nylon.

3.6.3 Sectioning and Staining

The biopsy was rinsed in phosphate buffered saline (PBS) to remove free blood, dried, and frozen at -20°C. This facilitated cutting in preparation for sectioning. The biopsy was divided with a sharp scalpel blade to ensure that the site of infection was sectioned.

Half the biopsy was fixed in AFA solution which is acetic acid (2%), formaldehyde (10%), alcohol (50%) and water (38%) (Humason 1972), prior to paraffin embedded sectioning. The remainder was kept frozen at -70°C ready for sectioning on the freeze microtome.

Six micrometre and three micrometre sections were cut in the Cryostat freeze microtome using the 'Tissue Tek' method. Many sections were cut and slides prepared. Where possible serial sections were made. These sections were then either fixed in cold acetone and stored at 4°C in readiness for fluorescent antibody staining or stored at 4°C (< one day) in readiness for haematoxylin and eosin staining.

Sections were stained in three ways for histological examination: Haemotoxylin and eosin (Baker *et al* 1973) (Figure 2), Jenner-Giemsa (Baker *et al* 1973) (Figure 3), and Fluorescent Antibody stain (Figure 4).

Figure 2: Halls Modified Harris Haematoxylin and Eosin Staining (Baker *et al* 1973)

Carnoy's Fixative	10 sec
80% Alcohol	60 sec
Distilled Water	wash
Halls Modified Harris Haematoxylin (Cater, Histologic 1980)	30 sec
Tap water	wash
Eosin	20 sec
90% Alcohol	30 sec
90% Alcohol	30 sec
Absolute Alcohol	30 sec
Absolute Alcohol	30 sec
Xylene	> 20 sec

then mounted in Eukitt.

Figure 3: Jenner Giemsa Stain (Baker *et al* 1973)

Fixed in methyl alcohol	
Stain Jenner stain	2 min
Drain	
Giemsa stain	12 min
Distilled water	wash
Drain and dry	

Figure 4: Fluorescent Antibody Staining

Hyperimmune sera, $\frac{1}{10}$ or $\frac{1}{20}$ in PBS	30 min at 37°C
Wash in PBS (3 times)	5 min
Drain	
FITC conjugated anti-sheep IgG	30 min at 37°C
Wash in PBS (3 times)	5 min
Rinse distilled water	2 min
Drain	

After observation and recording, some selected fluorescent antibody stained slides were restained with haematoxylin and eosin as described, but without fixing in Carnoy's fixative. This allowed accurate comparison of sites of fluorescence and histology of that site to be made.

3.6.4 Observation and Microphotography

Examination of stained sections was done using an Olympus Vanox microscope (Olympus Optical Co., Tokyo) at magnifications of 40 x, 100 x, 200 x and 400 x. Examination at 1,000 x magnification was used when identifying cell types but the quality of the sections was such that this magnification was of little use.

Light photomicrographs were recorded using a Karl Zeiss (West Germany) automatic photomicrographic camera mounted on the aforementioned microscope. Exposures were made on Ektachrome (Kodak, Australia) ASA 64, 100 and 400 rated films. Both print and slide films were used.

Examination of fluorescent antibody stained sections was done using a Karl Zeiss F1450 Ultraviolet microscope using barrier filters 53/44 and 50/44. A magnification of 100 x was generally used. Photomicrographs of these sections were recorded using a Wetzlar Leica (E. Leitz, West Germany) camera mounted on the aforementioned microscope. Ektachrome (Kodak, Australia) speed rating 400 ASA was the film of choice. Exposures were made for 2 minutes.

3.7 THE MITOGEN PHYTOHAEMAGGLUTININ (PHA) AND DELAYED TYPE HYPERSENSITIVITY (DTH) TO CONTAGIOUS PUSTULAR DERMATITIS (CPD) IN THE SHEEP

To determine whether phytohaemagglutinin has the same mitogenic effect in sheep as has been reported in guinea pigs (Stadecker and Leskowitz 1974) and the chicken (Graeves *et al* 1968), six sheep were tested. So far as was known, none had been previously exposed to phytohaemagglutinin.

Each animal was inoculated with 0.1 ml PHA solution by intradermal injection using a 26 gauge needle on either the woolless thigh or axilla region. The PHA solution was a concentration of 700 µg/ml of PBS (pers. comm. Huynh 1983). The thickness of a pinch of skin over the site of inoculation and the width of the visible response were recorded before inoculation and 24, 48, 72 and 96 hours PI. A control inoculation of 0.1 ml sterile phosphate buffered saline was made and measured 24 hours PI to ensure that any increase was not due to mechanical injury at the site.

To establish the magnitude of the DTH reaction to CPD virus in sheep, two animals were hyperimmunized by inoculating by scarification on the thigh and allowing the infection to run its course. After all signs of the lesion had gone, 50 doses of CSL-047-1 were injected intramuscularly. This was repeated three times at weekly intervals, and then again 7 days before skin testing or macrophage isolation.

After a DTH could be shown in hyperimmune animals, it was attempted in artificially inoculated animals, the equivalent of field vaccinated animals.

Initially a dose level had to be found. A stock solution of 50 doses/ml of CSL-046/51 was serially diluted to a dilution of 10^{-9}. Five sites of one hyperimmune animal were each inoculated with 0.1 ml of each dilution. These were recorded at 24 hours PI as either positive or negative, according to induration, erythema and size of reaction. A control of 0.1 ml phosphate

buffered saline was included. This enabled a sheep DTH eliciting dose of 50% to be calculated by the method of Spearman Karber.

The magnitude of DTH skin reaction was measured in 2 hyperimmune, 6 ordinarily positive (after vaccination), and 1 negative sheep, following the intradermal injection of 0.1 of 5 doses of CSL 047-1 vaccine strain as previously described. The skin reaction was measured at 3, 24 and 48 hours, in two ways. Firstly, a pinch of skin at the lesion was made and measured with calipers. The site before injection was measured as a control and a saline only injection was included as a control. The second method measured the width of the lesion on the skin surface, also with calipers.

An attempt to inhibit macrophage migration using PHA and CPD virus as antigen was made for both hyperimmune and serum negative animals. The method used to isolate lymphocytes from whole sheep blood was as described for the isolation of human lymphocytes using Ficoll-hypaque (specific gravity 1.077) (Pharmacia 1983) (see Figure 5).

Figure 5: Method for Isolation of Lymphocytes using Ficoll-hypaque (specific gravity 1.077).

1. Dilute whole blood 1:1 with PBS.
2. Put 3 ml Ficoll-hypaque in 10 ml centrifuge tube.
3. Carefully layer 4 ml blood solution on top of Ficoll-hypaque, ensuring the two solutions are not mixed.
4. Centrifuge for 40 minutes at room temperature, 400 g.
5. Remove plasma.
6. Remove white cells with bulb pipette.
7. Add cells and 6 ml PBS in centrifuge tube, mix.
8. Centrifuge 100 g for 15 minutes.
9. Remove supernatant, resuspend cells in 6 ml PBS.
10. Centrifuge 100 g for 15 minutes.
11. Remove supernatant and suspend cells in approximately $\frac{1}{2}$ ml PBS (37°C).
12. Fill haematocrit tubes, spin at 5000 rpm for 2 minutes.

The method of Bradley (pers. comm. 1983) was used to carry out macrophage migration inhibition tests (see Figure 6).

Figure 6: Method for Estimation of Macrophage Migration and Inhibition.

1. Put 2 small dobs of silicone grease into each petri dish.

2. Take haematocrit tubes, score each gently with file exactly at cell-fluid interface and break at that point. With forceps, seat the cristaseal plug end of each sample in the silicone grease, ensure that the other end is seated firmly on the bottom, clear of any excess grease, and as close as possible to the middle of the dish.

3. Gently pour 3 ml of culture medium, with or without antigen, into each petri dish taking care not to pour it directly onto the cells.

   Culture medium used: 90 ml Minimum Essential Medium
   1 ml Penicillin/Streptomycin
   10 ml New Born Calf Serum
   Sufficient Ha HCo_3 to turn mixture slightly orange

4. Incubate at 37°C in 5% Co_2 for 24 hours.

Reading results at 24 hours:

1. Transfer petri dishes very carefully onto microscope stage. Beware of knocks and splashes.

2. Estimate size of migration area
   a) using a planimeter after projection of image, and tracing around the shadow, or
   b) for rough estimation, put onto low power and use a calibrated eyepiece to measure height and width of migration area.

Cells from hyperimmune, ordinarily positive and naive animals were tested against PHA and CPD antigens. The CPD virus antigen was used at various dilutions from 10^{-1} to 10^{-9}.

3.8 FLUORESCENT ANTIBODY TECHNIQUE

Biopsied, Day 7 lesions were serially sectioned as described previously. Fluorescent antibody staining was also as described previously. All sera were test sera, except for hyperimmune sera and serum from an unexposed (naive) animal which functioned as positive and negative controls. All sera were used at a dilution of 10^{-1}.

A ranking scale was devised to qualitate the fluorescence shown by sections. This ranking was: - (negative), and + to +++++ (positives).

To find which sections were the most suitable to use, biopsies from days 1, 3, 5, 7, 9 and 11 PI were sectioned and stained. The day/s which showed the most consistent, easiest to find, and strongest fluorescence were used for fluorescent testing. These sections were cultured using CSL 048-12 vaccine strain. The sections were stained with 2×10^{-1} hyperimmune sera, negative sera, and unstained sections were included to assess auto fluorescence.

To define the fluorescence exhibited by hyperimmune sera, it was diluted from 10^{-1} to 1.28×10^{-3} in doubling dilutions. Each of these 8 dilutions, as well as a negative serum (control), were used to stain 3 sections from each of days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 PI.

To determine if positive animals would have a sufficient antibody level to cause fluorescence, serum was taken from each infected animal at various times PI and all animals were bled prior to inoculation. Two animals (numbers 3 and 4) were bled at 2-day intervals following inoculation until Day 20. These sera were used in fluorescence tests to determine, firstly, if convalescent sera had a sufficiently high antibody level of cause fluorescence and secondly, to follow antibody level in serum from inoculation and through convalescence.

The fluorescent antibody test was also used to test sera of samples of flocks that either had CPD, or had had it recently. Twenty sera were obtained from a flock from the property where the PEA virus isolate originated. Nineteen sera were obtained from a flock from the property where the Kwat strain originated. Twenty other sera were randomly selected from a flock of 300 in which suspected CPD had first been seen in a large part of the flock 5 days earlier. Twenty more sera were randomly selected from serum samples taken 26 days after the first samples.

3.9 VIREMIA

To determine whether a viremia was produced following a simple inoculation on the thigh, two animals were tested in the following manner.

The animal was divided, on paper, into quarters of the left thigh (LT), the right thigh (RT), the left axilla region (LAR) and the right axilla region (RAR). This is illustrated in Figure 7.

Inoculation was made on the left thigh and test scarifications were made adjacent to the inoculation site and in two sites remote from the

inoculation site. Test scarifications were made daily from 1 day pre-inoculation, to 20 days post inoculation.

Figure 7: Illustration of Regions of Sheep used as Adjacent and Remote Sites Relative to Inoculation Site.

Scarifications were made adjacent to the site of inoculation by scarifying scarifying once only, from approximately 5 cm from the inoculation site right up to the lesion in a straight line. This method is illustrated in Figure 8.

It was necessary to scarify in this direction, and only once, to eliminate any possibility of transferring virus from the site of inoculation to the line at scarification via the scarifying instrument.

Figure 8: Test Scarification Adjacent to the Inoculation Site.

Test scarifications were made using a sterile disposable 18 g x 40 mm needle.

A positive response following scarification was recorded by the appearance of pustules on Day 3 or Day 4 post scarification (PS). A negative response was one which healed as a superficial, uninfected scarification does, that is, in the same way as the control scarification had done. Control scarifications were previously done using PBS.

CHAPTER 4

RESULTS

4.1 ELECTRON MICROSCOPY

Electron microscopy was used to show whether Contagious Pustular Dermatitis (CPD) virions were present in scab material suspected of being a lesion of CPD origin. It was used on scab material from four primary lesions and on scab material produced on animals which had had prior experience of the virus (*eg* vaccinated and hyperimmune animals). The results are shown in Figure 9.

Figure 9: Results of Various Samples When Examined by Electron Microscopy.

Sample	Result	Previous Experience of Animal
CSL 047-1	+	-
CSL/9/23	+	Naive
Kwat/1/33	+	Naive
Kwat/1/53	-	Naive
Pea Isolate	-	Unknown
Goat Isolate	-	Unknown
CSL/4/87	-	Experienced (vaccinated)
CSL/6/96	+	Experienced (vaccinated)

Contagious pustular dermatitis virus particles were found in four of the nine samples examined. In some cases large amounts of cell debris was present, particularly in the unspun preparations. Virions were less conspicuous in these preparations. In all the positive cases, viral particles were found consistently in the cleared and uncleared preparations, and in both replicates. Virions were abundant and were found almost immediately after focussing.

From these results it can be seen that virions can be found in lesions cultivated in naive animals, at least until 33 days post inoculation (PI). It is of interest to note that virions were present in scab material from Kwat/1 on Day 33 PI, and yet scab material from the same lesion on Day 53 PI yielded no virions. This is a similar finding to that of Romero-Mercado *et al* (1973a, b) who also had difficulty in finding virus in scabs found later in the course

of the disease. This prompted them to suggest that this is in apparent conflict with the traditional view of the epidemiology, that is, scabs which are shed onto the ground act as a source of new infection. It is possible then that only scabs shed early in the course of the disease may be the source of new infection. An alternative view is that the source of outbreaks is latently infected carrier animals. This alternative view was proposed by Robertson (1976) (cited by Robinson and Balassu 1981) in an apparent extrapolation of the above results of Romero-Mercado *et al* (1973a, b) and also be Buddle (1981). At present there is unsufficient evidence supporting this view for it to be adopted as the current thought on the epidemiology of CPD.

It is interesting to note that no virions were found in the original scab material from Kwat isolate (15/1/82) when examined by electron microscopy at the N.S.W. Department of Agriculture's Regional Veterinary Laboratory, Glenfield. However, the inoculation of the scab material onto the skin of a susceptible animal (# 1) resulted in the clinical signs of CPD infection (Figure 10). When scab material from this infection was examined (Kwat/1/33) CPD virions were found.

Figure 10: Clinical Signs of CPD from Isolate Kwat on the Thigh of a Susceptible Animal.

This indicates that virions in the original scab material may not have been concentrated enough to be readily found by electron microscopy, but that they were in sufficient concentrations to cause infection. Another possibility is that the sample sent to Glenfield was too small to yield satisfactory results because, according to Harkness *et al* (1977) the most important factor in detection of virus is the preparation of a suspension from as large a sample as possible of scab material, and preferably from more than one individual case.

During the course of this work two types of viral particles were found. These two types are shown together in Figure 11.

Figure 11: Electron Micrograph of Both Types of Viral Particles Found (x 45000).

The first, most common, particle type encountered was a brick or ovoid shaped particle with a thick, well defined envelope. This form is also notable for its dark staining, hence electron dense, centre. This form of CPD virus is known throughout the literature as the 'C' or clear form (Figure 12) due to its permeability to phosphotungstic acid (Nagington and Horne 1962; Nagington *et al* 1964; Robinson and Balassu 1980; Martin and Palmer 1982). These viral

particles were indistinguishable from those 'C' form particles described by Kluge *et al* (1972) and Martin and Palmer (1982).

Figure 12: Electron Micrograph of 'C' or clear form, of CPD virus (x 110000).

The second form of the CPD virus encountered was the better-known, more photogenic, 'M' or mulberry form (Figure 13). These particles, which had a rotational elipsoid shape, had a surface showing a criss-cross pattern of fine tubular structures. This pattern has often been called basket weave or a whorled surface filament pattern. Nagington and Horne (1962) report that these threads are about 9 nm wide and frequently appear as double strands suggesting tubes with hollow centres about 3 nm in diameter. The 'M' form particles found in this work were indistinguishable from those 'M' form particles described by Gerstl (1923), Spradbrow (1969), Kluge *et al* (1972), Martin *et al* (1982), and many other authors.

No difference in size was found between the M and C forms. Both were between 140 and 170 nm wide by 180 to 250 nm long. Size appears to be variable but the above correlates with the findings of most authors.

Figure 13: Electron micrograph of 'M' or Mulberry Form of CPD Virus (x 110000).

The difference between the two forms has not yet been made clear. By analogy with the vaccinia virus (Westwood *et al* 1964) it is probable that the C form has been penetrated more by the strain than has the M form, therefore causing optical obliteration of the superficial M structure and revealing internal structure down to the surface of the neucleoid.

Simple morphological characteristics such as size, shape, symmetry, number of capsomeres (if present) and presence or absence of an envelope are useful for initial classification of a viral particle. This can generally be established rapidly by electron microscopy. The ease of this technique and the apparent reliability of this method makes it a valuable tool for rapid diagnosis of a number of viral diseases, including contagious pustular dermatitis.

Often complicated techniques of viral replication and/or concentration by dialysis or centrifugation are used before electron microscopy is attempted. In this case neither viral replication nor concentration was necessary prior to electron microscopy.

Although this technique is used for rapid diagnosis of CPD, it would be of particular use in areas where CPD outbreaks are uncommon, or when an atypical form occurs. In both of these instances CPD may be easily mistaken for another, more serious exotic disease. This technique allows rapid confirmation of a clinical diagnosis that vesicles, papules, pustules and/or scabs were of CPD origin and not an exotic disease such as foot and mouth or bluetongue. This could be valuable in Australia, where rapid diagnosis is essential if an outbreak of exotic disease is to be controlled. It is possible that a lot of money and resources would be spent on isolation, and perhaps slaughter, should the condition be thought serious enough, following the initial diagnosis of a veterinarian who has not had prior experience with CPD.

Several outbreaks of atypical CPD have been reported (Darbyshire 1961; Gardiner *et al* 1967) in which the clinical signs closely resemble those of bluetongue. Both these cases relied on diagnosis by clinical signs, histology and transmission experiments, including susceptible and immune sheep as well as other species. These diagnostic techniques take a long time, and as well as allowing time for suspected disease to spread this will cause unnecessary and costly quarantine procedures to be adopted. Both the above cases proved to be caused by CPD virus and both authors stated that because of the similarity to bluetongue, rapid differential diagnosis was essential. The abovementioned electron microscopic technique would have been extremely useful in rapid diagnosis of CPD in these cases.

4.2 GROSS APPEARANCE AND HISTOLOGY

For a full account of the histology of normal, uninfected sheep skin, the reader is referred to Abdussalam (1957), Jubb and Kennedy (1965), and various other texts on the subject.

An understanding of the histology of normal skin and the details of keratinization is necessary because the normal degenerative processes of keratinization have frequently been confused with histopathological changes due to disease (Abdussalan 1957). Normal skin is the standard for comparison of infected skin (Figure 25).

4.2.1 External Appearance

Although the gross appearance of the lesion could be characterized as papular, pustular and scab stages on the main part of the lesion, it was noted that in some cases a small area at the periphery of the site of inoculation had stages less advanced than those at the centre of the lesion.

In the first two to three days PI erythema was apparent (Figure 14) closely followed by erythematous papule elevated 2 to 3 mm (Figure 15). A distinct vesicle stage, although reported by a number of workers (Darbyshire 1961; Gardiner *et al* 1971) was not found. By the fifth day, pustules were forming from the papules (Figure 16). These pustules became heavily suppurated over the next two to three days.

By Day 13 PI a scab layer had formed from the superficial necrotic mass (Figure 17). This scab layer remained attached until Day 30 when it began to split and lift from the skin (Figures 18 and 19). These scabs came off over the next 10 to 12 days (Figure 20) revealing healed epidermis with no sign of scarring or other permanent effects (Figure 21).

Figure 14: Erythema of Epidermis Following Inoculation by Scarification with CPD Virus (Day 2 PI).

Figure 15: Elevated Erythematous Papule (Day 3 PI).

Figure 16: Formation of Pustules from Papules (Day 5 PI).

Figure 17: Heavily Suppurated Lesion with Exudate and Cell Debris Beginning to Form Scab Layer (Day 13 PI).

Figure 18: Scab Layer Fully Formed (Day 20 PI).

Figure 19: Scab Layer Splitting Prior to Lifting Off (Day 30 PI).

Figure 20: Scab Layer Lifting Off (Day 35 PI).

Figure 21: Completely Healed Epidermis (Day 40 PI).

This artificially cultivated lesion was on the relatively protected thigh, and the animal was caged, thus restricting movement which may have resulted in the scab remaining attached for longer than normal. It is thought that in field cases (Figures 22, 23 and 24), or vaccinated animals in the field, that the scab would not remain attached for the length of time as a result of the animal's greater movement and contact with brush, rocks, etc., which would help to dislodge the scab.

Figure 22: Field Case of Contagious Pustular Dermatitis.

Figure 23: Field Case of Contagious Pustular Dermatitis.

Figure 24: Field Case of Contagious Pustular Dermatitis.

4.2.2 Histopathology

Within 24 hours a slight infiltration of the corium with mononuclear cells (Figure 26) was apparent. The epidermis at the site of inoculation was broken with a small crust of dried blood (Figure 27). Little evidence of viral changes was apparent, and it is possible that the cellular infiltration was a result of the trauma of scarifications at inoculation by scarification. The only change that was apparent which may have been virus-caused was a very slight thickening of the stratum granulosum. Fluorescent staining revealed no accumulation of viral antigens at this stage.

By 48 hours degenerative changes developed in the more superficial nucleated cells of the epidermis, particularly the stratum granulosum's keratinocytes, and other outer malphigian layers. The cytoplasm became clear and in some cases vacuolated (Figure 28a,b) and the nucleus shrank. The normally flattened cells of the stratum granulosum swell and this results in thickening of the epidermis (Figure 29). There is some inflammation of the dermis with

with hyperaemia, oedema and the continuing accumulation of mononuclear cells (Figure 30). The vacuolar degeneration in the epidermis advances laterally and towards the deeper layers of the stratum spinosum (prickle cells) and stratum basale. The external root sheath becomes involved, with reticular degeneration, similar to the epidermis, progressing from the epidermis down the external root sheath. Accumulation of viral antigens in the keratinocytes and outer malphigian layers as well as the external root sheath is evidenced by fluorescent antibody staining (Figure 31a and b). There is increased proliferation of cells in the malphigian layer which is two to three times as thick, particularly as evidenced by acanthosis (hyperplasia of the cells in the stratum spinosum). There is a thickening and down-growth of rete pegs which resembles an invasive carcinoma (Figure 32). This down-growth continues and is most apparent by Day 5 PI.

Figure 25: Histological Appearance of Normal Sheep Skin (Thigh) (H + E x 100).

Figure 26: Section of Skin 24 Hours PI showing Infiltration of the Corium with Mononuclear Cells (H + E x 100).

Figure 27: Section of Skin at Site of Inoculation 24 Hours PI showing Small Crust of Blood, Broken Epidermis, Cellular Infiltration and Slight Thickening of the Stratum Granulosum (H + E x 100).

Figure 28a: Reticular Degeneration of the Prickle Cell Layer Showing Clear and in Some Cases Vacuolated Cytoplasma (H + E x 100).

Figure 28b: Reticular Degeneration of the Prickle Cell Layer Showing Clear and in Some Cases Vacuolated Cytoplasma (H + E x 400).

Figure 29: Swollen Cells of the Stratum Granulosum and Increased Thickness of the Epidermis (H + E x 100).

Figure 30: Continuing Accumulation of Mononuclear Cells in the Dermis (H + E x 100.

Figure 31a: Fluorescent Antibody Staining Showing Accumulation of Viral Antigens in Keratinocytes, Outer Malphigian Layers and External Root Sheath (FAS x 100).

Figure 31b: Transverse Section of Follicle Showing Involvement of Root Sheath with Enlarged Cells (H + E x 200).

Figure 32: Thickening and Down-growth of Rete Pegs (H + E x 100).

By 72 hours, cytoplasmic inclusions of various sizes had appeared on the swollen cells at the stratum granulosum which were polygonal, and not the normal columnar form (Figure 33a and b). These cytoplasmic inclusion bodies remained as long as the swollen cells remained (until about Day 6 PI). The dermis becomes heavily infiltrated with inflammatory cells, chiefly neutrophils (Figure 34).

On the fifth day PI, cells at the centre of the lesions were becoming necrotic and lymphocytes were appearing in the dermis with the neutrophils. After the fifth day, hyperkeratosis was obvious and the superficial layers had combined, forming a scab of coagulated exudates and cell debris (Figure 35a and b). Proliferations beneath the scab is intense resulting in parakeratosis, acanthosis and pseudoepithelial hyperplasia. Fluorescent antibody staining revealed accumulation of viral antigens beneath this scab layer and perhaps within the scab itself (Figure 35b). Incomplete keratinization resulting in some nuclei remaining in the horn cells contributes to the thickness of the scab.

Figure 33a: Cytoplasmic Inclusions in Swollen Cells of the Stratum Granulosum. Note Polygonal Form of Cells not Normal Columnar Form, and Increased Infiltration of Neutrophils (H + E x 100).

Figure 33b: Cytoplasmic Inclusions in Swollen Cells of the Stratum Granulosum. Note Polygonal Form of Cells not Normal Columnar Form, and Increased Infiltration of Neutrophils (H + E x 400).

Figure 34: Heavy Infiltration of the Dermis with Inflammatory Cells, Chiefly Neutrophils. (H + E x 100).

Figure 35a: Scab of Coagulated Exudate and Cell Debris. Note Proliferation Beneath Scab and Acanthosis (H + E x 100).

Figure 35b: Fluorescent Antibody Staining Revealing Accumulation of Viral Antigens Beneath Scab Layer (FAS x 100).

The epithelium, beneath the scab, is still inflammatory but is regenerating. Regeneration continues beneath the scab until complete, which is just prior to the scab sloughing off, at 30 days PI.

It is notable that a number of skin appendages were conspicuously unaffected by the CPD virus. The hair bulb, sebaceous gland, merocine sweat glands and the arrector pili were not seen to be affected during the course of the lesion development.

This study describes the response to the CSL vaccine strain of CPD on the thigh of the animal. This histological picture may differ for more severe field strains as it may differ on different sites in the animals, particularly around the mouth.

4.3 THE EFFECT OF THE INTRADERMAL INJECTION OF THE MITOGEN PHYTOHAEMAGGLUTININ IN THE SHEEP

Phytohaemagglutinin (PHA) was found to elicit a cutaneous cellular response in sheep. The skin reaction was of a transient nature, peaking between 24 and 48 hours. The reaction was completely gone by 96 hours.

The skin reaction was grossly characterized by erythema and non-oedematous induration and thickening of the dermis. Histology of the skin reaction was not examined.

The results are shown in Figure 36, which shows the reaction of six animals to intradermal inoculation of PHA. Figure 37a and 37b graphically represents the reaction measured by thickness and width respectively.

The data presented here clearly shows that a cutaneous response can be elicited in normal, non-sensitized sheep by an intradermal injection of 70 μg of PHA. As a histological examination of the skin reaction was not done, no comment can be made about the cells involved in such a skin reaction. However, PHA and other mitogens have been shown to elicit basophil rich skin reactions in guinea pigs (Stadecker and Leskowitz 1974). By analogy it is probably that similar cells are involved in causing the response in sheep.

It is of interest to note that the skin reaction to PHA in sheep, where the reaction was still apparent at 72 hours is of a less transient nature than the same reaction recorded in guinea pigs by Stadecker and Leskowitz (1974) where the reaction was completely gone by 72 hours.

Figure 36: Skin Reaction at 24, 48, 72 and 96 Hours After Injection of 70 µg Phytohaemagglutinin.

	Time PI (hours)	Sheep Number 0	1	3	4	6	7	$\bar{X}$	SD
Saline control (mm)	24	2.0	1.3	1.4	1.1	1.8	1.5	1.5	0.33
Site before PHA injection (mm)	-	1.8	1.2	1.6	0.9	1.8	1.4	1.5	0.35
Thickness (mm)	24	5.8	9.3	6.0	10.7	9.0	4.5	7.5	2.4
	48	4.2	9.0	4.0	17.0	4.5	5.0	7.4	5.4
	72	2.6	6.2	4.2	10.5	1.9	3.2	4.7	3.2
	96	1.8	1.0	1.7	1.0	1.8	1.6	1.5	0.38
Increase in thickness (mm)	24	4.0	8.1	4.7	9.8	7.2	3.1	6.1	2.6
	48	2.4	7.8	2.4	15.3	2.7	3.6	5.8	5.4
	72	0.8	4.0	2.6	9.6	0.1	1.8	3.2	3.4
	96	0.0	0.2	0.1	0.1	0.0	0.2	0.03	0.14
Width (mm)	24	20	25	25	26	22	9.3	21.2	6.3
	48	5.8	15.5	17.5	-	8.8	8.5	11.2	5.0
	72	5.6	10.2	13.7	11.3	0.0	6.2	7.8	4.9

Figure 37a: Graphical Representation of Thickness of Skin over Time After Injection of 70 µg of Phytohaemagglutinin.

Figure 37b: Graphical Representation of Width of Reaction Over Time After Injection of 70 µg of Phytohaemagglutinin.

4.4 DELAYED TYPE HYPERSENSITIVITY TO CONTAGIOUS PUSTULAR DERMATITIS VIRUS

4.4.1 Skin Reaction

The delayed type hypersensitivity (DTH) eliciting dose 50% was calculated in a hyperimmunized sheep using the method of Spearman-Karber and the titre was found to be $10^{-4.5}$/ml. The results of this experiment are set out in Figure 38 as is the calculation of the 50% end point titre.

Figure 38: Concentration of CPD Virus Solution Required to Elicit a DTH Skin Reaction in a Hyperimmune Sheep.

CPD Virus Dilution	Total Positive (out of 5) at 24 hours
0*	5
-1	5
-2	5
-3	4
-4	1
-5	0
-6	0
-7	0
-8	0
-9	0
Saline	0

DTH eliciting dose 50% by method of Spearman-Karber

$$= x + 0.5 - \frac{\Sigma r_1}{n}$$

$$= 9 + 0.5 - \frac{30}{5}$$

$$= 3.5$$

$\therefore$ titre is $10^{-3.5}$/0.1 ml, or $10^{-4.5}$/ml

* 50 doses/ml CSL 047-1 vaccine strain.

Delayed type hypersensitivity skin reactions, characterized by non-oedematous induration and erythema, could be elicited in hyperimmune animals and in ordinarily positive animals after intradermal injection of a 10^{-1} dilution of CPD virus solution. No such reaction could be elicited in an unsensitized animal.

On their first skin test hyperimmune animals gave, on average, a 4.3% thicker skin reaction than ordinarily positive animals (Figure 39). It is important to note that only two hyperimmune animals were tested.

Figure 39: Increase in Skin Thickness in two Hyperimmune, Five Ordinarily Positive and a Negative Sheep 24 Hours after Skin Testing with CPD Virus.

Treatment	Increase in skin thickness (mm)	Mean (mm)
Hyperimmune	2.2	3.2
2 animals	4.1	
Ordinarily positive	3.5	3.0
5 animals	3.0	
	2.2	
	2.6	
	3.8	
Negative	0.1	0.1
1 animal		

The negative animal showed no DTH skin response after intradermal testing with CPD virus. As expected the animal went on to express the clinical signs of CPD infection at the site of inoculation. This was expected as the animal was naive and the CPD virus used as antigen was in no way inactivated.

The DTH skin responses in hyperimmune and ordinarily positive animals were maximum at 24 hours and much reduced, although not gone, by 48 hours (Figure 40a).

After initial skin testing, hyperimmune animals were tested several times. They showed no propensity to increase or decrease the magnitude of the skin response as the number of skin tests increased (Figure 40b).

Figure 40a: Delayed Type Hypersensitivity Skin Reaction Measured by Thickness of Skin at 3, 24 and 48 Hours after Intradermal Injection of 10^{-1} Dilution of CPD Virus.

		Sheep Treatment and Number							
		HYP		Ordinarily Positive					Naive
	Hours PI	0	3	1	4	6	8	9	10
Site before injection		2.0	1.0	1.5	1.6	1.0	1.2	0.9	0.9
Saline control	24	-	1.5	1.5	1.7	1.2	1.3	0.9	-
	3	2.1	1.0	1.5	1.8	1.0	1.5	1.1	0.9
Thickness (mm)	24	4.2	5.1	4.8	4.6	3.2	3.8	4.7	1.0
	48	2.2	4.3	3.9	3.5	3.2	3.0	4.1	-
	3	0.1	0.0	0.0	0.2	0.0	0.3	0.2	0.0
Increase (mm)	24	2.2	4.1	3.3	3.0	2.2	2.6	3.8	0.1
	48	0.2	3.3	2.4	1.9	2.2	1.8	3.2	-

Figure 40b: Increase in Skin Thickness (mm) in Hyperimmune Sheep 24 Hours after Skin Testing with 0.1 ml 10^{-1} CPD Virus Solution.

Hyperimmune Sheep Number	Skin Test Number					
	1	2	3	4	5	6
0	2.2	3.7	2.6	-	-	-
3	4.1	3.7	4.0	4.1	4.5	3.8

The histology of the delayed type hypersensitivity skin reaction in sheep to CPD was similar to the histology of the same reaction in humans to tuberculin and in guinea pigs to various antigens: that is, extensive infiltration with mononuclear cells (Figure 41a and 41b) consisting mostly of macrophages, as

Figure 41a: Delayed Type Hypersensitivity Skin Reaction to CPD Biopsied 24 Hours PI (H + E x 100).

Figure 41b: Delayed Type Hypersensitivity Skin Reaction to CPD Biopsied 24 Hours PI (H + E x 1000).

well as some lymphocytes. These cells were found in the dermis as well as the connective tissue, but particularly around the blood vessels. No haemorrhagic arteritis nor any central necrosis was noted.

Although there was a slight increase in thickness, there was no reaction as characterized by erythema and induration evident three hours after skin testing. The increase in thickness is thought to be due to the bulk of the solution injected. This absence of the antibody mediated Arthus reaction is further evidence to suggest that the humoral immune system may have only a minor part to play in the recovery from, and subsequent protection to CPD. This is in agreement with the findings of Buddle (1981) who found that virus neutralizing antibody titres could not be used to predict the degree of protective immunity to CPD. This finding is similar for a number of other poxviruses including sheep pox (Srivastava and Singh 1980) and fowl pox (Morita 1973).

As the continued skin testing of hyperimmune animals did not cause an increase or decrease in the DTH skin response it would seem that skin testing itself did not have a further immunogenic effect, at least not one that was reflected by the DTH reaction, on the host. It is possible that the initial skin testing gave the maximum skin response obtainable in this way.

The delayed type hypersensitivity reaction, observed as a result of intradermal injection of CPD virus, suggests that cell mediated immunity (CMI) as manifested by DTH, may have an important role to play in the recovery from and subsequent resistance to CPD. Cell mediated immunity is thought to play an important role in the immune response to a number of other pox viruses, one of these being sheep pox virus where it has been reported that CMI is of "vital importance" in the immune response (Srivastava and Singh 1980). Morita (1973) suggests that CMI has a far more important role to play than humoral immunity in recovery from and protection to fowl pox. It is suggested that CMI may be even more important in the recovery from and protection to CPD than previously thought as the DTH response was elicited without the use of Freund's adjuvant and was apparent in ordinarily positive animals.

Although CMI has not been reported to play an important role in the recovery and subsequent protection from CPD it is known that Dr L. Fastier (cited by Robinson and Balassu 1980) used the CMI response, as manifested by DTH (to heat inactivated CPD virus) to predict the immune status of animals. Buddle (1981) attempted to predict the degree of protective immunity to CPD using virus specific lymphocyte stimulation indicies, but was unsuccessful.

These results indicate that the study of DTH to CPD may be a useful technique to acquire information about the immune status of an animal. By the use of more refined CPD antigens this technique may be improved; at least the use of refined antigens would not contribute to the spread of the CPD. It is also suggested that CPD in sheep may be a good model for the study of CMI in its own right, because of the apparent importance of CMI relative to the humoral immune system, in the recovery from and protection from, CPD.

4.4.2 Macrophage Migration and CPD Virus

The results of the following tests, carried out to see if macrophage migration can be inhibited *in vitro* by CPD virus, have been included although the validity of these tests is doubtful. The validity of the tests is doubtful because migration was not consistently obtained. When migration was obtained it was highly variable, even within replicates as can be seen in Figure 42a and 42b.

Figure 42a: Macrophage Migration Inhibition Tests in One Naive and Two Hyperimmune Sheep. All Measurements are Undefined Units of Area.

		MEM		MEM + Ag*	
Animal	Index	3 replicates	Mean	3 replicates	Mean
Naive	97	25	43.0	49	44.3
		49		35	
		56		49	
Hyp No. 0	82	1	9.6	8	11.6
		14		9	
		14		18	
Hyp No. 3	25	1	2.0	9	8.9
		1		4	
		4		10.5	

* 10% 10^{-1} dilution of CSL 047-1.

Figure 42b: Macrophage Migration Inhibition Tests in Two Naive and Two Hyperimmune Sheep. All Measurements are Undefined Units of Area.

		MEM		MEM + Ag*	
Animal	Index	3 replicates	Mean	3 replicates	Mean
Naive	4.4	57.5	8.0	3	4
		11.7		4	
		55.5		6	
Hyp No. 0	66	34.5	34	40	30
		30.8		18	
		37.8		-	
Naive	49	129.0	90	66	35
		100.0		30	
		30.0		15	
Hyp No. 3	199	54.5	40	136	60
		36.5		54.6	
		30.0		38.5	

* 10% 10^{-1} dilution of CSL 047-1

Bradley (1983, pers. comm.) states that migration must be consistently in the order of 100 units for the controls if the test is to be considered successful. As can be seen in Figure 42a and 42b, the migration in the controls was insufficient for use as a test for inhibition of migration.

It was initially expected that there may be a correlation between the immune status of an animal and the amount of macrophage migration inhibition. If this were the case, this may have been shown to be a useful *in vitro* technique for obtaining information about the immune status of an animal.

The time available did not permit a sufficient proficiency to be gained in using this technique and therefore to yield results. Two possibilities for the lack of success with this technique were:

1. inability to isolate sufficient macrophages in the initial isolation procedure, and
2. inability to keep the isolated cells viable throughout the isolation procedure.

4.5 FLUORESCENT ANTIBODY STAINING

Initially a series of biopsies were taken and sectioned as previously described. Biopsy sections taken at two-day intervals were stained with appropriately diluted sera and FITC conjugated anti-sheep IgG (Miles Labs, Elkhart, Indiana U.S.A), and were examined to find which sections were the best antigen source for subsequent fluorescent testing. Figure 43 shows the results of this initial examination.

Figure 43: Examination of Sections from Various Days: Unstained and Stained with Hyperimmune Sera and Naive Sera

CSL/3 Day	Unstained	Sera + FITC anti-sheep IgG	
		Naive	Hyperimmune $\frac{1}{20}$
1	√	-	-
3	√	-	+
5	√	-	+
7	√	-	+
9	√	-	+/-
11	√	-	?

√ = examined and autofluorescence recorded.

+ = positive, specific fluorescence.

- = negative.

From this examination it became clear that the lesions which provided the best sections for fluorescent testing were Day 5 and Day 7 PI. These were found best as they had sufficient antigen caused fluorescence, without high non-specific or autofluorescence due to hyperkeratinization. Consequently for all subsequent fluorescent testing for serum antibody to CPD, sections from Day 5 and/or Day 7 were used.

This initial examination also showed that in most cases transverse sections (TS) of hair follicles were the best place to record fluorescence. Even though longitudinal sections (LS) of hair follicles gave a brighter, more defined fluorescence (Figure 44). Transverse (or oblique) sections were found to be better for the following reasons:

Figure 44: Longitudinal Sections Through Follicles, Stained with $\frac{1}{10}$ Hyperimmune Sera Showing Strong Fluorescence.

1. Autofluorescence in the area was relatively low, except for the wool fibre itself which allowed the follicle area to be found easily and quickly (Figure 45).
2. The same follicle persisted through a large number of serial sections enabling the same follicle to be found easily in each section which allowed a more accurate and controlled comparison of sections stained with various sera.
3. The inner and outer root sheath had large concentrations of viral antigens resulting in strong specificity (Figure 46a and 46b).

Having done this it was necessary to define the fluorescence which resulted from hyperimmune sera. This was done by making doubling dilutions in PBS of hyperimmune sera, starting at $\frac{1}{10}$ and diluting through to $\frac{1}{1280}$. These dilutions and a $\frac{1}{20}$ negative sera were tested across sections biopsied from Day 1 to Day 21 PI. The results are shown in Figure 47.

Figure 45: Unstained Transverse Sections of Hair Follicle Showing Low Autofluorescence.

Figure 46a: Transverse Section of Follicle Stained with Negative Sera.

Figure 46b: Same Transverse Section of Follicle in Same Area as Above, but Stained with $\frac{1}{10}$ Hyperimmune Sera. Note Strong Fluorescence.

Figure 47: Results of Fluorescence Using Various Dilutions of Hyperimmune Sera on Sections Taken From Day 1 to Day 21 PI.

CSL/8 Day	Negative	Hyperimmune Dilutions							
		$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{520}$	$\frac{1}{840}$	$\frac{1}{1280}$
1	-	+	-	-	-	-	-	-	-
3	-	+	+	-	-	-	-	-	-
5	-	+	+	-	-	-	-	-	-
7	-	+	?	-	-	-	-	-	-
9	-	+	+	-	*	*	*	*	*
11	-	+	-	-	*	*	*	*	*
13	-	+	+	-	*	*	*	*	*
15†	-	+	?	-	*	*	*	*	*
17†	-	+	?	-	*	*	*	*	*
19†	-	-	-	-	*	*	*	*	*
21†	-	-	-	-	*	*	*	*	*

* = not done

- = negative

+ = positive

? = unknown

† = difficult to interpret due to autofluorescence.

It can be seen in Figure 47 that a $\frac{1}{10}$ dilution of hyperimmune sera resulted in a more definite and more consistent fluorescence than the recommended $\frac{1}{20}$ dilution. In the following experiments a $\frac{1}{10}$ dilution of hyperimmune sera was used.

It is clear that fluorescence only occurred when hyperimmune sera was not diluted past $\frac{1}{20}$. The large quantities of autofluorescence in lesions from Day 13 onwards made it difficult, if not impossible, to decide what was specific fluorescence.

To follow the rise and fall of antibody levels through infection and convalescence, the aforementioned fluorescent antibody technique was used. Sera was taken at various intervals from two infected sheep from Day 0 to Day 96 PI, diluted to $\frac{1}{10}$ and used to stain sections taken by biopsy from an animal

vaccinated with the same strain of CPD virus (CSL vaccine strain 047-1) five days PI.

In the first sheep, fluorescence was first seen at Day 7. This fluorescence persisted right through until Day 39, but was not present in sera taken 57, 76 or 97 Days PI. Sera from the second sheep showed no fluorescence at any time.

Sera which was positive at the $\frac{1}{10}$ dilution was not positive at a $\frac{1}{20}$ dilution. This indicates that it does not have as high an antibody level as hyperimmune animals (as expected). As the positive sera could not be diluted to, or past, a $\frac{1}{20}$ dilution, no comment can be made about antibody levels in the sera except to say that until Day 39 antibodies were present at a sufficient level to cause fluorescence. After this time, antibodies to CPD (if present) were not present in a sufficient quantity to cause fluorescence.

To ascertain if this method of fluorescence would be of diagnostic use, sera from five artificially inoculated, known positive animals and sera from Kwat property (19 sera), Pea property (20 sera) and the University farm (20 sera each from two separate visits) as well as hyperimmune sera and negative sera, were tested.

Field sera was taken from flocks which were thought, by the owner, to have had CPD in the recent past. Twenty sera from the University farm flock of 300 were taken randomly, initially from the fifth day after the first signs of the disease were noted. Twenty more sera were selected, independently of the first, 31 days after the disease was first recorded. The person who bled the flock said that "a large part of the flock was infected". Taking a conservative estimate of 50% to be infected gives the chance of missing a positive in the first or second bleeding as one in 10.5×10^5 and missing a positive in the first and second bleeding as being one in 9.09×10^{13}. The results of the test are shown in Figure 48.

Field sera were found to be negative in all cases. Two of the known vaccinated animals were positive and three were negative. This result is not consistent and it seems that this fluorescent antibody technique is not reliable as a diagnostic test, nor was it successfully used as a method for quantitative analysis of antisera.

Figure 48: Fluorescence From Sera Taken From Laboratory and Field CPD Infected Animals.

Sera	Fluorescence (number positive)	Days PI sera collected
CSL/3/10	1/1	10
Kwat/1/12	1/1	12
CSL/4/12	0/1	12
CSL/3/14	0/1	14
HYP No. 3	1/1	50
Kwat Sera	0/19	Unknown
Pea	0/20	Unknown
Kirby	0/20	≃ 5*
	0/20	≃ 31*
Naive	0/1	-

* Days after first signs were noted.

There are a number of possible reasons why this technique was found not to be definitive enough for diagnostic use. The sections themselves may have been a poor antigen source and, to complicate this, the antigen present in sections may have been antigenically dissimilar to the antigen that the sera was raised against, therefore resulting in less antigen-antibody complexes. For example, test sera raised at Kwat propery was raised against the Kwat strain of virus but, by necessity, tested against antigen from CSL vaccine strain. This would not be the case if the earlier held view of strain variation held true, *ie* that there is no strain variation (Glover 1932). However, a number of workers including Darbyshire (1961), Valder *et al* (1961), Trueblood *et al* (1963) and Buddle (1981), suggest that strain variation is a factor in CPD. This has been suggested on the basis of cross-protection (Trueblood *et al* 1963; Buddle 1981), differences in mortality and morbidity (Robinson and Balassu 1981) and the appearance of atypical forms of the disease such as the ulcerative-musocal form described by Darbyshire (1961) and the malignant form described by Valder *et al* (1961).

The most likely reason for this technique being unsuitable for a diagnostic test is that the antibody titre in test sera was too low to cause

fluorescence. This is in apparent agreement with the findings of Buddle (1981) who found that the antibody response to CPD was not great. As the hyperimmune sera had only sufficient antibodies to cause fluorescence at a titre of $\frac{1}{20}$ then it is logical that field sera would not give fluorescence as it is expected to have antibody levels much lower than hyperimmune sera.

The presence of CPD in the flocks 'Kirby' and 'Pea' was unconfirmed. It is possible therefore that it was not CPD and the tested animals had no serum antibodies to CPD as they were, in fact, naive.

The sheep that were selected from flocks were sheep thought to have been infected with CPD. However there was no certainty of this and it is possible, though unlikely, that they were naive through chance (*ie* random sampling).

A further possibility as to why the sera from Kwat and Pea flocks may have been negative is that the time of bleeding may have been too long since infection for the sheep to be retaining sufficient serum antibodies to cause fluorescence. This is possible, but unknown, as the time from infection until bleeding was not recorded.

The field sera being negative is in conflict with the findings of fluorescence in the laboratory infected animals. Two possible reasons for this fluorescence in laboratory animals are:

1. the sera was raised to CSL 046-51, the same antigen as in the sections
2. serum antibody levels in these animals may have been higher than those in field cases because of the large doses of virus used when inoculating.

Diagnosis of CPD with a fluorescent antibody technique has been performed on human orf by Erickson *et al* (1975) who used CPD virus grown in tissue culture as antigen source. This method may prove useful in the diagnosis of CPD in sheep. This technique is used with a large number of viruses including sheep pox (Davies and Otema 1981), where it was possible to dilute positive sera to 10 $\log_2$ units and still obtain fluorescence. This may reflect a greater antibody stimulating effect of SPV on the host, or it may reflect a greater sensitivity of the method in detecting serum antibodies.

4.6 VIREMIA

Figure 49 shows the results of the experiment carried out to determine if a viremia occurs following inoculation of CPD on the thigh. It can be seen from the table that no infection occurred following scarification at the two remote sites on the animal. Infection did occur in the immediate vicinity of the initial inoculation site following scarification from one day pre-inoculation to 15 days post inoculation. This localized viremia was observed up to a distance of 58 mm (Day 0) from the initial site of inoculation.

Figure 49: Infection at Local and Remote Sites Following Scarification on One Thigh as a Test for Viremia in Two Sheep (numbers 9 and 11).

	Adjacent			Remote Axilla		Remote Thigh		Challenge Axilla	
Day	9	11	$\bar{x}$	9	11	9	11	9	11
-2	ND	-	-	-	ND	-	ND	ND	ND
-1	ND	+ (50)	51	-	ND	-	ND	ND	ND
0	+ (52)	+ (58)	55	-	-	-	-	ND	ND
1	+ (44)	+ (50)	47	-	-	-	-	ND	ND
2	+ (48)	+ (49)	48.5	-	-	-	-	+	ND
3	+ (30)	+ (30)	30	-	-	-	-	+	ND
4	+ (50)	+ (42)	46	-	-	-	-	+	ND
5	+ (46)	+ (41)	42.5	-	-	-	-	+	ND
7	+ (51)	+ (38)	44.5	-	-	-	-	+	ND
9	- (0)	+ (46)	23	-	-	-	-	-	ND
11	+ (20)	+ (22)	21	-	-	-	-	-	ND
13	+ (38)	+ (3)	20.5	-	-	-	-	-	ND
15	-	+ (4)	2	-	-	-	-	-	ND
17	-	-	0	-	-	-	-	ND	ND
19	-	-	0	-	-	-	-	ND	ND
21	-	-	0	-	-	-	-	ND	ND
23	-	-	0	-	-	-	-	ND	ND

ND = not done

+ = positive (mm)

- = negative

It should be noted that the maximum distance recorded may not be the maximum extent of this localized viremia because this positive reaction was the full length of Day 0's scarification. This scarification length was limited because it abutted the wool bordering the thigh region.

These results suggest that a systemic viremia is not present, or, if it is, sufficient quantities of virus are not present in the circulatory system to initiate a lesion at remote sites. There is some evidence that a viremia does occur. Walker (1964) reported infection of the ears after earmarking infected sheep and Jubb and Kennedy (1963) report lesions occurring at the site of mulesing operations. In both these cases however, the appearance of lesions may be due to contaminated instruments rather than a viremia. More conclusive evidence is apparent in a report by Darbyshire (1961) who found CPD lesions on numerous internal organs of infected animals, including the heart, lungs and gastrointestinal tract. It should be pointed out that this was an atypical case in many ways including a high mortality (up to 78%) which was not reportedly due to any secondary infection, and the pathology of the lesion which exhibited an uncharacteristic ulcerative mucosal form.

These results need to be considered carefully before drawing conclusions. Each scarification line, although the positive result may have been 58 mm for example, was adjacent to the previous scarification. Consequently the last two or three test scarifications may have been close enough to the most recent scarification to be producing the viremia that was recorded, that is, the recorded viremia may have been emanating from previous positive test scarifications and not the initial inoculation site. If this is the case then this test is recording the animals' ability to suppress infection rather than viremia from the initial inoculation. This is considered unlikely in this case as the challenge dose on animal Number 9 showed resistance to challenge was not complete until Day 7, six days before positive scarifications were no longer recorded.

Despite the fact that no viremia signs were demonstrated in this way it is important to note that some resistance of the skin has been shown elsewhere by challenging and the fact that vaccination on the thigh confers at least some immunity to CPD.

A possibility as an alternative method to test for viremia in the host is to collect blood at intervals after infection and then attempt to isolate CPD virus from this blood. This method, being more direct, may be more

effective in yielding information about the presence or absence of a viremia in a CPD infected host.

It is important to note that in these trials, involving only two sheep, both were initially inoculated with CSL vaccine strain 046-51 and so these results are not necessarily a reflection on the viremia that may or may not be produced following infection with other strains of CPD virus. It is also possible that experimental lesions on the thigh region may not be equivalent to natural lesions on the mouth or other parts of the body, with respect to viremia production. Consequently it must not be assumed these results will necessarily apply to field cases.

4.7 OBSERVATIONS CONCERNING CHALLENGE AFTER PREVIOUS INFECTION

Five animals were challenged by scarification and inoculation at some time after the clinical signs of the initial infection had gone. Each animal was challenged at the same site that was initially infected, as well as at a site remote from the initial infection, *ie* on the opposite thigh to the initial infection. Figure 50 shows the results of these challenges.

Figure 50: Results of Challenge at the Same Site and at a Site Remote From the Initial Infection.

Sheep Number plus virus strain	Days PI to to Challenge	Challenge Site		Confirmation		
		Local	Remote	EM[+]	Inoculation[†]	Histology
1 Kwat	50	-	+*	-	ND	ND
3 CSL	87	-	-			
HYP 3 CSL	114	-	-			
4 CSL	86	-	+	-	+	ND
6 CSL	90	ND	+	+	+	+
8 CSL	47	-	-			

[+] EM = electron microscopy.

* Challenge site on axilla, not opposite thigh.

[†] Inoculation onto a susceptible animal followed by confirmation by EM.

ND = Not done.

It should be noted that challenges regarded as positive on the clinical signs were all very much reduced in severity. Perhaps this was the mild infection often called the 'accelerated' response, that has previously been recorded in experienced animals by Richter and Jansen (1968), Schmidt (1967) as cited by Robinson and Balassu (1981). It is also important to note that only two of the three positives were confirmed as being of CPD origin.

It can be seen in Figure 50 that of all the animals challenged none showed a positive response to challenge at the site of initial inoculation, however three of the five showed a positive response to challenge at the remote site. This casts some doubt on the efficacy of vaccination by scarification on the thigh.

It has been stated that "orf virus is highly epitheliotrophic thus inducing skin immunity" (Darbyshire 1961; Robinson and Balassu 1981). These statements have been criticized in the context of current immunological understanding as being unhelpful. However there is a general tendency to view the immune response as being of the whole animal, when in fact specific organs may be specifically resistant. This general outlook on immunity as invariably involving the whole animal may need to be revised. Buddle (1981) has suggested that the immune response to CPD is not systemic and that this explains the sometimes ineffectiveness of vaccination. These results, as well as the results of others previously cited, suggest that there may in fact be a propensity for the site of previous infection to have greater resistance to subsequent infection than other sites. The possible mechanisms for this are unknown.

Similar findings as these, and the fact that CPD virus can remain active in various leucocytes, prompted Buddle (1981) to suggest that vaccination may be more effective following the advent and use of a parenterally administered, fully active vaccine.

CHAPTER 5

GENERAL DISCUSSION

A brief discussion of each of the results sections has been included in the previous chapter. Here these discussions will be expanded upon.

This work poses a number of questions which remain unanswered. What are the differences between the 'M' and 'C' particle forms? To what extent, and how, are the cell mediated immune responses implicated in the recovery and subsequent protection from CPD? Why is the humoral response to CPD only slight and relatively short lived? If a viremia is not present, why then, and how, is there local migration of virus around the site of infection to nearby interruptions in the epidermis? If a local skin immunity exists, how does it occur compared with a systemic immunity? An effort will be made to discuss these questions and others in the light of the results obtained and those documented in the literature.

Two forms of the CPD viral particle, the 'M' or mulberry form and the 'C' or clear form, have been described here and elsewhere (Nagington and Horne 1962; Nagington *et al* 1964; Kluge *et al* 1972; Martin and Palmer 1982). The differences between these two forms of CPD viral particle have not yet been made clear. However, it is likely to be the same difference that is seen between 'M' and 'C' forms of other poxviruses, *eg* Vaccinia. By analogy with vaccinia virus, as described by Westwood *et al* (1964) it is probable that the 'C' form has been penetrated more by the electron dense negative stain than the 'M' form. This causes the optical obliteration of the more superficial layers of the 'M' form particle and reveals the internal structure down to, and within, the nucleoid. Westwood *et al* report that the 'C' form is larger than the 'M' form of vaccinia virus which allows for the theory of differing penetration of stain. No such size difference between 'M' and 'C' forms of CPD virus exists, so it is possible that the differing types of CPD virus are due to some factor other than differing penetration of stain. Nagington *et al* (1964) assumed that the two forms differ due to differing penetration of stain but fail to demonstrate this conclusively as did Westwood *et al* (1964) for the vaccinia virus.

The mitogen, phytohaemagglutinin has clearly been shown to cause a cutaneous cellular response in normal non-sensitized sheep when injected intradermally. A similar reaction has been investigated and demonstrated previously in a number of other experimental animals including the guinea pig

(Stadecker and Leskowitz 1974), the chicken (Graeves *et al* 1968) and the mouse (Stabo and Paul 1973) where it elicits a basophil-rich skin reaction. Phytohaemagglutinin has been shown to be a predominantly T-cell stimulant in the guinea pig (Stadecker and Leskowitz 1974) and the mouse (Stabo and Paul 1973) although the immunological significance of the in vivo transformation of these lymphocytes remains undefined. The reaction in sheep is probably the same as that in guinea pigs and mice, but the mechanism or mechanisms by which T cells attract basophils has not yet been demonstrated, although Stadecker and Leskowitz (1974) suggest that normal T cells may release a basophil chemotactic factor on contact with PHA or other suitable mitogen. Stadecker and Leskowitz (1974) had implicated the T cell in this reaction by abolishing the cutaneous basophil response with specific anti-guinea pig T cell serum. Graeves *et al* (1968) had earlier shown that the in vitro transformation of chicken lymphocytes in response to PHA depended on thymus derived lymphocytes by depressing or abolishing this transformation in neonatally thymectomized chickens when compared to normal or bursectomized chickens. In future work in this area it would be useful to investigate the histology of the reaction to establish the cells involved and to determine if PHA elicits a basophil-rich skin response, as it does in other species. It would also be useful to confirm the suspected role of the T lymphocyte in eliciting this skin reaction. This could easily be done by showing that the response can be abolished following the parenteral administration of specific anti-sheep T cell serum as done by Stadecker and Leskowitz (1974) in the guinea pig or following neonatal thymectomy as has been done in the sheep and mouse when investigating delayed type hypersensitivity reactions (Cole and Morris 1973).

The humoral immune response of sheep to CPD virus infection was monitored using an indirect fluorescent antibody staining technique. It was found that the antibody response to CPD infection was slight and lasted only five to six weeks. This is in line with the findings of other workers. Buddle (1981) found the antibody response to CPD to be low and it could not be used to predict the degree of protective immunity of the sheep to CPD. Abdussalam (1958) found only low levels of agglutinating, precipitating, complement fixing and neutralizing antibodies in the sheep three to four weeks after infection. These antibodies had disappeared by five months PI. Koptopoulos *et al* (1982) found that cytotoxic antibodies developed within one month of experimental infection and persisted for only two to four weeks. In this work it was also found that hyperimmunized sheep had only a marginally higher titre of antibody than normally infected experimental animals which is in

agreement with the findings of Koptopoulos *et al* (1982) who found that the titre of experimentally infected animals did not rise significantly after a first or second re-exposure nine to 16 weeks after the initial infection.

It is interesting to note that no antibody was detected by the fluorescent antibody staining technique in the sera of naturally (field) infected sheep, which suggests that field animals are not exposed to as large an amount of virus or that the differing route of infection results in a less (humoral) immunogenic effect. Koptopoulos *et al* (1982) however, found that antibodies could be detected in the sera of naturally infected sheep using an indirect fluorescent antibody technique but also found no cytotoxic antibodies in the sera of naturally infected sheep although both Koptopoulos *et al* (1982) and DeMartini *et al* (1978) found cytotoxic antibodies in the sera of experimentally infected animals. Other workers also have not found antibodies in the sera of naturally infected sheep (Trueblood *et al* 1963; Schmidt 1967), but Nagington and Whittle (1961), Poulain *et al* (1972) and Jan *et al* (1978) have all found neutralizing antibodies in the sera of normally infected sheep. Such incongruities in the literature suggest that antibody response of sheep to CPD virus is highly variable. Such variation is possibly due to individual variation between animals, strain variation and variation in the sensitivities of the tests used to detect such antibody levels.

Initially the indirect fluorescent antibody technique was thought to be relatively insensitive, however Koptopoulos *et al* (1982) thought it was probably the most useful technique for investigating the antibody response to CPD when compared with enzyme linked immunosorbent assay (ELISA), complement fixation and ^{51}Cr release assay of cytotoxic antibody. Erickson *et al* (1975) also used indirect immunofluorescent antibody staining in the diagnosis of human orf, using virus grown in tissue culture, and reported this technique to be successful.

Future work in this area would do well to compare all the possible techniques for estimation of serum antibodies to CPD with a view to finding a reliable serological technique, should one exist, for it can be seen that as yet, no generally reliable serological test has been proposed which can be used to predict with confidence the prior experience or immune status of an animal. It is possible though, that no such technique exists due to the apparent relative unimportance of the humoral immune system in recovery and subsequent protection from CPD.

Because it has been shown here and elsewhere that the antibody response in the sheep to CPD is not great it is suggested that the humoral immune system does not play a major role in the recovery and subsequent protection from CPD. This has also been suggested as a result of similar findings for other pox viruses, including sheep pox (Srivastava and Singh 1981) and fowl pox (Morita 1973).

By default it is probable that the cell mediated immune response is a major factor in recovery and protection from CPD and, in any case, it has been clearly implicated by the elicitation of a cutaneous delayed type hypersensitivity reaction with CPD. Such a delayed type hypersensitivity has been shown in various species to various antigens. A delayed type hypersensitivity reaction, dependent on lymphocytes of thymic origin, has been shown in the mouse to ovalbumin and tuberculin (Russe and Crowle 1965) and in the sheep to tuberculin (Cole and Morris 1973). Srivastava and Singh (1982) have shown a delayed type hypersensitivity reaction in sheep to sheep pox virus. These findings are in line with those of Blanden *et al* (1977) who suggest that cell mediated immunity is of major importance in the recovery and protection from pox virus infection.

The fact that no Arthus reaction, a humoral mediated immune reaction, was seen as a result of intradermal testing with CPD in hyperimmune sheep is further evidence that the humoral immune system is not a major factor in the recovery and protection from CPD.

It is suggested that the delayed type hypersensitivity as a result of skin testing with CPD viral antigen may well be used to gain information about the immune status of an animal or a flock of animals in the same way as skin testing with tuberculin gives information about the prior experience of a sheep (and other species) to bacillis Calmette-Guerin (BCG) or other Mycobacteria. A delayed type hypersensitivity reaction to CPD, so far as is known, has not previously been reported in the literature except for an unpublished report by Fastier (1981) cited by Robinson and Balassu (1981), who has reportedly used heat killed virus as an antigen to elicit a delayed type hypersensitivity reaction in experienced sheep. Because of the ease with which a delayed type hypersensitivity skin reaction was elicited, the apparent importance of cell mediated immunity in recovery and protection from CPD, the ease with which the CPD virus may be cultivated etc, and the importance of the sheep as an agricultural and experimental animal, it is suggested that CPD may be a particularly useful means of studying cell mediated immunity, as manifested by

a delayed type hypersensitivity reaction, in its own right. In future work looking at delayed type hypersensitivity in sheep or other experimental animals to CPD, it would be of particular interest to assess the use of inactivated viral antigens (heat killed, formalized or otherwise) in eliciting delayed type hypersensitivity reactions which would not therefore contribute to the spread of virus in the environment nor infect naive experimental animals. Future work would also do well to confirm the suspected role of thymus derived lymphocytes in evincing a delayed type hypersensitivity reaction by abolishing the reaction in sheep which have been thymectomized (either surgically or by irradiation) or have been treated with specific anti-sheep T cell serum as has been done for the delayed type hypersensitivity response to tuberculin in sheep (Cole and Morris 1973) and to tuberculin and ovalbumin in the mouse (Russe and Crowle 1965).

The question of whether a viremia occurs as a natural part of an infection with CPD has, as far as is known, never been questioned let alone resolved. A systemic spread of CPD virus (viremia) was not demonstrated, however, a localized transfer of virus along lines of scarification adjacent to an infected site was shown. It is suggested that this "localized viremia" is not actually a viremia but just cell to cell transfer of virus as a result of cell lysis and simple diffusion of virus in the regenerating epithelial layer, although this has yet to be shown. It has been reported by a number of authors that infection occurs at various sites (udder, rump following mulesing, ears following ear marking and other wool-free areas) (Walker 1964) in animals already infected on the mouth, yet no attempt has been made to distinguish whether this infection is due to normal mechanisms (breaks in skin and entrance of virus, contamination of instruments etc.) or whether this is the result of a viremia. It is important to note that in this trial, in which no viremia was demonstrated, only two animals and only the one vaccine strain of virus was used. It is possible also that the method of scarification used, although resulting in takes adjacent to the site of infection and when inoculating, may not be the most effective way of testing for viremia. Perhaps a more effective way of determining or refuting the presence of a viremia would have been to attempt to isolate virus from leucocytes collected at various intervals after infection, for Buddle (1981) reports that the CPD virus can replicate in buffy coat cells in vitro.

Should a viremia occur it is expected that a systemic immune response would result which may or may not result in protective immunity of the animal following vaccination or normal infection. Such protection has been reported

by a number of workers including Schmidt and Hardy (1935), Abdussalam (1958), and CSL (1981). However, the presence of a systemic immune response does not necessarily establish the existence of a viremia as such a response may be due to the normally occurring processes of T and B cell proliferation. Similarly, the absence of a measurable systemic immune response does not necessarily preclude the existence of a viremia as the viral antigen may naturally be of low immunogenicity. Minimal systemic immunity has been reported by Buddle (1981) who found both low levels of neutralizing antibody and minimal lymphocyte transformation responses and Schmidt (1967) who found only low levels of neutralizing antibody. Should a viremia be necessary to induce a good immune response then it seems, as the vaccine strain showed no viremia, that the efficacy of this strain as a vaccine may be less than optimal.

It remains possible that a viremia does exist in CPD. Future work in this this area should use a number of different strains of virus, and should examine other methods of establishing the existence of a viremia such as the aforementioned technique of attempting to isolate virus from leucocytes.

Following on from these findings, it becomes clear that a major question to arise from this work is that brought about by the apparent ability of different sites on the body to have a different propensity for resistance following infection. This shall be referred to as 'local skin immunity'. A 'skin immunity', the result of CPD virus being highly epitheliotrophic, has been stated by Aynaud (1923), Glover (1928), Boughton and Hardy (1935) and Darbyshire (1971). These statements have been criticized by Robinson and Balassu (1981) as being unhelpful in the light of current immunological understanding. Presumably such skin immunity, also found by Wittman and Mayr (1960) to fowl pox, refers to the ability to resist challenge via the cutaneous route of inoculation, while not necessarily implying that the skin has greater immunity than any other organ. It was found that two of six experimental animals were susceptible to challenge at sites remote from the initial infection (thigh) although resistant to challenge at the same site as the prior infection. Such a localized skin immunity has not been reported in the literature but it could certainly explain the numerous reports of the failure of vaccination, such as reported by Buddle (1981) who found 17% of vaccinated lambs were not fully protected when challenged four weeks later. The findings reported here on the differing immunity at different sites are at least partially supported by those of Schmidt (1967) who found that vaccinated sheep were resistant to infection of the lips for up to five months PI, but could be infected on the thigh (or udder, or genital region) as early as two months PI. Schmidt (1967),

when reporting this variation in duration of immunity for different sites found also that the duration of the length of immunity for the feet was longer than the thigh, udder or genital region. It is possible that these findings of Schmidt (1967) may support the findings reported here because Schmidt's animals while in the field, may have been continually challenged on the mouth and feet, the same sites reported by Schmidt (1967) to have the greatest resistance to challenge. Similar differences in sites and duration of immunity are reported by Kovalev *et al* (1971) cited by Robinson and Balassu (1981).

The scarification method of challenging although used successfully throughout the literature and in this work, may allow some variability to creep into the trial. Although this is a possibility it can only be pointed out that scarification as a method of challenging and inoculation was used successfully and without variation throughout this work, except for one animal which resisted inoculation in this way several times.

This work demonstrates a previously unreported local skin immunity which is in conflict with some previous work. What may be seen then is the enormous amount of variability which surrounds this aspect of CPD. There are similar findings for many other aspects of CPD including the humoral response to infection and the length of immunity conferred by vaccination. In any case this work does point out that challenging, if to be a valuable method of establishing immunity, must be done at a number of sites on the animal and that the large variation in the literature may possibly be due to variation in the site of challenge. It also points out that the time from vaccination until naturally occurring field infections (presumably via the mouth) cannot be considered parallel with data obtained from experimentally vaccinated animals challenged at some later time to determine duration of immunity, unless challenge is performed on the mouth. Similarly, comparisons of duration of immunity can only be made if the challenge sites are the same.

Although some question of the efficacy of the vaccine is raised here and elsewhere by finding low or no protective immunity following vaccination (Trueblood *et al* 1963; Buddle 1981), there are many reports of good immunity following vaccination (Boughton and Hardy 1935; Walker 1964; Zach 1979; CSL 1981) including life-long immunity, which in the field is most likely to be the result of continual challenge with endemic virus rather than the vaccination alone.

Future work should, of course, confirm and fully examine this question of differing immunity at different sites. Only with a more complete knowledge of this differing duration of immunity can any attempt be made to determine the possible mechanisms for such local immunity.

Many casual observations and thoughts about CPD have been reported. However, as can be seen, little experimental work or other conclusive evidence concerning CPD has been published. There is still conflict about the issue of strain variation, and the traditional view of epidemiology of CPD is in question. The role of cell mediated immunity in recovery and protection from CPD has barely been examined. In view of this a study in this area should prove to be particularly useful in many ways, both to improve knowledge of the immune response and virus in general, as well as to improve vaccination, treatment and control of the disease. These gaps in the knowledge of CPD such as epidemiology, strain variation and the role of cell mediated immunity are important in the understanding of the disease, and so until these questions have been more fully resolved, progress in the field of CPD will be hindered.

Although CPD in sheep is not a particularly serious disease, it is important because humans can contract the disease which causes irritation, if not dangerous skin lesions, and results in industrial relations problems. It is also important because current vaccination procedures are not always effective and contribute to the spread of virus. It is for these reasons that more research into the control and prevention of the disease is required.

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LITERATURE REVIEW: SHEEP POX

Sheep pox is a serious disease of sheep, caused by a pox virus, which remains a problem in many parts of the world. Currently sheep pox is found in Asia, Africa and Europe, particularly in the Middle East and around the Mediterranean. Sheep pox was reported several times in England last century but has not been seen since. It remains a notifiable disease in Great Britain (BVA 1955). It has never been recorded in Australia, New Zealand or the Americas (FAO 1972).

Sheep pox was first recorded in the second century AD, according to Huytra *et al* (1946) and it was possibly introduced to the western world with infected animals from Central Asia (Singh *et al* 1979). Sheep pox was first recorded as being a contagious disease in 1763 by Bourgelet (Singh *et al* 1979) who noted that uninfected animals would contract the disease when run with infected animals. The aetiology and pathogenesis of the disease were first studied by Chaveau (1868), Bollinger (1878) and Borrel (1902) (Singh *et al* 1979).

The disease remains important in many areas because of its highly infectious nature and because of the high mortality and large depression in productivity resulting in severe economic loss. As far as is known, no large scale estimates of economic loss have been made, possibly because of the difficulty in recording the losses due to sheep pox in many underdeveloped nations, which are often the nations primarily affected by sheep pox.

1. THE DISEASE AND HOST

1.1 Hosts

Sheep pox is primarily a disease of sheep but there are a number of reports of goats becoming naturally infected (Scott 1966). There is much conflict in the literature as to strain variation and whether sheep pox which infects goats is really goat pox which infects sheep. Such conflict is not constructive and further work is required on the basis of serological and other tests to establish the relationship between sheep and goat pox viruses and the other vertebrate pox viruses.

It is known that different breeds of sheep have differing susceptibilities to sheep pox. Plowright *et al* (1959) and Donatien *et al*

1947) have found that Merinos and Kivinik breeds are highly susceptible to sheep pox, while Algerian and other African breeds, particularly the Daglic, White Karaman and Red Karaman are relatively resistant.

1.2 Clinical Signs

Many authors have described the numerous clinical signs which may be a result of sheep pox virus infection. Pyrexia, skin nodules, coughing, diarrhoea and associated recumbency, depression, skin defects, mastitis, abortion, reduced milk yield, a high morbidity usually between 90% and 100% and a mortality about 30% are some of the signs (Huytra 1946; Plowright *et al* 1959; Wakeem 1960; Katiyar 1961; Marsh 1965; Scott 1966; Siegmund 1967; Leeman 1968; Murray *et al* 1973; Blood and Henderson 1974; Singh *et al* 1979; Asagaba and Nawathe 1980).

The lesions are widespread, appearing after an incubation period of four to seven days, usually following an acute systemic reaction. Lesions, although widespread, most commonly appear on the wool-free areas of the body, particularly the cheeks, nostrils and lips.

Sheep pox lesions are typical of a pox virus and exhibit the typical 'pock' stages. The following account of the appearance of the lesion is taken from the combined works of Plowright *et al* (1959), Jubb and Kennedy (1963), Murray *et al* (1973) and Singh *et al* (1979). After the four to seven day incubation period, a demarked macule appears which becomes a papule in the following one to two days. These papules are reported by Ivanoff (1941) as cited by Singh *et al* (1979) to be of four possible types. The first of these is known as a verrucoid papule and is often referred to as wart pocks. These resemble the common wart and are formed by hyperplasia and parakeratosis of the epidermis. The second type is the urticaria-like papule which results from severe cellular infiltration of the corium and thickening of the epidermis. The third type, the pelliculated papule is characterized by a layer of nectoric epithelium. The fourth type, the vesiculated papule, is also covered by a necrotic epithelium but resembles a vesicle as it is filled with serous fluid, leucocytes and cell debris. After this papule stage there is usually an umbilicate vesicular stage which may be haemorrhagic, although there are a number of reports indicating no vesicle formation but a generalized reaction. The vesicle stage is followed by the formation of pustules which later become scabs.

The healing of these lesions generally takes at least one month and the course of the disease depends on many factors. The breed of sheep (Donatien *et al* 1947), the strain of virus (Donatien *et al* 1947), and the status of the host, particularly in terms of other diseases such as brucellosis, tuberculosis, tendovaginitis and orchitis which may be aggravated by sheep pox (Scott 1966).

Secondary bacterial infection is important in the course of the disease, in severe cases it may lead to septicaemia resulting in death of the animal (Jubb and Kennedy 1963). Although no mention of fly strike is made in the literature it almost certainly is a problem and contributes to the prevalence of secondary bacterial infection.

1.3 Epidemiology

Sheep pox is usually contracted via the respiratory tract or skin following contact with infected animals or contaminated fomites (Singh *et al* 1979) and it has been reported that the virus can remain viable for up to six months in the scab form. Thus dust and sheep yards are likely to remain sources of outbreak long after infected animals have been held. Leeman (1968) reports that infected animals shed virus at all stages of the disease and for as long as eight weeks after the lesions have fully healed. Probably the most important factor in the epidemiology of sheep pox is the long-used and widespread practice of using fully virulent virus in scab material as a vaccine (BVA 1955).

1.4 Diagnosis

Initial diagnosis is nearly always made on the basis of the clinical signs which have been described previously. Such a preliminary diagnosis may be confirmed in a number of ways. Inoculation of immune and susceptible sheep with nodules from the skin made into a suspension (Asagba and Nawathe 1980), the presence of characteristic sheep pox virions in clinical material when viewed using electron microscopy (Muzichn and Babiker El Hag Ali 1979; Asagba and Nawathe 1980), serum neutralization in conjunction with tissue culture, or cultivation in eggs (Davies and Otema 1978), indirect immunofluorescence (Davies and Otema 1978), capillary agglutination (Dubey and Sawhney 1979), agar gel diffusion precipitation (Dubey and Sawhney 1979), complement fixation, conglutinin complement adsorption test, Coombs test and histological examination (Murray *et al* 1973) of the lesion have all been used in the diagnosis and study of sheep pox.

1.5 Cultivation

Sheep pox virus has been cultivated successfully by many workers, however there is much conflict in the literature as to whether or not is is possible to transmit to and cultivate in a large number of host organisms.

It seems that all workers have been able to cultivate sheep pox virus in sheep, however there is some variation as to the yields possible. Arik and Kurtul (1973) suggest that some of this variation is due to the breed of sheep as some (notably Merinos) give larger yields than others, for example, the Karaman breed.

A number of other organisms have been experimentally infected, including the horse, donkey, calf, pig, rabbit and guinea pig. Singh *et al* (1979) suggest that these reports should be accepted only with some reservations because in a number of cases these experiments were carried out in laboratories which were engaged in producing smallpox vaccine. Singh *et al* go on to say that because of this contamination of the inoculum with vaccinia virus was "notoriously easy". Adlakha *et al* (1971) could not adapt any of four strains to mice, rabbits or guinea pigs.

Attempted cultivation of sheep pox virus on the chorio-allantoic membrane (CAM) of embryonating chicken eggs has produced conflicting results. A large number of workers have failed to cultivate SPV in eggs. These workers include Sen and Uppal (1972) and Sharma and Dhanda (1972). Aygun (1955) however, observed odema, leucocytic infiltration and necrosis in the CAM. Cytoplasmic inclusions in the ectoderm of the CAM have also been observed (Tao 1938).

A number of workers have successfully grown SPV on the CAM of chick embryos. Sabban (1957) adapted a virulent strain of SPV from Cairo on the CAM of 12-day-old chick embryos and reported no loss of virulence for sheep after 12 alternate passages through sheep and 12-year-old chick embryos.

Yuan *et al* (1957) attenuated SPV after 90 passages on the CAM and used it as a vaccine. It was later found that this Chinese strain was harmless to sheep, yet when inoculated at either 0.2 ml ID or 0.5 ml SC it still induced a strong immunity.

Rao and Malik (1978) confirmed the adaption of the Ranipet strain of SPV and found attenuation for the final host and strong immunogenicity of this strain after 24 serial passages on 11-day-old chick embryo CAMs.

Other workers who have successfully cultivated SPV on the CAM of embryonating chick eggs include Borisovich (1962) and Arik and Kurtul (1974) who reported no change in viral characteristics after 25 passages on the CAM.

Sheep pox virus has been successfully cultivated in a variety of cell and tissue culture systems by a number of workers.

Cytopathic effects due to SPV occurs in a large number of cell culture types including tissue from sheep (Aygun 1955; Yuan *et al* 1957; Plowright and Ferris 1958; Hers *et al* 1963; Terrinha *et al* 1965; Pandey *et al* 1969; Pandey and Singh 1970; Martin *et al* 1973; Singh 1974; Goyal and Singh 1975; Srivastava 1975), goats (Plowright and Ferris 1958; Pandey and Singh 1970), cattle (Plowright and Ferris 1958; Martin *et al* 1973), and fowl (Aygun 1955).

Testes and kidney tissue have been the most successful for tissue culture, however the lung, liver, heart, embryonic muscle and skin have been used with success.

DNA cytoplasmic inclusion bodies have been reported in sheep, goats and cattle tissue (Aygun 1955; Plowright and Ferris 1958) and they appear as basophilic and/or eosinophilic inclusion bodies.

Anadan *et al* (1976) found that sheep thyroid monolayers at the 39th passage produced appreciable cytopathic effect, and that the virus was inocuous, safe and immunogenic.

1.6 Treatment, Prevention and Control

The treatment of sheep pox, like other viral diseases, is not often attempted as it is generally uneconomical, ineffective and impractical. Treatment with antiviral drugs has, as far as is known, not been attempted and traditional folklore treatments shall not be discussed here. Treatment is probably best accomplished by treating and preventing secondary infection, both bacterial and fly strike. As with most diseases, particularly viral diseases, prevention and control are a much better practice than treatment.

There are a large number of methods for vaccinating against sheep pox and consequently there is a large range of varying success. Hutyra *et al* (1946) describe ovination as being a method of immunization since the 1700s. An autogenous vaccine of ground-up scabs suspended in saline has been used in India for immunization for many years (Sharma and Malhotra 1959). However, there are problems with this in that contamination with other viruses and

bacteria is possible (Manninger 1948) and the immunization procedure has been known to be as severe as the disease, as well as contributing to the pool of endemic virus. The BVA (1955) state that this is the major cause of its persistence and spread. Munro (1947) suggested using desiccated infected scab tissue and Sabban (1955) reports that this gave satisfactory immunity for up to 14 months. Sero-clavelization, the simultaneous inoculation with convalescent sera and live virus was first attempted in 1896 by Duclert and was reported to give satisfactory immunity (Singh *et al* 1979). The length of this immunity however, was not stated.

Likhachev *et al* (1964), cited by Singh *et al* (1979) found that the precural lymph nodes as well as subcutaneous tissue was a good source of virus for vaccine production and that the virus from subcutaneous tissue was less virulent than virus obtained from internal organs. This may explain the success that nomadic herdsmen have had for many years by vaccinating with scab preparations and therefore inducing good immunity, yet not causing the severe disease in their flocks.

Other methods of immunization include sensitized virus (Martin 1939; Donatien *et al* 1947), gel adsorbed live virus (Anandan *et al* 1972), gel absorbed inactivated virus (Uppal 1963), inactivated virus (Uppal 1963; Sen 1967; Sharma and Dhanda 1970), live cell culture adapted virus (Singh *et al* 1979), and chick embryo adapted virus (Yuan *et al* 1957; Rao and Malik 1978).

Adjuvants which have also been used in immunization against sheep pox have been found to produce dependable immunity and have the desirable ability to be stored for long periods of time. Some problems with the use of adjuvants include blockage of needles, necrotic regions and abscesses at the inoculation site as well as requiring a large dose.

Much work has been done on the preparation of a vaccine against sheep pox virus. Lengths of immunity reported are conflicting and varied and vary from eight weeks to two years, depending on the vaccine type and schedule used (Singh *et al* 1979).

As sheep pox is an economically important disease, a successful eradication program would be particularly useful. With the range of vaccines available, the fact that there are few or no feral reservoir hosts and because SPV is spread through direct contact and fomites, while vectors are not an important source of infection, may indicate that an eradication program has a reasonable chance of success. Perhaps it would be as successful as the small-

pox eradication program, if problems such as the virus remaining viable in dust etc. for long periods of time, and problems that arise because sheep pox is mostly prevalent in third-world countries, can be overcome.

1.7 Histology

Histological changes to sheep pox infection have been well described by Murray *et al* (1973) and Plowright *et al* (1959). Most of the earlier histological work evolved in describing the nature and origin of the highly characteristic 'cellules claveleuses' or sheep pox cells which were first described in 1903 by Borrel. Most histopathological changes in the skin occur in the dermis and subcutis rather than the outer malphigian layers.

The following account of the histopathology of sheep pox lesions is taken from the combined works of Plowright *et al* (1959), Jubb and Kennedy (1963) and Murray *et al* (1973).

Four to five days post inoculation, a skin reaction in the form of a plaque with a thickened congested border appears, which is the result of intense inflammatory reaction in the dermis with congestion, extensive exudation and marked polymorphonuclear leucocyte infiltration from blood vessels. Over the next one to two days, cytolysis of the polymorphonuclear cells occurs and macrophages begin to appear in the dermis. At this time large cells resembling histocytes appear which become the cellules claveleuses. These are easily recognized by their large size, basophilia, stellate form and oval or irregular nuclei. It is at this time that granular and solid eosinophilic cytoplasmic inclusion bodies first appear, which have been shown to be the sites of viral replication (Martin *et al* 1973). Until this time the virus was underoing logarithmic increases but at this time (seven to eight days PI) the virus reaches its peak titre of 10^7 $TCID_{50}$ per gram of tissue. This titre persists for a further six to seven days and subsequently declines.

Accompanying the sheep pox cells are hyperplasia of the epithelium and proliferation of the endothelial cells lining the vessels of the micro-circulation. Also at this time there is increasing severity of the inflammatory oedema of the dermis which may extend to the subcutis. Subsequently the blood vessels develop an acute necrotizing vasculitis and thrombosis which results in necrosis of the dermis. Proliferation, acanthosis with a tendency to para-keratosis and hyperkeratosis, are present. From this point on, albeit slowly, the lesion regresses, infectivity drops and the lesion begins to heal through the mediation of fibroblasts.

Sheep pox virus can always be found in the lung and skin tissue (Balozet 1931) and has since been recorded in high titres (10^6) in the subcutaneous tissue and the precural lymph nodes and in lower titres (10^{-2}) in the spleen, liver, kidneys and lungs (Singh *et al* 1979).

1.8 Immunity

The mechanism of immunity to sheep pox is not fully understood, a more precise knowledge is bound to be of significance in the development of more suitable vaccines than those already available. Srivastava and Singh (1980a) have shown that both humoral and cellular factors play an active part in immunity to sheep pox by demonstrating that passive transfer of antiserum conferred mild protection, and that the cellular mechanism of immunity, manifested by a delayed type hypersensitivity reaction to viral preparations, is of vital importance in protection. Srivastava and Singh (1980b) also found that passive antiserum transfer against live virus conferred much better immunity than antisera against inactivated virus.

Davies and Otema (1981) demonstrated a classic antibody response curve using a fluorescent antibody technique and a micro-serum neutralization test but go no further in discussing the humoral immune response to sheep pox virus.

Typically, pox viruses are known to retain most progeny virus intracellularly with only a fraction released to become extracellular (Furness and Younger 1958; Smith and Sharp 1960; Easterbrook 1961). Sheep pox is no exception and Srivastava and Singh (1980b) found that 3% of total yield was released as extracellular virus while the remaining 97% was retained as intracellular virus. They also showed by cross-neutralization and cross-protection tests that intracellular and extracellular virus differ antigenically.

Srivastava and Singh (1980b) showed that lambs hyperimmunized with extracellular virus or released intracellular virus showed good immunity, in spite of a poor titre of neutralizing antibody in their antiserum. These findings are in agreement with those of other workers (Kaplan *et al* 1963; Boulter *et al* 1971) who also could find no correlation between titre of neutralizing antibody and the immune status of the animals.

Appleyard *et al* (1971) showed that extracellular virus was a better immunogen, and therefore conferred greater resistance to rabbit pox, than was intracellular virus. Srivastava and Singh (1980a) showed a similar effect with sheep pox virus. They explain this by saying that it is the extracellular

virus which is mainly responsible for spread of the disease apart from some intracellular virus which may spread by cellular fusion. They go on to comment that since extracellular virus is a better immunogen than intracellular virus, and that it is extracellular virus that is mainly responsible for the spread of disease, then efforts to produce more effective and safer vaccines for sheep pox should be concentrated on employing extracellular virus.

2. THE VIRUS

2.1 Morphology

It is generally agreed that pox viruses fall into two distinct morphological groups. One of these is brick-shaped, characterized by Vaccinia, and the other is ovoid with a criss-cross pattern on the particle surface (often called 'basket weave' appearance) as characterized by the parapoxvirus of contagious pustular dermatitis (Horne 1978).

The pox viruses are the largest and most complex viruses with large cubate or prolate virions being 250-300 nm long and 200-250 nm wide. They have multiple structural features and complex chemical components. They contain more DNA than the other DNA viruses with a single molecule of folded, tightly coiled double stranded DNA (Andrews 1978). This genome's length is 87 to 100 μm, its molecular weight is between 160 and 180 x 10 Daltons and the guanine plus cytosine content is between 26% and 40%. The large genome shows a potential to code for several hundred polypeptides (Fenner 1976) and at least 30 structural proteins are known, several other proteins, at least 10 antigens, one of which is common to all vertebrate pox viruses.

The genome is contained in a centrally positioned plate-like core which is often biconcave and surrounded by a complex series of membranes of viral origin (Fenner 1976). This core and two characteristic lateral protein bodies are enclosed in a lipoprotein outer coating possessing characteristic tubular structures (Andrews 1978). All pox viruses have a lipoprotein envelope at the surface with numerous hollow spikes on the virus periphery (Goodheart 1964).

The capripox viruses, of which sheep pox is the type species, is 113 nm by 194 nm (Abdussalam 1957) with large lateral bodies (Cohen *et al* 1971). The mature virion is therefore similar in shape to other pox viruses. It assumes a characteristic oval or rectangular profile with a typical bilobed biconcave core of which the dumb-bell shaped core is identical to that of other known pox viruses (Murray *et al* 1973).

The morphogenesis of sheep pox virus is similar to most pox viruses. The viroplasm of sheep pox virus forms into spherical particles limited by a single membrane, an eccentric nucleoid appears before the virion matures (Cohen *et al* 1971). Goat pox virus may produce paracrystalline arrays in macrophages (Andrews *et al* 1978), however it is as yet unknown whether the same phenomenon occurs with sheep pox.

2.2 Taxonomy

The taxonomy of viruses is generally based on expedient and useful characteristics rather than other methods due to the absence of a natural or phylo-genetic basis for classification (Wilner 1966).

The taxonomic criteria initially divide the viruses into a number of main subgroups or families, in particular: nucleic acid type, virus particle size, ether/chloroform susceptibility and virus morphology, which is usually based on virion symmetry, *ie* cubical or helical, enveloped or not, and the number of capsomeres if present (Virus Subcommittee on International Nomenclature Committee 1963); and so, the family Poxviridae, to which sheep pox virus belongs, is defined (Wilner 1966):

1. a predominantly double stranded DNA core
2. rounded quadrangular particles approximately 200 mμ x 130 mμ
3. mostly ether resistant, however the members which show resistance are susceptible to other solvents which suggests that all members may possess essential lipids
4. complex symmetry, enveloped

Other general characteristics of the poxviridae include:

1. apparently multiply wholly within the cytoplasm, therefore produce intra-cytoplasmic basophilic and eosinophilic inclusion bodies
2. pH labile, according to Ferriria who found pH3 and pH 11 for two hours would lead to complete inactivation, but relatively heat stable

In practice large particle size and a predilection for dermal epithelium with the characteristic pox virus lesion the 'pock' has often been the criteria which initially classifies these agents as a pox virus (Wilner 1966).

Subdivision of the family Poxviridae is based on host, serological activity, behaviour in host cells, susceptibility to chemical and physical agents, epidemiology and reactivation. The classification of the viruses is far from standard but it is generally accepted that there are seven major subvidivisions fo the poxviridae. These are:

Orthopoxvirus	Parapoxvirus	Capripoxvirus
Avipoxvirus	Lepripoxvirus	Entomopoxvirus

and a miscellaneous unclassified group containing a number of vertebrate poxviruses.

Sheep pox is caused by a virus belonging to the capripoxvirus group (Fenner 1976).

2.3 Growth and Replication

Nearly all the work on pox virus growth and replication has been done using the vaccinia group of viruses (Fenner 1968; Maramorosch *et al* 1971). However, some work has been done using sheep pox by Terrinha *et al* (1965); Murray *et al* (1973), Vigaria *et al* (1968) and Cohen *et al* (1971).

The whole sequence of infection, replication and dissemination is unclear but it is known to be rapid with complete virions appearing within five hours PI in cell culture (Horne 1965). It is also well known that in all pox viruses the whole process of replication occurs in cytoplasmic factories, that is, the previously mentioned cytoplasmic inclusion bodies, independently of nuclear function.

The penetration of the virus into the host cell is the first stage and it precedes intracellular uncoating (Maramorosch *et al* 1971). First the cell and the virus must come together by simple diffusion and the initial attachment probably involves reversible electrostatic forces (Goodheart 1969). Horne (1965) and Goodheart (1969) state that viropexis, the same process as pinocytosis are the major methods by which the pox viruses gain entry to a cell, so that viral entry may be an 'accident' on the part of the host cell.

When the virus is in the cell vacuole it is, strictly speaking, outside the cell as it is surrounded by cell membrane. Normally both the protein coat and the core of the virus enter the cell, however once the virion is in the cell the protein coat plays no further part in the infection (Goodheart 1969).

The process by which the protein coat is removed from the pox virus virion, thus releasing the viral DNA into the cytoplasm has been elucidated by treatment of the virion to remove the coat layer by layer (Marmorosch *et al* 1971). It was this method which led to the subsequent digestion of the lateral bodies with trypsin by Pogo and Dales (1969) and Horne (1978). It was found that the separation of the lateral bodies occurred 20 minutes PI and that simultaneously with the removal of these lateral bodies the neutral and acid DNA activities went up markedly. This led Pogo and Dales (1969) to suggest that the lateral bodies contained a specific inhibitor of the DNA-ases. It was then suggested that it may be significant that during penetration *in vivo* the lateral bodies separate from the core when the virus particles leave the viropexic vacuoles and enter the cytoplasm (Goodheart 1971). Goodheart (1971) went on to suggest that the dissociation of the lateral bodies from the core is the natural *in vivo* mechanism for activating the DNA-ases. The synthesis of the viral constituents of sheep pox virus is inhibited by 5-fluorodexyuridine which is thought to act by depriving the cell of a precursor necessary for DNA synthesis.

As soon as the viral genome is released, transcription of mRNA from the viral DNA occurs. This viral mRNA is associated with the polyribosomes in the cytoplasm and later translated to viral proteins (Fenner 1968).

Ten to eleven hours after the release of the viral genome into the cytoplasm of the host cell, a cytoplasmic inclusion body is formed, one per infectious particle as found by autoradiography and immunofluorescence by Cairns (1960) who also showed that clusters of ribosomes, which are the mechanisms of viral protein synthesis, DNA replication and synthesis begins and is followed by structural protein synthesis seven to eight hours later from the previously transcribed ribosomal mRNA. It must be remembered that as the time course and cytopathic effect differs between virus groups, strains and cell lines, some conflict between the results of different authors does occur.

Following protein synthesis, there appears folded isolated DNA structures. The appearance of an eccentric nucleoid precedes restructuring of the particle and formation of the mature virion (Singh *et al* 1979). These become enclosed within a lipoprotein membrane, complete with spicules within the inclusion body. Eventually core and polar bodies become clearly defined, this signifying the terminal stage in replication. Following this, large numbers of the virion are distributed throughout the cytoplasm ready for release (Murray *et al*

1973). It has been shown that different proteins are synthesized in sequential order and structural proteins are synthesized late in the growth cycle (Fenner 1968). Synthesis of the four well studied enzymes appear to be late functions (Maramorosch *et al* 1971), however Fenner (1968) states that these enzymes' synthesis is initiated early.

The maturation process has not been clearly defined. Following the core and polar bodies becoming clearly defined and enclosed in protein shells, the central area, perhaps the DNA genome, condenses and this immature particle differentiates (not changed by addition of further coats) to the mature form. This occurs asynchronously and over a prolonged period of time (Fenner 1968). These virions may move to the cell membrane and fuse, to be released to the cell exterior (Maramorosch *et al* 1971). However, most of the mature virions remain in the cell until the cell is destroyed.

3. CONCLUSION

Sheep pox remains a serious disease in many of the sheep raising areas of the world. Progres in the understanding of sheep pox is improving as are vaccine preparations and knowledge of the mechanisms of immunity. The technological ability to combat sheep pox is available but the major problem facing the potential control and eradication of sheep pox is that of disseminating the knowledge and ability to the people of the nations where sheep pox remains a problem. The difficulty of this is complicated because the nations involved are often very poor with many social and political problems.

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