A STUDY OF ORF VIRUS ISOLATES FROM SHEEP IN NEW ZEALAND

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STATEMENT

The gradients depicted in Figure 1 and the gel depicted in Figure 3 were done in collaboration with Dr. A.J. Robinson. The rest of the work was my own.

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ABSTRACT

A preliminary study of varicus orf virus isolates in New Zealand is presented. A method of isolating and purifying the virus from scab material is described. Thirteen orf virus isolates were compared by DNA restriction endonuclease analysis. There was extensive heterogeneity in the $EcoR_1$ restriction patterns, however, two of the isolates were similar.

Propagation of the virus *in vitro* was also attempted. Five of the isolates were successfully adapted to growth in lamb testis cells and two of these were plaque-purified twice. The characteristic cytopathic effect of cell-adapted virus is early rounding and clumping together of infected cells. This cell rounding could be seen as early as two hours post-infection and could be inhibited by cyclohexamide. There were two types of plaques exhibited by the isolates in lamb testis cell monolayers, the "open" and "closed" types. Inclusion bodies were also seen in stained infected monolayers.

^{32}P-labelling of a plaque-purified isolate was successfully performed. Low passage (23 passages) in cell culture of plaque-purified isolate No. 2 did not alter the $EcoR_1$, Bam H-I or Hind III patterns of the DNA when compared with the original viral DNA from scab material.

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INTRODUCTION

Orf is common among sheep in New Zealand and the incidence in man appears to be increasing. There is a vaccine available for sheep. Although the vaccine protects lambs from the disease it perpetuates the virus in the environment. There is a need for alternative vaccination methods.

It is the aim of this project to develop methods of isolating, propagating and purifying orf virus in cell culture and identifying isolates by restriction endonuclease analysis in preparation for vaccine studies.

CHAPTER ONE

REVIEW OF LITERATURE

CONTAGIOUS ECTHYMA IN SHEEP, GOATS AND MAN

Contagious ecthyma is a poxvirus disease of sheep, goats and man. It is also known by other names. In sheep, In England, it is called contagious pustular dermatitis (Glover, 1928); in Scotland, orf (Martin-Scott, 1955 as cited by Trueblood and Chow, 1963) from the Saxon for cattle (Hodgson-Jones, 1951). It is commonly known as scabby mouth in Australia (Ohman, 1941; Sutherland and Moule, undated) while in the United States, it is called soremouth, infectious dermatitis or contagious vesiculo-pustular dermatitis (Schmidt and Hardy, 1932; Trueblood and Chow, 1963). In New Zealand, the disease is known as scabby mouth to the farmer and ecthyma contagiosum or contagious pustular dermatitis to veterinarians (Purdy, 1955). In human medicine, it is more popularly referred to as orf (Purdy, 1955; Beck and Taylor, 1974).

The disease is prevalent in sheep-raising countries. It occurs at any time of the year but is more common during spring and summer mainly among lambs and kids (Glover, 1928; Schmidt and Hardy, 1932). Older animals are more resistant due to immunity from either vaccination or past infection (Bruner and Gillespie, 1973). Morbidity can be very high approaching 100% (Schmidt and Hardy, 1932) but mortality rate in uncomplicated cases rarely exceeds one percent (National Vet. Med. Publ., 1944 as cited by Hodgson-Jones, 1951). With secondary complications, mortality rate may range from 20-50% (Aynaud, 1923 and Jacotot, 1924 as cited by Howarth, 1929). Losses from scabby mouth in lambs are due to the lesions on the lips and mouth which interfere with feeding, or refusal of ewes with infected teats to nurse their young, subsequently affecting growth rates (Howarth, 1929; Bruner and Gillespie, 1973). Deaths are

usually due to complications caused by, for instance, the invasion of the lesions by the screwworm fly *Cochliomyia americana* (Boughton and Hardy, 1935) or the necrobacillus *Spherophorus necrophorus* (Newsom and Cross, 1931 and 1934a). A case of scabby mouth associated with *Dermatophilus congolensis* has been reported in New Zealand (Cooper et al., 1970). The authors also cited a case of secondary complications with Streptotrichosis among sheep in Kenya.

The virus enters the host through abrasions on the skin of the lips and face. Such abrasions are caused by thistles, hard stubbles or prickles of red-burr or similar plants. The virus can also be transmitted to the udder of ewes by suckling lambs. The incubation period of the disease is about 72 hours (Sutherland and Moule, undated).

The virus is highly epitheliotropic (Beck and Taylor, 1974) and the lesions produced are usually seen on the nostrils and commisures of the lips. In more serious cases it may extend to other parts of the body, for example the thigh, axilla, coronet, vulva or cornea (Glover, 1928; Howarth, 1929; Schmidt and Hardy, 1932); roof of the mouth, tongue, gums (Glover, 1928; Howarth, 1929; Valder et al., 1979); visceral organs (Newson and Cross, 1931). The last mentioned complications were due to *Actinomyces (Spherophorus) necrophorus*. A generalised case of the disease was reported among sheep in Kent by Darbyshire (1961). The infection persisted in the acute form for two months involving the gastro-intestinal tract and lesions extending to the lungs and heart. An average mortality rate of 18% was recorded. Bacterial organisms were isolated but none was *Spherophorus necrophorus*.

A serious outbreak occurred in Germany affecting both lambs and sheep. High mortality was noted among the young. In addition to foot and genital lesions, deep ulcerative stomatitis, pharyngitis and oesophagitis were observed. Many of the affected lambs developed lameness and lost their

hooves (Valder et al., 1979). No secondary infection was mentioned.

Grossly, the lesions in sheep start as discrete, reddened swellings on the lips followed by papules, vesicles, pustules and ulcer formation in three to four days. In uncomplicated cases, the disease is self-limiting. Scab formation occurs within one week and peels off the skin within three to four weeks, leaving no scar (Glover, 1928; Howarth, 1929; Schmidt and Hardy, 1932; Sutherland and Moule, undated). Sometimes continued proliferation of the epithelium occurs and a dense wart-like outgrowths appear, resembling papilloma (Glover, 1928; Selbie, 1944). The malignant form reported by Valder et al., (1979) produced cauliflower-like growths in the oral mucosa. With secondary complications, lesions become ulcerative and necrotic without scab formation (Newson and Cross, 1931; Darbyshire, 1961).

The microscopic appearance of skin lesions was described by Glover (1928) following artificial inoculation of lambs. He divided the disease process into three stages. The first stage, or papulo-vesicle stage, was characterised by proliferation of the cells of the rete Malpighi. The vesicles arose between the more superficial cells immediately beneath the stratum lucidum and were represented by collections of polymorphonuclear leucocytes. In the second stage, or vesico-pustule, there was further degeneration of cells forming an irregular network resembling the "ballonisante" stage of vaccinia. The vesicles became enlarged and developed into pustules and finally ruptured through the stratum lucidum. Crusts then formed from cellular debris, fibrin and portions of the stratum lucidum leading to the third stage, scab formation. Beneath the crust, the epithelium regenerated and healing occurred.

A more detailed description was given by Wheeler and Cawley (1956) also based on experimental inoculation of sheep. There was no evident change on the fourth day post-inoculation.

The earliest changes were "ballooning" degeneration of the uppermost layer of prickle cells seen on the 4th and 6th days and was almost complete by the 7th and 8th days. The remaining cell walls produced a "basket-weave" appearance. This resulted in multiloculated superficially placed vesicles and pustules. Between the 11th and 17th days, there was marked pseudo-epitheliomatous hyperplasia and granuloma formation which led to a papilloma phase. This feature was also described by Selbie (1944) in naturally occurring disease in lambs, but was not described by Glover (1928). Between the 22nd and 40th days, lesions gradually involuted. The papilloma appearance disappeared, dermal infiltrates gradually resolved, and regeneration occurred. There was only slight connective tissue proliferation and thus the healed lesions did not leave scars. Inclusion bodies were not described in these experiments but were described by Abdussalam (1957a). He described inclusion bodies that measured 4 to 8 microns in diameter and appeared in the cytoplasm of epidermal cells. The main microscopic feature he saw was proliferation and reticular degeneration of epidermal cells leading to necrosis. This was accompanied by the infiltration of the corium with polymorphonuclear and mononuclear inflammatory cells. In contrast to other authors, he did not observe "ballooning" degeneration, but he did observe the multilocular feature of vesicles.

The generalised cases reported by Darbyshire (1961) showed cardiac, pulmonary and gastro-intestinal involvement. This included myocardial haemorrhages, acute myocarditis, haemorrhagic pneumonia with necrotic foci in the apical lobes of the lungs, tracheitis, generalised degeneration of the liver and inflammatory changes in the gastro-intestinal tract. These lesions were mainly due to secondary complications.

As discussed briefly above, orf is also a zoonosis. Human orf usually follows contact with infected sheep (Hodgson-Jones, 1951) and is thus an occupational disease of farmers, sheep shearers, freezing workers and occasionally veterinarians (Purdy, 1955). It has, however, been seen in people not directly involved with handling sheep and goats (Kewish, 1951). These infections probably arose from fomites such as sheepyard rails, wool hooks and clothing. No man to man transmission has been reported (Moore, 1973; Leavell, 1968) but there is no theoretical reason why it could not occur. Presence of wounds and abrasions on the arms and hands increases risk of infection (Pask et al., 1951 as cited by Platt, 1958).

The disease in man has been reported and described in detail by many workers (Newsom and Cross, 1934b; Muir, 1951; Purdy, 1955; Hodgson-Jones, 1951; Nagington and Whittle, 1961). The incubation period ranges from three days (Carne et al., 1946; Hodgson-Jones, 1951; Muir, 1951) to seven days (Hodgson-Jones, 1951; Muir, 1951). It occurs most commonly as a single lesion on the arm or hand. Lesions appear as discrete, raised, circular, purplish-red papules (Carne et al., 1946) about 1-3 cm. in diameter (Carne et al., 1946; Hodgson-Jones, 1951). Often, the centre becomes ulcerated and surrounded by dead, white "soggy" skin which gives a granuloma-like appearance (Hodgson-Jones, 1951) and is sometimes described as "cigarette burn" lesion. In the early stages, the lesion is painless but later becomes sensitive to touch and in uncomplicated cases has resolved 5-8 weeks after infection (Hodgson-Jones, 1951).

A more severe form of the disease may sometimes occur. Kewish (1951) reported metastatic lesions on the face, arms and trunk accompanied by acute febrile reactions (Stevens-Johnston Syndrome?). A similar case was reported by Moore (1973) and Erickson et al. (1975). Blakemore et al., (1948 as cited by Platt, 1958) described an erythema-multiforme type of reaction. An unusual complication was reported by Royer et al. (1970). Extensive

vesiculo-pustular eruptions were seen on the patient's forearms, hands, feet and legs. There was swelling of regional lymph glands, and thrombosis of the ventral retinal vein occurred which resulted in permanent blindness of the right eye. These complications are, however, rare.

There are only a few reports of histological examination of lesions in man. Wheeler and Cawley (1956) described "ballooning" degeneration of the prickle cell layer in the early stages of the disease which led to vesiculation and sometimes changes associated with a chronic granuloma. The microscopic picture was consistent with the appearance of the gross lesions which are often multiloculated vesicles and at other times resemble pyogenic granuloma (Wheeler and Cawley, 1956). Inclusions may be seen (Blakemore et al., 1948, as cited by Wheeler and Cawley, 1956).

ORF IN OTHER HOSTS EXPERIMENTAL ANIMALS

Although orf is regarded as a disease primarily of domestic sheep and goats, it has also been reported as occurring naturally in other animals. The disease has been reported in the musk ox (Kummeneje and Krogsrud, 1978), reindeers (Kummeneje and Krogsrud, 1979), and also in bighorn sheep, chamois and thars (Davis et al., 1970 as cited by Beck and Taylor, 1974). These infections were believed to be of ovine or caprine in origin. There is also an unusual case in dogs reported by Wilkinson et al., (1970). The dogs could have been infected after being fed with unskinned sheep carcasses.

In the musk ox, lesions were characterised by cauliflower-like papillomas on the lips, muzzle, neck, eyelids, chest and perianal region. Grossly and histopathologically, these papillomas resembled common warts (Kummeneje and Krogsrud, 1978). The lesions seen in the dogs were essentially the same as those found in sheep. The

predeliction site was around the head. The lesions took the form of circular areas of acute moist dermatitis with ulceration and scab formation. Histologically there was "ballooning" degeneration of the prickle cell layer giving rise to small areas of acantholysis and intra-epidermal vesicle formation. In some areas, there was reticular degeneration of the prickle cells with sub-corneal vesicle formation. In some foci, these degenerated cells had been infiltrated with polymorphonuclear leucocyte giving rise to a spongiform pustule. The 'basket-weave" appearance due to vacuolisation of the cells of the stratum corneum was observed in some areas. There was ulceration and loss of epidermis. Heavy neutrophilic infiltration in the upper dermis and pustules within some of the hair follicles was also seen.

The situation as regards cattle is less uncertain. Orf is not seen in cattle that are run with infected sheep, and Howarth (1929) failed to reproduce the disease in a cow. However, Bennet et al., (1944) reported susceptibility of calves to experimental infection.

Many attempts have been made to experimentally infect other animals. These experiments have produced variable results. Glover (1928), Newsom and Cross (1934a), Boughton and Hardy (1935), Greig (1956), Plowright et al. (1959) and several others cited by Abdussalam (1957b) failed to infect rabbits. Abdussalam, however, claimed successful inoculation of rabbits by scarifying the skin with massive dose of the virus, and Darbyshire (1961) produced lesions in the rabbit by multiple injections of 0.1 ml of virus inoculum. Selbie (1944) and Wheeler and Cawley (1956) also described lesions in rabbits artificially infected with orf virus. Selbie (1944) at the time of writing claimed the virus had undergone 19 passages in rabbits.

The infection in rabbits was reported as a mild disorder. Wheeler and Cawley (1956) observed an incubation period of seven to nine days while Selbie (1944) reported 20 days which was reduced to seven days on the fifth passage. Abdussalam (1957b) saw first sign of infection on the 4th and 5th days. The first indication of infection was seen as tiny (1-2 mm), erythematous maculopapules and the disease ran a course of three to five days (Wheeler and Cawley, 1956). Abdussalam (1957b) observed erythema and papulation on the 4th day. The papulation became more marked and showed a well defined margin of congestion on the border of the lesion. On the 5th day, the papules became covered with a dull, yellowish-white scaly scab. In confluent lesions, central parts contained a serous or haemorrhagic exudate which was absent in discrete lesions. Scabs peeled off on the 6th day. The disease ran a course of about ten days. Selbie (1944) observed essentially the same lesions but gave emphasis to the appearance of scaliness of the lesion. The main microscopic feature of the infection in rabbits was mild chronic inflammatory changes and lack of the "ballooning" degeneration and the granulomatous and proliferative character of the disease in sheep (Selbie, 1944; Wheeler and Cawley, 1956; Abdussalam, 1957a). A reticular type of degeneration of the epidermal cells, as in sheep, (Abdussalam, 1957a) was observed but no cytoplasmic or nuclear inclusions were seen.

Unlike the orthopoxviruses, which readily grow on chick embryos, orf virus does not seem to be easily propagated in eggs (Newsom and Cross, 1934a; Greig, 1956; Webster, 1958; Darbyshire, 1961). Contrary to this, Abdussalam (1957b) reported a successful attempt, but lesions regressed and eventually disappeared after the fourth passage. Growth and multiplication in the chorioallantoic membrane of developing embryos was reported (Sawhney, 1966a as cited by Beck and Taylor, 1974) and later demonstrated by electron microscopy (Sawhney and Spasova, 1973).

Mice and guinea pigs were found refractory to infection. (Howarth, 1929; Newsom and Cross, 1934a; Greig, 1956; Abdussalam, 1957b; Darbyshire, 1961). Newsom and Cross (1934a) also failed to infect pigs.

In summary, it is evident that sheep and goats remain the most susceptible and the most suitable animals for experimental purposes. However, rabbits could be used to raise neutralising antibodies against orf virus by standard methods (Abdussalam, 1957b).

SCABBY MOUTH IN NEW ZEALAND

Although the disease is regarded as common in this country, there is no published data to show the extent of the problem. The incidence in sheep and the economic losses incurred by the infection have not been determined. There is, however, some data on the prevalence of scabby mouth affected lambs being presented for slaughter at New Zealand freezing works. Below are the results of a pilot survey done in two North Islands and one South Island works. These figures have been derived from the product of the average line size and the average number of affected animals in affected lines (Robinson, 1980).

Month	Auckland	Longburn	Oamaru
October	0	49	0
November	0	146	293
December	0	659	1464
January	0	342	1464
February	49	122	756
March	0	122	586
April	0	-	122

Although it seems to occur throughout the year, it is more prevalent during the summer months probably due to the seasonal nature of lambing. It is not normally a serious or devastating disease and can be controlled by vaccination. The availability and use of cheap but effective vaccines masks the risk of infection from naturally occuring or

residual vaccine virus as long as vaccination is done annually.

The importance of the disease can also be measured by its effect in man. The first human cases in New Zealand were reported by Muir and Purdy in 1951 and 1955 respectively. More recently, based on a report by the Accident Compensation Commission, there were increasing numbers of human cases reported, from two in 1975 to 143 in 1979. Compensation claims for orf in man from 1974 - 1978 have amounted to $75,000. Also, from this data, freezing workers comprised 90% of the compensation claimants, the remaining proportion included farmers, farm hands, shearers, bale press operators, food packers and retail butchers.

DIAGNOSIS

The proliferative lesions are characteristic for orf but should be differentiated from other infections such as sheep pox, goat pox and ulcerative dermatosis.

Laboratory confirmation can be achieved by one of several diagnostic techniques. Vesicular fluids from human patient or scab lesion from sheep can be inoculated onto sheep (Nagington, 1968), or onto cell cultures (Greig, 1957; Plowright et al., 1958; Sawhney, 1966c; Trueblood and Chow, 1963; Nagington, 1968) for fluorescent antibody test (Erickson et al., 1975). Agar-gel precipitation test can also be employed when using orf lesions to detect antigen but the technique is not sensitive in detecting antibodies from convalescent sera (Sawhney et al., 1973). Studies from experimental and natural cases in sheep have shown that the complement-fixation test is the most sensitive for detecting antigen (Romero-Mercado et al., 1973a & b). According to the same authors complement-fixing antigen persists in lesions or clinical material for 6 to 7 days or more whereas if by electron microscopy, virions may be masked by hyperplastic epithelium and

tissue debris in subacute and chronic cases. However, Harkness et al., (1977) described a method to obtain a maximal number of virus particles from scab materials to overcome some difficulties in recovering virus particles especially from old lesions when electron microscopy is employed in the rapid diagnosis of orf. Electron microscopy is probably the most rapid means of diagnosis.

TREATMENT AND CONTROL

Treatment of orf lesions in sheep is usually unsatisfactory and impractical except where secondary infection is occuring. However, there are a number of suggested treatments and these could be of value if only a few animals are involved. A 5% copper sulphate solution can be applied to the skin with a swab after removal of the scab (Sutherland and Moule, undated). Other medications that have been used are 7% iodine, creosote dip, and 3% phenol in vaseline (Beck and Taylor, 1974). Lithium antimony thiomalate (6%) also known as anthiomaline^{R} (May and Baker) can be used (Sanderson, 1976). Where problems of screw-worm flies occur, appropriate repellants or larvicides are recommended (Merck Vet. Manual, 1973).

Control is preferable to treatment and vaccines are available which produce a good immunity (Glover, 1928; Howarth, 1929; Schmidt and Hardy, 1932; Boughton and Hardy, 1935). Immunity could last for as long as two years. Also, it has been noted that vaccination of already affected lambs reduces the course and severity of the disease (Boughton and Hardy, 1935). Vaccines are also safe to use in suckling lambs (Kerry and Powell, 1971). Recurrence of the disease in vaccinated animals was observed by Trueblood et al., (1963) and Beck and Taylor (1974) but whether this was due to a different strain of virus, or poor vaccination technique was not determined.

Vaccines currently used consist of a suspension of live virus prepared from vesico-pustular lesions produced on the skin of healthy, 4 to 6 months old lambs. The lambs are killed and bled about the 5th day post-inoculation and the epidermal pulp is removed and ground up with a mortar and pestle. The ground-up lesions can either be dried and kept at low temperature or made into a 10% suspension in a suitable buffer. Buffers used are McIlvaine's citric acid and disodium hydrogen phosphate solution (pH 7.2) or a solution consisting of M/50 potassium dihydrogen phosphate solution, M/50 disodium hydrogen phosphate (pH 7.2) and 1/10 volume anaesthetic ether. With the latter buffer the suspension is incubated at 2^{o} to $4^{o}C$ for 12 to 24 hours. The ether is then removed by bubbling with filtered air. The suspension, prepared in either buffer, is stored at -20^{o} to $-70^{o}C$, tested for potency and diluted to 1:500 with mixture of 7.5 parts buffer and 2.5 parts glycerin and chloroform as a preservative (British Vet. Codex, 1953). In lambs, the vaccine is most commonly administered by the application of the suspension to the scarified skin on the inside of the hind leg. However, alternative vaccination sites can be used. The ventro-lateral surface of the tail or non-wooled area posterior to the axilla have been used in pregnant or lactating ewes (Beck and Taylor, 1974). Such vaccines produce a solid immunity but at the same time the scabs produced by vaccination contain live virus particles.

Attempts to use tissue culture-derived virus as vaccine as an alternative to the vaccine prepared as described above, have been reported. Ramyar (1973) suggested using virus passaged in primary lamb kidney cells for vaccine production. The protection conferred by such a vaccine against challenge with field virus was less than a year. Ecthyma virus passaged 28 times in calf kidney cells proved to be of low virulence and immunised lambs for six months (Ergin and Koklu, 1977). A strain of the virus grown in avian cells was reported to have lost its ability

to produce skin lesions in lambs and rabbits, however, its immunogenic properties were not tested. Zach (1979) reported producing a safe and effective vaccine from virus continuously passaged over a hundred times in sheep cells. However, no challenge test had been performed in his experiments even though a retrospective trial was encouraging. A tissue culture-derived vaccine containing interferon inducer was also reported by Valder et al., (1979) but there was not sufficient data to allow a critical evaluation of the effectiveness of the vaccine.

It has been suggested that passage in human amnion cells (Nagington and Whittle, 1961) and HeLa cells (Sawhney and Toschkov, 1971) has an attenuating effect on the virus as determined by lamb inoculation but again these claims have not been supported by published data.

Live virus vaccines, prepared from scab material are very effective but have some disadvantages. Tissue culture-derived vaccines have given promising results but their commercial application has not been adequately assessed and, as shown by two workers, immunity may be of short duration. Killed virus vaccines do not seem to be of value as immunising agents.

Knowledge of the mechanism of immunity to orf virus infection may be useful in the search for alternative vaccines. There are only a few reports of investigation into the mechanism of immunity to orf virus infection. It has been stated that orf virus is highly epitheliotropic thus inducing "skin immunity" (Aynaud, 1923 as cited by Trueblood et al, 1963; Glover, 1928; Boughton and Hardy, 1935; Darbyshire, 1961). In the context of current immunological understanding, such statements are unhelpful. There have, however, been some observations made which are helpful from the practical point of view and may give some insight into the type of immunity induced by orf virus. For example, the susceptibility to infection of lambs,

72 hours after birth, born to immune ewes, proves that no protective immunity is derived from the placenta or colostrum (Boughton and Hardy, 1935; Richter and Jansen 1968). This implies that antibody, if produced, in the affected animal is either of a class which does not pass through the placenta or into the colostrum, or it is not protective. It is stated in Buxton and Fraser (1977) that sera from immunised sheep contain precipitating and compliment-fixing antibodies to orf virus but the production of neutralising antibodies is variable. Nagington and Whittle (1961) reported detecting significant levels of neutralising antibodies in convalescent sheep and human patient's sera. On the other hand, Trueblood et al, (1963) failed to demonstrate significant level of neutralising antibodies in immune sheep sera. No investigations into cellular immunity induced by orf virus infection have been reported.

More is known about immunity to orthopoxviruses. *In vivo* and *in vitro* studies with vaccinia and rabbit pox showed that two forms of virus are produced during infection. About 90% of the virus progeny remains intracellularly and 10% are released from the cells, the extracellular forms (Boulter and Appleyard, 1973). These two forms of viruses act as separate antigens and immunity to one does not confer protection to the other, and this may be true in other pox viruses, e.g. orf. Where the extracellular form is invasive as in systemic poxvirus infections, resistance to infection would be an interplay of both humoral and cellular types of immunity. The humoral immunity is directed towards released virus and the cellular immunity attacks the infected cells. Antibodies against the released virus play a major role in the prevention of the spread of infection (Boulter and Appleyard, 1973). This aspect has not been investigated for orf virus and it may be of value to look into these two forms of virus in relation to the immunity developed. However, as orf is not a systemic disease, antibody directed against virus

particles may be of little significance in the protection from or pathogenesis of the disease. Rather, cellular immunity may be involved.

There are several speculations on the mechanism of cellular immunity to poxvirus infections. Cytotoxicity or direct attack on the infected cells is a function of the sensitised T-cells that recognise antigens expressed early on the surface of the cells (Blanden, et al., 1977). Direct attack on the cells by T-cells is sometimes blocked by the presence of low concentration of IgG antibodies on the surface of the cells. However, these antibody-coated cells can be destroyed by a mechanism termed antibody-dependent cell-mediated cytotoxicity (ADCC). This latter mechanism may be exhibited by both phagocytic and non-phagocytic myeloid cells and by weakly glass-adherent cells with Fc receptors known as "K-cells". Furthermore, interferon produced by either the sensitised T-cells or the lymphokine-stimulated macrophages might prevent the intercellular transfer of virus particles (Roitt, 1977).

PHYSICO-CHEMICAL PROPERTIES OF THE VIRUS

Contagious ecthyma is caused by a virus belonging to the family poxviridae. It has been grouped with papular stomatitis virus in the genus Parapoxvirus (International Committee on Taxonomy of Viruses, 1979). Its size is approximately 260 x 160 nm with an axis ratio of 1.6 (Nagington and Whittle, 1961). Electron microscopy of the virus particles stained with phosphotungstate reveals two interchangeable forms (Nagington and Horne, 1962). The type 1 or M (Mulberry) forms show the characteristic "ball of wool" appearance. The type 2 or C (clear) forms are phosphotungstate permeable. The "ball of wool" appearance of the type 1 forms is due to the criss-crossing patterns of the tubular protein threads on the surface of the virions (Nagington and Horne, 1962; Nagington et al., 1964).

By analogy with vaccinia virus (Westood et al., 1964) the type 2 forms are damaged type 1 virions which allow penetration of stain. The orf virion contains a double stranded linear DNA with a molecular weight of 89×10^6 (A.J. Robinson, pers. comm.).

The virus is very thermostable and resistant to changes in pH. It is completely inactivated at 60°C for 30 minutes but retains some infectivity when held at 55°C for 30 minutes (Sawhney, 1972; Buxton and Fraser, 1977). Infectivity is reduced by 1.5 - 2 $\log_{10}$ $TCID_{50}$ when the virus was kept at 36°C $\pm$ o.5°C for one week (Plowright et al., 1959; Sawhney, 1972). Scabs dried over sulfuric acid, powdered and stored in well-stoppered sealed glass tubes in ice box retained its potency for at least 32 months (Boughton and Hardy, 1935). In another experiment by the same authors, infectious dried scab material was divided into three portions and stored in stoppered glass bottles in the dark at 83°F. One of the three samples lost infectivity within 54 days, another within 64 days, and the third within 120 days. Scab material also dried over sulfuric acid, stored in amber bottle in the refrigerator at 45°F retained infectivity for 22 years, 8 months (Livingston and Hardy, 1960). Freeze-thawing does not affect the titre of the virus, instead, after the 4th freezing-thawing cycle, the titre is increased. This may be due to the disruption of viral aggregates and release of virus from cells (Plowright et al., 1959; Sawhney 1972).

When exposed to the summer heat in Texas, scabs remained infectious only for few months but when left on the ground in shaded areas, retained infectious particles for years (Boughton and Hardy, 1935). At room temperature in the laboratory, orf virus can be infective for as long as 15 years (Buxton and Fraser, 1977). It is only slightly sensitive to ether and fluorocarbon (Trueblood and Chow, 1963) but inactivated by chloroform (Buxton and Fraser,

1977). It is also resistant to glycerol (Sawhney, 1972).

In summary, it is evident that orf virus is typical of the poxvirus group, being very stable under normal environmental conditions.

MULTIPLICITY OF STRAINS

It has long been regarded that there is only one strain of orf virus and that strains from all over the world are antigenically similar. Cross-protection tests in sheep and serological studies by several authors support this view. Seddon and McGrath (1931 as cited by Boughton and Hardy, 1935) found that the English and Australian strains showed cross-protection. Glover (1932-33 as cited by Horgan and Hasseb, 1947) observed that virus isolates of ovine origin from England, France and California conformed to a single type. A caprine isolate from Tanganyika was identical with the English isolate but comparison between the caprine isolate from Cyprus and the English isolate was inconclusive. Horgan and Hasseb (1947) again by cross-protection tests in sheep showed that several of their isolates were immunologically indistinguishable while others were closely related. They described the difference of the reactions as being partial to complete but explained these different reactions as quantitative differences in antigen or antigens. However, they did not exclude the possibility that these minor differences were due to an expression of strain variance.

Trueblood et al., (1963) were of the opinion that strain variance had not been fully investigated. The occurrence of the disease in vaccinated animals led them to doubt the monovalence of the virus. Sawhney (1966b) cited Sarwar and Barya (1961) as having isolated a virus causing dermatitis in sheep in Pakistan which differed in some respect with the classical ecthyma virus. Also cited was

Bragazzi (1959) who observed outbreaks with mortality rate of as high as 30% and as low as 1% which indicated the possibility of different strains. However, although, these papers have not been cited by the author, the possible confusion with other diseases showing similar lesions such as sheep pox could account for such large difference in virulence. Valder et al. (1979) also described an outbreak of orf with clinical picture that differed from usual cases. They described this as a malignant form, and could be due to a different strain of the virus.

From the results of serum-neutralisation and crossprotection tests, Sawhney (1966b) concluded that there was a multiplicity of strains of contagious ecthyma virus. He tentatively classified Bulgarian B2 isolate and the English isolate in one antigenic group and the Rumanian and Slovakian isolates in another. He further showed that an Iranian isolate resembled the English isolate. However, Bulgarian isolate B1 differed from the B2 isolate. He stated further that although these strains did not differ in the type of Cytopathic effect they produced in cell culture, titres of the Rumanian and Slovakian isolates were always comparatively lower than those of the Bulgarian and English isolates (Sawhney, 1966c).

Another attempt to show strain variation was reported by Precausta and Stellman (1973). The five isolates studied were similar in their physico-chemical characteristics, however, they showed antigenic differences. Based on the neutralisation titres and indices, they placed four of the isolates under one antigenic subtype and the other in another subtype although the latter was very closely related to the other four. Wittek et al. (1980) in a comparison of eight pseudocowpox virus isolates and three orf virus isolates demonstrated differences in the restriction endonuclease patterns of these three orf virus DNAs.

It appears likely that strain variation does exist in orf virus as determined by serum-neutralisation and cross-protection tests. However, none of these studies are conclusive and the definitive experiments have yet to be done using plaque-purified virus.

GROWTH OF ORF VIRUS IN CELL CULTURES

Since Greig (1957) reported the first successful attempt to grow orf virus in cell cultures, in vitro studies of the virus have been often reported. Greig (1957) and Webster (1958) used primary embryonic skin cells but the latter did not observe cytopathic effect (CPE) with a New Zealand vaccine strain of virus. There was however, viral multiplication. Plowright et al, (1959) was able to propagate a virus strain in primary testis and kidney cells of ovine and bovine origin. Similar results were obtained by Nagington and Whittle (1961), MacDonald and Bell (1961) and Trueblood and Chow (1963). Primary human amnion cells could also be used to propagate the virus (Nagington and Whittle, 1961; MacDonald and Bell, 1961). Nagington and Whittle (1961) also used monkey kidney cells with success but failed to grow the virus in HeLa, MK2 and AM9 cell lines. Other successful attempts to propagate the virus in cell cultures were reported by Sawhney and Toschkov (1971, 1972) in HeLa and lamb testis cells; Ramyar (1973) and Precausta and Stellman (1973) in primary lamb kidney cells; and Rossi (1973) in chick and duck embryo fibroblasts. These findings show that cell culture-adapted orf virus can be propagated in a wide range of cell types.

There are divergent views on the cytopathogenic effect of orf virus on monolayer cultures. As mentioned earlier Webster (1958) did not observe CPE with a N.Z. vaccine strain of the virus inoculated onto embryonic sheep skin cells. However, he could demonstrate virus multiplication.

The authors that did observe a CPE reported a wide range in the time interval between inoculation and its appearance as well as the completion of CPE. For example, early signs of CPE were seen at 18 hours (Nagington and Whittle, 1961; Sawhney and Toschkov, 1972) at 20 hours (Ramyar, 1973; Precausta and Stellman, 1973) or as late as 36 hours (Greig, 1957; Rossi, 1973), and two days (Sawhney, 1966c). Observation of complete CPE also varied from 24 hours to 28 days. This wide variation could be due to the size of inoculum, type and batch of cells and age of culture (Nagington, 1968). Young and rapidly multiplying cells are more sensitive to virus infection than older cultures. Other factors that might affect production of CPE are passage and strain of virus (Sawhney, 1966c) and the origin of the serum used in the culture medium (Nagington, 1968; Zebrowski et al., 1974).

Inclusion bodies were seen and described by some of the authors cited above. Plowright et al. (1959) observed intracytoplasmic eosinophilic masses at twelve hours post-inoculation. These masses were oftentimes surrounded by an ill-defined halo. Sawhney and Toschkov (1971, 1972) and Rossi (1973) saw similar inclusions at two days post-inoculation, the former using HeLa and lamb testis cells and the latter, avian cells. Eosinophilic inclusions usually paranuclear were also seen by Nagington (1968) and Zebrowski et al. (1974). The behaviour of orf virus in cell cultures as observed by the above authors is summarised in Table I.

The virus seems to show a preference for primary cells of sheep or human origin, not more than four days old, and in the 1st to 10th subculture passages (Nagington, 1968). Nagington (1968) observed that orf virus from sheep would grow initially on sheep cells only. However, he found that this specificity was lost on subsequent passages or if the

TABLE I: CYTOPATHIC EFFECT OF ORF VIRUS AS DESCRIBED BY DIFFERENT AUTHORS

Cell Type	Virus Strain	CPE	Time of Appearance of CPE	Type of CPE	Author
Embryonic lamb skin	2 isolates from Canada 1 isolate from Sastratchewan	+	3-4 days	Enlargement rounding of cells. Increased granularity of cytoplasm with eventual fragmentation. Some cells detached from glass, other cells clumped together. Complete CPE in 7 days.	Greig (1957)
Suspended sheep skin	N.Z. vaccine Strain	-		no CPE observed but with possible replication	Webster (1958)
Sheep testis	Welcome Strain	+		nonspecific reaction on first day. Medium was changed. Shrinkage and rounding of remaining cells. Complete CPE in 4 days. Next passage, CPE complete in 7 days. CPE complete in 2-3 days on subsequent passages. Also mentioned detachment but did not mention clumping of cells.	Plowright et al (1959)
Sheep embryonic kidney	P3 from testis cells	+	Within 24 hrs	Complete CPE in 48 hours. Subsequent passages, Complete CPE between 2-4 days.	
Calf kidney	P3 from testis cells	+ +	Within 24 hrs	Rapid CPE 80-90% cell infection by 4th day next passage, still some normal cell-islets on 7th day. Subsequent passages showed similar extent till 4th day.	
	P4 from testis cells	+		Almost complete CPE on 2nd and third day, centripetal spread of infection. Similar changes on subsequent passages.	
Goat kidney	P4 from sheep kidney	+		Similar as in sheep kidney	

TABLE I: (continued)

Cell Type	Virus Strain	CPE	Time of Appearance of CPE	Type of CPE	Author
Lamb embryonic kidney	"Stock Strain" from Aberdeen University	+		Early rounding (time not specified) and swelling of cells followed by clumping. Using heavy inocula CPE seen in 24 hours. Almost complete CPE in 3 days.	MacDonald & Bell (1961)
Human embryonic kidney Human amnion Human embryonic liver	P3 from lamb kidney	+		Similar CPE as above.	
1^{o} Human amnion 2^{o} Monkey kidney Cell lives: HeLa MK_2 AM9	2 Human isolates	+ + Toxic Effect " "		Isolate #1 - rounded granular cells after 18 hrs, no further change until 15 days later. Then monolayer showed holes at the edge of the sheet. Marginal cells become refractile and in some the cytoplasm becomes granular. Isolate # 2 produced CPE more rapidly, 5 days. Good inclusion bodies are rare. In monkey cells, hole formation was less marked but more rounded cells.	Nagington & Whittle (1961)
Bovine fetal kidney, skin, muscle Ovine fetal kidney	Fort Dodge vaccine (USA)	+ +	3 days	Did not describe type of CPE CPE complete in 6 days	Trueblood & Chow (1963)

TABLE 1: (continued)

Cell Type	Virus Strain	CPE	Time of Appearance of CPE	Type of CPE	Author
1° lamb testis 1° lamb kidney 1° calf testis 1° calf kidney 1° pig kidney	Bulgarian B_2 Czechoslovakian United Kingdom Rumanian German Iranian	Complete Never complete Complete Never complete Not mention Inter-mediate	2 - 10 days	First sign. Loss of cellular boundary cells, rounding of the cells followed by appearance of gaps on holes in the monolayer, cells become more refactory before lysis. Inclusions seen in English and Bulgarian B_2 isolates only.	Sawhney (1966)
Bovine testis Sheep testis Human amnion	ORF Monedwin ORAFLAM vaccine Field strain	+		Rounding of cells with highly repactile cytoplasm and appearance of holes in the monolayer. Produced "open type" plaques	Nagington (1968)
Hela	Bulgarian strain (P5 from lamb testis)	+	48 hrs	Loss of cellular boundary in 48-72 hours. Pyknosis of nuclei, granulation of cytoplasm and appearance of vacuoles. Almost complete CPE in 6 days. Observed somewhat attenuating effect on virulence.	Sawhney and Toschkov (1971)
Lamb testis	Bulgarian strain	+	18 hrs	Cytotoxic effect in 24 hours. At 42 hours 60-90% cells lost normal shape. Gaps and holes appear in monolayer. There is cell rounding. Complete CPE in 4-5 days. Inclusions seen.	Sawhney and Toschkov (1972)

TABLE I: (continued)

Cell Type	Virus Strain	CPE	Time of Appearance of CPE	Type of CPE	Author
1° lamb kidney	Local isolate 4 vaccine strain	+ +	24 hours	Complete CPE in 3 days. Did not describe CPE	Ramyar (1973)
Duck embryo fibroblasts Chick embryo fibroblasts	Not specified	+	36 hours	First sign was cellular pyknosis, lateral displacement of the nuclius, enlargement of cellular body, vacuolation along cytoplasmic membrane. Inclusion bodies seen at 48th hour, Cells are rounded and swollen. Lysis occurred in some cells. Complete CPE in 96 hours. Virus showed loss of pathogenic activity.	Rossi (1973)
1° la mb kidney	Sheep isolate	+	w/in 24 hrs	Rounded cells with pyknosis. Produced "open type" plaques. Plaques - cellular lysis bordered by pyknotic cells seen in 24 hours. A multiplicity of infection of 10^7 TCID50/75 cm^2 flash produced CPE in 48 hours.	Precausta & Stellman (1973)
1° bovine cells	Fresh isolate from sheep	+	3-4 days	CPE in 3-11 days. More pronounced in testis cells. Irregular results in other ceel types.	Zebrowski et al (1974)
Caprine testis cells	Scab material from goats	+	3 days	Increased refractiveness and rounding of cells by 3rd day, with 70 - 90% CPE by 5th day. By 5th passage CPE was seen in 24 hours.	Renshaw and Dodd (1978)

virus isolate was from a human case. In contrast, Zebrowski et al. (1974) used primary bovine cells for isolation of the virus from scab material. While they claimed a successful attempt at isolation in testis cells, they pointed out that results in the other cell types were variable or no cytopathic effects were observed. They also observed that the virus appeared to prefer fibroblastic rather than epithelioid type of cells. This is in agreement with the findings of Plowright et al (1959) and Sawhney (1966c) in that fibroblastic testis cells were superior to epithelioid kidney cells.

There are only a few workers who have attempted to quantitate the yields of orf virus in cell cultures. In lamb testis cells, Plowright et al. (1959) observed the peak of virus multiplication at the 54th hour post-incoulation. The cell-associated virus gave a titre of 10^9 $TCID_{50}/80mm^2$ Carrel flask. The free virus was 0.4 to 1 log unit lower. In kidney cells, the titre was somewhat lower. The peak of multiplication was observed at 48 hours post-infection. The cell-associated virus had a titre of 10^8 $TCID_{50}/80mm^2$ flask and the free virus, of 0.2 to 1 log unit lower. They observed that freezing and thawing the culture several times resulted in higher titre probably due to the dispersion of viral aggregates and disruption of cells releasing more virus particles. MacDonald and Bell (1961) obtained 10^6 $TCID_{50}$ per 0.1 ml on repeated passage of the virus in embryonic lamb kidney.

Sawhney (1966c) observed that there was a difference in the titre depending on the strain of the virus, cell types and passage of the virus but there was a tendency of the virus to stabilise after the 12th passage. The titres he obtained varied from 2.5 - 4.8 $\log_{10}$ $TCID_{50}$ per 0.2ml at the 4th passage to 3.2 - 7.2 $\log_{10}$ $TCID_{50}$ per o.2ml at the 12th passage. Virus cultured in lamb testis cells gave higher titres than those grown in lamb kidney cells, except

for one Iranian strain and the Bulgarian GM_{53} strain which grew equally well in both cell types. Using primary lamb kidney cells, Precausta and Stellman reported a titre of $10^{6.5}$ to $10^{7.5}$ $TCID_{50}$ per ml.

Renshaw and Dodd (1978) obtained a titre of 10^3 to 10^5 $TCID_{50}$/ml of orf virus grown in caprine testicular cells. In avian cells, virus multiplication attained its maximum between the 72nd to 96th hour post-inoculation with a titre of $10^{7.8}$ pfus/ml (Rossi, 1973). Neither author however mentioned the passage number or initial size of virus inoculum.

The differences between these results do not conclude that one method of quantifying virus titre is superior to the other, nor that one virus strain is more virulent than the other, for there is no standard basis of comparison. The most important criteria for comparison would be the initial size of inoculum (or multiplicity of infection), the number of cells infected and the technique for assaying virus titre. Such factors are adsorption time and diluent used in adsorbing virus might also affect titres. Also, as shown with other viruses, use of overlay additives may enhance the number and size of plaques in the plaque assays for viruses (Tobita et al., 1975; Gil-Fernandez et al., 1976).

CHAPTER TWO

MATERIALS AND METHODS

Isolation and purification of orf virus

Origin of orf virus. Thirteen samples consisting of pooled scab material were each taken from a line of lambs awaiting slaughter. The samples were sent from a number of meatworks throughout New Zealand (see Table II). The samples were kept at -70°C until processed.

Samples were processed three at a time. Five grams of scab material from each sample were weighed and ground in a mortar and pestle in 50 mls. of 10nM Tris-HCl pH 7.5. A small amount of carborundam was added to aid disruption of the scab. The suspension was placed in a 30 ml-screwcap centifuge tube and centrifuged at 5000 RPM (3020g) for 30 minutes in the SS34 rotar at 4°C in the Sorvall RC5 centrifuge. A second centrifugation at this speed was sometimes necessary. The supernatant was then collected and 25 mls. was layered over 5 mls. of 36% sucrose in 0.1x ET buffer. This was centrifuged at 22,000 RPM (50,000g) for 30 minutes at 4°C in the Beckman Model L ultracentrifuge using Sw 25.1 rotor. The supernatant was discarded and the remainder of the sample was processed in the same manner using the same centrifuge tube still containing the first pellet. The resulting pellet containing the virus was resuspended with 1 ml. of 0.1x ET buffer and was layered onto a 25-50% sodium diatrizoate gradient. The preparation of the aryl gradients was as follows. Sodium diatrizoate solutions, 25 and 50% (w/v) were prepared in 0.1x ET buffer. Ten mls. each of the solution were placed into each of the cylinders of the gradient maker with a mixer inserted into one. The solutions were pumped slowly into a 1" dia. x 3" cellulose nitrate tube. Ten mls. of 10% Dextran T10 in 0.1x ET buffer was layered over the gradient.

TABLE II: ORIGIN OF SAMPLES

Sample No.	Owner/Farm	Location	Freezing works/sender
1	N.S. Chatfield	R.D. 2, Dannevirke	Longburn Freezing Works
2	S.E. Hill	R.D. 2, Dannevirke	Longburn Freezing Works
3	Nitske	R.D. 2, Dannevirke	Longburn Freezing Works
7	A. Reeves	R.D. 1, Hinerua, Onga Onga	Nelson N.Z. Ltd, Hastings
8	Trellinoe Station	Tepohue	Nelson N.Z. Ltd., Hastings
9	J.N. Briant	P.O. Box 3, Matawai	AFF Co., Rangiuru
10	Okere Corp.	P.O. Box 456, Rotorua	AFF Co., Rangiuru
11	T.B. McCone	13 R.D., Tapui, Oamaru	Waitaki Refrig. Ltd. Oamaru
12	W.J. Joneston	Fiveforks, 16 R.D., Oamaru	Waitaki Refrig. Ltd. Oamaru
13	M.I. Francis	Duntroon, 5 R.D. Oamaru	Waitaki Refrig. Ltd. Oamaru
14	Addington Saleyards		MAF, Islington
18	J.S. Robertson & Sons	Cadrona, 1 Rd., Wanaka	C.F.M. Co., Pareora Sth. Canterbury
19	E.D. Nicholson	3 R.D. Alexandra	C.F.M. Co., Pareroa Sth. Canterbury

For gradients suitable for the Beckman Sw 50.1 rotor, 2 mls. each of the 25 and 50% sodium diatrizoate and 0.8 ml. of DT10 were used in a ½" dia. x 2½" cellulose nitrate tubes.

The gradient with the partially purified virus was centrifuged overnight at 22,000 RPM (50,000g) using the Sw 25.1 rotor. The virus, if any, was visible as a white band in the middle of the gradient. (Fig. 1). The virus band was collected with the use of a peristaltic pump. The virus suspension was diluted with 0.1 X ET buffer at 1:1. This was layered over 1 ml. sucrose cushion and centrifuged at 22,000 RPM (30,000g) using the Sw 50.1 rotor for 30 minutes. The pellet was resuspended with 1 ml. of 0.1X ET buffer. Any residual sodium diatrizoate was removed by exhaustive dialysis for three days against 0.1x ET buffer pH 8-9, with frequent changes of the buffer. Removal of the last traces of diatrizoate was essential as it interferes with subsequent spectrophotometric analysis. Every step was done on ice or 4°C as appropriate.

Spray droplet counting of orf virus particles. Particle counting was done by the method of Backus and Williams (1950). To 15-30 ul of the virus suspension (either neat or diluted 1:10 depending on the concentration of the virus as determined by eye), 10 ul of 0.5% bovine serum albumin (BSA), 50 ul of 2% phosphotungstate pH 7.2 and 10 ul of 1:100 latex beads were added. The volume was made up to 100 ul with deionised distilled water and 50 ul of the solution was sprayed onto a formvar carbon-coated Cu grid using a spray gun. This was done inside a biohazard hood. The grids were examined under the electron microscope. At least eight photographs of suitable droplets from each sample were taken (Fig. 2). Average counts of orf particles and latex beads were taken. A 1:100 dilution of latex beads (Dow, 91 nm average diameter) in TE buffer from a stock of 10% solids solution represents 2.42×10^{10} beads per 10 ul. This was based on the assumption that the density of latex is 1.052.

The number of particles in the original virus suspension was then determined by a simple calculation.

Measurement of density of fractions from aryl gradients. The density relative to water of each gradient fraction was measured. Fractions were collected from the bottom of the centrifuge tube by passing a tube from the top of the gradient to the bottom. The gradient material was drawn through the tubes by a peristaltic pump and equal fractions were collected. An aliquot of each fraction was drawn into a 100 ul capillary tube, until filled and weighed. Two measurements were taken for each fraction. Tubes were rinsed with distilled water and dried with acetone in between sampling. To calculate relative density, the weight of each fraction was compared with the weight of an identical volume of water.

DNA extraction

To the virus suspension, 1/10 volumes 10 mg/ml pronase was added and incubated for 30 minutes at 37°C. Then 1/10 volume 5% SDS in 45% ethanol was added and incubated for a further 30 minutes at 37°C. Two to three volumes of phenol equilibrated with STE buffer was added, mixed gently for 2-5 minutes. The mixture was centrifuged at 2000 RPM for five minutes. The aqueous phase was carefully removed and phenol extraction was repeated twice. The extracted DNA was transferred into a dialysis tubing and dialysed exhaustively against TE buffer. The DNA concentration of each solution was calculated from the absorbance in a 1 cm light path quartz glass cell at a wave-length of 260 nm in a Model SP 500 series 2 spectrophotometer. An absorbance of 1.0 was taken to be 50 ug DNA/ml.

Restriciton endonuclease digestion and gel electophoresis of orf virus DNA

A 50 ul digest, containing 0.5 ug DNA, 10 ul of 5x R_1 buffer, 1 ul of $EcoR_1$ and water was incubated for an hour at 37°C. The digested DNA was precipitated with ethanol as follows: -

Ethanol precipitation. To the digest was added 1/10 volume 5M NaCl, two volumes 95% ethanol, mixed well and incubated at -20°C for at least 30 minutes. The mixture was then centrifuged for 5 minutes in a microfuge. The ethanol was tipped off and the precipitate was washed gently with cold 70% ethanol. After drying for 15-30 minutes, the precipitate was resuspended with 50 ul TE buffer.

The digested DNAs were subjected to electrophoresis in a 0.7% agarose slab gel containing ethidium bromide (0.5 ug/ml). Electrophoresis was done for 4-5 hours at 4 volts/cm of slab gel. The gel was placed on a filter plate measuring 15 x 15 cm in a light box. The pattern of DNA bands was visualised by illumination from below with 254 nM wavelength U.V. light and photographed with a 4" x 5" format plate camera through a wratten 23A filter onto Kodak Tri-X film.

Propagation of orf virus in cell culture

Culture Medium. Medium 199 (Tricine buffered) containing 20, 10 or 2% foetal bovine serum (FBS) as required and 100 I.U. penicillin, 100 ug Streptomycin and 100 ug Kanamycin per ml.

Cell Cultures. Low passage lamb testis, foetal ovine testis and bovine lung cells; ROK (ovine kidney cells that had undergone30 or more passages) and RK13 (rabbit kidney cell line). Primary cells were prepared by standard methods (Ferris and Plowright, 1958; Hess et al., 1963). Cells

were stored in 1 ml glass ampoules in liquid nitrogen at a concentration of about 40 x 10^6 cells per ampoule in T199 containing 20% FBS and 10% DMSO. For maximum recovery of viable cells from frozen stocks, thawed cells required gradual reduction of the DMSO concentration. This was done by progressive dilution of the cells with 1 ml. medium at 1 minute intervals for 3-8 dilution steps. Cells were finally diluted to the desired concentration in growth medium. Using this method, 90-100% of the cells remained viable as determined by trypan blue exclusion.

Detection of viable cells by trypan blue exclusion. One ml. of thawed stock cells or trypsinised cells were reconstituted with 9 mls. of growth medium. 0.2 ml. of the suspension was diluted with 1.8 mls. of 0.2% trypan blue in PBS. A small drop of the above was placed into each of the two haemocytometer counting chambers. Viable cells did not take up the stain while dead cells were stained blue. Viable cells were counted on eight corner squares of the chamber and the average count for one square was calculated. This count multiplied by 10^5 represented the number of viable cells per millilitre of the original suspension.

Propagation of cells in culture. Cell monolayers for virus inoculation were grown either in Linbro trays, 35mm x 10mm plastic dishes, or 25 cm^2 or 75 cm^2 plastic flasks. If plastic dishes were used, cells were incubated at 37°C in an atmosphere of 5% CO_2. Monolayers were usually confluent in 1-2 days if the cells were seeded at 1.5 - 2 x 10^5 cells/ml. With lamb testis cells, only cells up to the 5th subculture passage were used. Such cells were difficult to maintain after the fifth passage. Cells were subcultured at confluence by trypsinisation.

Virus inoculation of cell cultures. A standard procedure of virus infection of cell monolayers was followed. When monolayers became confluent, they were washed at least once with phosphate-buffered saline. The virus inoculum in T199 was allowed to adsorb for an hour at 37°C with tilting every 15 minutes. After adsorption, the medium was replaced with maintenance medium containing 2% FBS. Infected monolayers were incubated at 37°C and examined every 24 hours or as often as necessary for at least 14 days or until complete CPE was observed, after which they were frozen at -70°C. Different inocula and different methods of serial passage of infected monolayers were tried.

Plaque-purification of virus isolates. Attempts at plaque-purification were made with the tenth passage of isolates number 2, 3, 9, 10 and 12. Each of these virus preparations were derived from sodim diatrizoate-purified virus rather than directly from diluted scab material. Serial ten-fold dilutions of each isolate were prepared in T199 without serum. Lamb testis cells grown in 10mm x 35mm dishes were inoculated with 400ul of each dilution in duplicate. The virus was allowed to adsorb for one hour at 37°C. After adsorption, excess inoculum was removed by suction. Each dish was then overlayed with 2 mls. of equal quantities of double strength T199 (containing 4% FBS and antibiotics) and 2% sea-plaque agarose. When the overlay had gelled, the dishes were incubated in an inverted position in a CO_2 chamber at 37°C for a week. Five plaques from each isolate were picked with a pasteur pippete and resuspended in 500 ul of medium without serum. After freezing and thawing two or three times, 250 ul was inoculated into a 10 x 35 mm dish containing lamb testis cells. Each plaque was passaged four times before a second plaque-purification attempt.

^{32}P- labelling of orf virus DNA

Culture medium. Phosphate-free medium using Dulbecco's Modified Eagles Medium formulation was prepared. HEPES at 20 mM concentration, 2% FBS and antibiotics were added just before use.

Isolate No. 2 was used in radioisotope labelling experiments. Labelling was done after the first and second plaque-purification. A concentrated virus suspension was obtained from two 75cm^2 flasks of infected lamb testis cells. The virus was released by freezing and thawing the monolayers several times and sonicating for 1 minute. The cell debris was removed by low speed centifugation for 15 minutes. The supernatant was layered over a sucrose cushion in a U.V. light-sterilised cellulose nitrate tube. This was centrifuged at 22,000 RPM (50,000g) for 30 minutes. The pellet was resuspended in a phosphate-free medium without serum. Another method of preparing virus for concentration was done by pelleting the cells at low speed and grinding or crushing the cells in a Ten broeck tissue grinder. This and the infected fluid from low speed centrifugation was concentrated by centrifugation as described above.

For labelling, LT monolayers were grown in 75 cm^2 flasks. When the monolayers were confluent, they were starved of phosphorus overnight by substituting the growth medium for a phosphate-free medium. The following day the medium was removed by suction and the monolayer washed twice with phosphate-free medium without serum. The inoculum which was also resuspended in phosphate-free medium was allowed to adsorb to each monolayer for an hour at 37^{o}C. After adsorption, excess inoculum was removed and replaced with phosphate-free medium containing 2% FBS, PSK and 0.5 mCi ^{32}P (Orthophosphoric acid) per flask. The monolayers were incubated at 37^{o}C. When complete CPE was observed, usually after two days, the monolayers were frozen and thawed several times and sonicated. The ^{32}P-labelled virus was

concentrated as described earlier. The concentrated virus was was layered onto a 5 ml sodium diatrizoate gradient and centrifuged at 22,000 RPM overnight.

The gradient was fractionated dropwise (12 drops per fraction) and each fraction was assayed for radio-activity in a liquid scintillation counter. The fractions containing the peak of radioactivity were pooled, diluted with 0.1 x ET buffer, and centrifuged at 22,000 RPM for 30 minutes over a sucrose cushion. The pelleted radio-isotope-labelled virus was resuspended in 0.1x ET buffer and dialysed in 0.1x ET pH 8-9.

DNA was extracted essentially the same as described for unlabelled DNA except that 1 ul of 10 mg/ml yeast RNA was used as carrier to maximize recovery. The DNA was ethanol-precipitated following phenol extraction and resuspended in 100 ul TE buffer. About 2500 cpm (counts per minute, Cerenkov) of radiolabelled DNA was used for each restriction endonuclease digest. EcoR1, Bam HI and Hind III were each used in the analysis. Unlabelled DNA obtained from a proportion of the original isolate was included in each digest to enable comparison to be made with the ^{32}P-labelled DNA. The digested DNA was electrophoresed in 0.7% slab gel containing ethidium bromide. To locate the unlabelled DNA, the gel was illuminated from below with 254nM U.V. light and the DNA patterns photographed. The gel was then returned to the glass plate and covered with 3MM paper, a half-inch thick layer of paper towels and a weight to dry the gel down overnight. The dried gel was covered with a thin plastic film (Gladwrap). Two X-ray films were placed between the gel and another glass plate, clipped together, and wrapped in black plastic bag. In one experiment, a fast tungstate X-ray intensifying screen was used and autoradiography was done at -70^{o}C.

Effect of cyclohexamide on ofs CPE

The virus used in this experiment was a concentrated preparation of isolate No. 10 of the tenth passage from two 75 cm^2 flasks as described above. One concentrate was resuspended with T199 containing 300 ug/ml cyclohexamide, the other with T199 medium alone.

Four 25 cm^2 flasks of lamb testis cells were treated as follows. One flask was infected with a virus suspension containing cyclohexamide, another with a virus suspension without cyclohexamide, a third with cyclohexamide alone and the fourth with medium alone. After adsorption, medium was changed to the corresponding maintenance medium with or without cyclohexamide. In the same experiment, cyclohexamide was removed 5 hours post-inoculation and replaced with maintenance medium.

Cover slip preparations

Glass coverslips were washed in distilled water and autoclaved before use. Monolayers were grown in Linbro trays with a glass coverslip in each well. When monolayers were confluent, each well was infected with 200 ul of virus, concentrated by centrifugation as described. Duplicate coverslips were removed at 1, 3, 6, 9, and 24 hours post-inoculation, and uninfected coverslips for control. The coverslips were fixed in absolute alcohol and stained with May-Grunwald-Giemsa.

May-Grunwald-Giemsa stain. The following procedure was adopted.

1. Fix in absolute methanol for at least 5 minutes.
2. Flood slide with May-Grunwald stain and then mix in equal volume of $PO_4^{=}$ buffered water pH 6.6-6.9. Mix by blowing gently on the surface. Alternatively, mix solutions before flooding slide. Stain for 15 minutes.
3. Wash with buffered water. Do not tip off stain before

washing to avoid precipitation of stain.

4. Flood with aqueous working strength Giemsa stain for 20 minutes.
5. Flood off stain with buffered water.
6. Air dry. Dip in xylol.
7. Mount in Depex/xylol.

CHAPTER THREE

RESULTS

Virus purification

When partially purified scab material was centrifuged in a 25-50% sodium diatrizoate gradient, a band of virus appeared in the middle of the gradient. (see Fig. 1). The fraction containing the virus band had a relative density of 1.23. A less distinct band was sometimes seen below the main band. The main band consisted mainly of type 1 or M forms while the minor band was predominantly of the type 2 or C forms. Only the major band was used for DNA and isolation studies. It was observed that virus aggregated when dialysed at a pH of 6-7. This aggregation was not observed when a Tris-HCl buffer with a pH between 8-9 was used.

Particle counting

Results of the spray droplet counting of individual virus preparations are shown in Table III. Between 0.07 to 10^{11} particles can be recovered from one gram of scab material and 0.74 to 17 ug of DNA can be recovered from the virus. Also shown is the theoretical DNA content of each virus preparation estimated using the molecular weight of 89×10^6 for orf DNA.

Restriction endonuclease analysis

The DNAs of the thirteen orf virus isolates were digested with EcoR1. When the digested DNAs were subjected to electrophoresis in a 0.7% agarose slab gel, they showed varying fragment patterns as shown in Fig. 3.

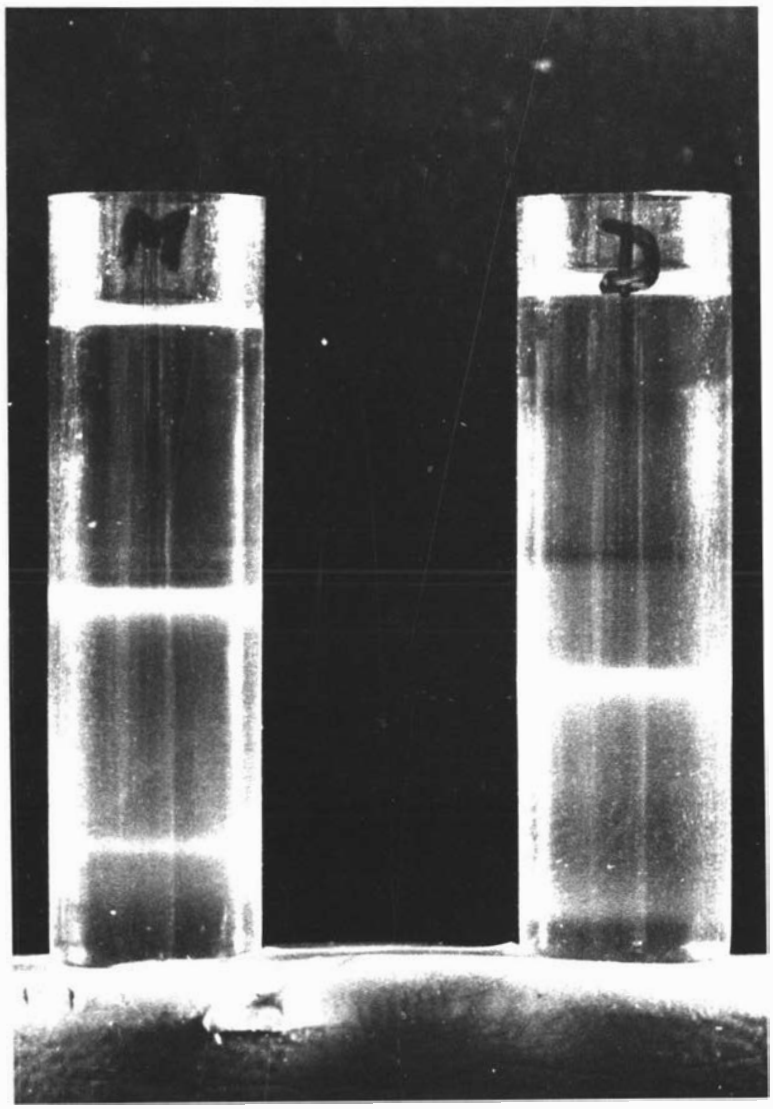

FIGURE 1: Na metrizoate (left) and Na diatrizoate (right) gradients containing virus bands. A minor band which is more distinct in Na metrizoate is seen below the main band.

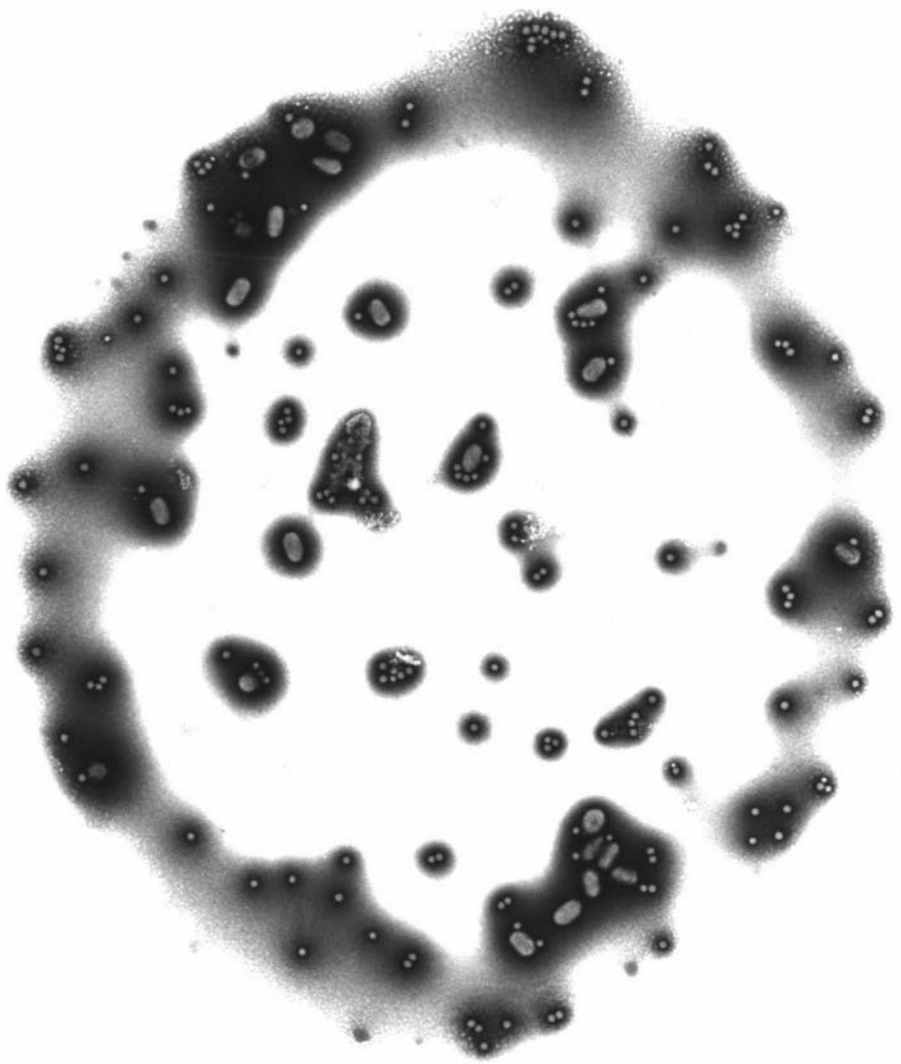

FIGURE 2: A spray droplet containing orf virus particles and latex beads. X 21,000

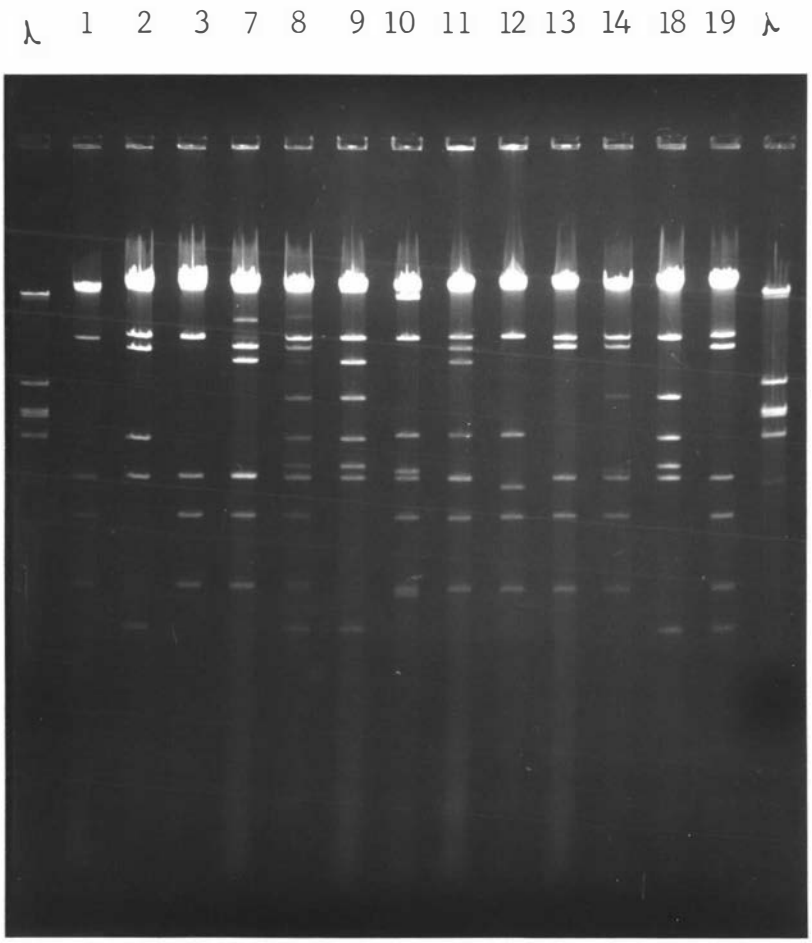

FIGURE 3: Agarose gel electrophoresis of thirteen orf virus DNAs digested with $Eco R_1$ restriction endonuclease. Electrophoresis was on a 0.7% agarose slab gel for 4 hours at 80V. Lambda DNA was used as control.

TABLE III RECOVERY OF ORF VIRUS AND ORF DNA FROM SCAB MATERIAL

Sample number	Weight of scab material (g)	Total particles ($\times 10^{11}$)	Estimated DNA content (µg)	Total DNA recovered (µg)
1	3	not done		4.5
2	5	0.98	11.97	14.9
3	5	5.00	64.30	68.5
7	5	0.58	7.00	7.5
8	4	1.00	15.20	8.23
9	5	1.57	16.70	12.9
10	5	1.74	21.30	25.8
11	5	0.37	5.49	3.85
12	5	0.43	6.40	6.6
13	5	0.47	7.00	4.2
14	5	0.40	5.80	3.7
18	5	0.71	10.50	9.2
19	5	0.78	11.70	18.9

When ^{32}P-labelled isolate No. 2 DNA was digested with EcoRl, Bam H-I and Hind III, the resulting patterns did not differ from the patterns produced from the viral DNA derived from the original scab material (Fig. 4). Isolate No. 2 had undergone two plaque-purification steps and 23 passages in lamb testis cells before labelling with ^{32}P.

Growth in cell culture

Trial 1: Using virus purified in sodium diatrizoate gradients.

Experiment 1: One ml. of a 1 in 10,000 dilution of virus from scab pool purified as described in methods was used to inoculate 75 cm^2 flask of foetal ovine testis cells. This was approximately 20 x 10^6 particles per 25 cm^2 flask. Only isolate No. 3 was used. The infected monolayer was incubated for a week and then blind passaged at weekly intervals using 1 ml. of the previous passage as inoculum. For the third and subsequent passages, lamb testis cells were used. This isolate was passaged ten times in lamb testis cells and after the 10th passage, used for attempts at plaque-purification.

Experiment 2: Sodium diatrizoate-purified virus scab pool Nos. 2, 3, 7, 8, 9, 10 and 11 were inoculated onto lamb testis cells grown in 25 cm^2 flasks at a multiplicity of 10 x 10^6 particles per flask. Infected monolayers were examined for 14 days or until complete CPE was observed, after which they were frozen and thawed twice. In subsequent passages, 1 ml. of the previous passage was used as inoculum. Only one virus was isolated and propagated, of the initial seven pools. This was from scab pool No. 10 and it was subsequently plaque-purified twice.

Experiment 3: Lamb testis cells were inoculated at a multiplicity of 100 x 10^6 particles/25 cm^2 flask. Subsequent passages were done as in the above experiments. Isolates were given at least five passages. All isolates except isolate No. 1 were tried in this experiment. Three

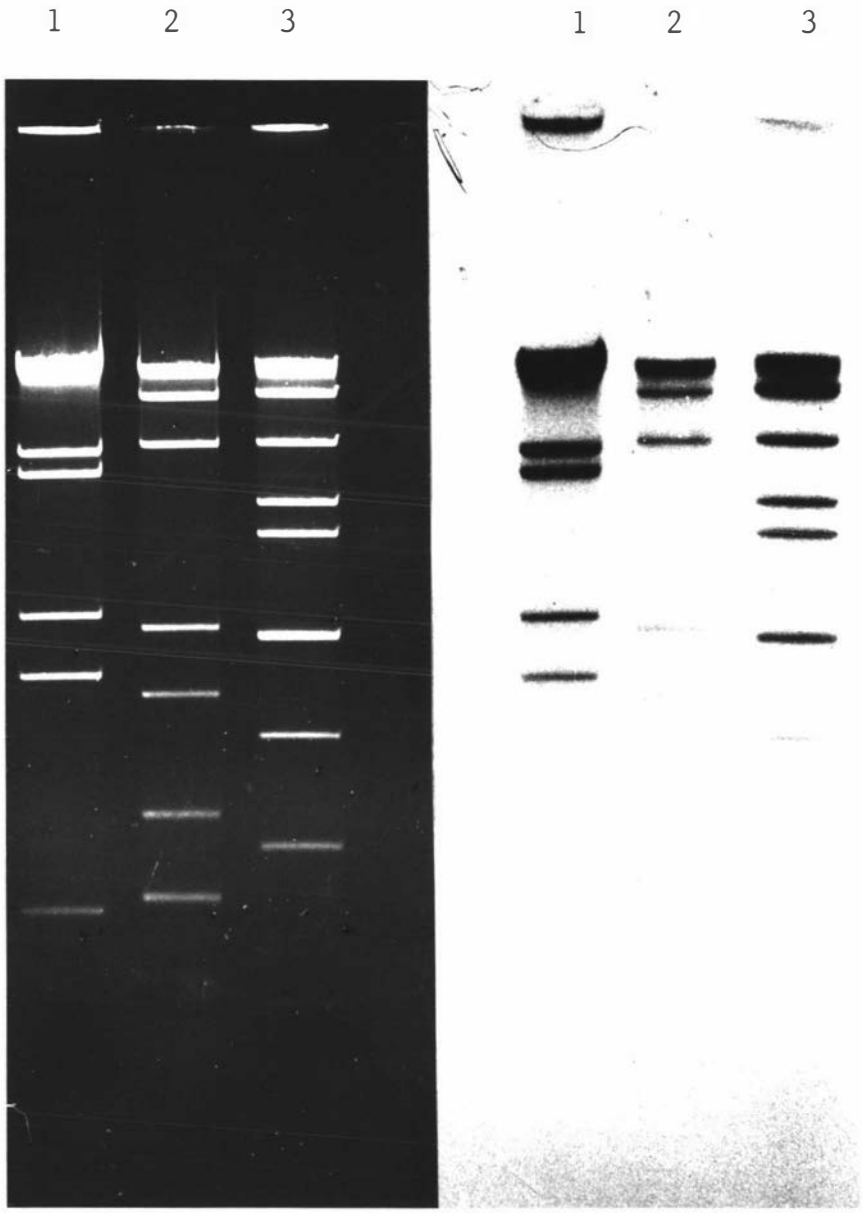

FIGURE 4: Agarose gel electrophoresis of isolate No. 2. Electrophoresis was on a 0.7% agarose slab gel for 4 hours at 80V. (A) Lanes contain virus DNAs derived from original scab lesions digested with $E_{co}R_1$(1), Bam H-I(2) and Hind III(3). (B) Lanes show an autoradiograph of the same gel containing corresponding ^{32}P-labelled DNA digests derived from a plaque-purified virus passaged 23 times in lamb testis cells.

of the virus inocula, Nos. 2, 9 and 12, adapted to cell cultures and plaque-purification was attempted on these. Isolate No. 2 had undergone plaque-purification twice at the time of writing.

Trial 2: Using virus directly from scab material.

Experiment 1: Two hundred milligrams of each of twelve scab pools (excluding isolate No. 1) were suspended in two mls. T199 containing antibiotics. A 1 in 10 dilution of the triturated scab material was filtered through 0.45 u millipore filter, and again diluted 1 in 10. One ml. of this 1:100 final dilution of scab material was inoculated onto a 25 cm^2 flask of LT cells. When complete CPE was observed, infected monolayers were frozen and thawed twice. Fresh LT monolayers were infected with one ml. of this culture and incubated for another week. Subsequent sub-cultures were performed in similar manner. No virus isolations were made by this method even though complete destruction of the cell sheets (CPE?) was seen in the first passage. No CPE was observed in the succeeding passages.

Experiment 2: The remaining cell lysates from passage one from the above experiment were frozen and thawed again and each culture was sonicated for 1 minute in E/MC ultrasonic cleaner. Cell debris was pelleted by centrifugation at 2000 RPM for 15 minutes and the virus was concentrated in the same manner as described in methods. The concentrated virus was resuspended in 0.5 millilitre T199 with PSK and used to infect 25 cm^2 flask of LT cells. By following this procedure a cytopathic effect was observed in seven of the initial 12 inocula up to the 5th passage. At the fifth passage the cultures were frozen at $-70^{o}C$ and stored for future reference.

In summary, five successful isolations were made from sodium diatrizoate-purified virus and seven from scab material. (See Table IVa)

Lamb testis adapted virus was able to be propagated and showed a characteristic cytopathic effect in ROK and foetal bovine lung cells. One attempt at propagation of plaque-purified isolate No. 2 on RK13 cells was made. A cytopathic effect was observed in the first passage but in the subsequent passages no changes were seen even though virus was concentrated by centrifugation from the previous infected fluid and cell lysate.

Cytopathic effect (CPE) on Cell Culture

Lamb testis cells were used for the isolation and propagation of orf virus. In the first passage a cytopathic effect was seen as early as four hours after adsorption. This consisted of rounding of cells which would eventually detach from the flask. If the inoculum was greater than 10×10^6 particles/flask complete destruction of the cell sheets often occurred within 24 hours. When the inoculum used was 10×10^6 or 20×10^6 particles/25 cm^2 flask, the development of CPE was slower and complete CPE was never attained. For most of the samples, fewer and fewer cells showed CPE on subsequent passage until no CPE could be observed. For isolates that did adapt to cells (No. 3 and No. 10), however, CPE became more rapid by the third passage and was complete in 6 to 7 days after inoculation. Such cultures showed a more rapidly developing CPE with each passage until complete CPE was attained in 1-2 days. No further changes were noted in subsequent passages. At higher infective doses (see Table IVb) complete cell destruction was rapid in the initial infection. In the next few passages CPE was slower in developing until in subsequent passages, a rapid CPE was again observed in isolates that did adapt to the cells. A rapid CPE was also seen when the virus was concentrated in between passages. (Table IVc).

TABLE IVa: SUMMARY OF VIRUS ISOLATES GROWN AND PROPAGATED IN LAMB TESTIS CELL CULTURE

Initial Inoculum (virus particles)	No. of virus Pools processed	Pool showing CPE after 5th passage	No. of cell Culture passages Obtained as of 1.12.80
Na diatizoate - purified virus			
1. $10.10^6/25$ cm^2 flask	7	1	23
2. $20.10^6/25$ cm^2 flask	1	1	10
3. $100.10^6/25$ cm^2 flask	12	3	10+
Scab-derived virus P1 = 1:100 dilution of scab			
1. P2 = 1:5 dilution of P1	12	0	
2. P2 = concentrated P1	12	7	5

TABLE IVb: GROWTH OF Na DIATRIZOATE-PURIFIED VIRUS IN LT CELLS

Isolate No.	Initial Inoculum (virus particles)	Days to Develop CPE						
		P1	P2	P3	P4	P5	P6	P10
10	$10.10^6/25\ cm^2$	+++(16)	++(10)	++++(6)	++++(4)*	++++(2)	++++(1)	++++(2)
3	$20.10^6/25\ cm^2$	++(6)	+(14)	++++(6)	++++(6)*	++++(5)	++++(5)	++++(3)
2	$100.10^6/25\ cm^2$	++++(4)	++++(6)	+++(3)*	++++(3)	++++(4)	++++(2)	++++(3)
9	"	++++(4)	++++(6)	++(14)	++(21)	+(20)	+(16)	++++(3)
12	"	++++(4)	++++(6)	+++(21)	+++(10)	++++(3)*	+++(3)	++++(2)

* Cell rounding and clumping

\+ - < 50% CPE at time of passage
++ - < 75% CPE "
+++ - < 90% CPE "
++++ - 90-100% CPE "

No. in parenthesis represents no. of days to attain complete CPE

TABLE IVc: GROWTH OF SCAB-DERIVED VIRUS IN LAMB TESTIS CELL CULTURE

Isolate Number	Days to Develop CPE				
	P1	P2	P3	P4	P5
3	++++(2)	++++(1)	++++(2)	++(6)	++++(4)
8	++++(1)	++++(2)	++++(5)	+(6)	++(8)
9	++++(1)	++++(1)	+++(5)	+(6)	++(8)
11	++++(2)	++++(2)	++++(5)	++(5)	++++(4)
14	++++(2)	++++(2)	++++(5)	++(5)	++(8)
18	++++(2)	++++(2)	++++(5)	++++(5)	++++(4)
19	++++(2)	++++(4)	++(9)	++++(5)*	++++(2)

Initial inoculum = 1 ml of 1:100 of scab pool

Subsequent passage = concentrated cell lysate

* cell rounding and clumping

\+ 50% CPE at time of passage

++ 75% CPE "

+++ 90% CPE "

++++ 90-100% CPE

No. in parenthesis represents no. of days to attain complete CPE

The distinguishing feature of adaptation of the virus to the cell culture was clumping of rounded cells. Clumping was not a feature of the cytopathic effect seen in cultures inoculated with first passage virus. In the LT-adapted virus, early rounding of infected cells were seen two hours post-adsorption depending on the infective dose. Rounding of cells became more generalised five hours after adsorption and rounded cells started to group together but clumping became more evident in 24 hours (see Fig. 6-8). Other cells in the culture that remained attached to the flask often became more epithelioid and enlarged but would eventually round up.

Another type of cellular response observed was the induction of rounded cells with numerous tiny projections on the surface. (See Fig. 9). These cells were seen in the sodium diatrizoate-derived virus cultures in LT and FBL cells but seem more common in some isolates derived from the scab material. In isolate No. 19 of the scab derived virus, these cells were seen as early as one hour post-absorption and they detached early from the flask.

The lamb testis adapted virus when inoculated onto ROK cells showed characteristic rounding and clumping of cells. Infected FBL cells with LT-adapted virus also showed CPE. Cells rounded up and many became surrounded with tiny processes but these rounded cells appeared much smaller than those seen in LT cells. Clumping of rounded cells as seen in LT cells was uncommon. Cells that remained attached to the flask for sometime became spindle-shaped. Cells with rounded central portions attached to the flask by two pointed processes at opposite poles were frequently seen. (see Fig. 10-12).

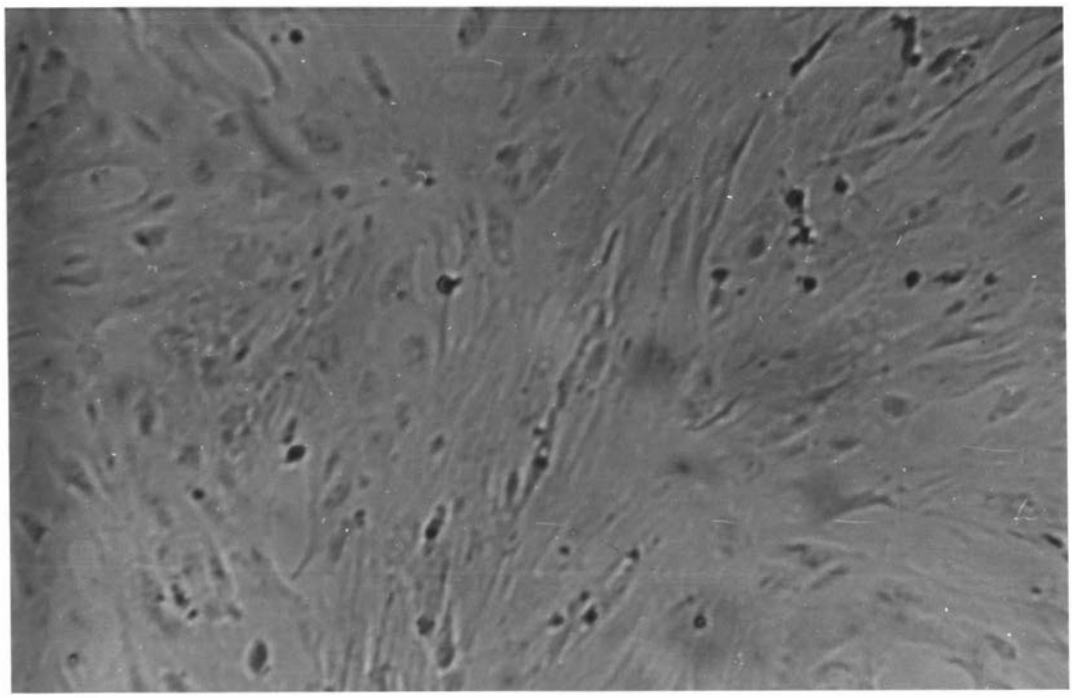

FIGURE 5: Uninoculated control lamb testis monolayer. X 134

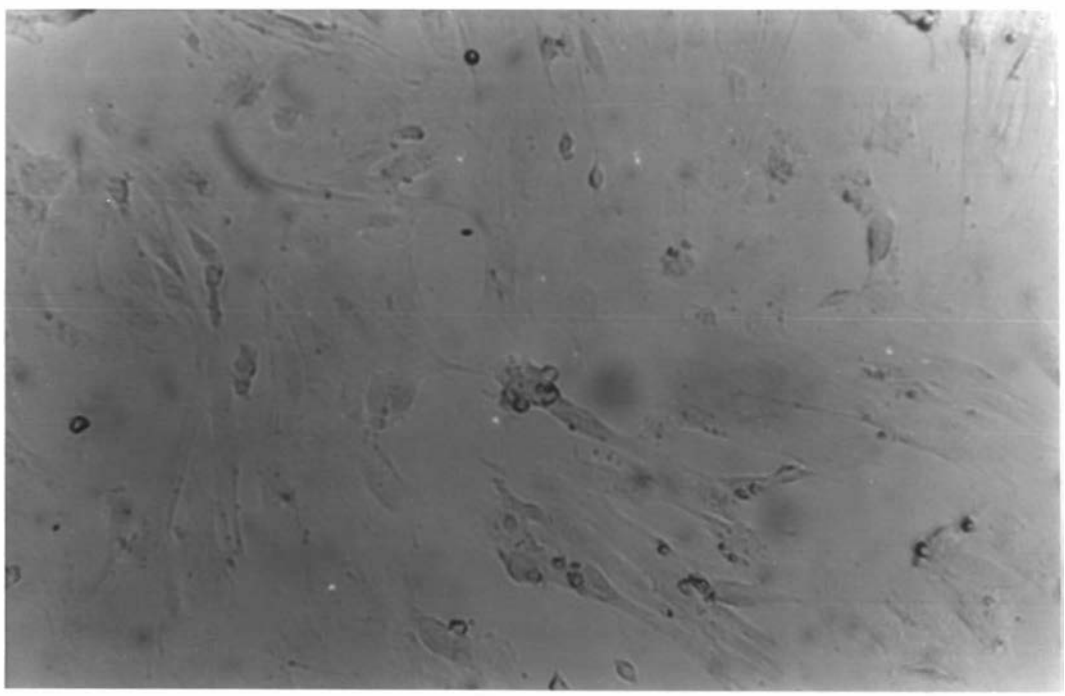

FIGURE 6: Lamb testis monolayer 2½ hours after infection with orf no. 10P10 showing early cell rounding. X 134

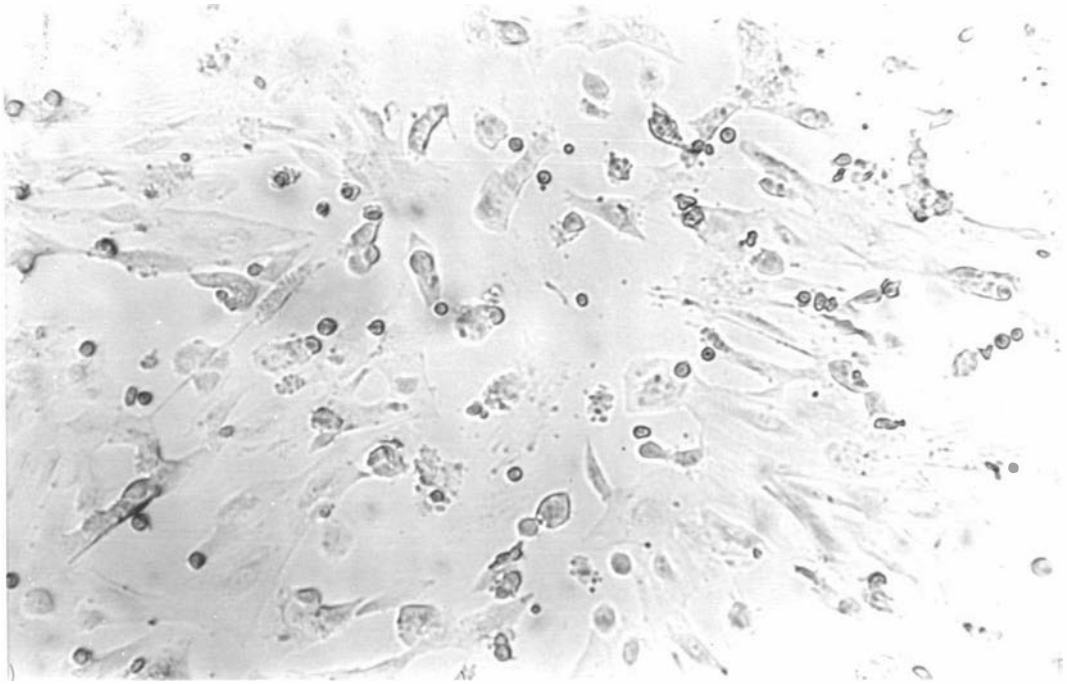

FIGURE 7: A more generalised cell rounding seen 5 hours after infection, lamb testis cells. X 134

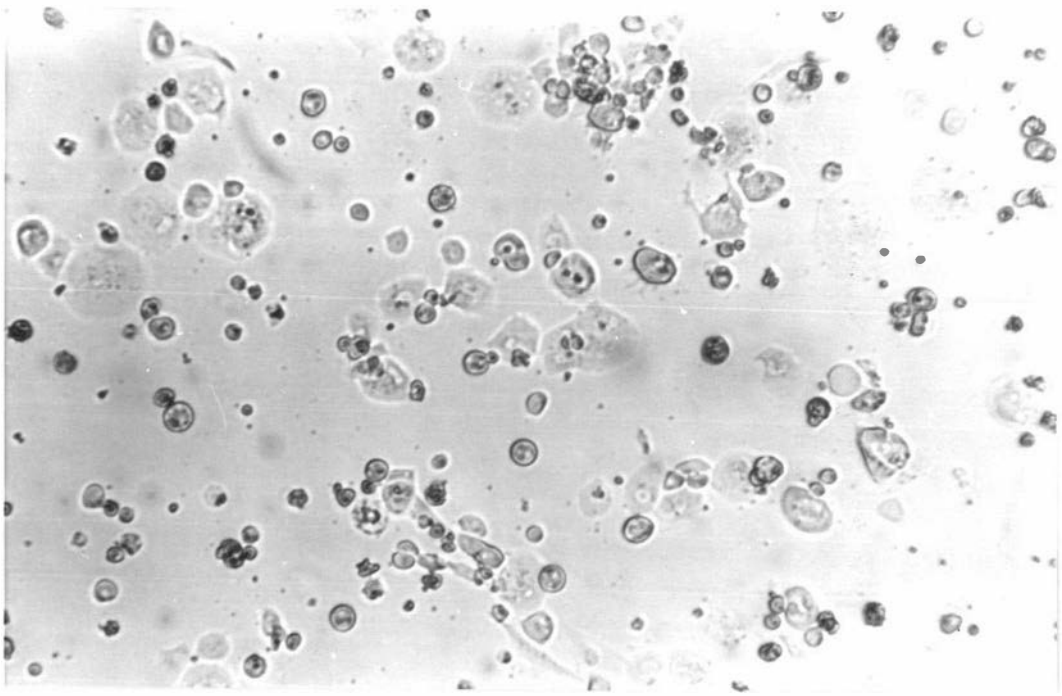

FIGURE 8a: LT, 48 hours post-infection. Most cells have detached from the flask. X 134

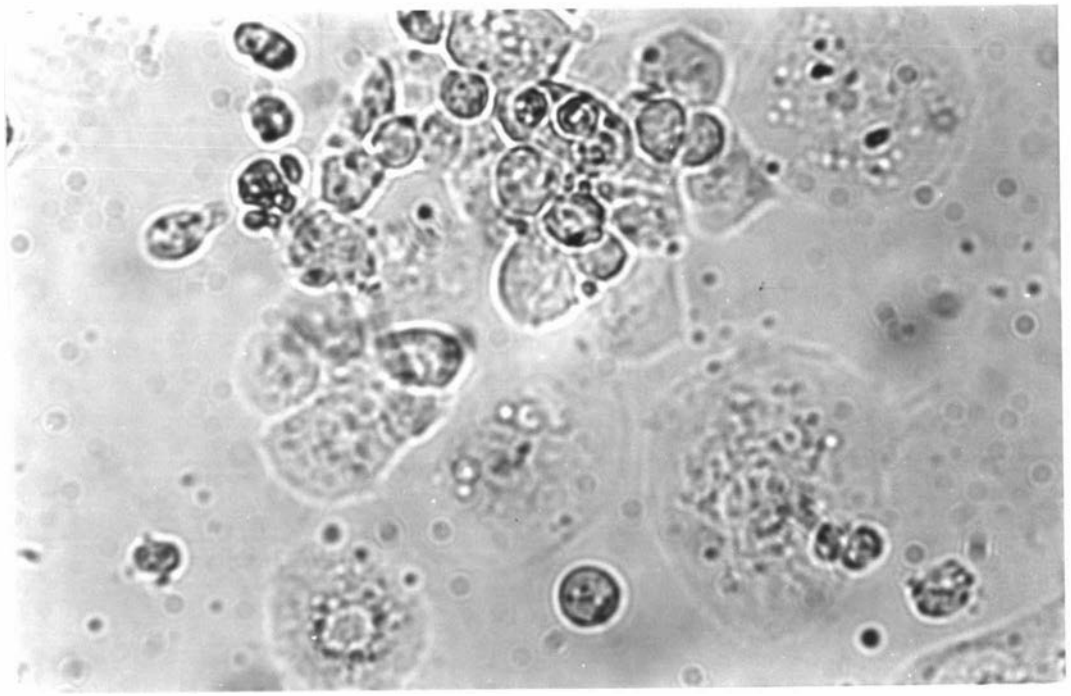

FIGURE 8b: LT, 48 hours p.i. showing clumps of rounded up cells. Note that some cells have become enlarged and epithelioid. X 500

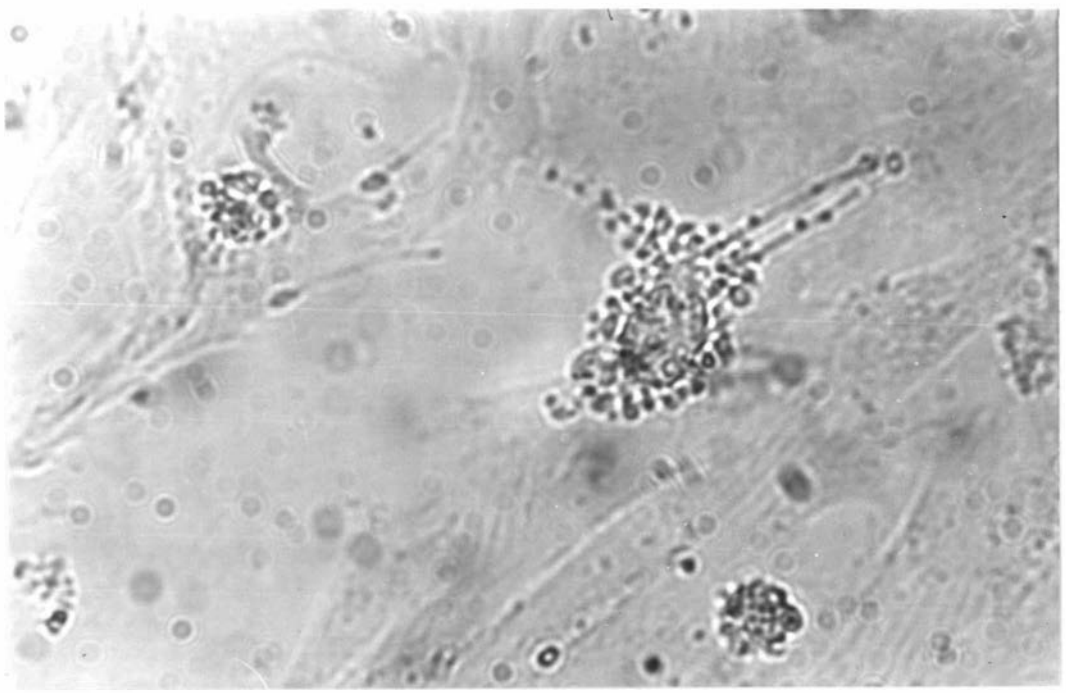

FIGURE 9: LT monolayer infected with scab-derived virus isolate No. 19P4. Note that some cells have numerous tiny cellular processes. X 500

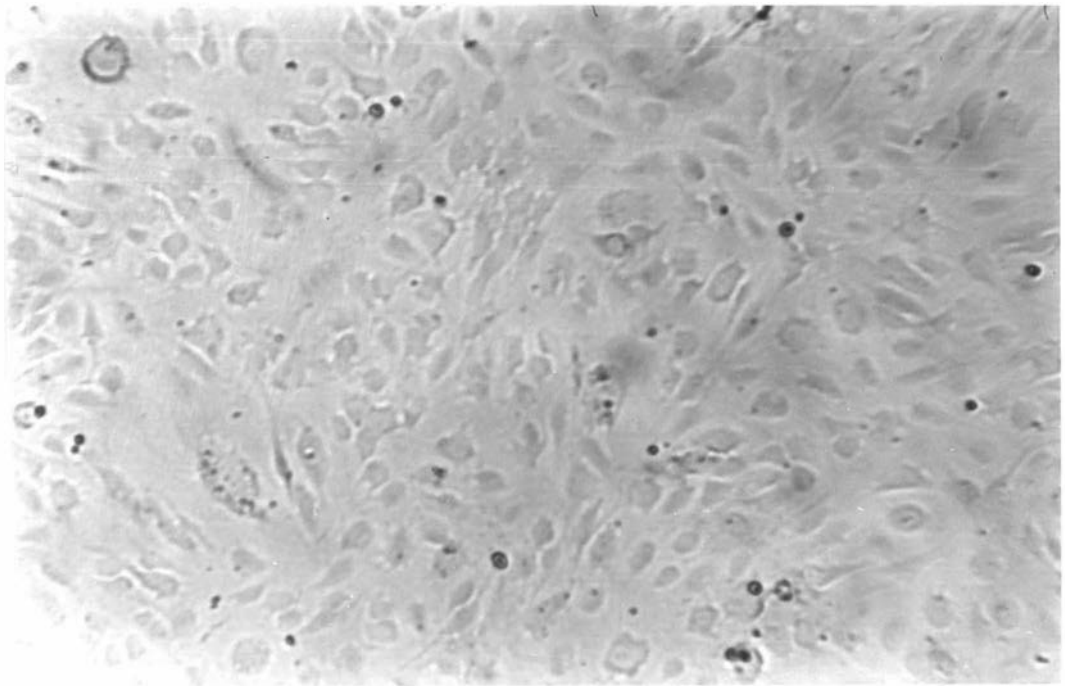

FIGURE 10: Uninoculated control foetal bovine lung monolayer (FBL). X 134

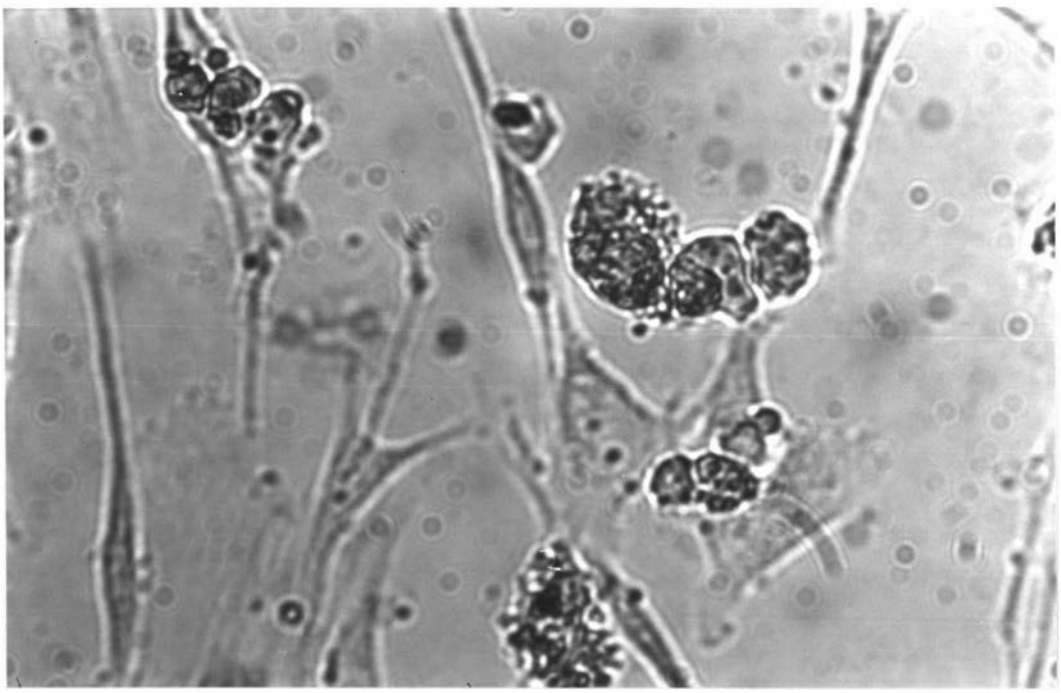

FIGURE 11: FBL, 5 hours after infection with scab-derived isolate no. 12P5, showing rounded cells with tiny processes. X 500

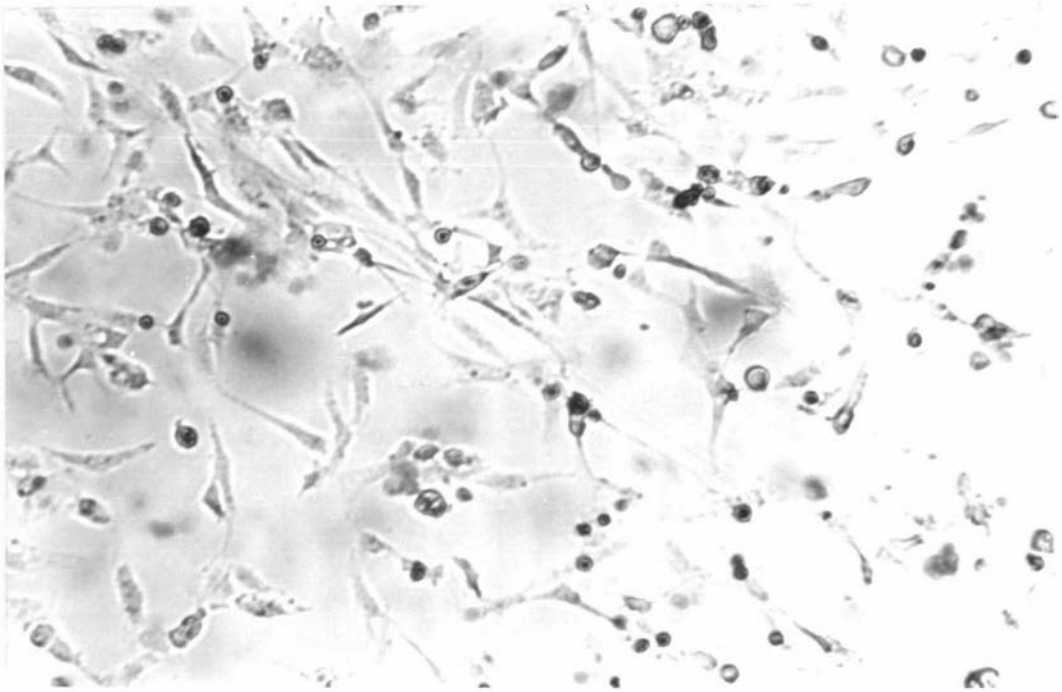

FIGURE 12a: FBL, 24 hours after infection. Note that there is less clumping of rounded cells and cells that remained attached to the flask become spindle-shaped. X 134.

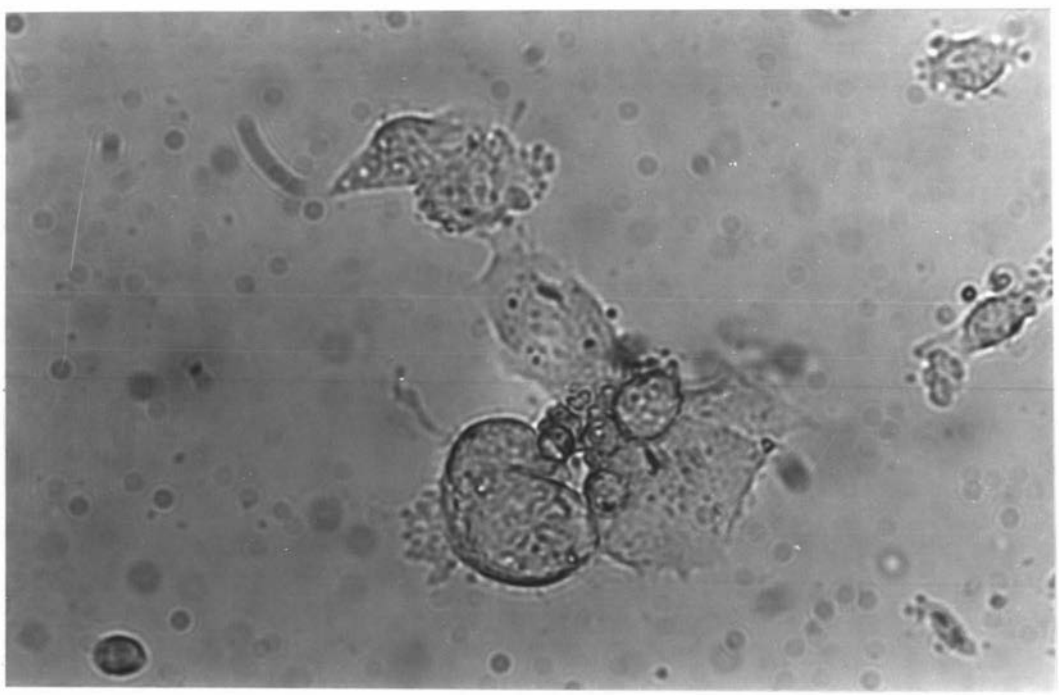

FIGURE 12b: FBL, 24 hours post-infection showing clumping of rounded up cells. X 500

Plaque-purification and titration of virus

The plaques produced by the five isolates tested could be easily detected only by microscopic examination. Two types of plaques were observed. One type appeared as an empty space surrounded by rounded cells. Sometimes this type of plaque could be seen as a group of rounded cells that clumped together creating spaces in the plaque. (Fig. 13a). Whereas, the other type was characterised by aggregates of refractile cells in an otherwise normal monolayer. (Fig. 13b). At the time of this writing only isolates No. 2 and 10 had been successfully plaque-purified twice. Isolates No. 3, 9 and 12 were plaque-purified once. Other isolates should also be amenable to plaque-purification.

Attempts were made to enhance the number and size of plaques. Incorporation of additives in the overlay and use of other overlays such as methylcellulose were tried. Such additives as trypsin and DEAE-dextran (Tobita et al., 1975) were also tried but did not enhance plaque formation. Trypsin at a concentration of 2 ug/ml caused detachment of lamb testis cells from the plastic dishes. Preliminary plaque titration of virus in infected cultures were undertaken. Titres of 10^4 to 10^6 pfus/ml were observed using different passages of LT-adapted isolate No. 10.

Examination of May Grunwald-Giemsa stained preparation

There were no evident changes seen in monolayers stained one hour post-inoculation. At 3 hours few rounded up cells were seen. Cultures stained six hours post-infection showed that most of the cells had rounded up and were staining more intensely than in the uninfected cultures. Some cells contained inclusion bodies surrounded by a halo. These inclusions are intracytoplasmic and appeared blue with May-Grunwald-Giemsa stain. Monolayers stained nine and 24 hours after infection showed the characteristic clumping of the rounded up cells. Inclusion bodies could also be seen in some cells at this time (Fig. 14-19). Cells containing a few nuclei (syncytia?) were also seen (Fig. 20).

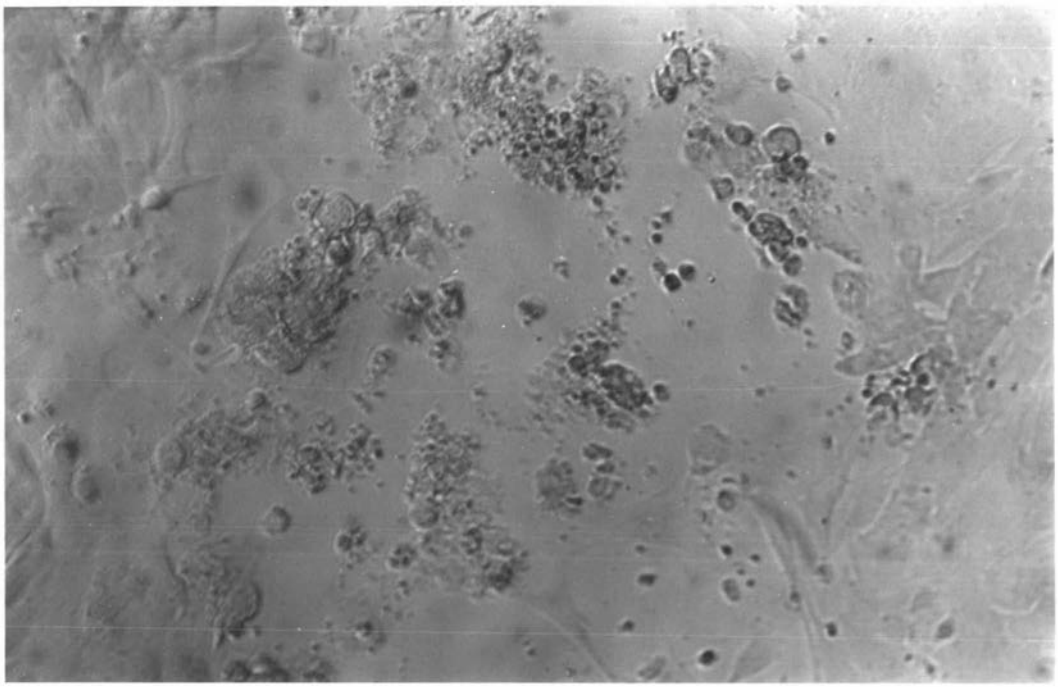

FIGURE 13a: Open type of plaque in LT monolayer seven days post-inoculation with isolate no. 10 (second plaque-purification). X 134.

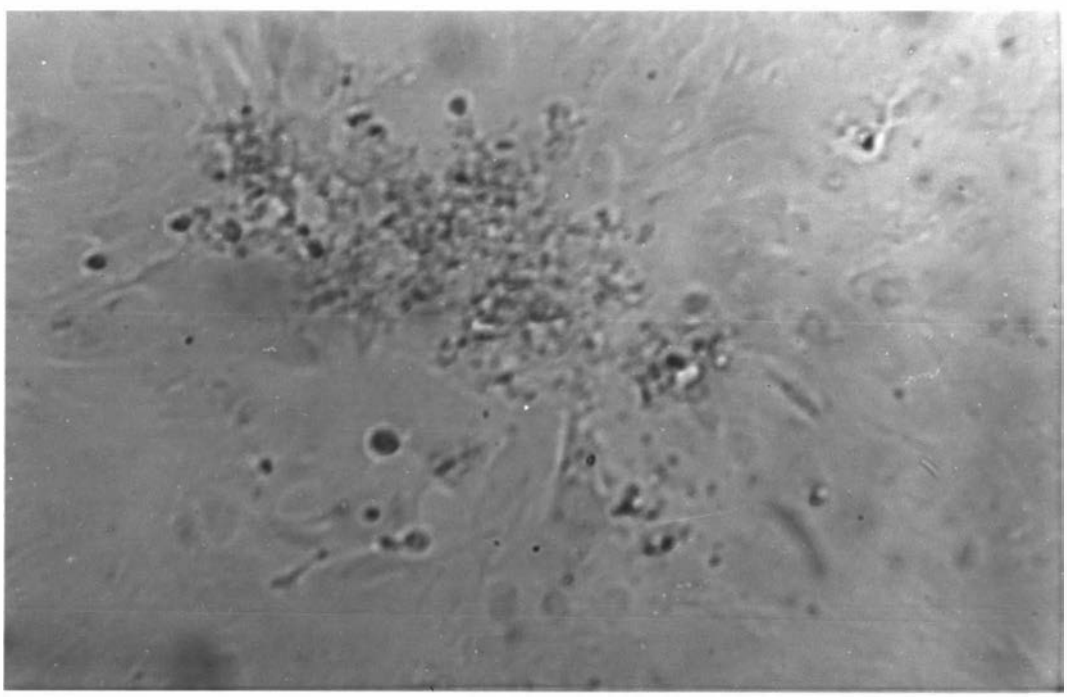

FIGURE 13b: Closed type of plaque in LT monolayer seven days after inoculation with isolate no. 12P11 (first plaque-purification). X 134.

Effect of cyclohexamide on orf CPE

It has been shown that early cell rounding induced by vaccinia virus can be inhibited by cyclohexamide (Bablanian et al., 1978 a and b). When cyclohexamide was present during virus absorption and subsequent incubation of the culture, cell rounding induced by orf was also inhibited. Cell rounding, however, was evident within an hour after withdrawal of the cyclohexamide, five hours after infection.

CHAPTER FOUR

DISCUSSION

There are no published procedures for the purification of orf virus from sheep scab material. The method described here, however, proved very satisfactory. In particular, it was found that sodium diatrizoate was very useful. It is preferable to sodium metrizoate due to its low cost. It is also preferable to ceasium chloride or sucrose in which the virus has a tendency to aggregate.

Virus particles purified in sodium diatrizoate gradients were intact as determined by electron microscopy. Some preparations did, however, contained amorphous material (see Fig. 2) but this did not appear to affect subsequent extraction and analysis of the viral DNA. Further purification may be necessary if other properties of the virus, such as protein components, were being investigated.

There was a wide range in the number of virus particles obtained from a given weight of scab material. This may have been due to losses of virus during the purification process (Joklik, 1962) but more likely was related to the inherent variability in the composition of the scab material. However, sufficient virus could be obtained from most of scab samples submitted to enable endonuclease analysis on extracted DNA. There was little difference between the theoretical and actual content of DNA recovered.

Digestion of the DNAs from thirteen different isolates with $EcoR_1$, showed there was considerable heterogeneity in the fragment patterns between different isolates. However, isolates No. 1 and 3 appeared identical while isolates No. 9 and 11 and 13 and 14 showed close similarity in pattern. The difference between the last two pairs is probably the gain or loss in one $EcoR_1$ cleavage site. Comparisons

between the other isolates showed extensive variation which could not be explained by gain or loss of one restriction endonuclease site. Wittek et al., (1980) have compared three orf isolates and have observed variations in the DNA patterns when digested with either $EcoR_1$, Hind III, Kpn I, Kho I or Sal 1. It is also interesting to note that the $EcoR_1$ patterns of the German strains do not seem to closely resemble any of the thirteen isolates analysed here. However, a comparison is difficult as no molecular weights were given for the fragments generated from the German strains. In their studies with papular stomatitis virus, they also observed variation between strains. In five isolates studied they found three distinct patterns in the $EcoR_1$ and Hind III digests. In contrast, endnuclease patterns of the DNAs of orthopoxviruses and in particular, vaccinia and smallpox, isolates do not show this degree of variation (Esposito et al., 1978). Also, only minor variations were observed when comparing different isolates of herpes simplex virus (Buchman et al., 1978).

Some isolates, for example, Nos. 7, 8 and 14, showed fragments that appeared to be represented in submolar amounts. This was probably due to the presence of two or more virus types in the scab material. This could possibly have been due to a change in virus type during the course of the disease or perhaps to a mixed infection within a line of lambs. Analysis of virus recovered from a number of plaques produced in cell culture from the original material would decide whether such digests are derived from a mixture of virus types.

Lamb testis cells were found very suitable for the isolation and propagation of orf virus. This is in agreement with findings of Plowright et al., (1959) and Sawhney (1966c). Other cell cultures such as ROK and FBL cells were found capable of supporting growth of LT-adapted virus. Their suitability for primary isolation of orf virus is yet to be

established. It is probable that any orf virus isolate could be permanently adapted to cell culture if the method of concentrating the virus inoculum particularly between each early passage is employed. Characteristic cytopathic effect was observed which is in contrast to the previous findings of Webster (1958) on his work on a New Zealand vaccine strain.

There were no apparent differences between the isolates in their behaviour in LT cells, except perhaps in the time of appearance and completion of CPE and ease of adaptation of the virus to cell culture. However, this difference could be explained on the basis of multiplicity of infection (m.o.i.). CPE observed in the first few passages was similar to the findings observed by Plowright et al., (1959) and Sawhney and Toschkov (1972). It was noted however, that when high titred inocula were used a rapid CPE developed within three hours of infection, only in the first two passages. In subsequent passages no CPE was seen or developed only after a period of some days. Three possible explanations can be given for this observation. Firstly, the CPE could have been due to defective virus. Secondly, most virus produced in the first few passages could have been defective. Thirdly, a cytotoxic effect of a high titred inoculum might have been inhibiting productive infection in early passages.

To expand on the first possibility, the initial inoculum, might contain only a small proportion of non-defective virus capable of producing progeny virus. Most of the virus particles in the defective population may be capable of some viral functions, possibly early protein expression, including cell rounding but going no further. Thus, even though early cell rounding is seen in most cells only a few cells go on to make virus. This would explain why second and subsequent passages appear to contain little or no infectious virus. It might be that not until enough

infectious virus is built up by passage that permanent establishment of virus is seen. This might also explain why concentration of the virus between passages help to establish virus in culture.

Another possible reason for the difficulty in establishing the virus in cell culture may be that virus progeny is not released from an infected cell until that cell degenerates. This is possibly why plaques are difficult to obtain in cell culture. If this were the case, there would be no advantage, when attempting isolation, in incubating cultures for longer than the time taken for one virus cycle. In the case of vaccinia virus, this is about 12 hours. Theoretically then, passage at twelve-hour intervals with a freeze-thaw cycle between passages should be as effective as weekly passage for rapidly adapting such a virus to cell culture.

A number of experiments could be designed that might explain the behaviour in culture of early passage orf virus. An experiment comparing the effect of increasing doses of U.V. light on virus yield and early cell rounding (CPE) would determine if cell rounding was more resistant to U.V. than was yield. If cell rounding was more resistant then a population of defective virus could give rise to CPE but little progeny virus. This situation would be analogous to what is observed with early passage virus.

Another worthwhile experiment would be to define how much virus is cell-associated and how much is released. This might indicate whether a short interval between passages would be preferable to a long interval.

Early cell rounding or CPE might be multiplicity dependent. Thus, it would be of interest to define multiplicity of infection more closely. It may be that a cell has to be infected with say 10+ virus particles to produce early cell rounding. The tendency of poxvirus particles to clump, however, might complicate any multiplicity of infection

experiments. This viral aggregation can however, be treated by sonication with the use of a probe disintegrator or an ultrasonic cleaner. Either method was found satisfactory in disrupting vaccinia and smallpox virus aggregates (Muhler, 1976). However, it was suggested by Muhler (1976) that ultrasonic cleaner was preferable to the use of a probe disintegrator. The usefulness of sonication on orf virus could be assayed by the following simple experiment. A sample of orf virus suspension is allowed to aggregate by long standing and subjected to sonication for one minute. By plaque-titration technique, titres of the suspension before and after sonication would then be compared. Also, the appearance of the virus in suspension before and after ultrasonic treatment can be compared by electron microscopy (E.M.).

Once the virus has established itself in cell culture, the characteristic CPE seen is clumping of rounded cells. This feature was also described by Greig (1957) and MacDonald and Bell (1961). It was also seen in avian fibroblasts (Rossi, 1973) and primary lamb kidney cells (Precausta and Stellman, 1973) infected with orf virus.

It was noticed that the CPE produced by scab-derived virus showed more cells with minute surface projections than did Na diatrizoate derived virus. One explanation for this is that the latter virus suspension contained a selective population of almost all type 1 form, whereas, the scab suspension would contain both types 1 and 2 forms in more equal proportions. Alternatively, it may be that, since virus inoculum in the subcultures of scab-derived virus is a concentrated virus from the previous passage, more virus particles might be infecting a single cell, leading to this type of reaction. The same might hold true in FBL cells where virus inoculum for passage was concentrated by cetrifugation. It would be interesting to see if this difference was maintained in high passage cultures.

It is worth mentioning that when uninfected cells are removed from their plastic surface by trypsin some of these cells show cellular projections which appear similar to those described in the infected cultures. It could be that detachment of cells from the flask as induced by virus infection is sufficient to produce the cell processes observed in infected cultures. The appearance of such cells therefore may not be peculiar to virus infected cells.

It has been shown that parapoxviruses produce two types of plaques in cell culture. Nagington (1968) observed that orf virus isolates produced the "open" type of plaques only while the bovine papular stomatitis virus produced mainly the "closed" type with few exhibiting both or intermediate type. He suggested that these plaques could be a distinguishing feature between the two groups. It was also shown by the works of Precausta and Stellman (1973) that orf virus produced the open type of plaques. However, in this study, both types were observed in the orf virus isolates. In the five isolates plaqued, three showed the "open" type and two produced the "closed" type. It may be that this characteristic is more useful in differentiating between isolates within rather than between the two groups. More studies should be undertaken to show if each isolate maintains this feature after subculture.

Analysis of DNA fragments produced from virus plaque-purified from isolate No. 2, using $EcoR_1$, Bam H-I and Hind III showed no difference between it and DNA pattern derived from virus purified from the original scab material. This means that passage of the virus in cell culture (23 passages) does not detectably alter the DNA structure. Wittek et al., (1980) however, has reported that 137 passages of plaque-purified orf virus produced a minor change in restriction endonuclease pattern when compared with the virus at passage 8. Further passages of virus No. 2 might also alter the DNA structure. The latter authors however, did not compare their passaged virus with the original scab material from

which the virus was derived. Thus the experiments described here represent the first report of the comparison of cell culture-adapted virus with the virus present in the lesion from which it was derived. It is encouraging to note that no changes can be detected albeit using only three restriction endonucleases in the comparison. Whether such a result shows that other virus-cell culture adapted systems do not alter the virus genome in early passage cannot be stated with certainty. However, it indicates that this might be the case. Unfortunately, most viral lesions do not yield sufficient virus for this experiment to be done.

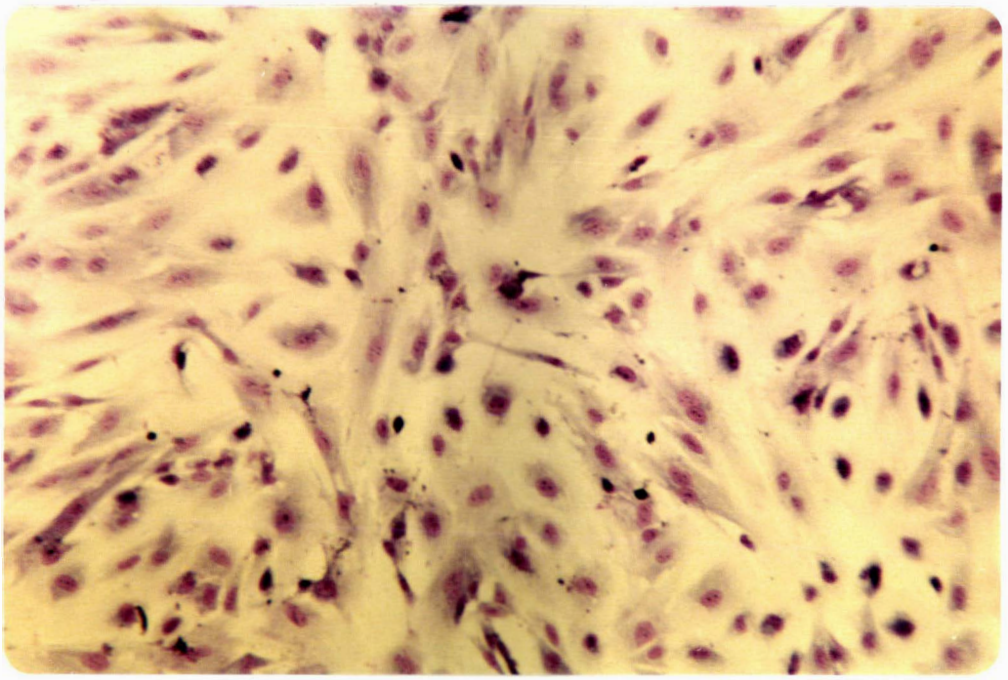

FIGURE 14: Uninoculated control LT monolayer. May-Grunwald-Giemsa, X 125

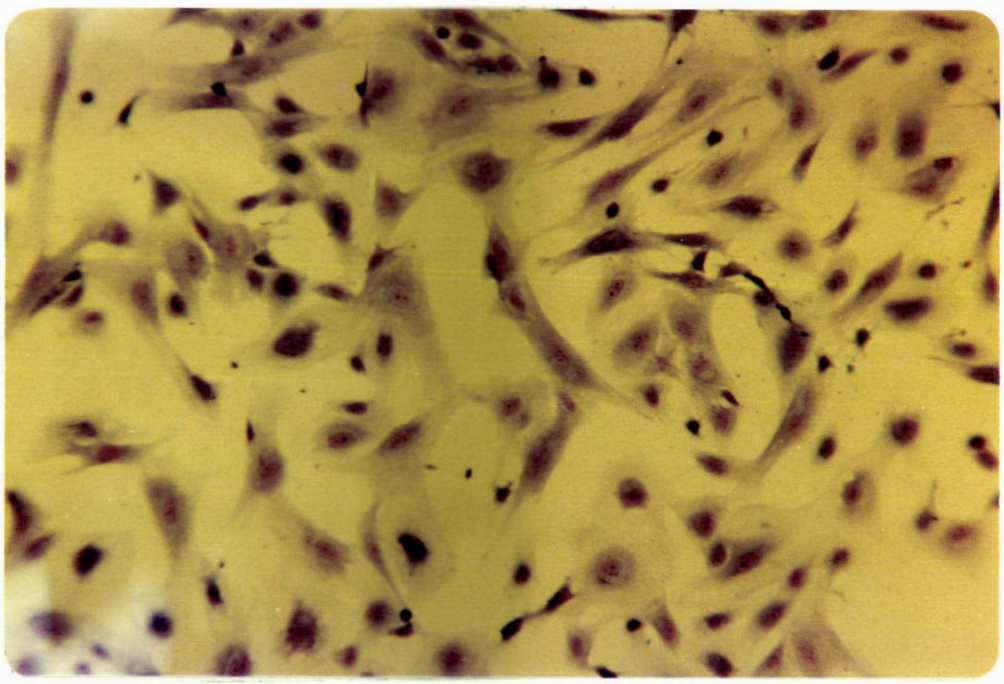

FIGURE 15a: LT, one hour post-inoculation with no. 10P10. May-Grunwald-Giemsa, X 125

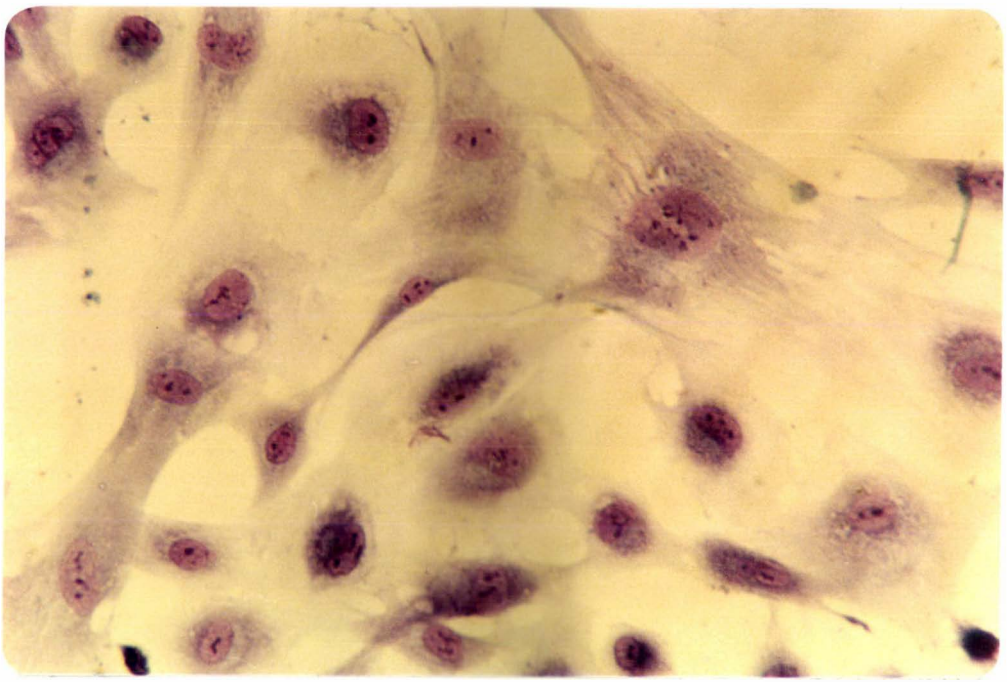

FIGURE 15b: LT, one hour post-infection at higher magnification. May-Grunwald-Giemsa, X 310.

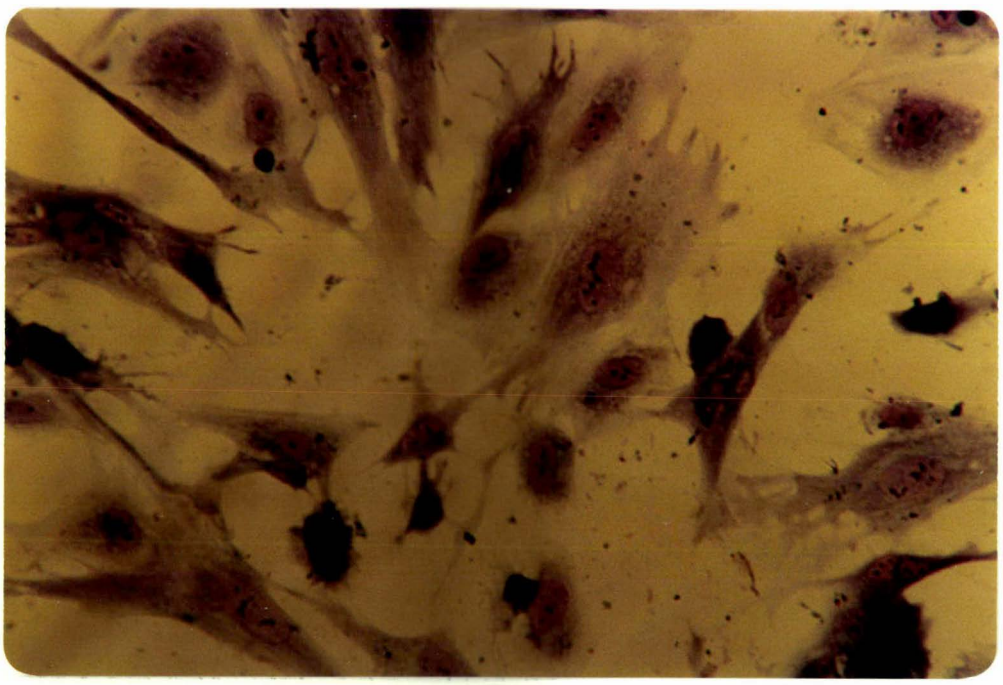

FIGURE 16: LT, three hours after inoculation, showing the appearance of a few rounded up cells. May-Grunwald-Giemsa, X 310.

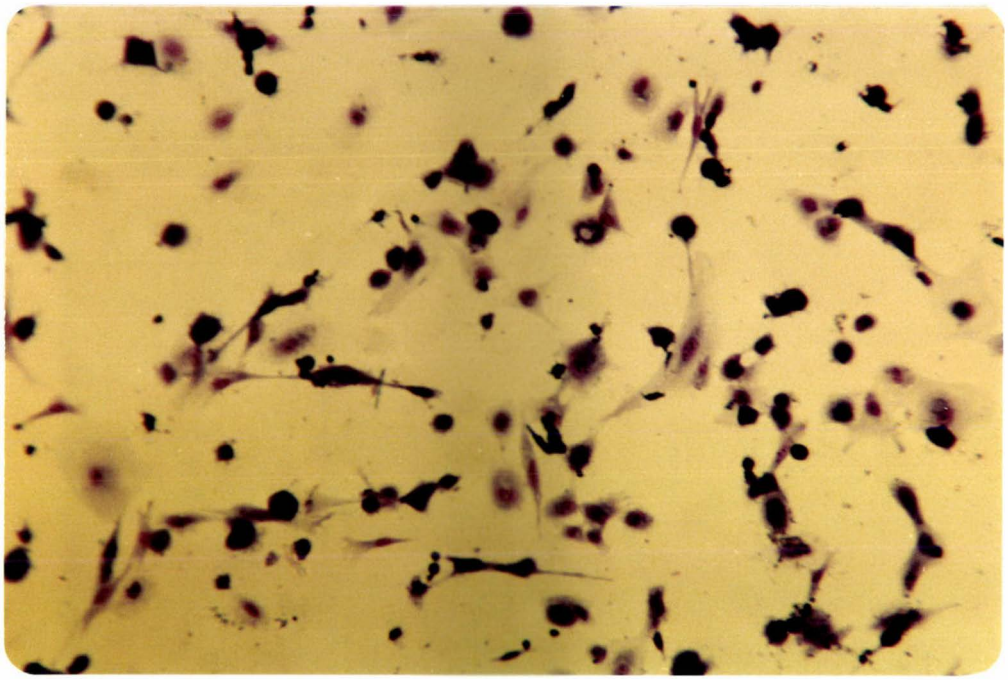

FIGURE 17a: LT, 6 hours post-inoculation. Note that most of the cells have rounded up and stained more intensely than the control. May-Grunwald-Giemsa, X 125

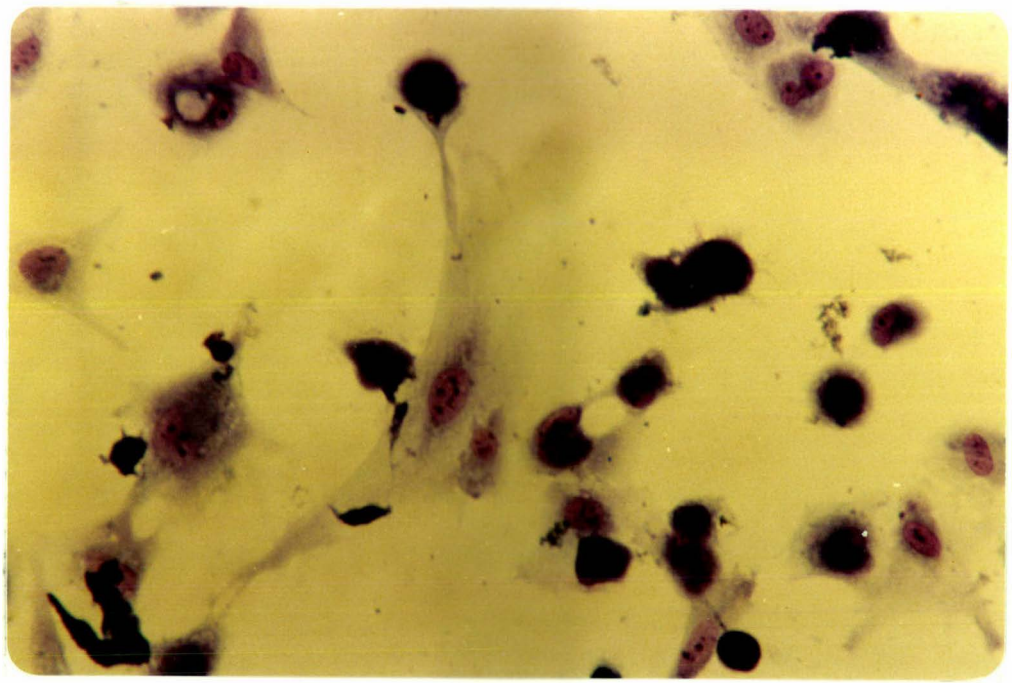

FIGURE 17b: Higher magnification of the above. X 310

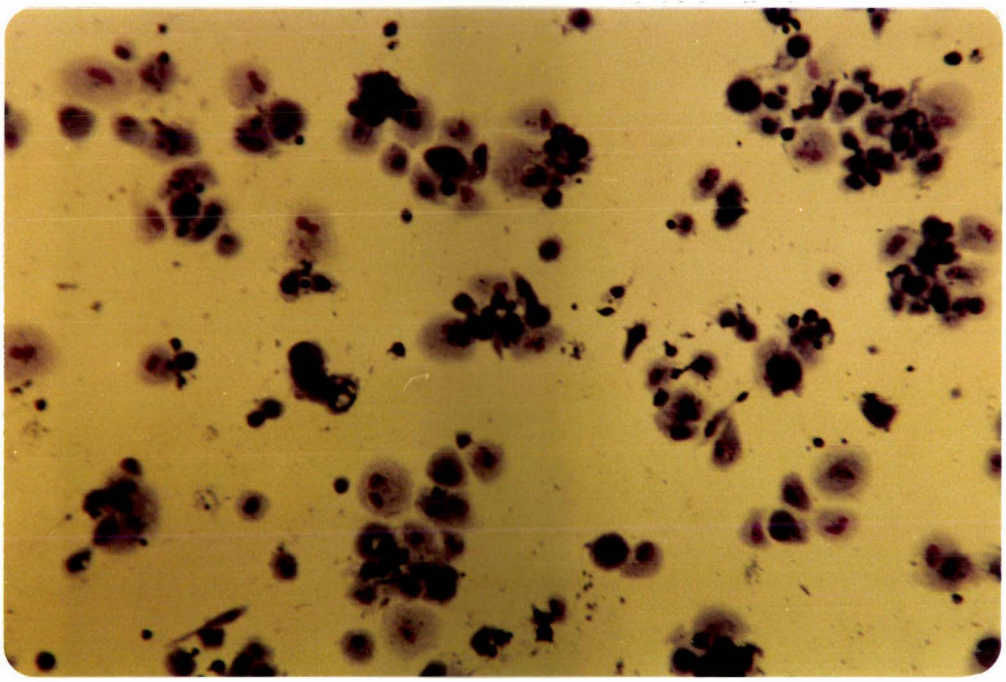

FIGURE 18a: LT, 24 hours after infection, showing clumping of rounded up cells. May-Grunwald-Giemsa, X 125

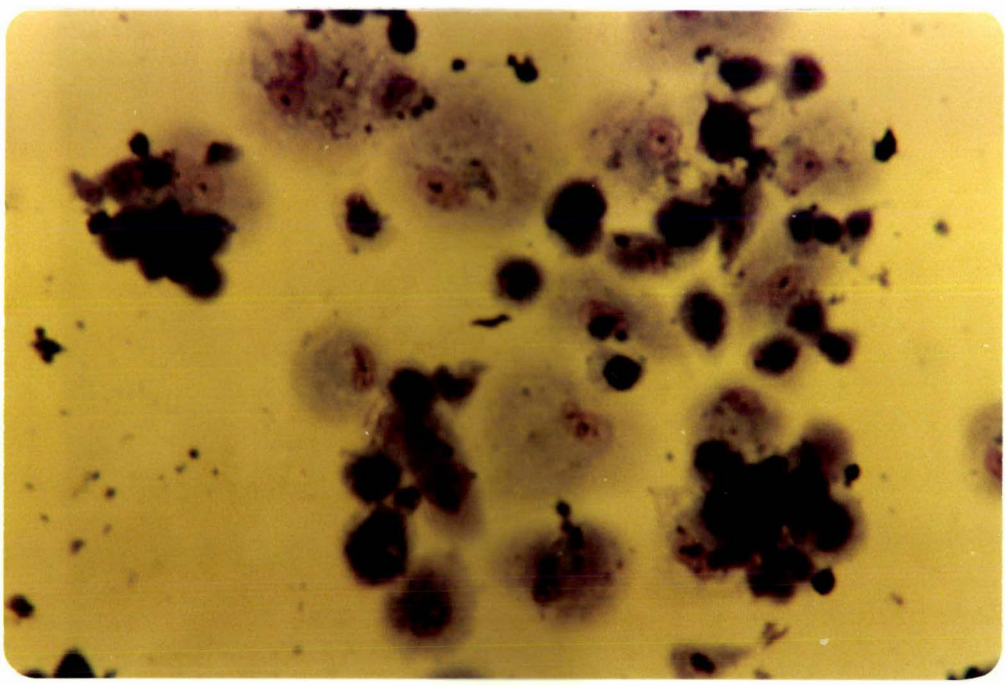

FIGURE 18b: Higher magnification of the above. X 310

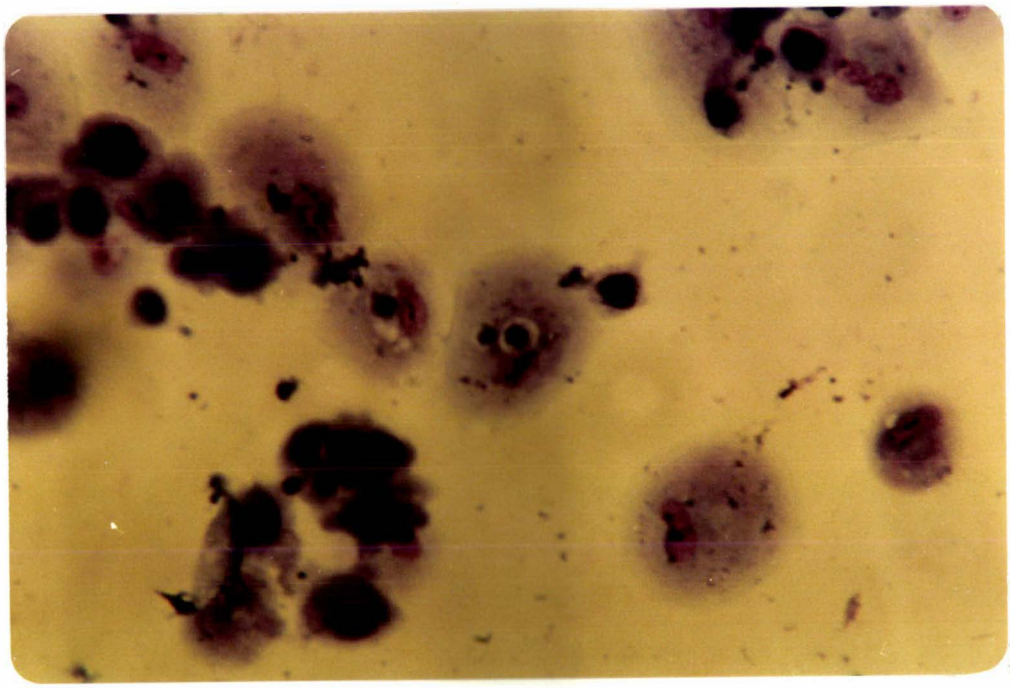

FIGURE 19: LT, 24 hours after infection. It can be seen that some cells contain paranuclear inclusion bodies surrounded by a "halo". May-Grunwald-Giemsa, X 310

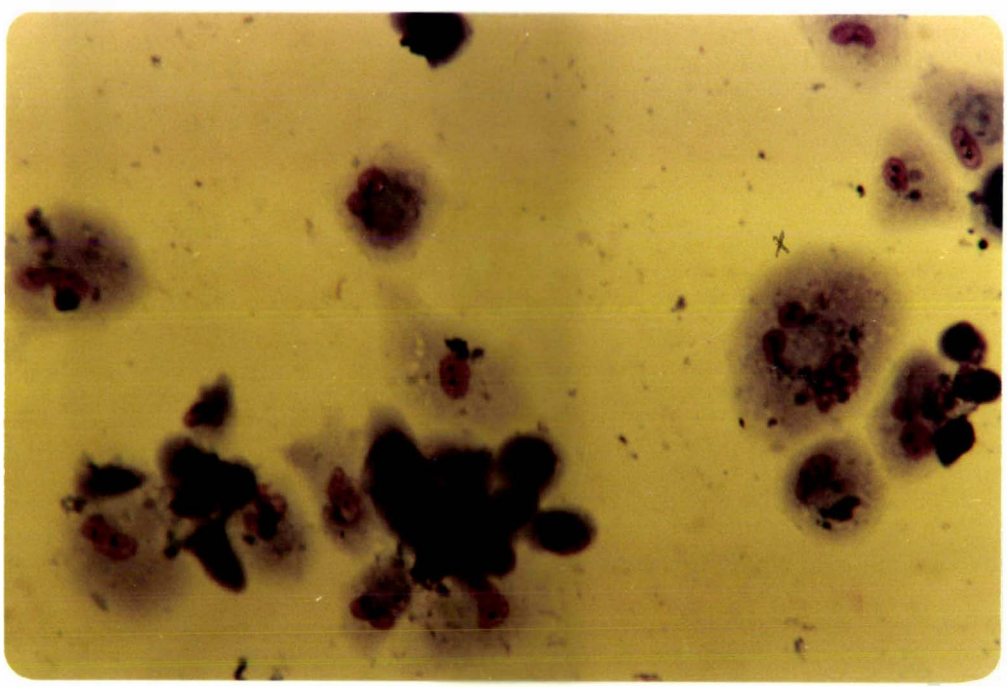

FIGURE 20: LT, 24 hours p.i. showing an enlarged cell (X) containing several nuclei (a syncytium?). May-Grunwald-Giemsa, X 310

CHAPTER FIVE

SUMMARY

Thirteen orf virus isolates were purified from scab material and identified by restriction endonuclease analysis. Most of the isolates showed considerable differences in their DNA patterns while two were identical.

The orf virus isolates could also be grown in cell culture. Five of these isolates were grown and propagated in lamb testis cells. They showed little difference in their behaviour in cell culture. Foetal bovine lung cells and ROK cells could also be used to grow orf virus. Both "open" and "closed" types of plaques in LT cells were seen. Early rounding of infected cells could be inhibited by cyclohexamide. Inclusion bodies were also seen in infected cells stained with May-Grunwald-Giemsa.

An isolate that had been passaged and plaque-purified in LT cell culture showed DNA "fingerprints" identical to the original DNA when digested with $EcoR_1$, Bam H-I and Hind III. This shows that 23 passages in cell culture did not alter the restriction patterns. It is suggested that DNA "fingerprinting" can be used in immunological and epidemiological studies to more closely define the strain of virus being used.

APPENDICES

APPENDIX 1

LIST OF MATERIALS

CHEMICALS	SOURCE AND ADDRESS
Agarose - Electrophoresis purity reagent, Std. low mr	Bio-Rad Laboratories 32nd & Griffin, Richmond, Ca. 94804 U.S.A.
Agarose - Sea plaque	Mci Biomedical P.O. Box 748 Rochland, Maine
Cyclohexamide	Sigma Chemical Co P.O. Box 14508, St. Louis, Mo.
Crystalline Trypsin	Sigma Chemical Co.
Dextran T10	Pharmacia Fine Chemicals UPPSALA, Sweden
Disodium hydrogen phosphate, anhydrous	BDH Biochemicals Ltd Poole, England
Dimethyl sulfoxide (DMSO) low spectrophotometry	BDH Biochemicals Ltd
Ethylene diamine tetra-acetic acid, disodium salts mw 336.2	Sigma Chemical Co
Mw 372.24	BDH Biochemicals Ltd
HEPES (N_2-hydroxyethyl piperazine N_2-ethane sulfonic acid) mw 238.3	Sigma Chemical Co
Phenol	BDH Biochemicals Ltd

CHEMICALS CONT'D	SOURCE AND ADDRESS
Pronase B-grade	Calbiochem San Diego, Ca.
Potassium dihydrogen phosphate	BDH Biochemicals Ltd
Sodium diatizoate, sodium salts	Sigma Chemical Co.
Sodium chloride, A.R.	BDH Biochemicals Ltd
Sodium dodecyl sulfate (sodium lauryl sulfate)	BDH Biochemicals Ltd
Sucrose - Analar	BDH Biochemicals Ltd
Tris Sigma 7-9	Sigma Chemical Co
Trypsin (Difco 1:250)	Difco Laboratories Detroit, Michigan
STAINS	
Giemsa Powder	George T. Gurr Ltd London, S.W. 6, England
May Grumwald Powder	Edward Gurr Ltd 42 Upper Richmond Road, West London, S.W. 14
Trypan blue CI 23850	BDH Biochemicals Ltd
CULTURE MEDIA	
Medium 199 Powder	Welcome Research Laboratory Beckenham, England
Dulbeco's Modified Eagles Medium (Phosphate-free)	Self-compounded
Foetal Bovine Serum	Laboratory Services Ltd Auckland, New Zealand

MISCELLANEOUS	SOURCE AND ADDRESS
Restriction Endonucleases (EcoRl; Bam HI; Hind III)	New England Biolab 283 Cabot Street, Beverly, MA.
^{32}P (Orthophosphoric acid)	New England Nuclear Boston, Mass.
X-ray film - Kodak (X-Omat RPM-l)	Kodak (Australasia) Pty Ltd Melbourne, Australia
X-ray intesifying screen (Cawofin-rapid)	Cawo West Germany
Tri-X pan professional film Estar thick base 4 x 5 in.	Eastman Kodak Co Rochester, New York 14650
Latex beads (DOW)	Sigma Chemical Co.

APPENDIX II

EQUIPMENT AND APPARATUS

EQUIPMENT & APPARATUS	MAKER OR SUPPLIER
Beckman Model L Preparative Ultracentrifuge; Sw 25.1, Sw. 50.1 rotors	Beckman Instruments Spinco Division Palo Alto, Ca.
Biohazard Biological Hood	Envirco Albuquerque, Mexio
CO_2 chambers	Billups - Rothenberg Inc., Box 977, del Mar, Ca.
Electron Microscope - EM 200 Phillips	Phillips Holland
Laminar Flow Cabinet	Gelman Clemco Pty. Ltd Artarmon, New South Wales
Liquid Scintillation Counter LS 700	Beckman Instruments
Microfuge	Beckman Instruments
Peristaltic pump	Buchler Instruments Fort Lee, N.J.
Spray gun	Custom built, DSIR after Backus and Williams (1950)
Sorvall RC5 Refrigerated Centrifuge (SS 34 rotor)	Dupont Instruments Newtown, CT.
Spectrophotometer Unicam SP500 Series 2	PYE Unicam Ltd York St., Cambridge, England
Tissue Culture Microscope (Wild)	Hoerbrugg, Switzerland

EQUIPMENT & APPARATUS	MAKER OR SUPPLIER
U.V. Lamp (germicidal, 15-w)	Phillips Eindhoven, Holland
Filter plate	Ultaviolet Products San Gabriel, Ca 91778, USA
Cellulose Nitrate tubes	Beckman Instruments
Electrophoresis box	Custom built
Gradient Maker	Custom built
Microfuge tubes - Eppendorf	2 Hamburg 63 West Germany
Micropippete (Gilson)	Villiers-Le-Bel France, 95400
Micropippete tips	Villiers-Le-Bel
Tissue Culture vessesl Linbro	Linbro Division Flow Laboratories Inc Hamden, Connecitut
Nunc	Inter Med Postbox 280, Kanstrup DK-400 Roskilde, Denmark
Falcon	Becton, Dickinson & Co Oxnard, Ca. 93030

APPENDIX III

COMPOSITION OF BUFFERS AND SOLUTION

Antibiotic-Trypsin-Versene (ATV)

0.5	gm	Trypsin (Difco 1:250)
0.2	gm	Versene (EDTA Sequestric acid)
8.0	gm	NaCl
0.4	gm	KCl
1.0	gm	Dextrose
0.58	gm	$NaHCO_3$
2×10^5	IU	Penicillin
100.0	mg	Streptomycin
0.02	gm	Phenol red

Made up to 1 L with deionised distilled water. Sterilise by filtration.

Bam H-I buffer (1X)

50.0	mM	NaCl
6.0	mM	$MgCl_2$
6.0	mM	Tris-HCl pH 7.4
100.0	mg/ml	Bovine serum albumin

Phosphate Buffered Water

$NaH_2PO_4H_2O$ (9.2g/l) Na_2HPO_4 (anhydrous) (9.5 g/l)
or KH_2PO_4 (9.08g/l)

PH units	mls	mls
6.6	63.0	37.0
6.7	56.6	43.4
6.8	50.8	49.2
6.9	44.8	55.2

This buffer is working strength - 0.05 M (Remember to check P^H of final working strength buffer if making up from high molarity solutions. A 0.5 M PO_4^- buffer PH 6.6 rises to 6.9 if diluted to 0.05M and rises to 7.1 if diluted to 0.00 5 M).

E. buffer (10x)

96.88 gm Tris 7-9
7.44 gm EDTA (mw 372.2)
8.20 gm sodium acetate

q.s.ad 2L deionised distilled water, p^H adjusted to 7.5 with glacial acetic acid.

E. buffer (working solution)

150.0 ml 10x E buffer
300.00 ul ethidium bromide 2.5 mg/ml

q.s.ad 1½L deionised distilled water.

ET buffer (10x)

0.25 M Tris-HCl pH 7.7
0.10 M EDTA

Hind III buffer (IX)

60.0 mM NaCl
10.0 mM Tris-H6l pH 7.4
7.0 mM $MgCl_2$
100.00 mg/ml BSA

Penicillin, Streptomycin, Kanamycin (PSK)

10.0 gm Streptomycin
10.0 1-mega vial Penicllin
10.0 gm Kanamycin

Made up to 1 L PBS. Sterilise by filtration.

Phosphate Buffered Saline (PBS)

8.0 gm NaCl
0.2 gm KCl
1.15 gm $Na_2{}^HPO_4$

q.s.ad 1 L with deionised distilled H_2O. Sterilised by autoclaving (15lbs for 15 minutes) Final pH 7.2 - 7.4.

Phenol

Redistilled under nitrogen and stored in the dark at -20°C under nitrogen.

Pronase

10 mg/ml (aqueous solution)

R_1 Buffer (1X)

100.0	mM	Tris-HCl pH 7.5
50.0	mM	NaCl
5.0	mM	$mgCl_2$
100 mg/ml		BSA

Sucrose Cushion

30% w/v in 0.1X ET buffer

STE buffer

0.15	M	NaCl
0.01	M	Tris-HCl pH 7.5
0.001	M	EDTA (mw 336.2)

Sodium dodecyl sulfate (SDS)

5% w/v in 45% ethanol

Tris-HCl pH 7.5

1 M in aqueous solution

TE buffer

0.01 M Tris-HCl pH 7.5
0.001M EDTA

Yeast RNA - 10 mg/ml (aqueous solution)

STAINS

Trypan blue

0.2 gm Trypan blue powder
100.0 ml PBS
dipense in 1.8 mls

Giemsa Stain (Stock)

1.0 gm Giemsa Powder
66.0 ml Glycerin
Heat at 60°C over 2 hrs
Add 66 mls. methanol

Giemsa - (working solution)

1.0 ml Giemsa stock solution
19.0 ml buffered water pH 6.6

May-Grunwald Stain (Stock solution)

0.3 gm powder
100.0 ml methanol

May-Grunwald Stain (working solution)

50 parts stock solution
50 parts buffered water

CULTURE MEDIA

Tricine buffered 199

10.0 gm Powdered media
1.8 gm Tricine
1.5 gm $NaHCO_3$ (closed vessel)
1 x 10^5IU Penicillin
100.00 mg Streptomycin

Made up to 1 L with deionised distilled H_2O. Sterilised by

Cont'd..

filtration. Stored at -4°C.

Modified Eagles Medium (Phosphate-free)

(Reference: Grand Island Biological Company, Grand Island, New York).

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